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N-terminal region of α-synuclein is essential for the fatty acid-induced oligomerization of the molecules

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Abstract Exposure of α-synuclein (αS), a major component of Lewy bodies in Parkinson's disease, to polyunsaturated fatty acids (PUFAs) triggers the formation of soluble αS oligomers. Here, we demonstrate that PUFA binds recombinant αS protein through its N-terminal region (residues 2–60). In HEK293 cells, αS mutants lacking the N-terminal region failed to form oligomers in the presence of PUFA. The PUFA-induced αS oligomerization was accelerated by C-terminal truncation or Ser129 phosphorylation of αS; however, this effect was abolished by deletion of the N-terminus. The results indicate that the N-terminus of αS is essential for the PUFA-induced αS oligomerization.

Keywords: Parkinson's disease; α-Synuclein; Oligomerization; Fatty acid; Truncation; Phosphorylation

1. Introduction

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra and the presence of intracytoplasmic filamentous inclusions called Lewy bodies (LBs) and Lewy neurites (LNs). Identification of missense mutations in the α-Synuclein (αS) gene (A30P, A53T, and E46K) [1–3] and the multiplication [4] of the locus in autosomal-dominant familial PD indicates a direct link of αS to the pathogenesis of familial PD. αS is also thought to play a critical role in sporadic cases of PD, because fibrillized αS is a major component of LBs and LNs [5]. In vitro experiments have shown that αS readily oligomerizes into fibrils that share ultrastructural features of fibrils in LBs [6]. Furthermore, overexpression of human wild-type [7] or A53T [8,9] αS in transgenic mice results in motor impairment with the development of αS inclusions. Feany and colleagues have also reported that overexpression of wild-type, A30P or A53T-αS in *Drosophila* shows loss of dopaminergic neurons with an increase in filamentous αS inclusions [10]. Accumulated evidence suggests that the oligomerization of αS into fibrils affects the toxicity of the molecule [11]. Previous studies have demonstrated that the oligomerization of αS is accelerated by oxidation and nitration of αS [12], phosphorylation of αS at Ser129 [13,14], C-terminal truncation of αS [15] or binding of phospholipids to αS [16]. However, the exact biochemical mechanisms for the formation of pathological αS oligomers remain to be elucidated.

Sharon et al. reported that the N-terminus (residues 2–19) and the C-terminus (residues 123–140) of αS have homology to the fatty acid (FA)-binding motif of fatty acid-binding protein (FABP) [17]. They also showed that recombinant αS directly binds to FA in a stochiometric manner, and that soluble, lipid-associated monomers and oligomers of αS are present in transgenic mouse brains expressing wild-type or A53T-αS [17,18]. On the basis of the facts, they concluded that αS has the properties of FABP [17,18]. They further demonstrated that exposure of monomeric αS to polyunsaturated fatty acids (PUFAs) promotes the formation of soluble αS oligomers in cultured mesencephalic cells [18]. In the brains of PD and dementia with Lewy bodies, the amount of soluble, lipid-associated αS oligomers increased [18]. Since the soluble αS oligomers seem to be precursors of fibrils [11], the findings suggest that the binding of αS to PUFA is one of the key events in the process for conversion from soluble monomers to pathological oligomers of αS.

The present study was carried out to determine the αS region responsible for the binding to FA by using a series of αS deletion mutants, and to assess a role of the binding of αS to PUFA in the oligomerization of αS. The results show that the binding of αS to PUFA in its N-terminal region is essential for the PUFA-induced oligomerization of αS.

2. Materials and methods

2.1. Plasmid construction

Wild-type human αS (Wt-αS) cDNA and human G protein-coupled receptor kinase 5 (GRK5) cDNA were cloned into the pcDNA3.1 vector (Invitrogen) [14]. αS deletion mutant cDNAs and αS familial PD-linked mutant (A30T, E46K and A53T) cDNAs were generated by the two-step PCR mutagenesis method. Nucleotide sequences of all constructs were confirmed.

