Synphilin-1A: An aggregation-prone isoform of synphilin-1 that causes neuronal death and is present in aggregates from α -synucleinopathy patients

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α -Synucleinopathies are a group of neurological disorders characterized by the presence of intracellular inclusion bodies containing α -synuclein. We previously demonstrated that synphilin-1 interacts with α -synuclein, implying a role in Parkinson's disease. We now report the identification and characterization of synphilin-1A, an isoform of synphilin-1, which has enhanced aggregatory properties and causes neurotoxicity. The two transcripts encoding synphilin-1A and synphilin-1 originate from the *SNCAIP* gene but differ in both their exon organization and initial reading frames used for translation. Synphilin-1A binds to α -synuclein and induces the formation of intracellular aggregates in human embryonic kidney 293 cells, primary neuronal cultures, and human dopaminergic cells. Overexpression of synphilin-1A in neurons results in striking cellular toxicity that is attenuated by the formation of synphilin-1A inclusions, which recruit α -synuclein. Synphilin-1A is present in Lewy bodies of patients with Parkinson's disease and Diffuse Lewy Body disease, and is observed in detergent-insoluble fractions of brain protein samples obtained from Diffuse Lewy Body disease patients. These findings suggest that synphilin-1A may contribute to neuronal degeneration in α -synucleinopathies and also provide important insights into the role of inclusion bodies in neurodegenerative disorders.

α -synuclein | cell death | Lewy body | Parkinson's disease

The first gene linked to Parkinson's disease (PD) encodes for α -synuclein, a presynaptic protein (1) with as yet unknown physiological functions. Three missense mutations in α -synuclein and gene locus triplication have been found to cause autosomal dominant PD (2–5). α -Synuclein was also identified as a major constituent of Lewy bodies in sporadic PD patients (6) and also in inclusions characteristic of other neurodegenerative disorders, such as Diffuse Lewy Body disease (DLBD) (7).

We described synphilin-1 as an α -synuclein-interacting protein (8). The two proteins were found to interact *in vivo* and, when coexpressed in human embryonic kidney (HEK)293 cells, caused the formation of eosinophilic cytoplasmic inclusions (8). In addition, we recently found that synphilin-1 is ubiquitinated by the E3 ubiquitin ligase SIAH, which is also present in Lewy bodies of PD patients (9). When synphilin-1 and SIAH are coexpressed in cells and proteasomal function is inhibited, ubiquitinated synphilin-1 inclusions are found in the vast majority of the cells (9), and this process is modulated by GSK3 β phosphorylation of synphilin-1 (10). Ubiquitination of synphilin-1 inclusion bodies was also shown to be mediated by parkin (11), an E3 ubiquitin ligase implicated in the development of autosomal-recessive juvenile PD (12). Two additional findings further highlight the importance of synphilin-1 in the study of PD. First, synphilin-1 is present in Lewy bodies of PD patients as well as in inclusion bodies characteristic of other α -synucleinopathies (13). Second, two sporadic PD patients were found to carry a missense mutation, R621C, in the gene encoding synphilin-1 (14).

We now report the cloning of a synphilin-1 isoform, denominated synphilin-1A. Synphilin-1A has a different start codon and initial reading frame due to alternative splicing and contains a previously

undescribed exon. Synphilin-1A is an aggregation-prone protein that causes neuronal toxicity. We demonstrate the presence of synphilin-1A in Lewy bodies and in the insoluble fraction of protein samples obtained from the brains of DLBD patients. Our findings suggest an important role for synphilin-1A in inclusion-body formation and its possible involvement in the pathogenesis of PD.

