Calpain-Cleavage of α -Synuclein

Connecting Proteolytic Processing to Disease-Linked Aggregation

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Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are both characterized pathologically by the presence of neuronal inclusions termed Lewy bodies (LBs). A common feature found in LBs are aggregates of α -synuclein (α -Syn), and although it is now recognized that α -Syn is the major building block for these toxic filaments, the mechanism of how this occurs remains unknown. In the present study, we demonstrate that proteolytic processing of α -Syn by the protease calpain I leads to the formation of aggregated high-molecular weight species and adoption of a β -sheet structure. To determine whether calpain-cleavage of α -Syn occurs in PD and DLB, we designed site-directed calpain-cleavage antibodies to α -Syn and tested their utility in several animal model systems. Detection of calpain-cleaved α -Syn was evident in mouse models of cerebral ischemia and PD and in a *Drosophila* model of PD. In the human PD and DLB brain, calpain-cleaved α -Syn antibodies immunolabeled LBs and neurites in the substantia nigra. Moreover, calpain-cleaved α -Syn fragments identified within LBs colocalized with activated calpain in neurons of the PD and DLB brains. These findings suggest that calpain I may participate in the disease-linked aggrega-

tion of α -Syn in various α -synucleinopathies. (*Am J Pathol* 2007, 170:1725–1738; DOI: 10.2353/ajpath.2007.061232)

The common pathological feature of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) is the presence of Lewy bodies (LBs), and the primary structural components of LBs are fibrils composed primarily of α -synuclein (α -Syn), a highly conserved 140-amino acid protein that is predominantly expressed in neurons and may play a role in synaptic plasticity and neurotransmission.^{1–3} Pathologically, both PD and DLB share LBs as a common feature; however, clinical features of these two diseases are distinct. PD is characterized as a neurodegenerative movement disorder presenting with rigidity, resting tremor, disturbances in balance, and slowness in movement.⁴ In contrast to PD, DLB is characterized as a neurodegenerative cognitive disorder. DLB is the second most common form of degenerative dementia, accounting for up to 20% of cases in the elderly, and LBs in DLB are found in a more generalized area than in PD.⁵ A key feature found in LB disorders such as PD and LBD is aggregates of α -Syn, and collectively, such diseases are referred to as α -synucleinopathies.⁶ Numerous studies now support the hypothesis that α -Syn aggregation is the key step driving pathology, cellular damage, and subsequent neuronal dysfunction.^{7–9} Although the majority of previous studies have focused on the aggregation of full-length α -Syn, recent studies suggest that truncated forms of α -Syn are of pathogenic significance: truncated species of α -Syn are found in PD and DLB brains,^{10,11} and they promote the ability of full-length

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α -Syn to aggregate^{11–14} and enhance cellular toxicity.^{11,15} Moreover, expression of C-terminally truncated α -Syn (1–120) in transgenic mice leads to the formation of pathological inclusions and to a reduction in striatal dopamine levels.¹⁶ The mechanisms governing the proteolytic cleavage of α -Syn are not firmly established, but a potential candidate protease is calpain I. Calpain I represents one of a large family of intracellular calcium-dependent proteases whose cleavage of specific proteins has been implicated in physiological pathways and in numerous pathological diseases including Alzheimer's disease and stroke.^{17,18} α -Syn is a substrate for calpain cleavage,^{19,20} and calpain-cleaved α -Syn species are similar in molecular weight to truncated α -Syn fragments that promote α -Syn aggregation and enhance cellular toxicity.^{12,14,21} Herein, we demonstrate that calpain I fits the criteria of a protease capable of converting α -Syn into its aggregated form after its cleavage in a cell-free system or in a cell model system consisting of SH-SY5Y neuroblastoma cells. Furthermore, we show that cleavage of α -Syn by calpain occurs in both PD and DLB brains and calpain-cleaved fragments of α -Syn colocalized with activated calpain. These data suggest that calpain I may be the molecular switch that turns on the aggregating properties of α -Syn, providing a general mechanism for the initiation and evolution of LB formation in various α -synucleinopathies.

Materials and Methods

Cell-Free Assay

A cell-free system was used consisting of human recombinant α -synuclein (α -Syn) and purified calpain I from erythrocytes (both from Calbiochem, La Jolla, CA). Recombinant α -Syn (20 μ g) was incubated with calpain I (2 U) in a buffer containing 40 mmol/L HEPES (pH 7.2) and 5 mmol/L dithiothreitol at 37°C. Reactions were initiated by the addition of calcium (1 mmol/L final). To stop the proteolysis, aliquots were removed from the reaction mixture and added to 5 $\times$ sample buffer (Pierce, Rockford, IL) at various times points, heated in a boiling water bath, and stored at –20°C until used for either N-terminal sequencing or Western blot analysis. For N-terminal sequencing experiments, a total of 10 μ g of recombinant α -Syn cleaved by calpain I was loaded in each well and separated using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Gels were transferred electrophoretically to a polyvinylidene difluoride filter and stained with Coomassie blue to visualize bands. Potential low-molecular weight bands of interest were excised, and N-terminal protein sequencing was accomplished (Midwest Analytical, Inc., St. Louis, MO). For circular dichroism (CD) and analytical ultracentrifugation (AUC) experiments, cell-free digestion was performed as described above, and samples were kept on ice until aliquots were removed for analysis.

CD Analysis

Digestion of α -Syn by calpain I was performed as described above in 20 mmol/L Tris buffer, pH 7.5, containing 100 mmol/L NaF, for 30 minutes at 37°C. Aliquots containing concentrations of α -Syn (0.2 mg/ml) or calpain I (0.15 mg/ml), or samples containing α -Syn and calpain I were added to 1-mm path-length suprasil quartz cuvettes (Hellma, Müllheim, Germany). CD spectra were recorded using a Jasco-810 spectropolarimeter (Jasco, Easton, MD) at 20°C in triplicate using a step size of 1 nm. Solvent blanks and spectra were collected from 190 to 250 nm and averaged, correcting for the buffer spectrum. Calpain I spectrum was subtracted from calpain plus α -Syn mixture to generate the α -Syn spectrum.

