Association of the Cytoskeletal GTP-binding Protein Sept4/H5 with Cytoplasmic Inclusions Found in Parkinson’s Disease and Other Synucleinopathies

Received for publication, February 6, 2003, and in revised form, April 11, 2003
Published, JBC Papers in Press, April 14, 2003, DOI 10.1074/jbc.M301352200

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α-Synuclein-positive cytoplasmic inclusions are a pathological hallmark of several neurodegenerative disorders including Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy. Here we report that Sept4, a member of the septin protein family, is consistently found in these inclusions, whereas five other septins (Sept2, Sept5, Sept6, Sept7, and Sept8) are not found in these inclusions. Sept4 and α-synuclein interact also in immunoprecipitated from normal human brain lysates. When co-expressed in cultured cells, FLAG-tagged Sept4 and Myc-tagged α-synuclein formed detergent-insoluble complex, and upon treatment with a proteasome inhibitor, they formed Lewy body-like cytoplasmic inclusions. The tagged Sept4 and α-synuclein synergistically accelerated cell death induced by the proteasome inhibitor, and this effect was further enhanced by expression of another Lewy body-associated protein, synphilin-1, tagged with the V5 epitope. Moreover, co-expression of the three proteins (tagged Sept4, α-synuclein, and synphilin-1) was sufficient to induce cell death. These data raise the possibility that Sept4 is involved in the formation of cytoplasmic inclusions as well as induction of cell death in α-synuclein-associated neurodegenerative disorders.

Parkinson’s disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are neurodegenerative disorders characterized by the formation of similar pathognomonic inclusions in the cytoplasm: namely, Lewy bodies (LBs) in PD and DLB (1–3), and glial cytoplasmic inclusions (GCIs) in MSA (4, 5). These inclusions contain α-synuclein as the major component of the filamentous aggregates. Hence these diseases are classified as synucleinopathies (6).

The importance of α-synuclein in neurodegeneration has been established by the findings that missense mutations in the α-synuclein gene cause familial PD (7, 8) and that transgenic animals overexpressing α-synuclein or its mutants (A30P or A53T) show some phenotypes resembling PD (9–12). LBs also contain ubiquitin (13) and ubiquitin carboxyl-terminal hydrolase-L1 (14), which may be involved in the pathogenesis of PD. Genetic studies on autosomal-recessive juvenile parkinsonism (AR-JP) led to the identification of the responsible gene, parkin, which encodes ubiquitin-protein isopeptide ligase (E3) (15). Loss-of-function mutations in parkin result in the accumulation of its substrates such as synphilin-1 (16), O-glycosylated α-synuclein (17), and Pael receptor (18) and cause neuronal damage without formation of visible inclusions. Synphilin-1 was first identified as an α-synuclein-binding protein (19) and was found to accumulate in LBs (20). Thus, impairment in the ubiquitin-proteasome system and accumulation of α-synuclein and other proteins in the neuronal cytoplasm may represent common molecular mechanisms underlying PD and AR-JP.

Septins are a family of filament-forming guanine nucleotide-binding proteins involved in cytokinesis, exocytosis, and other cellular processes (21–24). At least 10 septin genes can form co-polymers, their differential expression patterns in mammalian brains indicate some functional diversity among septins (26). Three septins (Sept1, Sept2, and Sept4) are accumulated in tau-based filamentous deposits known as neurofibrillary tangles and glial fibrils in Alzheimer’s disease (27). A splice variant of the Sept4 gene encodes a mitochondrial protein called ARTS (apoptosis-related protein in the transforming growth factor-β signaling pathway), which mediates a pro-apoptotic signal (28). These data support the hypothesis that accumulation of a class of septins may accelerate neurodegeneration. In addition, Sept5 was found to be a substrate of Parkin (29). Sept5 is associated with γ-aminobutyric acidergic synaptic vesicles (26), and excessive expression of Sept5 was found to interfere with regulated exocytosis (22). Thus, accumulation of Sept5 may affect dopamine release in the nigrostriatal system in AR-JP (29, 30).