Results

Cloning of Synphilin-1A, an Isoform of Synphilin-1. We identified several ESTs (BM984486, BM945433, and CF533728) at the National Center for Biotechnology Information site that contained an extra 71-bp sequence between the previously identified exons 9 and 10 of synphilin-1 (Fig. 1A) (15). We observed that this 71-bp sequence is found at the *SNCAIP* gene locus of human, mouse, and rat and that its 5' and 3' boundaries follow the AG/GT rule for intron/exon splicing (16). We carried out RT-PCR experiments using human brain mRNA and confirmed the presence of this 71-bp insertion in mRNA transcripts derived from the *SNCAIP* gene, indicating that it consists of a previously undescribed exon that was now termed exon 9A (Fig. 1B). The protein isoform containing this exon was named synphilin-1A.

RT-PCR studies aimed at identifying full-length synphilin-1A yielded a single amplification product that contained exon 9A and lacked exons 3 and 4. We did not isolate any cDNA products containing exons 3, 4, and 9A all together. A transcript similar to synphilin-1A was recently identified in a study of full-length human cDNA sequences performed by the National Institutes of Health Mammalian Gene Collection Program (GenBank accession no. BC094759), but no further analysis beyond sequencing was carried out with this cDNA (17).

To verify the expression of the synphilin-1A isoform in the brain, we generated an antibody that specifically recognized the amino acids encoded by exon 9A. Using this anti-synphilin-1A antibody (anti-Sph-1A), we identified endogenous synphilin-1A in the brain (Fig. 1D). This antibody specifically recognized a band of ≈ 75 kDa (Figs. 1D and 2), far less than the molecular weight predicated for synphilin-1 with the addition of exon 9A (≈ 100 kDa) but consistent with the lack of exons 3 and 4 of synphilin-1A.

Surprisingly, by analyzing the possible start codons, we found that the initial reading frame of synphilin-1A is different from that of synphilin-1. If translation of synphilin-1A would proceed by the use of the same start codon as that of the synphilin-1 transcript (Fig. 1C and D), the protein would contain only 66 amino acids because of a premature stop codon in exon 5, and this would not be

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Abbreviations: DLBD, Diffuse Lewy Body disease; HA, hemagglutinin; HEK, human embryonic kidney; PD, Parkinson's disease.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ27317).

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Fig. 1. Synphilin-1 and synphilin-1A differ in their exon organization and are translated from different start codons. (A) Exon organization of the *SNCAIP* gene, demonstrating the position of the previously unidentified exon 9A. (B) mRNA obtained from human brains was assessed for the presence of synphilin-1 and synphilin-1A transcripts. RT-PCR was carried out by using primers from exons 9 and 10 of synphilin-1. Two amplification products were obtained and verified by sequencing as corresponding to synphilin-1 and synphilin-1A. (C) A schematic representation of the exon organization of synphilin-1A and synphilin-1, with the different start codons used for translation. (C/I) Translation of the synphilin-1 transcript via start codon 1 results in the generation of a 919-aa protein (gray shading). (C/II) Predicted synphilin-1A product if start codon 1 is used for translation. The splicing out of exons 3 and 4 results in a frame shift (diagonal stripes), and translation is terminated by a stop codon after the 66th amino acid. (C/III) Translation of the synphilin-1A transcript via start codon 2 results in a different initial amino acid sequence (horizontal stripes), which, distal to the exons 2 and 5 splice junction, is identical to that of synphilin-1 (gray shading). The 51 amino acids present in the C terminus of synphilin-1A are encoded by exons 9A and 10 (dots). Lines above synphilin-1 and synphilin-1A indicate the epitopes of the antibodies used in this study. (D) Nucleotide sequence in the vicinity of the translation initiation site of the synphilin-1A transcript. Three ATGs that could serve as potential start codons of synphilin-1A are underlined. The known start codon of synphilin-1 is in bold type. HEK293 cells were transfected with cDNAs, each bearing a different ATG-to-CTG mutation at a potential start codon of synphilin-1A. Cell lysates were analyzed by Western blot for the presence of synphilin-1A by using the anti-Sph-1A antibody.