AUC Analysis

α -Syn (1 mg/ml) was incubated with calpain I (1.5 mg/ml) for 30 minutes at 37°C, and samples were placed on ice. Aliquots were used to determine sedimentation velocity coefficients using a Beckman Coulter XL-I analytical ultracentrifuge with an An-60 Ti rotor spun at 30,000 rpm at 20°C for 4 hours (Beckman Coulter, Fullerton, CA). Radial scans were recorded measuring the absorbance of the samples at 280 nm. The sedimentation coefficient of each sedimenting boundary was determined using UltraScan Analysis Software version 7.3 from Borries Demeler (University of Texas Science Center, San Antonio, TX). Each analysis incorporated 30 scans, and the values for the density and viscosity of the buffer relative to water were 1.0038 and 1.0227, respectively. Fringe displacement was then plotted as a function of radial distance within the cell, allowing for the determination of percent molecular weight changes of α -Syn after digestion by calpain I.

Generation of Site-Directed Calpain-Cleaved α -Syn Antibodies

Evidence suggests that there are no consensus peptide motifs for cleavage by calpains, and instead, it is the conformational determinants of the substrate, rather than primary sequence motifs, that are responsible for substrate recognition by calpains.²² Therefore, we sought to determine experimentally the cleavage sites within α -Syn after incubation with calpain I. To identify potential calpain cleavage sites within α -Syn, low-molecular weight bands were excised from polyvinylidene difluoride filters, and N-terminal sequencing was performed. After digestion by calpain I, a consistent 9- to 10-kD fragment appeared. When sequenced, the N-terminal sequence of KAKEG was identified. KAKEG corresponds to the beginning amino acid sequence of a fragment of α -Syn generated after cleavage between amino acids 9 and 10. Based on these preliminary results, we chose the peptide KAKEGVVAAGGGGGC for immunization in rabbits using the same strategy to generate similar antibodies developed in our laboratory.²³ KAKEGVVA represents the neoepitope region of the fragment that would be generated after cleavage of α -Syn by calpain between amino acids 9 and 10. In addition, we used a string

of glycine residues to increase the length of the peptide and to enhance the probability of an antigenic response. Affinity purification of antibodies was accomplished using a sulfink column (Pierce) coupled with the peptide used as the immunogen (KAKEGVVAAGGGG). Hereafter, the affinity-purified antibody will be referred to as the α -Syn calpain-cleavage product antibody (CCP Ab). For this antibody, synthesis of peptides, injections of immunogens, and collection of antisera were contracted to Invitrogen Corporation (Carlsbad, CA). In addition, we also synthesized a site-directed antibody to the C terminus after cleavage of α -Syn between amino acids 122 and 123. A peptide was synthesized corresponding to the upstream neopeptide fragment that would be generated after cleavage of α -Syn at this site (CPVDPDN), conjugated to keyhole limpet hemocyanin, and injected into rabbits as described above. After collection of antisera, the antibody was affinity purified. This antibody is referred to as the C-terminal α -SynCCP Ab. For this antibody, synthesis of peptides, injections of immunogens, and collection of antisera were contracted out to Bethyl Laboratories (Montgomery, TX). Figure 1 depicts the regions within α -Syn to which synthetic peptides were synthesized to manufacture these two site-directed antibodies.

Cell Culture

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were grown on 12-well plates to confluency (about 2×10^6 cells per well) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin and incubated at 37°C with 5% CO₂. The calcium ionophore A23187 (Sigma, St. Louis, MO) was prepared as a 4 mmol/L stock solution in dimethylsulfoxide. To activate calpain, SY5Y cells were incubated for various times in serum-free media supplemented with 12 mmol/L CaCl₂ containing 3 μ mol/L calcium ionophore A23187. Calpain I activation has been shown to be enhanced in SY5Y cells when treatment with the calcium ionophore is combined with an elevation of the extracellular calcium concentration.²⁴ To block calpain activation, the calpain inhibitor III (EMD Biosciences, San Diego, CA) was prepared as a 10 mmol/L stock solution in dimethylsulfoxide, diluted in serum-free media, and preincubated with cells at a final concentration of 10 μ mol/L. To permit adequate cellular loading, the calpain inhibitor was added 1 hour before the addition of A23187. After various treatments, SY5Y cell extracts were prepared by adding ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.25% deoxycholate, and 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH 7.4, with protease inhibitor cocktail), followed by centrifugation and the addition of sample buffer.

Preparation of Human Brain Lysates

Case demographics are presented in Table 1. Frozen human temporal cortex tissue from age-matched control or DLB cases were homogenized in a Tris extraction buffer with 1% sodium dodecyl sulfate and protease in-

Figure 1. Peptide sequences used to generate site-directed calpain-cleavage Abs and locations of the epitopes for the full-length anti- α -Syn Abs used in the present study. Sequences between amino acids 10 and 18 or 117 and 122 were used to synthesize peptides and generate polyclonal Abs as described in Materials and Methods. The N-terminal site was chosen based on sequence analysis after digestion of recombinant α -Syn with calpain I, whereas the C-terminal site was chosen based on a previous report demonstrating the cleavage of α -Syn between amino acids 122 and 123.¹⁹ Reported epitopes for the two full-length anti- α -Syn Abs (LB509 and AB5038) used in this study are also shown.

hibitors (MP Biomedicals Inc., Solon, OH). After homogenization, samples were centrifuged for 1 hour at 4°C at 130,000 $\times$ g, and the supernatant was collected (representing the soluble fractions). Protein content was measured using the bicinchoninic acid method (Pierce Biotechnology Inc.), and equal protein amounts were analyzed by Western blot.

Western Blot Analysis and Immunoprecipitation

Human recombinant α -Syn digested with calpain I, SY5Y cell extracts, or human brain lysates was processed for Western blot analysis. Briefly, proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were incubated with α -SynCCP Ab (1:100 to 1:500), and primary antibody was visualized using a goat anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA), followed by enhanced chemiluminescence detection. Blots developed in the absence of secondary antibody were completely negative.