In this study, we have conducted an immunohistochemical examination of brain tissues from patients afflicted by Parkinson’s disease or two other synucleinopathies. We found that Sept4 was co-localized with α-synuclein in LBs/GCIs in all cases. We also analyzed the physical and functional interaction among Sept4, α-synuclein, and synphilin-1 in cultured cells. Our data suggest that these proteins are involved in the formation of cytoplasmic inclusions as well as induction of cell death.
EXPERIMENTAL PROCEDURES

Tissues—Postmortem brain samples were obtained from five patients with PD (age: range, 68–79 years; mean, 74.6 years), two with DLB (65 and 69 years), five with MSA (71–78 and 75.0 years), and four with non-neurological diseases (68–81 and 74.5 years) from the Department of Neurology, Kyoto University Hospital. The brain specimens were used for neuropathological investigation after informed consent was obtained from the patients’ relatives. The diagnoses of PD, DLB (pure form), and MSA were established on the basis of clinical and neuropathological data according to widely accepted criteria (31–33). After fixation with 4% paraformaldehyde, tissues were dissected. The midbrain and cerebellum were embedded in paraffin and sliced (6 μm in thickness). The frontal lobe, pons, caudatoputamen, and thalamus were freeze-sectioned (20 μm). Mouse brains were obtained from adult male C57Bl/6 mice (25–30 g) after deep anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal) and transcardial perfusion with phosphate-buffered saline (PBS), using procedures approved by the Animal Use and Care Committee of Kyoto University.

Anti-septin Antibodies, Immunoblot Assay, and Immunohistochemistry—The following polyclonal antibodies were generated as described elsewhere (host animal and antigen are given in parentheses) (21, 26, 27): H5C-2 (rabbit; oligopeptide corresponding to residues 466–478 of mouse Sept4), CRC-3 (guinea pig; residues 358–369 of human Sept5), C10C-3 (guinea pig; residues 400–418 of human Sept7), N5N-1 (rabbit; residues 1–18 of mouse/human Sept2), S6C-3 (guinea pig; residues 413–427 of mouse/human Sept6), and STC-1 (rabbit; residues 415–429 of human Sept8). The authenticity and specificity of these antibodies were confirmed by antigen adsorption experiments and in some cases by co-immunoprecipitation using cells overexpressing the corresponding gene. Human and mouse brain homogenates (50 μg) were analyzed by standard immunoblot assays (26, 27) using peroxidase-conjugated secondary antibodies and an ECL chemiluminescence kit (Amersham Biosciences). Anti-septin antibodies (see above) or goat anti-a-synuclein antibodies (Santa Cruz Biotechnology) were used as the primary antibodies. Brain sections were stained by immunoperoxidase techniques using Vectastain ABC kits (Vector) with diaminobenzidine tetrahydrochloride as a chromogen or by immunofluorescent staining procedures using fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies (Molecular Probes).

Plasmids—The coding region of human a-synuclein cDNA was cloned in-frame between the EcoRI and XhoI sites of the pCMV-Myc vector (Clontech) to express a-synuclein tagged with the Myc epitope at its amino terminus (Myc-a-syn). The coding region of mouse Sept4 cDNA was cloned between the EagI and SalI sites of the pFLAG-CMV-2 vector (Sigma) to express Sept4 tagged with FLAG epitope at its amino terminus (FLAG-Sept4). The coding region of Sept2 was cloned between the XhoI and XbaI sites of the same vector to express Sept4 tagged with MYC epitope at its amino terminus (Myc-Sept4/H9251). Two mouse cell lines, NIH3T3 and N18, were transiently transfected with three plasmids (each expressing Myc-a-syn, FLAG-Sept4, or V5-synphilin-1) in various combinations were incubated in the absence or presence of 10–50 μM lactacystin for 24 h and immunostained as described previously (21). Mouse monoclonal antibodies against Myc (Sigma), V5 (Invitrogen), or FLAG or rabbit polyclonal antibodies against a-synuclein, FLAG (Sigma), or ubiquitin (Sigma) were used as primary antibodies. Fluorescein isothiocyanate- or rhodamine-conjugated anti-mouse IgG or anti-rabbit IgG was used as secondary antibodies. Dead cells were scored after brief staining with 0.2% trypan blue (Invitrogen) or with 1.0 μg/ml annexin V-enhanced green fluorescent protein (Clontech) plus 2.5 μg/ml propidium iodide (Clontech).

Ubiquitination Assay—NIH3T3 cells (~5 × 10^4) transfected with 1 μg of FLAG-Sept4 expression vector were incubated in growth medium for 24 h and then in medium with or without 20 μM lactacystin for an additional 16 h. Fractionated cell lysates were analyzed directly, or after immunoprecipitation with anti-FLAG antibodies, by immunoblot assay using antibodies against ubiquitin or FLAG.