compatible with the 75-kDa protein identified by the anti-Sph-1A antibody (Fig. 1C). Moreover, because of its short size, translation of the transcript using the start codon of synphilin-1 would most probably target it for degradation via the mechanism of nonsense-mediated mRNA decay (18). Only if translation of synphilin-1A occurs via an alternative start codon (Fig. 1C and D), a 75-kDa protein is produced. Interestingly, the translation of synphilin-1A using this alternative start codon is predicted to generate a different N terminus, because the frame is different from that of synphilin-1. However, synphilin-1A would contain the same amino acids as synphilin-1 in exons 5 to 9 because of a frame shift caused by the

absence of exons 3 and 4 (Fig. 1C). To confirm this possibility, HEK293 cells were transfected with the isolated synphilin-1A cDNA including the 5' untranslated region. Western blot analysis of transfected cells using anti-Sph-1A revealed the presence of a 75-kDa protein with the same molecular mass as endogenous synphilin-1A (Fig. 1D, compare lanes 1 and 5). This indicates that synphilin-1A is produced from a transcript that, in addition to the presence of exon 9A, lacks exons 3 and 4 of synphilin-1 and is translated by using a start codon different from that of synphilin-1 and in a different initial frame.

In the synphilin-1A transcript, there are three ATGs that can

Fig. 2. Specificity of anti-synphilin antibodies and expression pattern of synphilin-1A. (A) Western blot of synphilin-1 and synphilin-1A expressed in HEK293 cells demonstrates the isoform specificity of the two different antibodies used for the characterization of the two isoforms. (B) Lysates prepared from different rat tissues (50 μ g) were probed with the anti-Sph-1A antibody, which specifically recognizes synphilin-1A. (C) Western blot analysis, showing expression of synphilin-1A in different brain regions of P14 and P28 rats by using the anti-Sph-1A antibody. The membranes were probed against actin to ensure equal protein loading (Lower).

Fig. 4. Overexpression of synphilin-1A in neurons results in cellular toxicity. (A) Rat cortical neurons were transfected with different constructs and examined for dendritic arborization by immunofluorescence against MAP2, a marker for neuronal proximal dendrites. Neurons transfected with synphilin-1A exhibit retraction of processes (AA–AC). In contrast, neurons transfected with either synphilin-1 or GFP exhibit abundant processes (AD–AI). (Scale bar, 20 μ m.) (B) Quantification of the reduction in the length of processes in neurons transfected with synphilin-1A compared with neurons transfected with either synphilin-1 or GFP. (C) Neurons transfected with synphilin-1A, synphilin-1, or GFP were assessed for cell death by examining the appearance of nuclear condensation or fragmentation. Neurons transfected with synphilin-1A exhibit a significant increase in cell death, whereas cell death in neurons overexpressing synphilin-1 or GFP is negligible. ***, significantly different from control at $P < 0.001$.

in the increase of synphilin-1 in the triton-insoluble fractions of transfected HEK293 cells (Fig. 3E).

We have recently reported that the E3 ubiquitin ligase SIAH interacts with and ubiquitylates synphilin-1, promoting its degradation through the ubiquitin proteasome system (9). Because synphilin-1A recruits synphilin-1 to cytosolic aggregates, we investigated whether synphilin-1A affects the degradation of synphilin-1 promoted by SIAH-1. HEK293 cells were transfected with HA-

synphilin-1, myc-SIAH-1, and increasing amounts of synphilin-1A and were analyzed by Western blot to determine the steady-state levels of synphilin-1. We found that synphilin-1A inhibits the degradation of synphilin-1 by SIAH-1 in a concentration-dependent manner (see Fig. 9A, which is published as supporting information on the PNAS web site). Pulse-chase experiments confirmed that synphilin-1A inhibits the degradation of synphilin-1 by SIAH-1 (Fig. 9B).