Immunoprecipitation (IP) experiments were performed using recombinant α -Syn after digestion with calpain I. After incubation of α -Syn (20 μ g) in the presence or absence of calpain I for 30 minutes, samples were incubated under nondenaturing conditions [phosphate-buffered saline (PBS) only] or under denaturing conditions (radioimmunoprecipitation assay buffer) together with 8 μ g of the C-terminal α -SynCCP Ab overnight at 4°C. Samples were then incubated for 2 hours at room temperature with immobilized protein G (Pierce Biotechnology Inc.) to pull down immune complexes. Samples were centrifuged and washed three times in ice-cold PBS, and the supernatant was discarded. Pellets were solubilized with 5 $\times$ sample buffer and boiled for 5 minutes before loading on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. After transfer onto nitrocellulose and blocking, blots were incubated overnight with polyclonal FL anti- α -Syn (AB5038; 1:1000). Bands were visualized using enhanced chemiluminescence detection system.

Table 1. Case Demographics for Immunohistochemistry and Western Blot Analyses

Case	Neuropathological diagnosis	Sex	Age (years)	PMI (hours)
1 (IH)	DLB	M	88	2.0
2 (IH)	DLB	M	75	6.0
3 (IH)	DLB	M	67	2.5
4 (IH)	DLB	M	72	5.5
5 (IH)	DLB	F	77	2.8
6 (WB)	DLB/AD	F	77	5.4
7 (WB)	DLB	M	85	4.9
8 (WB)	DLB	M	71	2.6
9 (WB)	DLB	M	70	3.6
10 (WB)	DLB	M	83	9.5
Mean			76.5 ± 6.93	4.48 ± 2.28
1 (IH)	PD/AD	F	88	3.0
2 (IH)	PD	F	86	13.5
3 (IH)	PD/AD	M	64	2.3
4 (IH)	PD	M	69	4.5
5 (IH)	PD	M	71	3.5
Mean			75.6 ± 10.7	5.36 ± 4.62
1 (WB)	CTL	F	73	4.0
2 (WB)	CTL	M	64	7.0
3 (WB)	CTL	M	77	6.5
4 (WB)	CTL	F	74	2.8
Mean			72 ± 5.59	5.08 ± 2.00

DLB cases 1 to 5 were used for IH analysis, whereas cases 6 to 10 and CTL cases were used for Western blot analysis (WB). PMI, postmortem interval; M, male; F, female; AD, Alzheimer's disease; CTL, age-matched control cases.

Cerebral Ischemia Produced by Middle Cerebral Artery Occlusion

C57BL/6 mice (20 to 23 g) were subjected to middle cerebral artery occlusion (MCAO) as described previously.²⁵ In brief, under anesthesia, mice were subjected to MCAO using an intraluminal filament for 1 hour. After 1 hour of MCAO, the filament was removed, and blood flow was restored for 24 hours, at which time animals were sacrificed. Mouse brains were perfused with 10% formalin in PBS and then postfixed in 10% formalin for 4 hours and cryoprotected overnight in phosphate buffer containing 30% sucrose at 4°C. After fixation, brains were sectioned into 50- μ m free-floating sections to be processed by immunohistochemistry. Ischemic infarct areas were identified by Hoechst staining as described previously.²⁵

Human Subjects

Autopsy brain tissue from the substantia nigra of five neuropathologically confirmed PD and 10 DLB cases was studied. Case demographics are presented in Table 1. Age at death was not significantly different between PD (mean 76 ± 10), DLB (mean 77 ± 7), and controls (mean 72 ± 6). Autopsy brain tissues used in this study were generously provided by the Institute for Brain Aging and Dementia Tissue Repositories at the University of California (Irvine, CA).

Immunohistochemistry and Immunofluorescence of Human Postmortem Brain Sections

Free-floating 40- μ m-thick serial sections were used for immunohistochemical and immunofluorescence studies

as previously described.²⁶ Antibody dilutions were as follows: α -SynCCP, 1:100; C-terminal α -SynCCP, 1:100; full-length polyclonal anti- α -Syn AB5038, 1:1000 (Chemicon International, Temecula, CA); mouse full-length mAb anti- α -Syn LB509, 1:200 (Zymed Labs, San Francisco, CA); and anti-calpain-1, C-terminal, human, 1:100 (EMD Biosciences). Antigen visualization was determined using avidin-biotin-peroxidase complex (ABC Elite immunoperoxidase kit; Vector Labs, Burlingame, CA), followed by 3,3'-diaminobenzidine (DAB) substrate with nickel chloride, which generates a black product, or NovaRed, which generates an orange product (Vector Labs). For immunofluorescence studies, antigen visualization was accomplished using an Alexa Fluor 488-labeled tyramide (green, excitation/emission = 495/519) or streptavidin Alexa Fluor 555 (red, excitation/emission = 555/565), both from Invitrogen (Carlsbad, CA).

Quantification

Substantia nigra from five neuropathologically confirmed PD and DLB were examined by bright-field immunohistochemical microscopy after labeling with either α -SynCCP Ab or full-length mAb anti- α -Syn (LB509) as described above. The full-length anti- α -Syn mAb was used to identify Lewy bodies in brain sections because full-length α -Syn is the most sensitive marker known for Lewy bodies.⁸ To ensure that cross-reactivity to reagents was not a factor in double-labeling experiments, experiments were replicated with antibodies in reverse without consequence. The total number of Lewy bodies and neurites with and without α -SynCCP labeling was counted in each of the five cases. Subsequently, the number of Lewy bodies and neurites containing α -SynCCP was counted separately to determine the relative percentage of Lewy

bodies containing calpain-cleaved α -Syn. Raw counts were summed and analyzed using a Pearson rank correlation coefficient (Microsoft Excel, Redmond, WA) to examine a possible relationship between these two variables. Data were plotted and fitted using a nonlinear regression to the second power.

Immunohistochemistry of Transgenic Mouse and *Drosophila* Tissue Sections

PD A30P mice brains were processed for immunohistochemical analysis as previously described.²⁷ In brief, after fixation of brains, 50- μ m coronal free-floating sections were prepared using a vibratome and stored in sodium azide/PBS buffer at 4°C. Age- and strain-matched nontransgenic control mice brains were processed in a similar fashion.