Statistics—Quantitative data were collected in at least three independent experiments. Significance of difference between data groups was assessed by Student’s t test using StatView II software (version 5.0 for Macintosh, SAS Institute). p values smaller than 0.05 were considered significant.

RESULTS

Expression of Six Septins in Human and Mouse Brains—We first examined the expression of six septins in the normal human frontal lobe and mouse whole brain by immunoblot assay using specific antibodies (Fig. 1). All six septins were detected in the human and mouse brains. The anti-Sept4 antibody (H5C-2) detected three major bands of 53, 51, and 49 kDa in the human brain; this antibody does not recognize the 32-kDa pro-apoptotic Sept4 variant, ARTS (28), because ARTS lacks the carboxy-terminal region of Sept4 where the epitope for H5C-2 resides.

Sept4 Is Commonly Associated with a-Synuclein-based Cytoplasmic Inclusions—Having established the expression of six

| MW (kDa) | Sept2 | Sept4 | Sept5 | Sept6 | Sept7 | Sept8 |
|---------|-------|-------|-------|-------|-------|-------|
| H       | M     | H     | M     | H     | M     | H     |
| 175.1   |       |       |       |       |       |       |
| 82.3    |       |       |       |       |       |       |
| 62.0    |       |       |       |       |       |       |
| 47.5    |       |       |       |       |       |       |
| 32.5    |       |       |       |       |       |       |
| 26.0    |       |       |       |       |       |       |
| 18.5    |       |       |       |       |       |       |
Sept4 neurons with Sept4-positive (yet none of them except Sept4 was found to be accumulated in examined are expressed in normal substantia nigra (26), and in normal control brains (Fig. 2, H–J). Immunostaining for Sept4 and α-synuclein was low in the corona of LB where staining was most intense (Fig. 2, D). However, Sept4 staining was strongly expressed in the core of LBs (Fig. 2, E). Double staining revealed that most of the Sept4-positive LBs in the cingulate cortex (Fig. 2, E) and the merged image (F). Scale bar = 100 μm (A and E), 40 μm (C, D, and H–J), and 20 μm (B, F, and G).

Our previous studies indicate that Sept4 is expressed in both neurons and glial cells (26). Consistent with this observation, we found Sept4-positive GCIs in several regions including the cerebellum,pons,caudatoputamen, and thalamus in brains with MSA (n = 5, Fig. 3). These findings demonstrate common and selective association of Sept4 with the inclusions in synucleinopathies.

Physical Interaction between Sept4 and α-Synuclein in the Brain and in Cultured Cells—To test the possible interaction between Sept4 and α-synuclein in vivo, human brain homogenate was immunoprecipitated with anti-HA (negative control), anti-α-synuclein, or anti-Sept4 antibodies and then subjected to immunoblot analysis with anti-α-synuclein antibodies (Fig. 4A). A fraction of α-synuclein was consistently pulled down with Sept4, indicating direct or indirect physical interaction between Sept4 and α-synuclein in vivo.