Fig. 5. Synphilin-1A aggregates within neurons, and its inclusions formed by proteasome inhibition are correlated with decreased cell death. (A) Immunofluorescence of transfected neurons, showing intracytoplasmic aggregates in cells transfected with HA-tagged synphilin-1A. (Scale bar, 25 μ m.) (B) Quantification of aggregate formation in neurons transfected with HA-synphilin-1A, HA-synphilin-1, and GFP by immunofluorescence with anti-HA antibody. (C) Neurons overexpressing HA-synphilin-1A, HA-synphilin-1, or GFP were treated with 1 μ M lactacystin for 24 h and examined by immunofluorescence with anti-HA for the presence of intracellular inclusions. Inclusion formation was evident only in neurons overexpressing synphilin-1A or synphilin-1. (D) Neurons transfected with HA-synphilin-1A were assessed for inclusion formation by immunofluorescence with anti-HA and for cell death by nuclear staining with Hoechst 33342. Nuclear condensation reflecting apoptotic process is evident in a neuron lacking intracellular inclusions (DA and DB), whereas a neuron containing inclusions appears to be healthy (DC and DD). (Scale bar, 20 μ m.) (E) A graph depicting the inverse correlation between inclusion formation and cell death in neurons overexpressing synphilin-1A. ***, significantly different from control at $P < 0.001$; **, significantly different from control at $P < 0.01$.

Fig. 6. Synphilin-1A interacts with α -synuclein and is aggregated in patients with DLBD. (A) Pull down of synphilin-1A with α -synuclein. Extracts of HEK293 cells transfected with HA-synphilin-1A were incubated with indicated GST fusion proteins. Binding was analyzed by using an anti-HA antibody. (Lower) The GST fusion proteins by Coomassie blue staining. (B) Synphilin-1A coimmunoprecipitates with α -synuclein. HA- α -synuclein was immunoprecipitated from extracts of HEK293 cells by using an anti-HA antibody. Coimmunoprecipitation was determined by Western blot using an anti-myc antibody. (C) α -Synuclein was found to colocalize with synphilin-1A aggregates in HEK293 cells transfected with myc-synphilin-1A (CA) and HA- α -synuclein (CB). Nuclei were revealed with TOPRO-3. (Scale bar, 25 μ m.) (D) Immunofluorescence of neurons cotransfected with synphilin-1A (DA) and α -synuclein (DB) reveals the presence of α -synuclein in the inclusions formed by synphilin-1A (DC). Neurons were treated with samples of DLBD patients. Protein insoluble fractions. Western blot analysis. Synphilin-1 is observed mostly in the protein loading. (F) Synphilin-1A is present in anti-Sph-1A antibody (FA) and anti- α -synuclein antibody (FB). Staining of Lewy bodies counterstained with hematoxylin.

Synphilin-1A Causes Neuronal Toxicity. We next investigated the cellular effects of synphilin-1A in neurons. Primary neuronal cultures were transfected with HA-synphilin-1A, and immunocytochemistry was carried out by using an anti-HA antibody. We found that neurons overexpressing synphilin-1A display a drastic decrease in the length of their processes (Fig. 4*A* and *B*). This decrease is not due to impaired distribution of synphilin-1A, because staining with anti-MAP2a antibody, which recognizes proximal dendrites, is also drastically reduced in neurons overexpressing synphilin-1A (Fig. 4*A*). Overexpression of synphilin-1 or the control protein GFP does not promote any decrease in the length of neuronal processes (Fig. 4*A* and *B*).

To verify whether synphilin-1A causes neuronal toxicity, we assayed transfected neurons for nuclear condensation and fragmentation, which are markers of apoptotic cell death. We found that overexpression of synphilin-1A promotes the death of $\approx 20\%$ of the transfected neurons after 72 h (Fig. 4C). In contrast, overexpression of synphilin-1 or GFP does not promote toxicity under the same conditions.