Two additional Tg PD mouse models including one line expressing human wild-type α -Syn under the platelet-derived growth factor (PDGF) promoter²⁸ and one line expressing wild-type human α -Syn under the Thy-1 promoter²⁹ were examined by IH and Western blot analysis using the C-terminal α -SynCCP Ab. In this case, purified antibody was shipped to the laboratory of Dr. Eliezer Masliah (University of California, San Diego, CA), and experiments were independently performed by his transgenic mouse team. Immunoreactivity of the C-terminal α -SynCCP Ab was also assessed in a transgenic *Drosophila* model of PD as previously described.³⁰ In brief, 20-day-old adult flies were fixed in formalin and embedded in paraffin, and sections were processed using the avidin-biotin-peroxidase system and DAB to visualize staining. Sections were counterstained with hematoxylin. Purified C-terminal antibody was shipped to the laboratory of Dr. Mel Feany (Harvard Medical School, Boston, MA) where the staining was performed.

Results

Calpain Cleavage of α -Syn Results in Aggregation and Adoption to a β -Sheet Configuration

To examine a potential role for calpain I in the proteolytic processing of α -Syn, cell-free assays were undertaken using soluble human recombinant α -Syn and purified calpain I. α -Syn was efficiently digested by calpain I, leading to the disappearance of full-length α -Syn (14 kd) and formation of both low- and high-molecular weight bands (Figure 2A). These results confirm a previous report indicating that α -Syn is a suitable substrate for calpain I cleavage.¹⁹ Because truncation of α -Syn has been shown to enhance its self-aggregation and lead to the adoption of a β -sheet structure,^{12,13,31} we examined whether similar changes occur to calpain-cleaved α -Syn by using AUC and CD analyses. As shown in Figure 2B, digestion of α -Syn by calpain I led to the formation of high-molecular weight species as determined by AUC.

Figure 2. Calpain cleavage of α -synuclein results in aggregation and adoption of a β -sheet configuration. **A:** After incubation of α -Syn with calpain I for various periods of time in a cell-free assay, samples were subjected to gel electrophoresis, transferred to nitrocellulose filters, and probed with an antibody to FL α -Syn (AB5038). Over time, the appearance of low- and high-molecular weight bands were evident in addition to the disappearance of FL α -Syn (14 kd). **B:** Analytical ultracentrifugation analysis after incubation of α -Syn alone (line on the left) or after digestion with calpain I (line on the right) for 30 minutes indicated the presence of high-molecular weight species. **Insets** represent the 30 raw data scans used for data analysis. **C:** CD spectra after incubation of α -Syn with calpain I indicated a change in structure from random coil to a β -sheet configuration. CD spectra of α -Syn alone (top line) and mixture of α -Syn incubated with calpain I with the calpain spectrum subtracted (bottom line).

Sedimentation analysis revealed that $96 \pm 1.8\%$ of the molecular species present after digestion of α -Syn by calpain I exhibited a molecular mass of 40 kd. In addition, CD analysis indicated that calpain-cleaved α -Syn resulted in a change in secondary structure that was consistent with a β -sheet conformation (Figure 2C). CD results obtained were similar to those published in a

Figure 3. Calpain cleavage of α -Syn results in aggregation and formation of high-molecular weight species. **A:** After incubation of α -Syn with calpain I for various periods of time in a cell-free assay, samples were subjected to gel electrophoresis, transferred to polyvinylidene difluoride filters, and stained with Coomassie blue. Over time, the appearance of low- and high-molecular weight bands were evident in addition to the disappearance of full-length α -Syn (14 kD). **B:** Western blot analysis reveals the presence of high-molecular weight bands after digestion of α -Syn with calpain I in a cell-free system. α -Syn was incubated with calpain I for the indicated times, and samples representing the soluble fractions were immunoblotted using the α -SynCCP Ab. The arrowhead indicates the immunoreactivity of the α -SynCCP Ab with a 10-kD fragment of α -Syn. **C:** Western blot analysis using the α -SynCCP Ab demonstrated the presence of calpain-cleaved aggregates of α -Syn after treatment of SY5Y neuroblastoma cells with the calcium ionophore A23187 (**C**, lanes marked 2 and 4 hours), which were prevented after pretreatment of cells with a calpain I inhibitor (**D**, far right lane). **E:** Detection of numerous high-molecular weight calpain-cleavage aggregates of α -Syn in the DLB brain by Western blot analysis. Human brain lysates from age-matched controls or DLB cases were probed with the α -SynCCP antibody. Arrowheads above 10 kD indicate the presence of bands between 30 and 80 kD.

previous study examining the secondary structure changes after expression of truncated α -Syn species in *Escherichia coli*.³¹

Calpain Cleavage of α -Syn Leads to the Formation of High-Molecular Weight Species

To determine whether calpain cleaves α -Syn in the PD or DLB brain, we designed a site-directed calpain-cleavage antibody that specifically detects cleaved but not full-length (FL) α -Syn. To accomplish this, N-terminal sequencing was performed on the 10-kD band that formed after digestion of α -Syn by calpain I (arrowhead in Figure 3A, lane marked 5 minutes). This sequence was used to synthesize a corresponding peptide that could be used

to generate polyclonal antibodies (for details, see Materials and Methods). After affinity purification of this antibody, hereafter termed the α -Syn CCP Ab, specificity was determined by Western blot analysis. The α -SynCCP Ab recognized the predicted 10-kD fragment of α -Syn but did not detect FL α -Syn (14 kD) (Figure 3B). In addition to recognizing the predicted 10-kD calpain-cleaved fragment of α -Syn, the α -SynCCP Ab immunolabeled a prominent band at ~40 kD (Figure 3B), which corresponds to the exact size of the species calculated after AUC analysis (Figure 2B). The α -SynCCP Ab identified high molecular calpain-cleaved aggregates of α -Syn with no immunoreactivity to FL α -Syn in an *in vitro* model system (Figure 3, C and D) as well as in DLB brain extracts (Figure 3E). The α -SynCCP Ab also detected the 10-kD cleavage fragment of α -Syn in all DLB subjects examined (Figure 3E).