To analyze the molecular nature of this interaction, mouse cell lines (N18 neuroblastoma and NIH3T3 fibroblast) were co-transfected with a plasmid vector expressing α-synuclein tagged with Myc epitope (Myc-α-syn) and a second vector expressing either the normal Sept4, a mutant Sept4 (Sept4G154V), or the normal Sept2, each tagged with the FLAG epitope (Fig. 4, B and C). We chose to use short epitope tags (Myc and FLAG) to reduce the probability of generating insoluble fusion proteins, as has been reported for the green fluorescent protein tag (34). Sept4G154V has a missense mutation in its P-loop domain and shows reduced guanine nucleotide-binding activity (21, 28, 35). The transfected cells were lysed in a buffer containing 1% Nonidet P-40, and the subcellular components were separated into Nonidet P-40-soluble and -insoluble fractions. We subjected each fraction directly or after immunoprecipitation with anti-FLAG antibodies to the immunoblot assay using anti-Myc antibodies (to detect Myc-α-syn) or anti-FLAG antibodies (to detect FLAG-septins). From the Nonidet P-40-soluble fraction, Myc-α-syn co-precipitated with FLAG-Sept4 (Fig. 4B; compare lanes 4, 6, and 8) even though the total amount of Myc-α-syn in the sample of FLAG-Sept4G154V or FLAG-Sept2-transfected cells was comparable with, if not greater than, that in the sample of FLAG-Sept4-transfected cells (Fig. 4B, top panel; compare lanes 6 and 8 with lane 4). This may reflect the difference in the levels of FLAG-tagged septins present in these samples and/or the difference in their ability to be incorporated into the molecular complexes. Nevertheless, the wild type FLAG-Sept4 construct was the most efficient among the three septin constructs we tested in bring-
The amount of Myc-α-syn found in the Nonidet P-40-insoluble fraction was small when expressed alone (Fig. 4C, top panel, lane 2) but was increased when FLAG-Sept4 or FLAG-Sept4G154V was co-expressed (Fig. 4C, top panel, lanes 4 and 6); this effect was minimal when FLAG-Sept2 was co-expressed (Fig. 4C, top panel, lane 8). Interestingly, immunoprecipitable FLAG-tagged protein and the associated Myc-α-syn came to the Nonidet P-40-insoluble fraction only when FLAG-Sept4 and Myc-α-syn were co-expressed (Fig. 4C, bottom two panels, lane 4), suggesting that FLAG-Sept4 and Myc-α-syn become insoluble to Nonidet P-40 in a co-operative manner. In contrast, FLAG-Sept4G154V and FLAG-Sept2 failed to bring Myc-α-syn to the Nonidet P-40-insoluble fraction (Fig. 4C, bottom two panels, lanes 6 and 8). We obtained similar results with NIH3T3 cells (data not shown).

**Fig. 4.** Sept4 and α-synuclein form insoluble complexes. A, human brain homogenates were subjected to immunoprecipitation (IP) with anti-HA (negative control), anti-α-synuclein, or anti-Sept4 antibodies followed by immunoblot (IB) detection with anti-α-synuclein antibodies. B and C, co-immunoprecipitation of FLAG-Sept4 and Myc-α-synuclein (Myc-α-syn). Two mice neuroblastoma cells were co-transfected with the indicated expression vectors. After a 36-h incubation, the cells were lysed and fractionated with buffer containing 1% Nonidet P-40. The Nonidet P-40-soluble (S) and insoluble (P) fractions were subjected to immunoblot analysis using anti-FLAG (to detect FLAG-Sept4 or FLAG-Sept2) or anti-Myc antibodies (to detect Myc-α-syn). Note that the proteins in the Nonidet P-40-insoluble fractions were solubilized directly with SDS sample buffer in this experiment, which is different from the procedure employed in the experiments shown in Fig. 4 (see “Experimental Procedures” for more detail).

Myc-α-syn showed little effects on the solubility of FLAG-Sept4G154V or FLAG-Sept2 under these conditions (Fig. 5, compare panels 4, 5, and panels 7 and 8). Reciprocally, expression of FLAG-Sept4, but not FLAG-Sept4G154V or FLAG-Sept2, brought more Myc-α-syn into the insoluble fraction (Fig. 5, compare panels 3, 6, and 9). These data demonstrate the bidirectional nature of the interaction between FLAG-Sept4 and Myc-α-syn in bringing these proteins into the Nonidet P-40-insoluble fraction.

**Localization and Biological Activity of Overexpressed FLAG-Sept4 and Myc-α-syn in Cultured Cells**—To determine the subcellular localization of FLAG-Sept4 and Myc-α-syn, we performed immunofluorescent staining of transfected cells using anti-FLAG and anti-Myc antibodies (Fig. 6A). FLAG-Sept4 and Myc-α-syn were diffusely distributed in the cytoplasm with apparent accumulation and co-localization at the cortex or periphery of the cells (Fig. 6A, top row). After treatment with a proteasome inhibitor, lactacystin, a subset (~30%) of the cells contained punctate aggregates at the cell periphery and/or rods in the cytoplasmic protrusions (Fig. 6A, middle row). Interestingly, a fraction (~5%) of doubly transfected, lactacystin-treated cells contained large, double-positive structures reminiscent of LBs (Fig. 6A, bottom row). Such a structure was not found in cells expressing FLAG-Sept4 alone or Myc-α-syn alone. Aggregates were rare in the cells expressing Myc-α-syn alone (Fig. 6B), FLAG-Sept4 alone (~0.5%, data not shown), or Myc-α-syn plus FLAG-Sept4G154V or FLAG-Sept2 (~5%, data not shown).

