Biochemical Characterization of the Core Structure of α-Synuclein Filaments*

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Hiromoto Mikaesh, Hidehiro Mizusawa§, Takeshi Iwatsubo¶, and Masato Hasegawa‡§

From the ‡Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, the $Department of Neurology and Neurological Science, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, and the ¶Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan

Intracellular filamentous aggregates comprised of α-synuclein such as Lewy bodies and glial cytoplasmic inclusions are the defining hallmarks of a subset of neurodegenerative diseases including Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy. We have analyzed biochemical and structural properties of α-synuclein filaments assessed in vitro or extracted from brains of patients with multiple system atrophy and found that both types of filaments are insoluble to detergents and partially resistant to proteinase K digestion. Immunoelectron microscopy and immunoblot analysis showed that both amino and carboxyl termini of α-synuclein in in vitro assembled filaments were degraded by proteinase K treatment, whereas the central portion of α-synuclein is resistant to proteinase K and retains filamentous structures. Protein sequencing and mass spectrometric analyses of the proteinase K-resistant, minimal fragment of 7 kDa revealed that amino acid residues 31–109 of α-synuclein constitute the core unit of the filaments. These observations suggest that the central half of the α-synuclein polypeptide, containing five tandem repeats as well as a part of the carboxyl-terminal acidic region, forms the core structure of α-synuclein filaments, which is coated by the amino- and carboxyl-terminal portions at the periphery.

Filamentous cytoplasmic inclusion bodies comprised of α-synuclein in neurons or glial cells are the hallmark lesions of a group of neurodegenerative diseases collectively referred to as synucleinopathies (1). In Parkinson’s disease (PD)1 and dementia with Lewy bodies (DBL), α-synuclein is deposited as Lewy bodies and Lewy neurites that accumulate in cell bodies or neuronal processes (2–4), whereas filamentous α-synuclein aggregates are predominantly found in oligodendrocytes as glial cytoplasmic inclusions (GCIs) in multiple system atrophy (MSA) (5–7). The following evidence strongly implicates the deposition of α-synuclein in the pathogenesis of these neurodegenerative disorders. 1) Two missense mutations (A53T and A30P) in the α-synuclein gene that cosegregate with the onset of PD have been identified in kindreds of autosomal dominantly inherited familial PD (8, 9). 2) Immunohistochemical and biochemical analysis of PD, DBL, and MSA brains have revealed widespread deposition of α-synuclein in the brains of patients with either sporadic or familial forms of PD, as well as in DLB and MSA (10, 11), in which α-synuclein has been shown to form the major filamentous component of inclusion bodies (6, 10, 12). 3) Recombinant α-synuclein proteins assemble into filaments in vitro that closely resemble those found in LB and GCIs, whereas other members of synuclein family proteins, i.e. β-synuclein and γ-synuclein, neither deposit in brains nor assemble into filaments (13–15). 4) Missense mutations (A53T and A30P) identified in familial PD have been shown to increase the propensity of α-synuclein to form filaments or oligomers (16–20).

α-Synuclein is a 140-amino acid, heat-stable protein, harboring seven imperfect tandem repeat sequences in the amino-terminal half (Fig. 1A), followed by a hydrophobic central region (referred to as the NAC portion) and an acidic carboxyl terminus. α-Synuclein is abundantly expressed in neurons as a cytosolic protein that is localized to presynaptic terminal, although it has been shown that a proportion of α-synuclein is associated with membranes (21, 22). Circular dichroism spectroscopy analysis of recombinant proteins revealed that α-synuclein is a natively unfolded protein with little ordered secondary structure (23). Further structural analyses have shown that full-length or carboxyl-terminal-truncated recombinant α-synuclein can assemble into straight filaments 5–10 nm wide that closely resemble filaments isolated from PD, DBL, or MSA brains (13–15). X-ray fiber diffraction and electron diffraction analyses have shown that a transition from random coil to a cross-β-sheet structure underlies the assembly of α-synuclein into filaments (15). Recent studies have shown that residues 71–82 of α-synuclein, which are absent in β-synuclein, play a crucial role in its assembly into filaments (24). However, γ-synuclein also harbors a hydrophobic stretch similar to that of α-synuclein (and especially a homologous portion to residues 71–82 of α-synuclein) although γ-synuclein poorly assembles into filaments (14, 15).