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Abbreviations: PD, Parkinson's disease; αS, alpha-synuclein; LB, Lewy body; LN, Lewy neurite; FA, fatty acid; PUFA, polyunsaturated fatty acid; FABP, fatty acid-binding protein; OA, Oleic acid; ALA, α-linolenic acid; GRK, G protein-coupled receptor kinase; NAC, non-amyloid β-protein component

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2.2. Expression and purification of recombinant αS

Wild-type and deletion mutants of human αS cDNAs were cloned into the pGEX-4T-1 vector (GE Healthcare) to generate glutathione S-transferase fusion protein. The fusion proteins were expressed in *Escherichia coli*. Recombinant αS was purified from the fusion protein by the method described previously [14].

2.3. Oleic acid (OA)-binding assay

OA-binding assay was carried out as previously described with slight modifications [17,19]. Hydroxyalkoxypropyl-Dextran type IV (known as Lipidex 1000, Sigma) was known to bind to free FA at 0–4 °C and FA-bound proteins at 37 °C [19]. To collect FA-unbound proteins, recombinant αS proteins purified from *E. coli* were incubated for 30 min with 5% Lipidex 1000 at 37 °C. After centrifugation at $20000 \times g$ for 5 min, the supernatant containing FA-unbound αS was recovered and stored at −80 °C. To determine the binding of αS to OA, 5 μM αS protein was incubated with various amounts of [^{14}C] OA (Moravek Biochemicals, 54 mCi/mmol) in 100 μl of phosphate-buffered saline (PBS; 100 mM phosphate, pH 7.4, 150 mM NaCl) for 1 h at room temperature. The mixture was kept on ice for 10 min, and 10 μl of 50% (v/v) Lipidex 1000 was added. The mixture was incubated for 10 min on ice. After centrifugation at $15000 \times g$ for 5 min, protein-bound [^{14}C] OA in the supernatant was measured by scintillation counting.

2.4. In vitro oligomerization of αS

One micromolar recombinant αS proteins in PBS were incubated at 37 °C for 12 h with or without α-linolenic acid (ALA, Sigma) of indicated concentrations [14]. Samples (100 ng protein) were analyzed by immunoblotting using anti-αS monoclonal antibody (Syn-1, which recognizes residues 91–99 of human αS; BD Transduction Laboratories) [20].

2.5. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. HEK293 cells were transfected with αS cDNAs using Lipofectamine Plus reagents (Invitrogen), according to the manufacturer's protocol.

2.6. PUFA-induced αS oligomerization in the cells

For the PUFA-induced αS oligomerization, the transfectants were recovered at 32 h after transfection, and the cells were further incubated with serum-free medium containing 500 μM ALA and 100 μM FA-free bovine serum albumin (BSA, Sigma) for 16 h [14,18].

2.7. PUFA-induced oligomerization of phosphorylated αS in the cells

HEK293 cells were transiently co-transfected with GRK5 cDNA and either wild-type or mutant αS cDNA [14]. Twenty-four hours after the transfection, 20 nM okadaic acid (Wako) was added to the culture and the cells were incubated for 16 h. The cells were further incubated for 9 h in fresh serum-free medium containing ALA/BSA complexes and okadaic acid and the cell lystaes were analyzed by immunoblotting [14].

2.8. Protein fractionation and immunoblotting

Transfectants of HEK293 cells were suspended in homogenization buffer [20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.32 M sucrose, 43 mM 2-mercaptoethanol, 1 × protease inhibitor cocktail (RocheDiagnostic), 20 mM NaF, 1 mM Na_3VO_4] and were disrupted by brief sonication [14,17]. Samples were sequentially centrifuged at $8000 \times g$ for 15 min and at $370000 \times g$ for 1 h. The resultant supernatant was recovered and stored as the cytosol fraction. The post-$370000 \times g$ pellet was disrupted by sonication in homogenization buffer containing 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% SDS, and the homogenates were incubated on ice for 30 min. After centrifugation at $12000 \times g$ for 30 min, the supernatant was stored as the pellet fraction. Since the detection of lipid-associated αS oligomers by immunoblotting requires delipidation procedures, we treated the cytosol fractions at 60 °C for 16 h for delipidation prior to gel-loading. The effect of the delipidation for detection of αS oligomers was described previously [14,17,18].

