Synthetic filaments assembled from C-terminally truncated α-synuclein

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Received 24 August 1998

Abstract Recently two point mutations in the α-synuclein gene have been found in familial Parkinson's disease. The characteristic fibrous neuropathological lesions of Parkinson's and other neurodegenerative diseases have been shown to stain strongly with antibodies against α-synuclein and extracted filaments have been labelled with anti-α-synuclein antibodies. In view of the close involvement of α-synuclein filaments with pathology, it was important to establish an in vitro assembly system. We report here that C-terminally truncated recombinant α-synuclein readily assembles into filaments resembling those isolated from diseased brain and suggest that truncation by proteolysis may play a role in the pathological process.

Key words: α-Synuclein; Filament assembly; Parkinson's disease; Dementia with Lewy bodies; Multiple system atrophy

1. Introduction

α-Synuclein is a presynaptic protein, of presently unknown function, found abundantly in human brain and at lower levels in other tissues [1,2]. It contains 140 amino acids and has five imperfect repeats with consensus sequence KTKEGV in the N-terminal half and a very acidic C-terminal region. The protein is thermostable, with little or no detectable secondary structure, and is believed to be natively unfolded [3].

Two point mutations in the α-synuclein gene have recently been found in rare cases of familial Parkinson's disease (PD) (Ala^{53}Thr [4] and Ala^{30}Pro [5]). PD is characterised neuropathologically by the presence of Lewy bodies and Lewy neurites, predominantly in the substantia nigra and other subcortical brain regions [6]. Lewy bodies and neurites consist of dense cytoplasmic inclusions of fibrous material. Abundant Lewy bodies and Lewy neurites in cerebral cortex are also the defining features of a common late-life dementia, known as dementia with Lewy bodies (DLB) [7]. The linkage of familial PD to the α-synuclein gene suggested that α-synuclein might itself form a component of Lewy bodies and neurites both in PD and in DLB and this was demonstrated to be the case by antibody staining at the light microscope level [8]. Subsequently, dispersed filaments extracted from the brains of patients with DLB were shown by electron microscopy to be strongly labelled by antibodies against α-synuclein [9], suggesting that such filaments form an important component of Lewy bodies in both diseases. Another neurodegenerative disease, known as multiple system atrophy (MSA), was also shown to have extractable α-synuclein-containing filaments in the characteristic glial cytoplasmic inclusions that constitute the predominant pathology [10].

The pathological assembly of α-synuclein into filaments and its association with a range of neurodegenerative diseases suggested that it would be important to establish an in vitro assembly system to aid study of the filaments. Recombinant human α-synuclein can be efficiently expressed in *E. coli* [2]. Besides producing proteins with the mutations reported in familial PD, various C-terminally truncated fragments of the wild-type protein had been generated for mapping the epitopes of anti-synuclein antibodies. When incubated under suitable conditions, we observed that C-terminally truncated proteins, particularly α-synuclein (1–120), assembled into filaments much more readily than the full-length wild-type or mutated proteins. It has been reported that α-synuclein extracted from Lewy bodies in cases with DLB is partially truncated [11]. These observations suggest that C-terminal truncation of α-synuclein may play a role in assembly of α-synuclein into filaments in the various diseases.

2. Materials and methods

2.1. Expression and purification of recombinant wild-type, mutated or truncated human α-synuclein

The open reading frame of human α-synuclein was subcloned into M13. Site-directed mutagenesis was used to change Ala^{30} to proline or Ala^{53} to threonine. Following primer extension, the mutated cDNAs were subcloned into the bacterial expression vector pRK172. The carboxy-terminally truncated α-synuclein (1–110), α-synuclein (1–120) and α-synuclein (1–130) were amplified by PCR from full-length α-synuclein (1–140) and subcloned into pRK172. All constructs were verified by DNA sequencing. The full-length α-synuclein construct has been described [2]. Recombinant proteins were expressed in *E. coli* BL21(DE3), as described [2]. Bacterial pellets were sonicated in 50 mM Tris, pH 8.0, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged for 15 min at 15 000 rpm. 30–50% ammonium sulfate cuts of the supernatants were applied to an S-200 Sephacryl column equilibrated in sonication buffer. For full-length wild-type and mutated α-synucleins, peak protein fractions were applied to a Mono Q column equilibrated in sonication buffer, and α-synuclein eluted using a 0–500-mM NaCl gradient. For truncated α-synucleins, peak protein fractions from an S-200 Sephacryl column equilibrated in 50 mM MES, pH 6.25, 0.1 mM DTT, 0.1 mM PMSF, were applied to a phosphocellulose column equilibrated in the same buffer. Truncated α-synucleins were eluted by salt step elution. Immunoblot analysis was carried out with anti-α-synuclein serum PER4 (diluted 1:1000) [9] and developed using a Vectastain kit (Vector Laboratories, Peterborough, UK). SDS-polyacrylamide gels (15%) were used to monitor protein purification and for immunoblot analysis.