Detection of Calpain-Cleaved α -Syn in Vivo

To confirm further the specificity of the α -SynCCP Ab and to demonstrate that calpain cleavage of α -Syn does not represent an entirely artificial phenomenon, experiments were undertaken using an animal model of ischemia/reperfusion injury. C57BL/6 mice were subjected to MCAO as described previously.²⁵ This model has been previously used to demonstrate calpain activation and cleavage of cellular proteins²⁵ and has the advantage that the ischemic infarct is confined to one side of the brain, leaving the other side intact and damage-free, which allows for an internal control. Because the protein sequence of α -Syn in the region used to generate the α -SynCCP Ab is conserved between murine and human sequences (Basic Local Alignment Search Tool alignment; data not shown), we anticipated the α -SynCCP Ab would recognize murine calpain-cleaved α -Syn. We performed IH/IF on brain sections from MCAO mice using the α -SynCCP Ab and detected calpain-cleaved α -Syn only in the ischemic infarct areas (Figure 4, B and D). Labeled neurons appeared shrunken and damaged. No staining was observed on the contralateral side of the brain (Figure 4, A and C) nor in controls or shams (data not shown). These data suggest that calpain cleavage of α -Syn occurs *in vivo* under conditions known to activate calpain I and can be specifically detected using our α -SynCCP Ab.

To test whether the α -SynCCP Ab could detect calpain-cleaved α -Syn in an animal model of PD, application of the α -SynCCP Ab was performed on fixed sections using Tg mice overexpressing mutant α -Syn. These mice overexpress human α -Syn harboring the A30P mutation (line Tg5093) under the control of the prion protein promoter.²⁷ Heterozygous mice develop severe motor dysfunction characterized by rigidity, dystonia, gait impairment, and tremor after high expression of the transgene in numerous areas of the brain.²⁷ Figure 5 depicts results after application of the α -SynCCP Ab in age-matched non-Tg controls or from 4- and 12-month-old heterozygous PD mice. Interestingly, we detected strong punctate staining localized in clusters within the hippocampus of

Figure 4. Detection of calpain-cleaved α -Syn in an animal model of cerebral ischemia. Mice were subjected to 1 hour of MCAO, followed by reperfusion for 24 hours. Sections at 50 μ m were subjected to either IF or IH analysis using the α -SynCCP Ab (1:100) to detect calpain-cleaved α -Syn. Staining of shrunken, damaged neurons in the cortex of MCAO mice was evident only in ischemic infarct areas (**B**, fluorescence, and **D**, bright-field DAB labeling) and was not evident on the contralateral side of the brain (**A** and **C**). Scale bars = 10 μ m.

aged non-Tg mice sections (Figure 5A). This staining most likely represents so called “Wirak bodies” that are typical age-associated inclusions associated with C57BL/6 mice.³² In the cortex, however, tissue sections from non-Tg age-matched control mice were completely devoid of any labeling (Figure 5B). In 4-month-old asymptomatic Tg PD mice, little staining was observed in the cortex (Figure 5C) or any other brain regions including the hippocampus (data not shown). In contrast, strong neuronal staining in the cortex was seen in 12-month-old Tg PD mice (Figure 5D). Staining of neurons

was also observed in a subset of neurons in the hippocampus (data not shown).

Calpain Cleavage of α -Syn Is Evident in PD and DLB Brains

To determine *in situ* whether calpain cleaves α -Syn in PD or DLB, immunohistochemical (IH) and immunofluorescence (IF) experiments were undertaken using the α -SynCCP Ab. Calpain-cleaved α -Syn was evident within Lewy

Figure 5. Detection of calpain-cleaved α -Syn in a transgenic mouse model of PD. Fixed-tissue brain sections from age-matched non-Tg controls in the hippocampus (A) or cortex (B) immunolabeled with the α -SynCCP Ab (1:100). C and D: Cortical staining from 4-month-old asymptomatic PD Tg mice (C) or 12-month-old PD Tg mice (D) was immunolabeled with the α -SynCCP Ab (1:100). Scale bars = 10 μ m.

bodies and Lewy neurites in the substantia nigra of both DLB and PD brains (Figure 6). No neuritic labeling was evident, nor did we observe labeling of axonal spheroids after application of the α -SynCCP Ab. Two consistent features were apparent after labeling with the α -SynCCP Ab. First, calpain-cleaved α -Syn seemed to localize primarily within the core of Lewy bodies (Figure 6, B and I–N). Second, we often observed staining that appeared granular and sheet-like (Figure 6, C and E), suggesting that calpain-cleaved α -Syn is in an aggregated configuration. Importantly, staining with the α -SynCCP Ab was completely prevented after preadsorption with free peptide (data not shown).

Quantification was performed to determine a possible relationship between calpain-cleaved α -Syn and the number of Lewy body structures in DLB and PD. Both LBs and neurites with and without calpain-cleaved α -Syn products were quantified. There was a positive relationship between these two variables; as the number of LB structures increased, so did the appearance of calpain-cleaved α -Syn (Figure 6, G and H). In this regard, we found the presence of calpain-cleaved α -Syn in $70 \pm 8.9\%$ of the total number of LBs and neurites identified in DLB (799 of 1131). In PD, $90 \pm 3.4\%$ of the total number

of LBs and neurites identified had the presence of calpain-cleaved α -Syn (542 of 603). It is noteworthy that we also detected calpain-cleaved α -Syn in nonhyaline LBs in the cortex of DLB subjects (Supplemental Figure 1, see <http://ajp.amjpathol.org>). In addition, widespread staining within degenerating astrocytes was also observed in cortical white matter of DLB subjects (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

We next examined whether calpain-cleaved α -Syn colocalized with the enzyme calpain I in PD and DLB brains. As shown in Figure 6, O–T, we detected activated calpain I surrounding Lewy bodies containing calpain-cleaved α -Syn.