To learn more about the mechanisms underlying the assembly of the natively unfolded α-synuclein protein into β-sheet-rich filaments, we studied the biochemical properties of α-synuclein filaments, especially their structural stability to protease digestion. Here we have shown that α-synuclein filaments assembled in vitro or recovered from MSA brains that...
are morphologically similar to each other share the following biochemical characteristics: (i) insolubility in detergents ( Triton X or Sarkosyl) but high solubility in urea or SDS, and (ii) resistance of a subdomain of α-synuclein against proteinase K treatment. We propose that the proteinase K-resistant 7-kDa fragment comprised of residues 31–109 of α-synuclein may represent the core unit of α-synuclein filaments, which contributes to the structural stability of these filaments.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal antibody LB509 was raised against isolated Lewy bodies, and the epitope was localized to residues 121/122 of α-synuclein (25). Syn102 was raised against recombinant α-synuclein with an epitope within residues 131–140 (7, 26). No. 36 is an anti-serum against a synthetic peptide corresponding to residues 1–10 of α-synuclein. NAC1 raised against a synthetic peptide corresponding to residues 75–91 of α-synuclein is a gift from Dr. Jakalas (27).

**Extraction of Dispersed α-Synuclein Filaments from MSA Brains—** α-Synuclein filaments derived from glial cytoplasmic inclusions were extracted from frozen cerebella from patients with MSA or from normal individuals as controls. 0.5–1 g of brain tissue was homogenized in 10 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM dithiothreitol) and centrifuged at 1,000 g for 10 min. The supernatants were ultracentrifuged at 350,000 × g for 20 min, and the resulting pellets were sequentially extracted by homogenization followed by ultracentrifugation in buffer A containing 1% Triton X-100 and then in 8 M urea containing 2% SDS and analyzed by immunoblotting with LB509, which immunoreactive polypeptides migrating at similar positions to those in other synucleinopathies were observed in the same fractions of control brains. Immunoelectron microscopic analysis of the Sarkosyl-insoluble fraction from MSA brains showed filaments that were labeled by multiple anti-α-synuclein antibodies. The extraction patterns of α-synuclein in these fractions were slightly smaller in MSA brains compared with control brains. In contrast, LB509 immunoreactive polypeptides migrating at similar positions to normal α-synuclein were detected in the Sarkosyl-insoluble, urea-soluble fraction of MSA brain (Fig. 1D), whereas no α-synuclein immunoreactivities were observed in the same fractions of control brains. Immunoelectron microscopic observation of the Sarkosyl-insoluble fraction from MSA brains showed filaments that were labeled by multiple anti-α-synuclein antibodies. The extraction patterns of α-synuclein aggregates in MSA brains were similar to that observed by Dickson et al. (11) as well as to those in other synucleinopathies including DLB (30) and Hallervorden-Spatz disease, suggesting that filamentous α-synuclein aggregates deposited in synucleinopathy brains exhibit similar insolubility profiles (i.e. Sarkosyl-insoluble and urea-soluble). The amounts of Sarkosyl-insoluble α-synuclein appeared to correlate with the density of GCIs as judged by semiquantitative evaluation of the amount of α-synuclein-positive aggregates by immunostaining of the smears of the brain homogenates (data not shown).

Similarly, in vitro assembled α-synuclein filaments were sequentially extracted by Triton-HCl, 1% Triton X-100, 1% Sarkosyl, and 8 M urea, together with unassembled α-synuclein incubated without shaking and analyzed by immunoblotting. As shown in Fig. 1E, unassembled α-synuclein was totally recov...
ered in Tris-HCl and Triton X-soluble fractions, without any immunoreactive substances detected in Sarkosyl-insoluble fractions. In sharp contrast, a 15-kDa protein as well as additional polypeptides migrating at ~25–30 kDa in a Sarkosyl-insoluble, urea-soluble fraction of in vitro assembled α-synuclein showed strong immunoreactivity for LB509, the latter presumably representing α-synuclein dimers. Electron microscopic observation of the Sarkosyl-insoluble fraction of in vitro assembled α-synuclein confirmed the preservation of filamentous structures after extraction with 1% Triton X and 1% Sarkosyl. Taken together, synthetic α-synuclein filaments share a number of biochemical as well as morphological characteristics (i.e. solubility profiles, molecular size, and ultrastructure) with those recovered from inclusion bodies in synucleinopathy brains.