2.2. Assembly of α-synuclein filaments and electron microscopy

Purified recombinant human α-synuclein, Ala^{30}Pro α-synuclein, Ala^{53}Thr α-synuclein, α-synuclein (1–110), α-synuclein (1–120) or α-synuclein (1–130) was incubated in 25 μl of 30 mM MOPS, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem), pH 7.4, at 37°C for 48 h. The α-synuclein concentrations ranged from

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PII: S0014-5793(98)01146-6

5–10 mg/ml. The samples were checked by SDS-PAGE immediately prior to microscopy to see that there had been no protein degradation.

Aliquots of assembly mixtures were placed on carbon-coated 400-mesh grids and stained with 1% lithium phosphotungstate, and micrographs recorded at a nominal magnification of ×40 000 on a Philips model EM208S microscope. Procedures for immunoelectron microscopy were as described [12]. The primary anti-α-synuclein antisera used were PER1 (raised against amino acids 11–34 of α-synuclein [9]), diluted 1:50, or PER4 (raised against full-length recombinant human α-synuclein [9]), diluted 1:100. After reaction with the appropriate secondary gold-conjugated antibody (Sigma), the grids were stained with 1% lithium phosphotungstate.

Micrographs of filaments extracted from brains of cases with DLB or MSA and labelled with anti-α-synuclein antibodies were selected from previously published studies [9,10], to allow comparison with the filaments assembled in vitro.

3. Results

When the various C-terminally truncated α-synuclein constructs (Fig. 1A) were run on SDS-PAGE, the expected ladder of bands was observed by Coomassie staining (Fig. 1B) and by immunoblotting with the PER4 antiserum (Fig. 1C). In the latter, the bands for α-synuclein (1–120) and α-synuclein (1–110) were more weakly labelled than those for full-length α-synuclein and α-synuclein (1–130). This suggests that some of the stronger epitopes which the PER4 antiserum recognises are in the C-terminal region but the results also indicate that PER4 can still be used to label the truncated proteins.

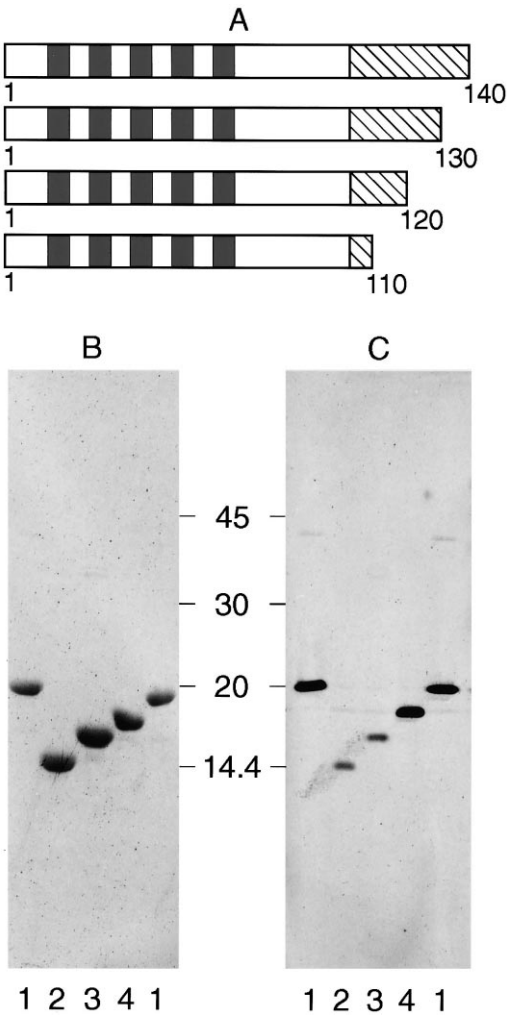

Fig. 1. α-Synuclein and various C-terminally truncated constructs. A: Diagram of the full-length 140 amino acid protein, showing repeats and acidic C-terminal region, together with truncated proteins of lengths 130, 120 and 110 amino acids. B: Coomassie brilliant blue stained blot of purified α-synuclein constructs. C: Immunoblot with antiserum PER4 similar to that shown in B. Lanes 1: Full-length protein; 2: 1–110; 3: 1–120; 4: 1–130.

