Increasing evidence suggests that α-synuclein is a common pathogenic molecule in several neurodegenerative diseases, particularly in Parkinson’s disease. To understand α-synuclein pathology, we investigated molecules that interact with α-synuclein in human and rat brains and identified tubulin as an α-synuclein binding-associated protein. Tubulin co-localized with α-synuclein in Lewy bodies and other α-synuclein-positive pathological structures. Tubulin initiated and promoted α-synuclein fibril formation under physiological conditions in vitro. These findings suggest that an interaction between tubulin and α-synuclein might accelerate α-synuclein aggregation in diseased brains, leading to the formation of Lewy bodies.

The non-β-amyloid (Aβ) component of Alzheimer’s disease amyloid, or NAC, originally detected in an amyloid-enriched fraction, was shown to be a fragment of its precursor, NACP, by cloning of the full-length cDNA (1). Later, NACP turned out to be a human homologue of Torpedo synuclein (2). Therefore, it is also referred to as human α-synuclein (3). α-Synuclein is abundantly present in presynaptic terminals of neurons (4). Recently, two missense mutations in the α-synuclein gene (5) were discovered in certain pedigrees with familial Parkinson’s disease and were shown to segregate with the illness (6, 7). Shortly thereafter, α-synuclein was identified as the major filamentous component of Lewy bodies (LBs) in Parkinson’s disease (8, 9) and of cytoplasmic inclusions in multiple system atrophy (MSA) (10–12).

Thus, α-synuclein appears to be a common pathogenic molecule in these diseases. Although the physiological role of α-synuclein is unknown, α-synuclein has the property of forming fibrils by itself in vitro, and mutations of α-synuclein accelerate the fibril formation (13, 14). However, the vast majority of cases of neurodegenerative diseases associated with LBs or with α-synuclein pathology, such as Parkinson’s disease, dementia with Lewy bodies (DLB), MSA, and the LB variant of Alzheimer’s disease, are sporadic, where wild-type α-synuclein has shown to be abnormally accumulated as fibrillar structures. It is therefore likely that at some stage(s) in the fibril formation of α-synuclein, either the nucleation and/or the elongation steps should be somehow accelerated in diseased brains, or alternatively, some degeneration process(es) of abnormal structures of α-synuclein might be defective in these patients (15).

With respect to the amyloidogenesis of Alzheimer’s disease, it was demonstrated in vitro that a seed of NAC can accelerate Aβ fibril formation, and conversely, a seed of Aβ can promote NAC fibril formation (16). Similarly, heterogeneous molecules could also be involved in the formation of α-synuclein fibrils, leading to pathological structures of α-synuclein such as LBs.

In this study, we performed a biochemical investigation of molecules that interact with α-synuclein in the human brain, and we identified tubulin as one of the α-synuclein binding-associated proteins. This interaction was confirmed by co-immunoprecipitation experiments. Further, α-synuclein was co-purified with microtubules. Double labeling immunofluorescence revealed that tubulin co-localized with α-synuclein-positive pathological structures such as LBs, Lewy-related neurites in Parkinson’s disease and DLB, and glial cytoplasmic inclusions (GCIs) in MSA. In vitro studies demonstrated that α-synuclein fibril formation was initiated and accelerated in physiological media containing a small amount of tubulin.

To our knowledge, this is the first demonstration of the initiation and promotion of α-synuclein fibrillogenesis by an intrinsic neural protein under physiological conditions. We speculate that the interaction between tubulin and α-synuclein may be linked to α-synuclein-associated neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Expression and Purification of α-Synuclein—α-Synuclein cDNA clone phBS 6-1 (1) was digested with NcoI and BglII, and the DNA fragment was ligated into an expression vector, pET-15b (Novagen), previously linearized with NcoI and BamHI. The resultant pET-α-synuclein was used for transformation of Escherichia coli BL21(DE3) pLysS, and expression was induced by isopropyl-β-D-1-thio-β-D-galactopyranoside. The recombinant α-synuclein was purified using two-dimensional HPLC methods as described previously (15). The identity was confirmed and the purity was shown to be >98% by SDSPAGE, immunoblotting, MALDI/TOF-mass spectrometry, and microsequencing analyses (15).
**Preparation of Human Brain Extract**—A human brain neocortex (75 g) was homogenized in 9 volumes of 20 mM phosphate buffer, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 10 μM leupeptin, and 10 μM pepstatin. Insoluble materials were precipitated by centrifugation at 140,000 × g at 4 °C for 60 min. Soluble proteins from three cycles of homogenization and centrifugation were pooled.