We next examined the structural stability of α-synuclein filaments by treating them with proteinase K. Tris-soluble fractions from control brains containing abundant normal α-synuclein and Sarkosyl-insoluble fractions from MSA brains rich in insoluble α-synuclein filaments were treated with 1% proteinase K.
μg/ml proteinase K, and digestion of α-synuclein was monitored by immunoblotting with LB509. Immunoreactivities for α-synuclein in Tris-soluble fractions disappeared after proteinase K treatment for 30 min. In contrast, the amount as well as banding patterns of α-synuclein in the Sarkosyl-insoluble fraction remained almost unchanged until 60 min of treatment (Fig. 2A). Soluble α-synuclein added to Sarkosyl-insoluble fractions from control brains was also readily degraded by proteinase K treatment (data not shown), indicating that the stability of Sarkosyl-insoluble α-synuclein to proteinase K is not due to interference by contaminants in this fraction. These results suggest that the filamentous form of α-synuclein in Sarkosyl-insoluble fractions is resistant to proteinase K digestion.

Sarkosyl-insoluble α-synuclein from MSA brains was further analyzed by immunoblotting with an additional anti-α-synuclein antibody NAC1, which recognizes the central hydrophobic region (i.e. NAC domain, residues 75–91) of α-synuclein, using higher concentrations of proteinase K (Fig. 2B). Prior to proteinase K treatment, NAC1 reacted with the major ~7–9-kDa polypeptide (migrating at the same position as full-length α-synuclein), as well as with additional minor bands migrating at ~6–12 kDa. After treatment with 100 μg/ml proteinase K, NAC1 exclusively reacted with ~7–9-kDa polypeptides (Fig. 2B, arrowheads), whereas α-synuclein immunoreactive bands migrating at higher molecular weight ranges (including full-length α-synuclein) were almost completely abolished. The ~7–9-kDa bands were not recognized by LB509, the epitope of which is located around residues 121/122 at the carboxyl terminus of α-synuclein. The 7-kDa band was still detected after the treatment with 500 μg/ml proteinase K with NAC1, although it disappeared by the 1,000 μg/ml proteinase K treatment (Fig. 2C), suggesting that these ~7–9-kDa polypeptides corresponded to the core portion of α-synuclein filaments that acquired high resistance to protease digestion. To further characterize the proteinase K-resistant α-synuclein fragments, in vitro assembled filaments were treated with 1 μg/ml proteinase K for 30–60 min and analyzed by immunoblotting with Syn102, LB509, and NAC1. Immunoreactivities of Syn102 or LB509 were almost completely abolished by the proteinase K treatment for 60 min, whereas ~7–11-kDa bands were detected in the assembled filament fraction with NAC1 (Fig. 2D). Similar results were obtained in the experiment with 10 μg/ml proteinase K treatment for 30 min. NAC1 detected ~7–9-kDa polypeptides in the assembled filament fraction (Fig. 2E, ar-
to proteinase K treatment, the filaments were positively labeled by antibodies against the amino- (Fig. 3A, no. 36) and carboxyl- (Fig. 3E, Syn102) terminal portions of α-synuclein, whereas NAC1 failed to label them (Fig. 3C). After proteinase K treatment, these filaments still retained their filamentous nature, but the mean diameters were decreased by ~20% (untreated, 12.3 ± 0.5 μm; proteinase K-treated, 9.8 ± 2.3 μm), and immunoreactivities for the amino (no. 36) and carboxyl (Syn102) termini were abolished. In sharp contrast, the filaments became immunoreactive for NAC1, which recognizes the mid-portion of α-synuclein. These results suggest that both the amino- and carboxyl-terminal regions of α-synuclein are structurally labile and cleaved off from the filaments by proteinase K digestion, whereas the central region containing the hydrophobic NAC portion represents the core structure of filaments that is resistant to protease treatment.