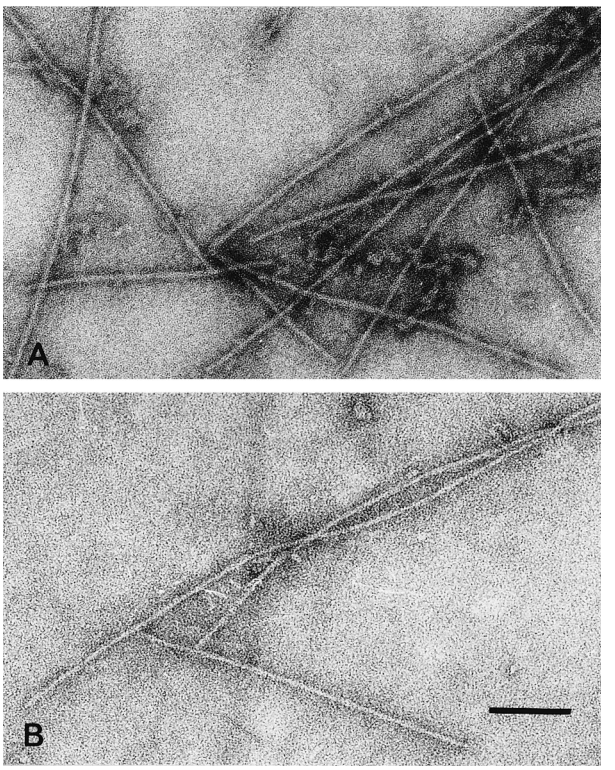

Fig. 2. Electron micrographs of in vitro assembled filaments. A: α-Synuclein (1–120); B: α-synuclein (1–110). Scale bar 100 nm.

When the full-length wild-type or mutated proteins were incubated at 37°C, little aggregation was seen. For the α-synuclein (1–130) construct, small irregular wavey assemblies were formed. However, for the α-synuclein (1–120) and (1–110) constructs long regular filaments were produced, more abundantly for the former than the latter (Fig. 2). The filaments were about 6–9 nm in width and could be up to several microns long. In some filaments the overall width appeared to vary slightly along the length of the filament but in general rather little substructure could be seen, apart from some irregular long pitch helical strands in places. The filaments were of very similar appearance to those found in the various α-synuclein-linked neurodegenerative diseases.

The in vitro assembled α-synuclein (1–120) filaments were labelled strongly by PER4 antiserum (Fig. 3A). The appearance of individual filaments (Fig. 3B,C) was very similar to that of PER4 labelled filaments extracted from brains with DLB (Fig. 3D) or MSA (Fig. 3E). The labelling was apparent along the whole length of the filament. By contrast PER1 antiserum predominantly labelled a single end of the in vitro assembled filaments (Fig. 3F–H), as has been described for filaments from cases of DLB or MSA (Fig. 3I). For the in vitro assembled filaments, some gold particles were also seen at the sides of filaments (e.g. Fig. 3G) but this is not surpris-

