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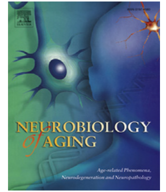

Differential aggregation properties of alpha-synuclein isoforms

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ABSTRACT

Pathologic aggregation of α-synuclein is a central process in the pathogenesis of Parkinson's disease. The α-synuclein gene (*SNCA*) encodes at least 4 different α-synuclein isoforms through alternative splicing (*SNCA140*, *SNCA126*, *SNCA112*, *SNCA98*). Differential expression of α-synuclein isoforms has been shown in Lewy body diseases. In contrast to the canonical α-synuclein isoform of 140 amino acid residues (*SNCA140*), which has been investigated in detail, little is known about the properties of the 3 alternative isoforms. We have investigated the aggregation properties of all 4 isoforms in cultured cells and analyzed fibril-formation of 3 isoforms (*SNCA140*, *SNCA126*, and *SNCA98*) in vitro by electron microscopy. Each of the 3 alternative isoforms aggregates significantly less than the canonical isoform *SNCA140*. Electron microscopy showed that *SNCA140* formed the well-known relatively straight fibrils while *SNCA126* formed shorter fibrils, which were arranged in parallel fibril bundles and *SNCA98* formed annular structures. Expression analysis of α-synuclein isoforms in different human brain regions demonstrated low expression levels of the alternative isoforms in comparison to the canonical *SNCA140* isoform. These findings demonstrate that α-synuclein isoforms differ qualitatively and quantitatively in their aggregation properties. The biological consequences of these findings remain to be explored in vitro and in vivo.

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1. Introduction

Parkinson's disease (PD) is one of the most common movement disorders. PD is in most cases a sporadic, genetically complex disorder with late onset. Monogenic variants of the disease, caused by mutations in at least 11 different genes have contributed eminently to our understanding of the PD pathomechanism. Mutations in the α-synuclein (*SNCA*) gene were the first to be discovered and it turned out that α-synuclein is central in the pathogenesis of monogenic as well as sporadic PD (Polymeropoulos et al., 1997). Alpha-synuclein protein is abundant in Lewy bodies, which are pathognomonic proteinaceous inclusions found in the brains of sporadic PD patients as well as in some forms of monogenic PD (Spillantini et al., 1997). Point mutations but also duplications and triplications of the *SNCA* gene cause monogenic PD and polymorphisms in the *SNCA* gene are strongly associated with sporadic PD (Ahn et al., 2008; Chartier-Harlin et al., 2004; Simon-Sanchez et al., 2009; Singleton et al., 2003). Alpha-synuclein aggregates spontaneously in vitro and in vivo and α-synuclein aggregation is believed to be central in the pathogenesis of PD (Uversky, 2007). Point mutations in the *SNCA* gene causing PD are associated with altered aggregation properties (Opazo et al., 2008). The *SNCA* gene contains 5 protein-coding exons (2–6) and a 5′-UTR exon (1). The canonical α-synuclein isoform contains all 5 coding exons and encodes a protein of 140 amino acid residues (*SNCA140*). At least 3 alternative isoforms of 126, 112, and 98 amino acid residues (aa) are generated by alternative splicing (Fig. 1; *SNCA126* lacking exon 3, *SNCA112* lacking exon 5, and *SNCA98* lacking exons 3 and 5). It has been shown that *SNCA* gene isoform expression is differentially regulated in the brains of patients with PD and other Lewy body disorders (Beyer et al., 2004, 2006, 2008a, 2008b). However, the physical, biochemical, and biological properties of alternative *SNCA* isoforms have not been investigated to date. We hypothesized that alternative *SNCA* isoforms might differ in their aggregation properties compared with the canonical *SNCA140* isoform (Fig. 1). Therefore, we investigated the aggregation properties of *SNCA* isoforms in 2 paradigms as well as the expression levels in different brain regions. First, we used a modified version of an established cell system to quantitatively investigate the aggregation properties

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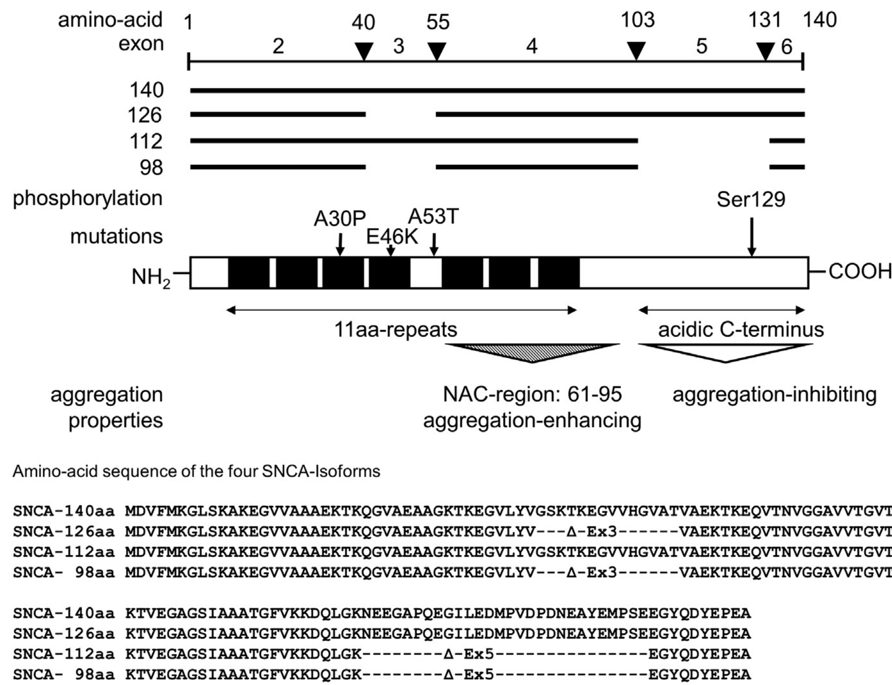