Interestingly, a dye exclusion assay using trypan blue showed that the cells expressing both FLAG-Sept4 and Myc-α-syn were more sensitive to cytotoxicity by lactacystin as compared with the cells expressing one of the proteins alone or transfected with empty vectors (Fig. 6C). Separate experiments employing annexin V-enhanced green fluorescent protein and propidium iodide to stain dying cells also showed similar results (data not shown). Hence, FLAG-Sept4 and Myc-α-syn were co-operative in making cells vulnerable to the stress induced by proteasome inhibition.

The FLAG(Sept4)/Myc(α-syn) double-positive aggregates
Fig. 6. FLAG-Sept4 and Myc-α-syn synergistically induce aggregate formation and make cells vulnerable to the cytotoxic effects of lactacystin. A, NIH3T3 cells co-transfected with plasmids expressing FLAG-Sept4 and Myc-α-syn were incubated in the absence (−) or presence (+) of lactacystin (Lc) followed by immunostaining with anti-FLAG (red) or anti-Myc (green) antibodies. Merged images are also shown. Scale bar = 5 μm (upper six panels) and 7.5 μm (lower three panels). B, NIH3T3 cells co-transfected with a vector expressing Myc-α-syn plus either FLAG-vector (−) or a vector expressing FLAG-Sept4 (+) were treated with lactacystin. After a 24-h incubation and immunofluorescent staining with anti-Myc antibodies, the proportion of cells harboring fluorescent aggregates among the total of 800 Myc-α-syn-positive cells was scored. Error bars represent S.D. from three independent experiments. Scale bar = 20 μm. *, p = 0.0016 comparing cells expressing both Myc-α-syn and FLAG-Sept4 to those expressing Myc-α-syn alone. C, NIH3T3 cells co-transfected with the indicated expression vectors (−−− indicates empty vector) were treated with lactacystin for 24 h and then stained with trypan blue. The proportion of blue cells among the total of 1000 cells was scored under a phase-contrast microscope. Error bars represent S.D. from three independent experiments. *, p < 0.002 comparing cells co-transfected with Myc-α-syn/Sept4 and FLAG-Sept4 to those transfected with other indicated combinations. Scale bar = 100 μm. All experiments were repeated three times with similar results.

also contained ubiquitin (Fig. 7A), and ubiquitinated FLAG-Sept4 was found predominantly in detergent-insoluble fractions (Fig. 7, B–E). Thus, FLAG-Sept4 can be ubiquitinated; this may occur in insoluble molecular complexes, or once ubiquitinated, FLAG-Sept4 may rapidly be incorporated into such complexes.

We also generated and tested the activities of a Myc-α-syn expression plasmid harboring a missense mutation (A30P) that has been found in familial PD cases (8). Interestingly, the amounts of FLAG-Sept4 and Myc-α-syn were in the anti-FLAG-immunoprecipitates from Nonidet P-40-soluble fractions slightly but consistently greater when Myc-α-synA30P was used (Fig. 8A). Furthermore, cells co-expressing FLAG-Sept4 and Myc-α-syn were more sensitive to lactacystin toxicity than cells expressing FLAG-Sept4 plus wild type Myc-α-syn (Fig. 8C).

Effects of V5-Synphilin-1—It has been reported that synphilin-1 induces cytoplasmic inclusions when co-expressed with α-synuclein in HEK293 cells (19). When Myc-α-syn and synphilin-1 tagged with another oligopeptide epitope (V5) were co-expressed in NIH3T3 cells, α-syn/V5 double-positive cytomembrane inclusions were found slightly but consistently greater when Myc-α-synA30P was used (Fig. 8A). Furthermore, cells co-expressing FLAG-Sept4 and Myc-α-syn were more sensitive to lactacystin toxicity than cells expressing FLAG-Sept4 plus wild type Myc-α-syn (Fig. 8C).
An important finding in this study is that Sept4 is accumulated in cytoplasmic inclusions found in three major synucleinopathies, PD, DLB, and MSA. In nigral LBs, Sept4 is found predominantly in the central cores and α-synuclein in the peripheral portions. Possible explanations for this finding include: (i) indirect association between Sept4 and α-synuclein; (ii) selective association of one of the proteins with a subset of the other due, for instance, to differential modification or conformational change; and (iii) epitope masking in specific area of LBs. In cultured cells, FLAG-Sept4 and Myc-α-syn were cooperatively incorporated into the Nonidet P-40-insoluble fraction, and upon proteasome inhibition or expression of V5-synphilin-1, they participated in the formation of LB-like cytoplasmic inclusions and promotion of cell death. Taken together, our findings in vivo and in vitro support the idea that endogenous Sept4 and α-synuclein are actively involved in LB formation and promotion of cell death. Furthermore, our finding that pale body-like cytoplasmic aggregates did not contain V5-synphilin-1 (Fig. 9) suggests that interaction between Sept4 and α-synuclein may be an early event during LB formation. In fact, a recent report indicates that synphilin-1 is negative in pale bodies (36).