Evidence for C-Terminal Truncation of α -Syn in the DLB Brain

Because numerous studies have documented the presence of C-terminal truncated species of α -Syn in PD and DLB,^{10–12,33} we examined whether calpain may mediate this proteolytic role. *In vitro*, our results suggested that a major fragment generated after cleavage of α -Syn by calpain I was approximately 10 kd in size (Figures 1 and

2). FL α -Syn has a molecular mass of 14 kd; this suggests an additional cleavage event near the C terminus of α -Syn. Based on this and a previous study,¹⁹ we hypothesized that calpain I also cleaves α -Syn between amino acids 122 and 123. We synthesized a peptide corresponding to the upstream neopeptide fragment that would be generated after cleavage of α -Syn at this site (PVDPDN) and generated C-terminal α -SynCCP Abs (see Materials and Methods for details). Figure 7 depicts the results after application of this antibody showing that it immunolabels the predicted 10-kd fragment of α -Syn (Figure 7A). This supports the conclusion that calpain I is able to cleave α -Syn both at the N- and C-terminal ends of α -Syn and that these cleavage events happen simultaneously. Unlike the N-terminal α -SynCCP Ab, the C-terminal α -SynCCP Ab seemed to immunolabel FL α -Syn under denaturing conditions (Figure 7A). However, it should be noted that ample levels of FL α -Syn were still present in all lanes even after digestion with calpain I (Figure 3A), yet there was no detection of FL α -Syn under these conditions. Application of this antibody by bright-field microscopy or by immunofluorescence of represented DLB cases indicated a similar staining pattern as with the N-terminal α -SynCCP antibody; namely, a staining pattern that was found within Lewy structures and was fibrillary in its appearance (Figure 7, B and C). Double-labeling experiments confirmed the colocalization of C-terminal α -SynCCP labeling with a marker for LBs (Figure 7, D–K). Figure 7D (left) was overexposed purposely to better see the separation of the two colors. Similar results were obtained in PD cases (data not shown).

To evaluate further the specificity of the C-terminal α -SynCCP Ab, IH and Western blot experiments were performed using several Tg mouse models of PD. IH experiments provided evidence for calpain-cleaved α -Syn in two unique Tg mouse models of PD, including one line expressing human wild-type α -Syn under the PDGF promoter²⁸ and one line expressing human wild-type α -Syn under the Thy-1 promoter.²⁹ In this case, labeling within neurons was evident in the neocortex for PDGF α -Syn Tg mice and Thy-1 α -Syn Tg mice, respectively (Figure 8, B and C), and staining was comparable

Figure 6. Detection of calpain-cleaved species of α -Syn in PD and DLB. **A** and **B**: Representative immunohistochemical single-label staining in the substantia nigra of a DLB (**A**) and PD (**B**) case after application of the α -SynCCP Ab demonstrates the presence of calpain-cleaved α -Syn within Lewy bodies. **C** and **D**: Double-label immunohistochemical analysis with α -SynCCP Ab (black) together with a marker for Lewy bodies (LB509, orange) reveals colocalization in Lewy bodies from a representative DLB (**C**) or PD (**D**) case. Colocalization was also observed in Lewy neurites in DLB (**E**, arrows) or PD (**F**) cases. **G** and **H**: Graphical representations depicting the relationship between calpain-cleaved α -Syn and the number of LBs/neurites in five different DLB (left) or PD (right) cases. Data indicate a high degree of association between the total number of LBs/neurites and the presence of calpain-cleaved α -Syn. **I–N**: Double-label immunofluorescence images for α -SynCCP (green) and an antibody to full-length α -Syn (red) confirmed the colocalization of calpain-cleaved α -Syn within Lewy bodies in a representative DLB (**I–K**) or PD (**L–N**) case. Yellow/orange coloring in **K** and **N** indicate areas where markers are overlapping. **O–T**: Double-label immunofluorescence images for α -SynCCP (green) and calpain I (red) demonstrating the presence of active fragments of the enzyme surrounding Lewy bodies containing calpain-cleaved α -Syn. **O–Q** and **R–T**: Representative staining of a DLB or PD case, respectively. Yellow/orange coloring in **Q** and **T** indicate areas where markers are overlapping. Asterisks in **N** denote regions containing neuromelanin. All scale bars = 10 μ m.

Figure 7. Evidence for C-terminal truncation of α -Syn in the DLB brain. **A** and **B**: Characterization of the C-terminal α -SynCCP Ab by Western blot analysis. Recombinant human α -Syn (20 μ g) was incubated for various periods of time with calpain I (2 U), separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, and probed with an antibody directed toward a predicted C-terminal calpain-cleavage site (PVDPDN) of α -Syn (**A**). The antibody immunolabeled the predicted 10-kd fragment of α -Syn. **B**: Representative immunohistochemical single-label staining of LBs in the substantia nigra of two DLB cases after application of the C-terminal α -SynCCP Ab. **C**: Identical to **B**, showing labeling with a single Lewy neurite. **D** and **E**: Double-label immunohistochemical analysis in a representative DLB case showing localization of the C-terminal α -SynCCP Ab within LBs and neurites (nickel DAB), which colocalized with a marker for LBs (Nova Red). **F–K**: Immunofluorescence with the C-terminal α -SynCCP Ab shown in green (**G** and **J**), FL α -Syn Ab (LB509) in red (**F** and **I**), and the overlap image (**H** and **K**). All scale bars = 10 μ m.

with a FL α -Syn Ab (Figure 8, E and F). No staining was observed in non-Tg age-matched control mice after application of the C-terminal α -SynCCP Ab (Figure 8A). A similar staining profile was observed in the hippocampus (data not shown). We were also able to document immunoreactivity of the C-terminal Ab in a *Drosophila* model of PD³⁰ (Figure 8H). As depicted in Figure 8H, staining was apparent in both the cellular cortex (arrows) and inner neuropil areas (asterisks). In flies, the cell bodies of neurons and glia are concentrated in the peripheral cortex (arrows), whereas the inner neuropil (asterisks) contains the processes of neurons and glial cells. The increased staining apparent in some cortical and neuropil areas represents the normal pattern of the *elav-GAL4* driver.³⁰ Neuropil staining was somewhat more punctate than typically seen with an antibody that recognizes full-length

α -Syn (not shown), consistent with preferential inclusion of C-terminally truncated α -Syn in inclusions.