Overexpression of synphilin-1A in neurons leads to the formation of large aggregates, like those observed in HEK293 and SH-SY5Y, in $\approx 8\%$ of transfected neuronal cells (Fig. 5 *A* and *B*). These synphilin-1A aggregates are observed mostly in the soma of the neurons and much lower amounts of aggregates are formed in cells overexpressing synphilin-1 (Fig. 5*B*). Treatment with the proteasome inhibitors lactacystin or MG132 turned synphilin-1A aggregates into more organized inclusions, which are observed in both the soma and processes of neurons and appear in $\approx 40\%$ of synphilin-1A-transfected neurons (Fig. 5*C*). Upon proteasome inhibition, neurons transfected with HA-synphilin-1, but not GFP, also developed significant amounts of inclusion bodies.

Under our experimental conditions, proteasome inhibition in GFP- or HA-synphilin-1-transfected neurons does not increase cell

death when compared with untreated transfected cells (data not shown). However, in neurons transfected with HA-synphilin-1A, proteasome inhibition causes a slight increase in cell death that is observed only in neurons that lack inclusion bodies (Fig. 5*E*, compare with Fig. 4*C*). Interestingly, cell death is markedly reduced in neurons containing inclusions (Fig. 5*D* and *E*). Thus, in synphilin-1A-overexpressing neurons, there is an inverse correlation between cell death and inclusion formation.

α -Synuclein Interacts with and Is Recruited to Inclusions Formed by Synphilin-1A. Synphilin-1 was first discovered by its interaction with α -synuclein (8). We now show that synphilin-1A specifically interacts with α -synuclein. We found that GST- α -synuclein pulls down synphilin-1A in a specific manner (Fig. 6A) and also that synphilin-1A specifically coimmunoprecipitates with α -synuclein from cotransfected HEK293 cells (Fig. 6B). To examine a possible colocalization of α -synuclein with synphilin-1A aggregates, HEK293 cells were cotransfected with myc-synphilin-1A and HA- α -synuclein. Immunofluorescence of transfected cells revealed the presence of α -synuclein in the aggregates formed by synphilin-1A (Fig. 6C). Furthermore, in neurons cotransfected with synphilin-1A and α -synuclein and treated with a proteasome inhibitor, α -synuclein was found to colocalize with synphilin-1A inclusions (Fig. 6D).

To examine the possibility that synphilin-1A might be implicated in the pathogenesis of α -synucleinopathies, we analyzed its distribution in protein extracts of DLBD patients and age-matched controls. Western blot analysis revealed that synphilin-1A is present in the Triton X-100-insoluble fraction of brain samples from DLBD patients but not in control samples (Fig. 6E). By contrast, synphilin-1 was not significantly present in the triton-insoluble fraction of DLBD patients (Fig. 6E). The level of triton-soluble synphilin-1A is decreased in one DLBD patient (case 2) but no correlation with

disease stage can be inferred because of the limited number of patients.

To further investigate the nature of synphilin-1A insolubility in α -synucleinopathies, we carried out immunohistochemistry experiments using PD and DLBD tissues. We found that synphilin-1A is present in Lewy bodies of PD and DLBD patients in a specific manner (Fig. 6F). Preabsorbed anti-synphilin-1A antibody gave no specific labeling of Lewy bodies in PD and DLBD patients (Fig. 6F and data not shown).

Discussion

The major finding from this study is the ability of synphilin-1A to cause neuronal toxicity that is attenuated by the formation of inclusion bodies, suggesting that synphilin-1A may be a key player in the pathogenesis of neurodegenerative disorders characterized by α -synuclein-containing inclusion bodies, as is corroborated by our finding that synphilin-1A is insoluble in brain samples of DLBD patients and present in Lewy bodies of PD and DLBD patients.

Synphilin-1A is an alternative splice variant of synphilin-1 that lacks exons 3 and 4 and contains a previously unidentified exon 9A of the *SNCAIP* gene. However, the transcripts of synphilin-1A and synphilin-1 differ not only by their exon content but also by their start codon and initial reading frame. To our knowledge, we here show a previously uncharacterized mechanism where two totally functional alternatively spliced transcripts originating from the same gene are translated by the use of two different initial reading frames. The possibility that this is a general mechanism used by cells has a profound impact on cell biology, because it markedly increases the number of proteins that can be generated from a single gene.

