Communication

Binding of α-Synuclein to Brain Vesicles Is Abolished by Familial Parkinson’s Disease Mutation* (Received for publication, July 10, 1998)
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The presynaptic protein α-synuclein has been implicated in the pathogenesis of Parkinson’s disease. First, two missense mutations A30P and A53T cause inheritable early onset Parkinson’s disease in some families. Secondly, α-synuclein is present in Lewy bodies of affected nerve cells in the predominant sporadic type of Parkinson’s disease as well as in dementia with Lewy bodies. We demonstrate in the rat optic system that a portion of α-synuclein is carried by the vesicle-moving fast component of axonal transport and that it binds to rat brain vesicles through its amino-terminal repeat region. We find α-synuclein with the A30P mutation of familial Parkinson’s disease devoid of vesicle-binding activity and propose that mutant α-synuclein may accumulate, leading to assembly into Lewy body filaments.

Parkinson’s disease is a common neurodegenerative disorder that affects approximately 0.2% of the population. Neuropathologically, it is characterized by filamentous Lewy bodies and Lewy neurites, in dopaminergic nerve cells of the substantia nigra and other nerve cell populations (1). Their presence may cause neurodegeneration, but the mechanisms underlying their formation are unknown. Two separate missense mutations (A30P and A53T) in α-synuclein have been identified in some families with early-onset Parkinson’s disease (2, 3). α-Synuclein is an abundant 140-amino acid neuronal phosphorylated protein that is localized in the presynaptic terminals (for a review, see Ref. 4). This normal localization is perturbed in idiopathic Parkinson’s disease and in dementia with Lewy bodies (5–7), a common late-life dementia that is clinically similar to Alzheimer’s disease (8). In these diseases, α-synuclein accumulates in the cell bodies and neurites of degenerating neurons as a major component of Lewy bodies and Lewy neurites. Here we report that in rat optic nerve a portion of α-synuclein is carried in the vesicle-moving fast component of axonal transport. Fast axonal transport represents the movement of tubulovesicular structures along microtubules driven by motor proteins (9). Accordingly, we show that α-synuclein binds to vesicles from rat brain through its amino-terminal repeat region. α-Synuclein with the A30P mutation of familial Parkinson’s disease is devoid of significant vesicle-binding activity. As a result, it may accumulate, leading to its assembly into Lewy body filaments.

EXPERIMENTAL PROCEDURES

Axonal Transport—Adult female rats received a bilateral intracerebral injection of 0.5 mCi of [125I]Iodoamphetamine (DuPont) (10). The animals were killed by cervical dislocation after 4 h, and their optic nerves, optic chiasmata, optic tracts, and lateral geniculate bodies were dissected. The proximal 3 mm were removed from the samples, to avoid labeled material moving in slow component b of axonal transport (SCb). Tissues were homogenized in 8 × urea, 0.5% SDS, 2% mercaptoethanol, and insoluble material was removed by centrifugation. For immunoprecipitation, supernatants were diluted 25-fold in phosphate-buffered saline (PBS), in the presence of a mixture of protease inhibitors (Complete, Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany), cleared of endogenous IgG by incubation with 60 μl of protein A-Sepharose slurry for 1 h, and incubated with 20 μg of affinity-purified rabbit prealbumin IgG or 2 μl of mouse monoclonal anti-SNAP25 (Affinity Bioreagents Inc.) for 16 h at 4 °C. IgG was collected by the addition of 40 μl of protein A-Sepharose slurry for 1 h, followed by 7 × 10 ml washes in PBS, 0.1% Triton X-100. The synuclein/IgG/protein A-Sepharose was heated to 95 °C for 5 min in 1% SDS, 20 mM dithiothreitol (DTT), 20 mM Tris-HCl, pH 6.8, and eluted synuclein was recovered by centrifugation in a spin column (Bio-Rad Laboratories). The eluate was brought to 20% glycerol and loaded directly on a 10–20% gradient SDS-polyacrylamide gel that was further processed for fluorography at −80 °C. Control experiments showed that the ratio of anti-α-synuclein IgG/optic nerve resulted in the quantitative precipitation of α-synuclein. The α-synuclein antibody was raised in a rabbit using recombinant human α-synuclein as the immunogen. By immunoblotting of rat brain extract, it recognized α-synuclein and the related β-synuclein. It similarly recognized recombinant human α-synuclein and β-synuclein.