Western blot analysis was performed using brain extracts from Tg mice or representative DLB subjects (Figure 9, A and B). Immunoreactivity of the C-terminal α -SynCCP Ab was confined primarily to the soluble fraction (Figure 9A). In contrast to LB509, the C-terminal α -SynCCP Ab detected a C-terminal fragment running just under FL α -Syn in both Tg mouse models of PD and in human DLB extracts (Figure 9A, lanes 3–6). In addition, the C-terminal α -SynCCP Ab detected a prominent band running at approximately 40 kd, which is similar to the results observed after application of the N-terminal α -SynCCP Ab. No staining was seen in non-Tg control mice either by IH or by Western blot analysis (Figures 8A and 9). This probably reflects the fact that the peptide used as an immunogen to generate the C-terminal α -SynCCP Ab is not conserved in mouse.

In a final set of experiments, IP studies were performed to determine whether the C-terminal α -SynCCP Ab is preferentially a conformational-specific Ab. To accomplish this, cell-free assays using recombinant α -Syn were performed in the presence or absence of calpain I. Samples were then incubated with the C-terminal α -SynCCP Ab overnight at 4°C either under nondenaturing conditions (PBS only) or under denaturing conditions (radioimmunoprecipitation assay buffer). After the addition of protein G to pull down immune complexes, samples were analyzed by Western blot analysis using FL α -Syn AB5038. Figure 9C depicts the results of such an experiment and shows that the C-terminal α -SynCCP Ab did not immunoprecipitate FL α -Syn under nondenaturing conditions but did pull down several calpain-cleaved fragments. In contrast, if IP experiments were performed under denaturing conditions now, the C-terminal α -SynCCP Ab preferentially recognized FL α -Syn but not calpain-cleaved fragments (Figure 9C). These results suggest that the C-terminal α -SynCCP Ab may recognize a conformational epitope of calpain-cleaved α -Syn under native conditions. However, after denaturation of α -Syn to a linear peptide, specificity to calpain-cleaved fragments of α -Syn is lost, and now the antibody seems to recognize both FL α -Syn and C-terminal fragments.

Discussion

The present report demonstrates that calpain can cleave α -Syn *in vitro*, leading to its aggregation and adoption of a β -sheet conformation. The cleavage of α -Syn at either the N- or C-terminal end of α -Syn could be detected in the PD brain and in the DLB brain using two site-directed calpain-cleavage antibodies. These data suggest that calpain may link α -Syn processing to its disease-linked aggregation and formation of LBs in various α -synucleinopathies. Although numerous studies have demonstrated the presence of truncated species of α -Syn in PD and DLB,^{10–12,33} the identity of the protease responsible for this proteolytic processing is unknown. A likely candidate for this processing role of α -Syn is the ubiquitin proteasome, and several studies have supported a role for the proteasome in the metabolism of

Figure 8. Comparison of C-terminal calpain-cleaved α -Syn immunoreactivity from several different animal models of PD. **A–F:** Panels are from vibratome sections from the brains of mice immunostained with either the C-terminal α -SynCCP Ab (**A–C**) or a commercially available FL α -Syn Ab from Chemicon AB5038 (**D–F**). After application of the C-terminal α -SynCCP Ab, immunoreactivity within neurons of the neocortex was observed in two different transgenic mice expressing wild-type α -Syn under a PDGF promoter (**B**) or in a line expressing α -Syn under Thy-1 promoter (**C**). **E** and **F:** A similar staining profile was observed using the FL α -Syn Ab. **A** and **D:** No staining with either antibody was observed in non-Tg control mice. **G** and **H:** Detection of the C-terminal calpain-cleavage fragment of α -Syn in a *Drosophila* model of PD. Paraffin-embedded frontal brain sections at the level of the mushroom bodies from 20-day-old flies were immunostained with the C-terminal α -SynCCP Ab (DAB staining) and counterstained with hematoxylin. **G:** The control genotype (*elav-GAL/+*). **H:** α -Syn expressed in a pan-neural pattern under the control of the *elav-GAL* driver. **Arrows**, the peripheral cortex; **asterisks**, the inner neuropil.

α -Syn.^{11,34} However, this is still subject to debate because other studies have failed to show any relationship between α -Syn processing and the proteasome.^{35–37} In addition, numerous studies have demonstrated a diminished activity of the proteasome in PD.^{3,38–41} Based on this observation, it is difficult to reconcile how a compromised proteasome could lead to enhanced proteolytic processing of the α -Syn.

Another potential protease that may be involved in proteolytic processing of α -Syn is caspase-3. Several studies have demonstrated a role for caspase activation and the cleavage of cellular proteins in PD.^{41–43} How-

ever, examination of the protein sequence of α -Syn did not reveal any consensus caspase sequences, and furthermore, we were unable to digest α -Syn in the presence of activated caspase-3 (data not shown). This together with the lack of any studies demonstrating α -Syn is a substrate for caspase-mediated cleavage suggests that this to be an unlikely role for this family of proteases.

In the present study, we demonstrate a role for the ubiquitous family of proteases known as calpains as potential proteolytic mediators of α -Syn. Calpain activation has been demonstrated to occur in the PD brain⁴⁴ and in

Figure 9. Detection of the C-terminal calpain-cleavage fragment of α -Syn by Western blot analysis. Cytosolic and membrane fractions were prepared from nonTg mice (lanes 1 and 2), Tg mice overexpressing wild-type or mutant A53T α -Syn under the PDGF promoter (lanes 3 and 4), or from two representative DLB cases (lanes 5 and 6). Blots were probed either with the C-terminal α -SynCCP Ab (A) or with mAb LB509 (B). The C-terminal fragment of α -Syn was detected by α -SynCCP Ab in soluble fractions but not by LB509 (band running just under full-length, 14 kD). C: IP experiments with the C-terminal α -SynCCP Ab incubated with control α -Syn (lane 1) or digested with calpain I (lane 2) under nondenaturing or denaturing conditions. Data suggest the C-terminal α -SynCCP Ab is a conformational-specific Ab whose epitope is lost after denaturation of α -Syn.

animal models of PD.^{45,46} Moreover, inhibition of calpains prevents behavioral deficits in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD.⁴⁷ Previous studies have demonstrated that α -Syn is a suitable substrate for calpain.^{19,20} Our studies provide evidence that calpain fits the criteria as the protease linking α -Syn to its pathological form: 1) α -Syn is rapidly and simultaneously cleaved at the N- and C-terminal regions by calpain; 2) cleavage of α -Syn by calpain leads to the generation of high-molecular weight species and converts α -Syn from a random coil to a β -sheet structure, a key step that enhances the ability of α -Syn to aggregate⁷; and 3) calpain-cleaved species of α -Syn were present in the preponderance of LBs quantified in the human PD and DLB brain and colocalized with active calpain in neurons. In this manner, we detected activated calpain I surrounding Lewy bodies containing calpain-cleaved α -Syn. These data suggest that after cleavage of α -Syn by calpain I, α -Syn aggregates may be deposited within Lewy body structures. Taken together our findings suggest that calpain may be the molecular switch turning on the toxic gain of function of α -Syn.