Fig. 1. *SNCA* gene, alternative isoforms, domains, and amino-acid sequences; amino-acid (aa): numbering of the aa of the *SNCA* gene; cds-exon: coding exons 2–6; phosphorylation–shows Ser129 which is subject to phosphorylation; mutations: mutations causing monogenic PD; 11aa repeats: repeats in the n-terminus of *SNCA140* represented by black boxes; acidic c-terminus: c-terminus rich in acidic aa; aggregation properties: central non-amyloid-component (NAC) region, which is aggregation enhancing and c-terminus which is aggregation inhibiting. Bottom: amino-acid sequence of the 4 alternative α-synuclein isoforms. Abbreviation: PD, Parkinson's disease.

in cultured cells and second, we aggregated bacterially expressed *SNCA140*, *SNCA126*, and *SNCA98* in vitro and examined the fibrils by electron microscopy (EM) (Opazo et al., 2008).

2. Methods

2.1. Quantitative assessment of α-synuclein aggregation in HEK293T cells

Alpha-synuclein fused to a fluorescent protein does not aggregate readily in cultured cells. To circumvent this problem, we used a bicistronic expression strategy previously described by Opazo et al. 2008 (Fig. 2A). In short, the first cistron consisted of red-fluorescent cherry protein fused to the PDZ1 domain of S-SCAM (NP_446073). This cistron was followed by an internal ribosomal entry site element and a second cistron coding for the *SNCA* isoforms fused in frame to the corresponding PDZ binding motif (HSTTRV, from neuroligin-1, NP_446320). The small PDZ binding motif does not seem to impede aggregation. Aggregates are labeled in red by the highly specific interaction between PDZ domain and PDZ binding motif. A c-terminally truncated α-synuclein containing the amino acid residues 1–108 of *SNCA140* (*SNCA108*) was used as a positive control for aggregation because it has previously been shown that this variant aggregates strongly (Opazo et al., 2008). A construct containing only the cherry protein fused to the PDZ domain served as a negative control for aggregation (pPDZ). All cloning procedures were performed using the MultiSite Gateway Pro System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The PDZ domain and the *SNCA*-PDZ binding motifs were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All elements were polymerase chain reaction (PCR)-amplified and cloned into the pDONR221 P1-P4 (PDZ domain, element 1), pDONR221 P4r-P3r (internal ribosomal entry site, element 2), and pDONR221 P3-P2 (*SNCA140*, *SNCA126*, *SNCA112*, *SNCA98*, *SNCA108*, all fused to the PDZ binding motif, element 3) plasmids. These 3 elements were then simultaneously transferred to the pcDNA-DEST53Cherry plasmid vector. pcDNA-DEST53Cherry was derived from pcDNA-DEST53 (Invitrogen, Carlsbad, CA, USA) through replacement of the green-fluorescent-protein by the cherry protein. All primer sequences are available upon request. The DNA sequence of all final constructs was verified by Sanger sequencing. Plasmid maxipreparations were performed using the Jetstar NoEndo plasmid purification kit (GENOMED, Löhne, Germany). HEK293T cells were cultured in DMEM Glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin. Cells were transiently transfected using Turbofect reagent (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA). HEK293T cells (25,000) were seeded on coverslips in 6-well plates and transfected with 3 μg of plasmid DNA after 72 hours. After 48 hours of transfection, cells were washed once with phosphate-buffered saline (PBS), fixed for 10 minutes in 3.5% paraformaldehyde in PBS, washed thrice with PBS. Nuclei were stained with DAPI followed by 2 further washing steps with PBS. Coverslips were mounted on glass slides using Immumount (Thermo Fisher Scientific, Waltham, MA, USA). After 24 hours cells were examined by fluorescence microscopy on a Zeiss Axio Observer A1 microscope. Cherry staining was classified as "diffuse", "aggresome", or "aggregates" (Fig. 2B) according to the definitions published by Opazo et al. 2008. Consistence of the principal features and consistent classification of findings was assured by a personal visit of one of the authors (Gregor Kuhlenbaumer) at P. Opazo's lab including a joint microscopy and classification session. Alpha-synuclein aggregation was quantified by classifying 300 cells on each slide into the previously mentioned categories by a blinded observer. All results are expressed as mean and standard deviation. The aggregate counts of alternative isoforms, negative and positive control, were compared with the ones of the canonical isoform, *SNCA140*. The statistical significance of deviations in aggregate formation from *SNCA140* was assessed using the 2-sided Student *t* test with a heteroskedacity correction. The

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