Results with overexpression of tagged proteins in cultured cells cannot be directly extrapolated to the pathological conditions in the brain. Such experiments in vitro, however, have their own advantages, and if supported by pathological evidence, they may shed light on the role of particular molecules in human disorders. The most obvious and important limitation of pathological evidence is that it completely depends on the availability of samples and that it is always retrospective. Furthermore, immunohistological evidence heavily depends on the quality of the antibodies used; thus the possibility of cross-reaction or epitope masking is often hard to exclude. Antibodies against epitope tags (e.g. FLAG, Myc, and V5) are high in affinity and specificity, so that clear-cut experimental evidence can be obtained. Although the effects of the extra peptide on the conformation of tagged protein cannot be completely excluded, we found no evidence that the tagged proteins tended to be insoluble when expressed alone. Our immunohistochemical evidence (Figs. 2 and 3) as well as the data obtained with specific antibodies (Fig. 4A) make it further unlikely that the observed interactions between the tagged proteins represent mere artifacts.

The primary structure of Sept4 is unique among septins in that it has an extended amino-terminal region (37). The largest form of Sept4 (478 amino acid residues) is more than 100 residues longer than Sept5 (369 residues). This extended region, which shares no homology with any other protein in the current data base, may confer unique biochemical properties on Sept4. In contrast, the remaining portion of Sept4 is highly homologous (78% identical) to Sept5, a known target for Parkin (29, 30). Our data indicate that upon proteasome inhibition, Myc-α-syn and ubiquitinated FLAG-Sept4 accumulate in the detergent-insoluble fraction (Fig. 7), which probably corresponds, at least in part, to the visible cytoplasmic inclusions (Fig. 6). Whether the FLAG-Sept4 ubiquitination in this system is mediated by endogenous Parkin or by some other ubiquitin-protein ligase remains to be clarified.

Sept4 and synphilin-1 share three properties in common: they can be co-immunoprecipitated with α-synuclein (19), ubiquitinated by Parkin (16) or unknown ubiquitin ligase, and concentrated in the LB cores (20). In the cell culture model, we demonstrated that FLAG-Sept4 and V5-synphilin-1 can co-localize in the Myc-α-syn-based cytoplasmic inclusions and that the three proteins can synergistically exert cytotoxicity. The relationship between inclusion body formation and cell death in synucleinopathies is currently unknown, but we can envisage two possibilities: 1) Sept4, α-synuclein, and synphilin-1 syner-

![Fig. 8. Co-immunoprecipitation of Sept4 and wild type or A30P α-synuclein.](image-url)
Fig. 9. FLAG-Sept4 cooperates with Myc-α-syn and V5-synphilin-1 in inducing inclusions and cell death. A, NIH3T3 cells co-transfected with plasmids expressing Myc-α-syn and V5-synphilin-1 (V5-sph) were immunostained with anti-α-synuclein (red) or anti-V5 antibodies (green). The merged image is also shown. Scale bar = 5 μm. B, NIH3T3 cells transfected with plasmids expressing V5-synphilin-1, Myc-α-syn, and FLAG-Sept4 were immunostained with anti-α-synuclein (red), anti-V5 (green), or anti-FLAG (H9262, red). Merged images are also shown. Scale bar = 5 μm. C, NIH3T3 cells transfected with the indicated expression vectors (+ indicates empty vector) were treated with lactacystin (LC) for 24 h and then stained with trypan blue. The proportion of blue cells among the total of 1000 cells was scored under a microscope. Error bars represent S.D. from three independent experiments. Differences were significant (p < 0.05) between the following data sets: bars 1 versus 5, 1 versus 7, 1 versus 9, 2 versus 4, 2 versus 6, 2 versus 8, 4 versus 8, and 8 versus 10. All experiments were repeated three times with similar results.