Our data demonstrating the cleavage of α -Syn by calpain in a cell-free system are in complete agreement with Li et al,¹² who also reported an increase in aggregated α -Syn after C-terminal cleavage. However, our results are in contrast to those reported by Mishizen-Eberz et al,²⁰ who showed that rather than promoting aggregation, calpain cleavage of soluble α -Syn prevents fibrillization. In this regard, Mishizen-Eberz et al²⁰ demonstrated that calpain cleavage near the middle of soluble α -Syn can generate fragments of α -Syn that are unable to self-fibrillize and that also prevent FL α -Syn from fibrillizing. At the present time, we are unsure of the reason for the discrepancy between our results and the study of Mishizen-Eberz et al. One possibility is that data supporting

their conclusion consisted of a Western blot using a monoclonal antibody (Syn 303), which recognizes epitopes at amino acids 2 to 4 of α -Syn. Our results suggest that calpain cleaves α -Syn between amino acid 9 and 10, and therefore, this antibody would be unable to detect aggregates of α -Syn because the epitope for the antibody would have been lost. Another possibility is the difference in starting material (soluble versus fibrillized α -Syn) between the studies. Collectively, these studies and our present results suggest that the action of calpain on α -Syn may be more nuanced than originally thought depending on the initial state of α -Syn (soluble versus fibrillized). Therefore, before calpain I can become a target for the treatment of α -synucleinopathies, further studies are warranted to clarify these different patterns of calpain cleavage of α -Syn.

Demonstration of the calpain-cleavage of α -Syn was confirmed using two different site-directed Abs. Extensive characterization of these antibodies was performed using cell-free assays and *in vitro* as well as *in vivo* models of calpain activation. Although both antibodies labeled LBs and Lewy neurites in PD/DLB brains, there were distinct differences between the two Abs. The N-terminal antibody seemed to be highly selective for the calpain-cleaved fragment of α -Syn by IH and by Western blot analysis. In contrast, the C-terminal Ab seemed to react with FL α -Syn as well as with C-terminal fragments. Thus, we cannot say with certainty that the staining observed with the C-terminal Ab in IH/IF experiments was only attributable to immunoreactivity to calpain-cleaved fragments of α -Syn. However, several pieces of data suggest the C-terminal α -SynCCP Ab is a conformational epitope-specific antibody, and therefore, the staining we observed might reflect a preferential immunoreactivity to the calpain-cleaved fragments of α -Syn. First, we compared the immunoreactivity of the C-terminal Ab with LB509 in DLB tissue sections. Coincidentally, the epitope for LB509 is identical to that of the C-terminal Ab with the exception of two additional terminal amino acids (Figure 1). Because the LB509 Ab is specific for FL α -Syn, it would be expected that double-label experiments using these two Abs would indicate a high degree of overlap after IH or IF analysis. However, this was not the case: although there were regions of overlap where colocalization was evident, there was a clear spatial resolution between the two Abs in labeled structures. For example, in LBs, C-terminal α -SynCCP Ab immunoreactivity was predominantly within the core of the LB, whereas that of LB509 was throughout the entire LB. A spatial separation between these two Abs was also observed in Lewy neurites. Second, in single-labeled experiments using the C-terminal α -SynCCP Ab, we often observed staining that was rough and aggregated in its appearance (Figure 7B, right panel). This was never the case for LB509, which always gave a homogenous staining pattern. This suggests that the two Abs are recognizing distinct epitopes within α -Syn. Third, only the C-terminal α -SynCCP Ab and not LB509 detected a C-terminal 12-kD fragment of α -Syn by Western blot analysis in brain tissue extracts from Tg PD mice or DLB subjects. Finally, IP experiments using the C-terminal α -SynCCP Ab demonstrated it to be a

conformational epitope-specific Ab. These results suggest that *in situ*, the C-terminal α -SynCCP Ab may preferentially recognize calpain-cleaved α -Syn and, taken together with the data using the N-terminal α -SynCCP Ab, support a role for calpain cleavage of α -Syn in DLB and PD.

Although this report focuses on proteolytic processing of α -Syn as a mechanism leading to the aggregation of α -Syn, various other factors can transform natively folded α -Syn into β -folded structures, which have the propensity to aggregate (see review⁷). Of particular importance in this regard is the phosphorylation of α -Syn, which has been shown to be a critical step leading to fibrillization. Selective phosphorylation of α -Syn at Ser129 has been demonstrated in DLB and PD brains and enhances the aggregation of FL α -Syn *in vitro*.⁴⁸ Moreover, mutation of Ser129 to alanine blocks subsequent phosphorylation and prevents dopaminergic neuronal cell loss in a *Drosophila* model of PD.⁴⁹ These same authors, however, found that blocking phosphorylation of Ser129 enhances the aggregation of α -Syn. The authors concluded, therefore, that aggregation of α -Syn may actually protect cells from neurotoxicity because phosphorylation of α -Syn "favors the maintenance of α -Syn in a soluble, toxic form."⁴⁹ In the present study, we have demonstrated the calpain-cleavage of α -Syn leading to its aggregation. It is interesting to speculate whether such modification prevents the phosphorylation of α -Syn at Ser129 and thus serves a protective role. Further studies examining the relationship between calpain cleavage of α -Syn and its phosphorylation are warranted to address this question.

In summary, we have demonstrated that cleavage of α -Syn by calpain I leads to the formation of calpain-cleaved aggregates of α -Syn in DLB and PD. Our findings suggest that calpain I may participate in the disease-linked aggregation of α -Syn, and these data provide a general mechanism for the initiation and the evolution of Lewy body formation in various α -synucleinopathies.

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