Protein samples were denatured by boiling for 5 min in Laemmli's sample buffer containing 2.5% 2-mercaptoethanol. Immunoblotting was performed as described previously [14]. The transferred membrane was probed with the antibodies as follows: αS specific, Syn-1, LB509 (monoclonal IgG, which recognizes residues 115–122 of αS; Zymed) [21] and 211 (monoclonal IgG, which recognizes residues 121–125 of αS; Sigma) [22]; phosphorylated αS at Ser129 specific, psyn#64 (monoclonal IgG; Wako) [23]; β-actin (Sigma); or superoxide dismutase 1 (SOD1, Stressgen). Bands were visualized by enhanced chemiluminescence (ECL) or ECL plus (GE Healthcare). Relative intensities of detected bands were scanned and quantified with NIH Image J, version 1.33 or Quantity one (Bio-Rad) software.

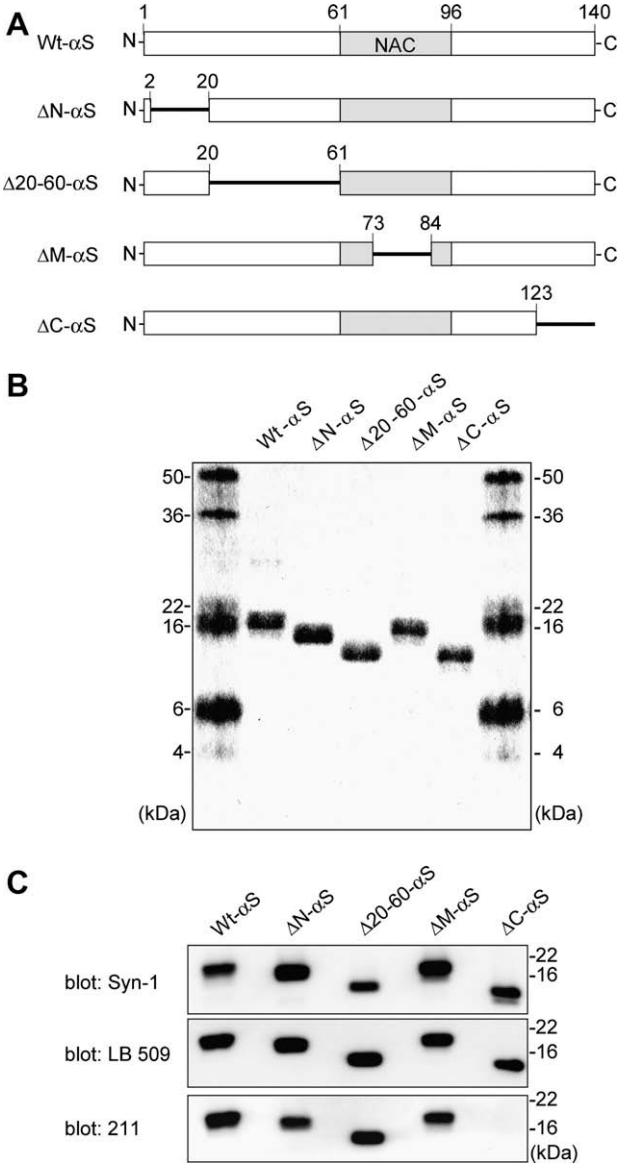

Fig. 1. αS deletion mutants. (A) Schematic diagrams of wild-type αS (Wt-αS) and αS deletion mutants. (B) The purity and electrophoretic pattern of recombinant αS mutants. Recombinant proteins (2 μg) were analyzed by SDS–PAGE using 15% polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250. (C) Recombinant αS proteins (100 ng) were loaded to the 12.5% polyacrylamide gel and analyzed by immunoblotting using three different monoclonal antibodies. Monoclonal antibodies Syn-1, LB509 and 211 recognize residues 91–95, 115–122 and 121–125, respectively.