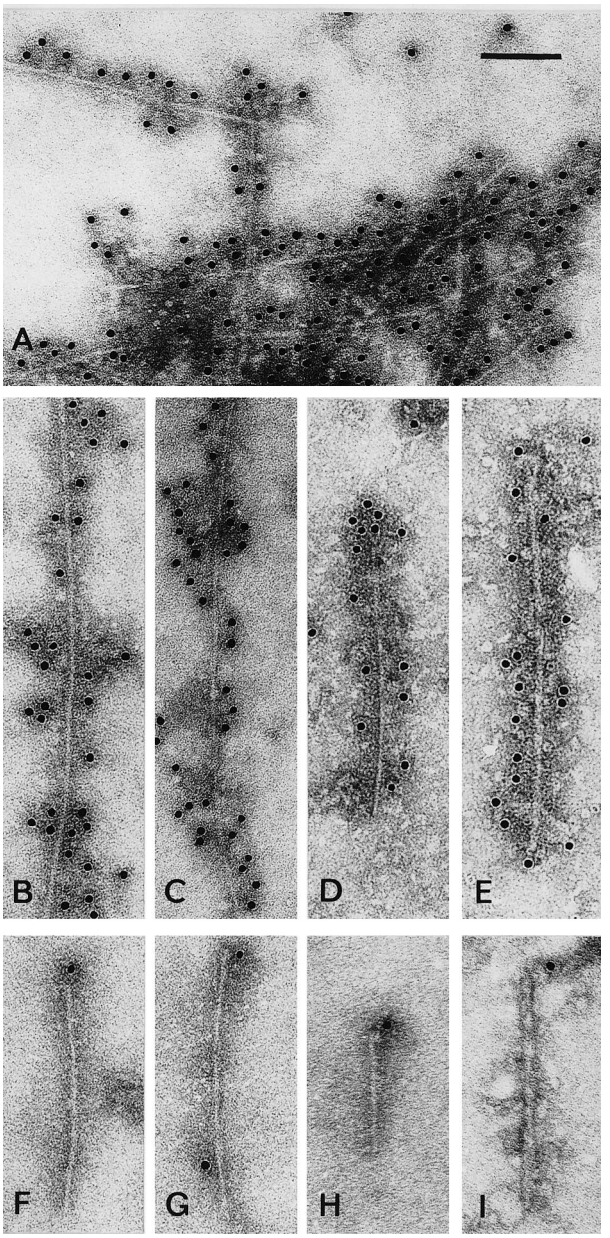

Fig. 3. Immunolabelling of synthetic α-synuclein (1–120) filaments and filaments extracted from diseased brains, labelled with PER4 antiserum (A–E) and labelled with PER1 antiserum (F–I). A–C: α-Synuclein (1–120) filaments showing a labelled clump and individual filaments. D–E: Filaments extracted from brains with diffuse Lewy body disease (D) or multiple system atrophy (E). F–I: Filaments end-labelled with PER1 antiserum, showing α-synuclein (1–120) filaments (F–H) and a filament from a brain with multiple system atrophy (I). The 10-nm gold particles attached to the secondary antibody appear as black dots. Scale bar 100 nm.

ing, as the filaments were assembled from quite high concentrations of α-synuclein, so unassembled molecules could well have casually adhered to the filaments.

4. Discussion

Point mutations in the α-synuclein gene have been identified in rare cases of familial PD [4,5]. In sporadic PD and DLB the characteristic Lewy bodies and Lewy neurites have been shown to stain with anti-α-synuclein antibodies [8,9,11,13–15], as have glial cytoplasmic inclusions in MSA [10,16–18]. Furthermore, sarkosyl extracted individual filaments with a clear morphology have also been shown to be labelled by anti-α-synuclein antibodies in cases of DLB [9] and MSA [10]. These observations strongly link the presence of aggregates of abnormal α-synuclein containing filaments with the neurodegenerative process in these diseases.

We show here that C-terminally truncated α-synuclein, particularly α-synuclein (1–120), assembles into filaments morphologically very similar to those seen in the various neurodegenerative diseases. Additionally the pattern of labelling of the in vitro assembled filaments with anti-α-synuclein antibodies is very similar to that seen with filaments extracted from diseased brain [9,10]. Antiserum PER4, raised against full-length α-synuclein, decorated the whole length of filaments, whereas PER1, raised against amino acids 11–34 of α-synuclein, decorated only one end of the filaments. This indicates that the packing of α-synuclein molecules into the in vitro formed filaments is very similar to that in the filaments extracted from brain. Under the conditions defined here, full-length α-synuclein, wild-type or mutated as in familial PD, failed to give filaments.

A large proportion of the α-synuclein extracted from partially purified Lewy bodies in DLB has been found to be truncated [11]. In conjunction with the in vitro results reported here, this suggests that proteolytic degradation of α-synuclein may be an important factor in the assembly of α-synuclein in the various neurodegenerative diseases. Viewed in this way, the mutations in familial PD may cause partial loss of function of α-synuclein [19], which in turn leads to a toxic gain of function as the proteolysed α-synuclein assembles into aggregates with detrimental effects for the cells in which assembly occurs. The situation may be similar to that of tau protein in the familial frontotemporal dementias, where mutations may lead to a partial loss of function for tau protein in its interactions with microtubules and a subsequent toxic gain of function, as tau protein assembly leads to neurodegeneration [20–22].

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