**Isolation and Characterization of α-Synuclein-binding Proteins from a Human Brain Extract**—An affinity column was prepared by the conjugation of cyanogen bromide-activated Sepharose-4B resin (Pharmacia) and recombinant α-synuclein (27 mg) according to the manufacturer’s instructions. Soluble proteins from the human brain were loaded onto the column, and the column was then washed with a large volume of loading buffer, 50 mM PBS, pH 7.0, at 4 °C. The adsorbed proteins were eluted with 50 mM sodium citrate, pH 3.0, containing 0.5 M NaCl. An identical control experiment was performed using a glycine-Sepharose-4B column. α-Synuclein-binding proteins were purified by the adsorbed fraction obtained from the α-synuclein affinity column by standard gel-cast methods followed by a reversed phase HPLC and characterized by immunoblotting, mass spectrometry, and microsequence analysis.

**Isolation of Microtubules and Tubulin from Rat Brain**—Microtubules were isolated from rat brains by two cycles of a temperature-dependent assembly and disassembly method (17) in PIPES buffer (0.1 M PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl₂) containing 1 mM GTP, 1 μM pepstatin, and 10 μM leupeptin, and tubulin was purified from the microtubule fraction by phosphocellulose (Whatman P11) column chromatography (18). The purity of the tubulin was determined to be >98%, α-tubulin was co-immunoprecipitated (IP) from the cytosolic fraction of rat brain with anti-α-synuclein antibodies PQE3 and EQV1. The immunoprecipitated proteins were separated by 12% SDS-PAGE, analyzed by immunoblot and quantified (Fig. 1). After purification of the upper band (arrowhead), amino acid sequencing analysis revealed the protein to be α-tubulin (see text).

**Immunoprecipitation and Immunoblotting**—Primary antibodies against the N-terminal region of α-synuclein (MDV2), the NAC domain (EQV1), and the C-terminal region of α-synuclein (PQE3), and β-synuclein-specific antibody (REE1) were described previously (9, 10). Stringent immunoprecipitation was performed using a fraction of rat brain in radioimmunoprecipitation buffer (50 mM PBS, pH 7.4, 1 mM EGTA, 1% Nonidet P-40, and 0.5% sodium deoxycholate). Antibodies PQE3, EQV1, and anti-α-tubulin (mouse monoclonal clone DM 1A, Sigma) were added and incubated at 4 °C for 2 h. Antigen-antibody complexes were collected with protein A-Sepharose (Sigma). The beads were washed five times by centrifugation with 1% Triton X-100 in PBS and then boiled for 5 min in SDS-sample buffer. Following SDS-PAGE and immunoblotting, the signals were detected with chemiluminescence reagents (PerkinElmer Life Sciences) and Hyper-ECL film (Amersham Biosciences) as described (9, 10). Anti-β-tubulin (clone TUB 2.1, Sigma), TAU-2 (Neomarkers), and anti-MAP5 (identical to MAP1b) (Chemicon International) antibodies were also employed. It was confirmed that EQV1 and PQE3 did not cross react with tubulin, whereas PQE3 and finally with 5-nm diameter immunogold conjugate with anti-rabbit IgG (1:80, BIOSOURCE). Antibodies used were anti-α-tubulin (1:200). To prove the binding or association of α-synuclein with α-tubulin, we subjected human brain proteins to α-synuclein affinity column chromatography. Employing a recombinant α-synuclein column, several proteins were isolated as possible α-synuclein-binding/associated proteins (Fig. 1a). After purification of the α-synuclein bound to the α-synuclein column by a reversed phase HPLC, several proteins were determined from their amino acid sequences. One of them with an apparent molecular weight of 50 kDa (Fig. 1a, arrowhead) was sequenced as MRE-CISHVG for the N terminus, and VQINYPPTVPGGDLAK for a peptide from the Achromobacter lyticus protease digest. In a data base search (SWISS-PROT), both sequences were found to belong to human α-tubulin. To confirm the interaction between α-synuclein and α-tubulin, immunoprecipitation was carried out, and α-tubulin was co-precipitated with endogenous α-synuclein in the cytoplasmic fraction of the rat brain (Fig. 1b) and vice versa (Fig. 1c).