Vesicle Binding—A modification of the flotation assay of Brown and Rose (11) was used. All procedures were carried out at 4 °C. One adult rat cerebral hemisphere was Dounce-homogenized in 2.5 ml of 5 mM dithiothreitol, 2 mM EDTA, 9% sucrose, 25 mM MES, pH 7.0, in the presence of a mixture of protease inhibitors (Complete, Boehringer Mannheim GmbH). Nuclei and debris were removed by a 5-min centrifugation at 2,500 rpm, and a crude vesicle fraction was isolated by ultracentrifugation of the supernatant at 100,000 × g for 1 h. The resulting pellet was resuspended by Dounce homogenization in the above buffer. Vesicle binding was performed by incubating 100 μl of resuspended vesicles (approximately 7 mg of protein/ml) with 1 μl biotinylated probe for 2 h. The solution was brought to 55% sucrose in a volume of 0.35 ml, placed into a 4-mL ultracentrifuge tube, and overlaid with 3 ml of a 45–20% sucrose gradient. Flotation was carried out for 16 h at 100,000 × g in a SW60 swinging rotor. Following ultracentrifugation, the gradient was divided into 9 fractions, which were collected from the top. A sample of each fraction was used for determining the sucrose concentration by refractometry and the protein concentration (Bio-Rad Protein Assay, Bio-Rad Laboratories). For localizing α-synuclein in the gradient, an aliquot of each fraction (200 μl) was precipitated with 20% trichloroacetic acid, run on 10–20% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Endogenous rat α-synuclein was identified with affinity-purified rabbit anti-synuclein IgG, followed by HRP-conjugated donkey anti-rabbit IgG (Amersham). Biotinylated human α-synuclein proteins were identified with HRP-conjugated streptavidin (Boehringer Mannheim). HRP was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

α-Synuclein Constructs—Human α-synuclein was expressed and purified as described (12). Site-directed mutagenesis was used to produce

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† The abbreviations used are: SCb, slow component b of axonal transport; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; MES, 4-morpholinoolthanesulfonic acid.
Axonal transport of $\alpha$-synuclein was investigated in the rat visual system (10). Retinal ganglion cells were pulse-labeled by an intraocular injection of $[^{35}\text{S}]$methionine, and movement of synucleins was monitored by quantitative immunoprecipitation. Animals were sacrificed 4 h after the injection, the first 3 mm of the optic nerve were discarded, and $\alpha$-synuclein was immunoprecipitated from the remainder of the visual system. Proteins labeled at 4 h are transported by fast axonal transport (100–400 mm/day) (10). As shown in Fig. 1, two immunoprecipitated labeled bands were detected at 4 h; they migrated with recombinant human $\alpha$-synuclein and the related $\beta$-synuclein (12, 13), indicating that both synucleins move in the fast component of axonal transport. Comparison of the amounts of immunoprecipitated labeled synucleins at 4, 30, and 96 h showed that approximately 15% of the total pool of $\alpha$-synuclein and $\beta$-synuclein moves by fast axonal transport, with the remainder moving in SCb.2

Fast axonal transport of a portion of optic nerve $\alpha$-synuclein suggested that it may be associated with the vesicles and motor proteins that are transported at this speed. We therefore used a flotation assay to study the association of wild-type and mutant $\alpha$-synuclein with vesicles. Upon ultracentrifugation, vesicles and their associated proteins float in the sucrose gra-

FIG. 1. Fast axonal transport of $\alpha$-synuclein. Rat retinal neurons were labeled by intraocular injection of 0.5 mCi of $[^{35}\text{S}]$methionine. Axonally transported synucleins were immunoprecipitated from extracts of optic nerve, optic chiasm, optic tract, and lateral geniculate body 4 h later, resolved by SDS-polyacrylamide gel electrophoresis, and probed for fluorography. Based on co-migration with the recombinant human proteins, the labeled bands were identified as $\alpha$-synuclein and $\beta$-synuclein. Molecular markers are shown to the left ($M_r \times 10^{-3}$).

FIG. 2. Binding of $\alpha$-synuclein proteins to rat brain vesicles. Adult rat brain extract was fractionated into postnuclear supernatant, cytosol, and vesicles. Binding of $\alpha$-synuclein to vesicles was measured in a flotation assay. The gradient was divided into 9 separate fractions (ranging in density from 1.0690 to 1.1634 g/ml), and $\alpha$-synuclein was visualized in each fraction. Endogenous rat $\alpha$-synuclein was measured in the postnuclear fraction (panel 1), the cytosol (panel 2), and the crude vesicle fraction (panel 3). Endogenous rat SNAP25 was measured in the crude vesicle fraction (panel 4). Binding of biotinylated human $\alpha$-synuclein proteins to crude vesicles was measured in panels 5–9: wild-type $\alpha$-synuclein, panel 5; $\alpha$-synuclein-(30–140), panel 6; $\alpha$-synuclein-(55–140), panel 7; A30P $\alpha$-synuclein, panel 8; A53T $\alpha$-synuclein, panel 9. Similar results were obtained in four separate experiments. Molecular weight markers are shown to the left ($M_r \times 10^{-3}$).