ACKNOWLEDGMENTS—We thank S. Tanaka for NACP/α-synuclein cDNA, K. Kato for H5/Sept4 cDNA, P. J. McLean for synphilin-1 cDNA, E. Nishimoto and H. Nakabayashi for technical assistance, and M. Fukuda and A. Miyazaki for secretarial assistance. We also thank B. T. Hyman and D. B. Alexander for the critical review of our manuscript.

REFERENCES

1. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) Nature 388, 839–840
2. Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998) Am. J. Pathol. 152, 879–884
3. Irizarry, M. C., Crowdon, W., Gomeza-Isla, T., Newell, K., George, J. M., Clayton, D. F., and Hyman, B. T. (1998) J. Neurochem. Exp. Neurol. 57, 334–337
4. Tu, P. H., Galvin, J. E., Baba, M., Giasson, B., Tomita, T., Leight, S., Nakajo, S., Iwatsubo, T., Trojanowski, J. Q., and Lee, V. M. (1998) Ann. Neurol. 44, 415–422
5. Spillantini, M. G., Crowther, R. A., Jakes, R., Cairns, N. J., Lantos, P. L., and Goedert, M. (1998) Neurobiol. Aging 19, 253–260
6. Galvin, J. E., Lee, V. M., and Trojanowski, J. Q. (2006) Arch. Neurol. 53, 186–190
7. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubinstein, J., Breyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvivier, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
8. Krueger, R., Kuhn, W., Muller, T., Woltz, D., Gierbrecht, M., Kosel, S., Przuntek, H., Epplen, J. T., Schulz, L., and Riess, O. (1998) Nat. Genet. 18, 106–108
9. Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) Science 287, 1265–1269
10. Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2002) Neuron 34, 521–533
11. Lee, M. K., Stirling, W., Xu, X., Xu, Q., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8968–8973
12. Neumann, M., Kahle, P. J., Giasson, B. I., Ozmen, L., Borroni, E., Sporen, W., Muller, V., Ocky, S., and Trojanowski, J. Q., Kretzschmar, H. A., and Haass, C. (2002) J. Clin. Investig. 110, 1429–1439
13. Kurahara, S., Mori, H., Iizumiya, N., Yoshihara, M., and Ibara, Y. (1998) Acta Neuropathol. 75, 345–353
14. Lowe, J., McDermott, H., Landon, M., Mayer, R. J., and Wilkinson, K. D. (1990) J. Pathol. 161, 153–160
15. Shimura, H., Hattori, N., Kudo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) Nat. Genet. 25, 302–305
16. Zhang, K. K., Zhang, Y. L., Lim, K. L., Tanaka, Y., Huang, H., Gao, J., Ross, C. A., Dawson, L. V., and Dawson, T. M. (2001) Nat. Med. 7, 1144–1150
17. Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K. S., and Selkoe, D. J. (2001) Science 293, 263–269
18. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001) Cell 105, 891–902
19. Engelen, S., Kaminsky, Z., Guo, X., Sharp, A. H., Amaravadi, R. K., Kleider, J. J., Margolis, R. L., Troncoso, J. C., Lanahan, A. A., Warley, P. F., Dawson, L. V., Dawson, T. M., and Ross, C. A. (1999) Nat. Genet. 22, 110–114
20. Wakabayashi, K., Engelen, S., Yoshimoto, M., Tsuchi, S., Ross, C. A., and Takahashi, H. (2000) Ann. Neurol. 47, 521–523
21. Kinoshita, M., Kumar, S., Mizoguchi, A., Ide, C., Kinoshita, A., Haraguchi, T., Hiraoaka, Y., and Noda, M. (1997) *Genes Dev.* 11, 1535–1547

22. Beites, C. L., Xie, H., Bowser, R., and Trimble, W. S. (1999) *Nat. Neurosci.* 2, 434–439

23. Field, C. M., and Kellogg, D. (1999) *Trends Cell Biol.* 9, 387–394

24. Kinoshita, M., Field, C. M., Coughlin, M. L., Straight, A. F., and Mitchison, T. J. (2002) *Dev. Cell* 3, 791–802

25. Macara, I. G., Baldarelli, R., Field, C. M., Glotzer, M., Hayashi, Y., Hsu, S. H., Kennedy, M. B., Kinoshita, M., Longtine, M., Low, C., Maltais, L. J., McKenzie, L., Mitchison, T. J., Nishikawa, T., Noda, M., Petty, E. M., Pener, M., Pringle, J. R., Robinson, P. J., Roth, D., Russell, S. E., Stuhlmann, H., Tanaka, M., Tanaka, T., Trimble, W. S., Ware, J., Zeleznik-Le, N. J., and Zieger, B. (2002) *Mol. Biol. Cell* 13, 4111–4113