**RESULTS**

**Tubulin Is a Binding/Associated Protein of α-Synuclein**—To detect molecules that interact with α-synuclein in the human brain, we subjected human brain proteins to α-synuclein affinity column chromatography. Employing a recombinant α-synuclein column, several proteins were isolated as possible α-synuclein-binding/associated proteins (Fig. 1a). After purification of the α-synuclein bound to the α-synuclein column by a reversed phase HPLC, several proteins were determined from their amino acid sequences. One of them with an apparent molecular weight of 50 kDa (Fig. 1a, arrowhead) was sequenced as MRE-CISHVG for the N terminus, and VQINYPPTVPGGDLAK for a peptide from the Achromobacter lyticus protease digest. In a data base search (SWISS-PROT), both sequences were found to belong to human α-tubulin. To confirm the interaction between α-synuclein and α-tubulin, immunoprecipitation was carried out, and α-tubulin was co-precipitated with endogenous α-synuclein in the cytoplasmic fraction of the rat brain (Fig. 1b) and vice versa (Fig. 1c). β-Tubulin was also co-precipitated using anti-α-synuclein antibodies (not shown), indicating the interaction of α-synuclein with β-tubulin heterodimers.

**α- and β-Synucleins Are Associated with Microtubules**—To prove the binding or association of α-synuclein to microtubules, we purified microtubules from rat brains by two cycles of polymerization and depolymerization (17, 18). First, the presence of α-synuclein, β-tubulin, tau, and MAP2 (MAP1b) was confirmed in the purified microtubules by immunoblot analyses (Fig. 2a). α-Synuclein was also detected in the same sample with three kinds of anti-α-synuclein antibodies (MDV2, EQV1, and PQE3) that interact with epitopes covering the entire α-synuclein molecule (Fig. 2, b and c). Doublet-like bands with molecular mass of about 19 kDa were detected with MDV2 (anti-α- and β-synuclein), and a band corresponding to the upper one was...
Tubulin Seeds α-Synuclein Fibrillogenesis

To test this potential, microtubule assembling cofactors, such as MgCl₂ and GTP, were added in a separate reaction at minimal concentrations (the commonly used concentrations times 10⁻³), and the fibril formation was found to be accelerated by these cofactors (Fig. 4, a and b). α-Synuclein was assembled into fibrillar structures only in the presence of 1 μM tubulin as shown by light scattering (Fig. 4, a–c) and by EM (Fig. 4, d–f). Over the course of several hours filaments 10 nm in diameter and about 1 μm in length were formed (Fig. 4d). α-Synuclein fibrils thus produced were examined by immuno-EM with anti-α-synuclein antibodies MDV2 (not shown), EQV1 (Fig. 4e), and PQE3 (not shown), which were previously used to study LBs in Parkinson’s disease (9) and GCIIs in MSA (10). These filaments were also decorated with anti-α-tubulin (Fig. 4f) and anti-β-tubulin (not shown), but not with negative controls, such as anti-β-synuclein REE1 (not shown) or anti-αβ (not shown). The dimensions and morphology of these structures are similar to those of α-synuclein filament in the brain sections of Parkinson’s disease (9) (Fig. 4g). α-Synuclein filament were produced only in the presence of 1 μM tubulin (Fig. 4, a–d) not in solutions containing α-synuclein alone (Fig. 4c, C1) or in other controls (Fig. 4c, C2, C3, and C4). It is notable that the additives alone were not able to stimulate production of α-synuclein fibrils (not shown) but enhanced the fibril production in the presence of tubulin. The morphology of the fibrils produced in the presence of tubulin with additives was identical (not shown) to the morphology of fibrils produced without additives (Fig. 4, d–f). These results indicate that tubulin is able to induce and promote α-synuclein fibril formation under physiological conditions in vitro.

**Tubulin Co-localizes with Pathological Structures of α-Synuclein**—We subsequently investigated the in vivo co-localization of tubulin and α-synuclein in LBs in the dorsal motor nucleus of the vagus nerve of a patient with Parkinson’s disease and cervical sympathetic ganglia of that with DLB, as well as in GCIIs of that with MSA using double labeling immunofluorescence methods. The rhodamine-labeled anti-α-synuclein antibody (PQE3) decorated LBs, pale bodies, Lewy-related neurites, and GCIIs intensely (Fig. 5, a, d, and g). FITC-tagged anti-α-tubulin stained these structures as well (Fig. 5, c, f, and i). These signals overlapped completely or partially when merged (Fig. 5, b, e, and h). β-Tubulin was also detected in these pathological structures by a similar method (not shown).