Under our experimental conditions, synphilin-1A, but not synphilin-1, was found to aggregate in transfected HEK293 and SH-SY5Y cells, even without proteasome inhibition. A possible explanation for this difference is that aggregation of synphilin proteins is mediated by their ankyrin-like domains, which, in synphilin-1, might be masked by the presence of the 350-aa N terminus. In contrast, synphilin-1A lacks a long N-terminal tail, and, hence, its ankyrin-like domains might be more exposed and able to drive aggregation. Also, interaction of synphilin-1 with synphilin-1A through their ankyrin-like domains enabled synphilin-1 to coaggregate with synphilin-1A, strengthening the idea that exposure of the ankyrin-like domains of synphilin might be essential for its aggregation. In addition to HEK293 and SH-SY5Y cells, we also found that synphilin-1A aggregates within neurons, suggesting that it may contribute to the formation of Lewy bodies.

Although synphilin-1A aggregated much more effectively than synphilin-1 in neurons, similar amounts of synphilin-1A and synphilin-1 inclusions were observed in the presence of proteasome inhibition. This finding could be explained by the much higher ubiquitylation of synphilin-1 in comparison with synphilin-1A (A.E. and S.E., unpublished observations). Because we analyze inclusion formation in transfected neurons with proteasome inhibition at the steady state, the strong tendency of ubiquitylated synphilin-1 to

aggregate into inclusions (9) may compensate the natural propensity of synphilin-1A to aggregate.

We observed marked neurotoxicity with synphilin-1A but not with synphilin-1, indicating that synphilin-1A is directly involved in cell death. The attenuation of synphilin-1A toxicity by the formation of intracellular inclusions should shed some light on the controversial role of inclusion bodies in neurodegenerative disorders. To date, it is still debatable whether inclusion bodies promote or inhibit neuronal toxicity. Our findings clearly suggest a cytoprotective role for inclusion bodies.

Finally, our findings that α -synuclein coimmunoprecipitates with and colocalizes to synphilin-1A inclusions and that synphilin-1A is insoluble in brain samples of DLBD and is present in Lewy bodies of both PD and DLBD patients suggest that this protein might be involved in the pathogenesis of α -synucleinopathies. Our identification of synphilin-1A increases the understanding of the molecular mechanisms that underlie protein aggregation and toxicity in these neurodegenerative disorders.

Methods

Further experimental details are provided as *Supporting Text*, which is published as supporting information on the PNAS web site.

Primary Neuronal Cultures and Transfections. Embryonic day (E)18 primary cortical cultures were prepared from Sprague–Dawley rats. The animals were killed by decapitation according to the protocol approved by the committee for animal experimentation at the Technion–Israel Institute of Technology. Neurons were maintained in neurobasal medium supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine (Biological Industries, Beit-Haemek, Israel). For immunostaining of endogenous proteins, neurons were cultured for 3 weeks. For the transfection experiments, neurons were transfected with 6–9 μ g of endo-free cDNA per well at 5 days *in vitro* by the calcium phosphate method as described in ref. 20. After transfection, cells were grown for an additional 72–96 h before fixation for immunocytochemical studies as described for HEK293 cells.

Biochemical Fractionation of Brain Tissues. Human cerebral cortices from DLBD patients and age-matched controls were obtained from Harvard Brain Tissue Resource Center (Boston), McLean Hospital (Belmont, MA), and Massachusetts General Hospital (Boston). For the preparation of Triton X-100-soluble and -insoluble fractions, human cortical tissues were homogenized in 5 ml/g buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 30 μ M MG132, and protease inhibitors mixture (Complete; Roche) and processed as described for HEK293 cells.

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