P. H. Jensen and C. G. Dotti, unpublished observation.
α-Synuclein A30P Mutation Abrogates Vesicle Binding

![Diagram](https://example.com/diagram.png)

**Fig. 3. Biotinylated recombinant human α-synuclein proteins.** Top panel, schematic diagram of the 140-amino acid human α-synuclein, with the seven core repeats shown as black boxes. The borders of α-synuclein-(30–140) and α-synuclein-(55–140) are indicated, as are the positions of the familial Parkinson’s disease mutations A30P and A53T. Bottom panel, recombinant wild-type biotinylated human α-synuclein, lane 1; biotinylated A53T α-synuclein, lane 2; biotinylated A30P α-synuclein, lane 3; biotinylated α-synuclein-(30–140), lane 4; biotinylated α-synuclein-(55–140), lane 5. The weaker band in lane 3 corresponds to dimerized A30P α-synuclein. Molecular weight markers are shown to the left (M, × 10−6).

α-synuclein and A53T α-synuclein to bind to vesicles. Both mutations, which are located in the repeat region of α-synuclein, lead to early-onset Parkinson’s disease, by as yet unknown mechanisms. Purified recombinant A30P and A53T α-synuclein were biotinylated (Fig. 3) and used in the vesicle-binding assay. As shown in Fig. 2, panel 8, A30P α-synuclein was devoid of significant vesicle-binding activity. By contrast, A53T α-synuclein bound as well to vesicles as wild-type α-synuclein (Fig. 2, panel 9).

**DISCUSSION**

Axonal transport studies are useful for identifying specific associations of proteins with intracellular structures. Thus, proteins associated with tubulovesicular structures move within the fast component, whereas proteins interacting with non-tubulovesicular structures, such as cytoskeletal elements, move within slow component a and/or SCb (10). Proteins with multiple associations move at multiple rates. α-Synuclein belongs to the latter class, as it moves within both the fast component and SCb. Movement of α-synuclein in the fast component suggested that it might bind to vesicles. This was investigated directly using a flotation assay of vesicles from rat brain. A portion of α-synuclein was associated with vesicles, to which it bound through its amino-terminal four repeats. These findings demonstrate a function for the repeat region of α-synuclein. It is unclear whether α-synuclein binds to lipids or to vesicle proteins. Previous work has shown an affinity of endogenous α-synuclein for synaptosomes, and similarities between the repeats in α-synuclein and in apolipoproteins have been described, suggesting an interaction between vesicle lipids and the hydrophobic repeats of α-synuclein (14, 15, 17). Moreover, a recent study has shown the binding of α-synuclein through its repeats to small synthetic unilamellar liposomes that are rich in phospholipids (18). Biophysical studies have shown that recombinant human α-synuclein is a natively unfolded molecule, with only little secondary structure (19). However, like other natively unfolded molecules, it is likely that it becomes structured upon binding to vesicles (18, 19).

The mutations in α-synuclein that lead to familial Parkinson’s disease are located in the amino-terminal repeats, the same region that binds to vesicles. We therefore investigated their influence on the binding of human α-synuclein to rat brain vesicles. Recombinant human A53T α-synuclein bound in a similar manner to recombinant wild-type human or endogenous rat α-synuclein. Rat α-synuclein has a threonine at position 53, like the mutated human protein (14, 15). Except for this difference, human and rat α-synucleins are identical in sequence in their vesicle-binding repeats but differ in another six amino acids downstream of the amino-terminal four repeats. This renders binding of human A53T α-synuclein to rat brain vesicles difficult. Understanding the effects of this mutation may require studies on primate or human brain. Similar complications do not arise with the second mutation in α-synuclein, which changes alanine residue 30 to proline. This residue is alanine in all known α-synuclein sequences (12, 14–17). In the vesicle-binding assay, recombinant human A30P α-synuclein was devoid of significant binding activity. In nerve cells A30P α-synuclein may thus not move in the fast component of axonal transport, but only in SCb. The redistribution of A30P α-synuclein resulting from a loss in vesicle-binding activity may be a major deleterious effect of the mutation. Over time, it will lead to a slow build-up of protein and, upon reaching a critical concentration, A30P α-synuclein may assemble into Lewy body filaments. In addition, A30P α-synuclein may also have a higher tendency to assemble into filaments than the wild-type protein. α-Synuclein is an abundant presynaptic protein (20, 21), whereas Lewy bodies and Lewy neurites are found in nerve cell bodies and abnormal neurites (1, 5–7), indicative of an abnormal localization of the assembled protein. In familial Parkinson’s disease, assembly into filaments may result from a redistribution of A30P α-synuclein, due to a lack in vesicle-binding activity. In idiopathic Parkinson’s disease and in dementia with Lewy bodies, unknown post-translational modifications of α-synuclein or its putative “receptor” molecules in the axonal transport apparatus may have a similar effect.

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