**DISCUSSION**

The discovery of two missense mutations in the α-synuclein gene in certain autosomal dominant familial Parkinson’s disease pedigrees (6, 7) suggested a pivotal role of α-synuclein in the onset and progression of Parkinson’s disease. This has been substantiated by the following findings. (i) Anti-α-synuclein antibodies detect the major filamentous component of LBs, Lewy-related neurites, and pale bodies (a possible early phase of LB-formation) (9) in Parkinson’s disease and DLB (8, 9) and LB variant of Alzheimer’s disease (23, 24). (ii) Insoluble α-synuclein filaments are recovered from DLB brains (25). (iii) Recombinant α-synuclein forms LB-like filaments (26), and familial Parkinson’s disease mutations accelerate the fibril formation (13, 14). Because familial Parkinson’s disease patients are rare, unknown epigenetic factors may induce fibril formation of wild-type α-synuclein, leading to pathological structures such as LBs. In this study, we searched for potential intrinsic factors that may initiate and/or promote fibril formation of α-synuclein using an α-synuclein column, and we found that tubulin was one of the α-synuclein-binding/associated proteins. Co-localization of tubulin with α-synuclein in LBs and other pathological structures suggested that tubulin might be such a factor.

Employing affinity column chromatography using recombi-
FIG. 3. Electron microscopic immuno-negative staining of purified rat microtubules. α–f, the rat microtubules were incubated with different primary antibodies, and the immunoreactions were visualized using 10-nm immunogold particles. Anti-α-tubulin (α), TAU-2 (b), MDV2 (c), PQE3 (d), and REE1 (e) labeled the microtubules, but anti-Aβ did not (f). Bar represents 100 nm.

FIG. 4. A small amount of tubulin can initiate and promote fibril formation of α-synuclein under physiological conditions in vitro. Solutions of α-synuclein (0–700 μM) in 25 μl of PBS were incubated at 37 °C with a fixed concentration (1 μM) of tubulin. In a separate reaction, 1 μM each MgCl₂ and GTP were used as cofactors to induce formation of oligomers of tubulin (see text). Control experiments were performed by incubating either α-synuclein or tubulin alone, with or without BSA (300 or 1 μM). The ordinate represents the relative amount of α-synuclein aggregates shown as A₄₀₀ nm, and the abscissa in a represents time, and in b and c the concentration of α-synuclein. The value represents the average of three determinations. a, the amount of α-synuclein aggregates was measured by quasi-elastic light scattering methods using 1 μl of each reaction mixture at 400 nm with a spectrophotometer (Hitachi, Gene Spec III). α-Synuclein and tubulin at a molar ratio of 300:1 in the presence or absence of cofactors, including four controls (C1–C4), were incubated. The light scattering was monitored at various times (0, 6, 12, 24, 48, 72, and 96 h). The filamentous structures began to form after 6 h of incubation, and this formation steadily increased and reached a plateau in 3 days. b, different concentrations of α-synuclein containing 1 μM tubulin were incubated for 1 week and the light scattering (A₄₀₀ nm) was determined as described in a. The highest amount of assembly was observed in the presence of cofactors (CFs). The amount of aggregates was proportional to the concentration of α-synuclein. In a and b, an open square represents four kinds of controls that were completely flat as shown in c. c, bar graphs of the data obtained in the experiment described in b. Tubulin can promote the fibrillogenesis of α-synuclein (open columns), and the presence of cofactors accelerates the process (gray columns). Controls; C1: 300 μM α-synuclein; C2: 1 μM tubulin; C3: 300 μM α-synuclein + 1 μM BSA; C4: 300 μM BSA + 1 μM tubulin. Different concentrations (100–700 μM) of α-synuclein plus 1 μM tubulin. d, an electron micrograph of representative filamentous aggregates of this polymerization reaction. A number of long, 10-nm-wide, amyloid-like filaments were formed. Bar represents 100 nm. e and f, isolated filaments immunolabeled with EQV1 and anti-α-tubulin antibodies, respectively. Bar represents 100 nm. g, filaments of Lewy bodies are 9–10 nm in diameter in post-embedding EM, labeled with PQE3 and probed by 5-nm diameter immunogold particles.
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FIG. 5. α-Tubulin co-localizes with α-synuclein in cytoplasmic inclusions. A double labeling immunofluorescence study of brain tissues demonstrated co-localization of α-tubulin and α-synuclein (PQES) epitopes. a, d, and g, PQES is labeled with rhodamine and shown in red. c, f, and i, α-tubulin is detected by the FITC-tagged secondary antibody and shown in green. b, e, and h, when the two signals are merged, the overlap is indicated by yellow signals. Dual immunolabeling was demonstrated in Lewy bodies (arrows), pale bodies (double arrows), Lewy-related neurites (arrowheads) in the dorsal motor nucleus of the vagus nerve of a patient with Parkinson’s disease (α-c), and in the cervical sympathetic ganglia of that with dementia with Lewy bodies (d-f), as well as in glial cytoplasmic inclusions (arrows) of that with multiple system atrophy (g-i). Bar represents 10 µm.