26. Kinoshita, A., Noda, M., and Kinoshita, M. (2000) *J. Comp. Neurol.* 428, 223–239

27. Kinoshita, A., Kinoshita, M., Akiyama, H., Tomimoto, H., Akiguchi, I., Kumar, S., Noda, M., and Kinoshita, M. (2000) *Am. J. Pathol.* 153, 1551–1560

28. Larisch, S., Yi, Y., Lotan, R., Kerner, H., Eimerl, S., Tony Parks, W., Gottfried, Y., Birkey Reffey, S., de Caestecker, M. P., Danielpour, D., Book-Melamed, N., Timberg, R., Duckett, C. S., Lechleider, R. J., Steller, H., and Schlesinger, M. (2000) *J. Cell Biol.* 150, 915–921

29. Zhang, Y., Gao, J., Chung, K. K., Huang, H., Dawson, V. L., and Dawson, T. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 13354–13359

30. Chung, K. K., Dawson, V. L., and Dawson, T. M. (2001) *Trends Neurosci.* 24, Suppl. 1, 87–914

31. Gelb, D. J., Oliver, E., and Gilman, S. (1999) *Arch. Neurol.* 56, 33–39

32. McKeith, I. G., Perry, E. K., and Perry, R. H. (1999) *Neurology* 53, 902–905

33. Gilman, S., Low, P. A., Quinn, N., Albanese, A., Ben-Shlomo, Y., Fowler, C. J., Kaufmann, H., Klockgether, T., Lang, A. E., Lantos, P. L., Litvan, I., Mathias, C. J., Oliver, E., Robertson, D., Schatz, I., and Wenning, G. K. (1998) *J. Auton. Nerv. Syst.* 74, 189–192

34. Melean, P. J., Kawamata, H., and Hyman, B. T. (2001) *Neuroscience* 104, 901–912

35. Zhang, J., Kong, C., Xie, H., McPherson, P. S., Grinstein, S., and Trimble, W. S. (1999) *Curr. Biol.* 9, 1458–1467

36. Wakabayashi, K., Engelender, S., Tanaka, Y., Yoshimoto, M., Mori, F., Tsuji, S., Ross, C. A., and Takahashi, H. (2002) *Acta Neuropathol.* 103, 209–214

37. Katz, K. (1990) *Eur. J. Neurosci.* 2, 704–711

38. Takahashi, H., Ohama, E., Suzuki, S., Horikawa, Y., Ishikawa, A., Morita, T., Tsuji, S., and Ikuta, F. (1994) *Neurology* 44, 437–441

39. Xu, J., Kao, S. Y., Lee, F. J., Song, W., Jin, L. W., and Yankner, B. A. (2002) *Nat. Med.* 8, 600–606

40. Rideout, H. J., Larsen, K. E., Sulzer, D., and Stefani, L. (2001) *J. Neurochem.* 78, 899–908

41. Masliah, E., Rockenstein, E., Weinbergs, I., Sagara, Y., Mallory, M., Hashimoto, M., and Mucke, L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 12245–12250

42. Schlossmacher, M. G., Frosch, M. P., Gai, W. P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharron, R., Hattori, N., Langston, J. W., Mizuno, Y., Hymam, B. T., Selkoe, D. J., and Kosik, K. S. (2002) *Am. J. Pathol.* 160, 1655–1667

43. Gearing, M., Junco, J. L., Proccacio, V., Gutekunst, C. A., Marinorodriguez, E. M., Gyure, K. A., Oso, S., Santuani, B., Krawieccki, N. S., Wallace, D. C., and Wainer, B. H. (2002) *Ann. Neurol.* 52, 465–476

44. Minamide, L. S., Striegl, A. M., Boyle, J. A., Meberg, P. J., and Bamberg, J. R. (2000) *Nat. Cell Biol.* 2, 629–636

45. Perl, D. P., Olanow, C. W., and Calne, D. (1998) *Ann. Neurol.* 44, Suppl. 1, S19–S31