To unequivocally define the structure of the minimal fragment that constitutes the protease-resistant core of α-synuclein filaments, in vitro assembled α-synuclein filaments were treated with various concentrations of proteinase K ranging from 2 to 1,000 μg/ml. The NAC1 immunoreactive 7-kDa polypeptide remained undigested even by treatment with 1,000 μg/ml proteinase K, whereas other fragments including the 6-kDa species disappeared with increasing concentrations of proteinase K (Fig. 4A). This strongly suggested that the 7-kDa fragment corresponds to the highly stable, protease-resistant core unit of the α-synuclein filaments. To determine the exact structure of the proteinase K-resistant 7-kDa polypeptide, assembled α-synuclein filaments and unassembled α-synuclein proteins were treated with 500 μg/ml proteinase K, and the digests were dissolved in 6 M guanidine HCl and separated by reverse-phase-HPLC. When the HPLC profiles of unassembled and filamentous α-synuclein were carefully compared after treatment with 500 μg/ml proteinase K, one peak was unique to assembled filament digests (Fig. 4B, peak 9); other peaks were derived from fragments of proteinase K, because identical peaks were observed by incubation without α-synuclein (data not shown). Immunoblotting of these HPLC fractions with NAC1 confirmed that the 7-kDa α-synuclein fragment was recovered in fraction 9 (Fig. 4, B and C). MALDI-TOF mass analysis of fraction 9 gave signals corresponding to a molecular mass of 7873.5, which nearly matched to that of residues 31–109 of human α-synuclein (predicted average mass: 7869) (Fig. 4D). These results strongly suggested that residues 31–109 of α-synuclein represent the proteinase K-resistant core unit of α-synuclein filaments.

**DISCUSSION**

The mechanisms by which α-synuclein is assembled into highly ordered filaments and forms intracellular inclusions in the brains of patients with synucleinopathies including PD, DLB, and MSA are unknown. It has been shown that recombinant α-synuclein can assemble into filaments that closely resemble the abnormal α-synuclein filaments in synucleinopathy brains (12). Thus, in vitro modeling of α-synuclein assembly is a useful strategy for the study of the molecular mechanisms of α-synuclein fibril formation as well as for screening of small molecules that affect the formation of pathological α-synuclein filaments.

In this study, we have shown that the in vitro assembled α-synuclein filaments closely resemble the pathological filaments of synucleinopathy brains in their biochemical and structural characteristics. In *vitro* assembled α-synuclein filaments and those from synucleinopathy brains shared very similar solubility profiles, *i.e.* insolubility in detergents (Triton X and Sarkosyl) and effective solubilization in high concentrations of urea. Furthermore, proteinase K treatment revealed...
that these two types of α-synuclein filaments show very similar resistance profiles to protease digestion.

Biochemical analysis of the proteinase K-resistant α-synuclein filament cores strongly suggested that the ~7-9-kDa fragments truncated at amino and carboxyl termini constitute the core portion of α-synuclein filaments. Giasson et al. (24) have reported similar proteinase K-resistant α-synuclein fragments, of which F1 and F2 fragments may correspond to our 8 and 7-kDa fragments, respectively. Conway et al. (20) have also reported a similar proteinase K-resistant α-synuclein fragment that may correspond to our 7-kDa fragment. We have further extended the characterization of the proteinase K-resistant α-synuclein core fragments by two complementary strategies, i.e. immunoelectron microscopy and protein chemical analysis.

Immunoelectron microscopic analysis showed that intact α-synuclein filaments are labeled by antibodies that recognize the amino or carboxyl termini of α-synuclein (no. 36 and Syn102, respectively), whereas an antibody that recognizes the central region of α-synuclein (NAC1) failed to label them. In sharp contrast, proteinase K treatment abolished the immunoreactivities for the amino- and carboxyl-terminal portions, whereas NAC1 immunoreactivity was retrieved, probably because removal of the surface structures exposed the antigen buried at the filament cores. Taken together with the immu-
Proteinase K-resistant Core of α-Synuclein Filament

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