...related neurites, and GCIs in the central and peripheral nervous systems. These results indicate that tubulin co-localizes with pathological structures of α-synuclein in Parkinson’s disease, DLB, and MSA.

Tubulin may be a physiological binding partner of α-synuclein. These results suggest that tubulin may be a physiological binding partner of α-synuclein. Further studies will be needed to know the physiological role of α-synuclein and tubulin in association with tubulin. Because the brain is a rich source of tubulin, we speculate that the interaction between α-synuclein and free tubulin may occur in vivo and that under pathological conditions tubulin might initiate and promote the fibril formation of α-synuclein, leading to pathological structures such as LBs. To test this hypothesis we performed in vitro studies, which showed that α-synuclein fibril formation was in fact initiated and promoted by tubulin. Most importantly, these α-synuclein fibrils were produced only in the presence of a small amount of tubulin under physiological conditions. Immuno-EM studies revealed that the dimensions and morphology of these fibrils made in vitro closely resembled those of the α-synuclein filaments observed in autopsied brain sections of Parkinson’s disease and were obviously different from those of microtubules. These results indicate that tubulin is capable of seeding the fibril formation of α-synuclein.

We then investigated the in vivo co-localization of tubulin and α-synuclein in autopsied brains using double labeling immunofluorescence, and we showed that both α-synuclein and α-β tubulin epitopes are co-localized in LBs, pale bodies, Lewy-related neurites, and GCIs in the central and peripheral nervous systems. These results indicate that tubulin co-localizes with pathological structures of α-synuclein in Parkinson’s disease, DLB, and MSA.

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Tubulin is a well-characterized MAP, and abnormal aggregates of tau, or tau pathology, are thought to be an early event in the development of neurofibrillary lesions (27, 28). The C terminus of α-synuclein is reported to be reactive with the microtubule binding region of tau (29) and MAP5 (MAP1b) (22). In our in vitro experiments (Fig. 4) purified tubulin produced α-synuclein fibrils in the absence of tau and MAP5 (MAP1b). More importantly, tau does not co-localize with α-synuclein in LBs in general (30), but tubulin does as shown in Fig. 5, suggesting that the interaction between α-synuclein and tubulin may not be mediated by tau.

The precise mechanism of neurodegeneration in Parkinson’s disease brain is unknown. Accumulating evidence suggests that the aggregation of α-synuclein may play a critical role in the pathogenesis of Parkinson’s disease (31), although the mechanisms by which α-synuclein is preferentially aggregated in the Parkinson’s disease brain remained elusive. As shown here, tubulin is apparently able to initiate the polymerization of α-synuclein, resulting in the formation of α-synuclein fibrils, which may eventually lead to pathological structures such as LBs in diseased brains. Thus, it is possible that some epigenetic elements (e.g. drugs, chemicals, additives in food, or environmental toxins) may affect the assembly/disassembly equilibrium of microtubules. Abnormally increased free tubulin thus produced may trigger α-synuclein fibril formation. If so, those microtubule-disrupting elements can be risk factors for α-synuclein-associated degenerative diseases such as Parkinson’s disease, DLB, MSA, and LB variant of Alzheimer’s disease. Further investigations of the role of tubulin in the aggregation of α-synuclein will be needed to clarify the mechanisms of neurodegeneration in α-synuclein-related diseases.

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Tubulin Seeds α-Synuclein Fibril Formation
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