Synphilin-1 associates with α-synuclein and promotes the formation of cytosolic inclusions

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Parkinson disease (PD) is a neurodegenerative disease characterized by tremor, bradykinesia, rigidity and postural instability. Post-mortem examination shows loss of neurons and Lewy bodies, which are cytoplasmic eosinophilic inclusions, in the substantia nigra and other brain regions[1,2]. A few families have PD caused by mutations (A53T or A30P) in the gene *SNCA* (encoding α-synuclein; refs 3–5). α-synuclein is present in Lewy bodies of patients with sporadic PD (refs 6,7), suggesting that α-synuclein may be involved in the pathogenesis of PD. It is unknown how α-synuclein contributes to the cellular and biochemical mechanisms of PD, and its normal functions and biochemical properties are poorly understood[8–10]. To determine the protein-interaction partners of α-synuclein, we performed a yeast two-hybrid screen. We identified a novel interacting protein, which we term synphilin-1 (encoded by the gene *SNCAIP*). We found that α-synuclein interacts *in vivo* with synphilin-1 in neurons. Co-transfection of both proteins (but not control proteins) in HEK 293 cells yields cytoplasmic eosinophilic inclusions.

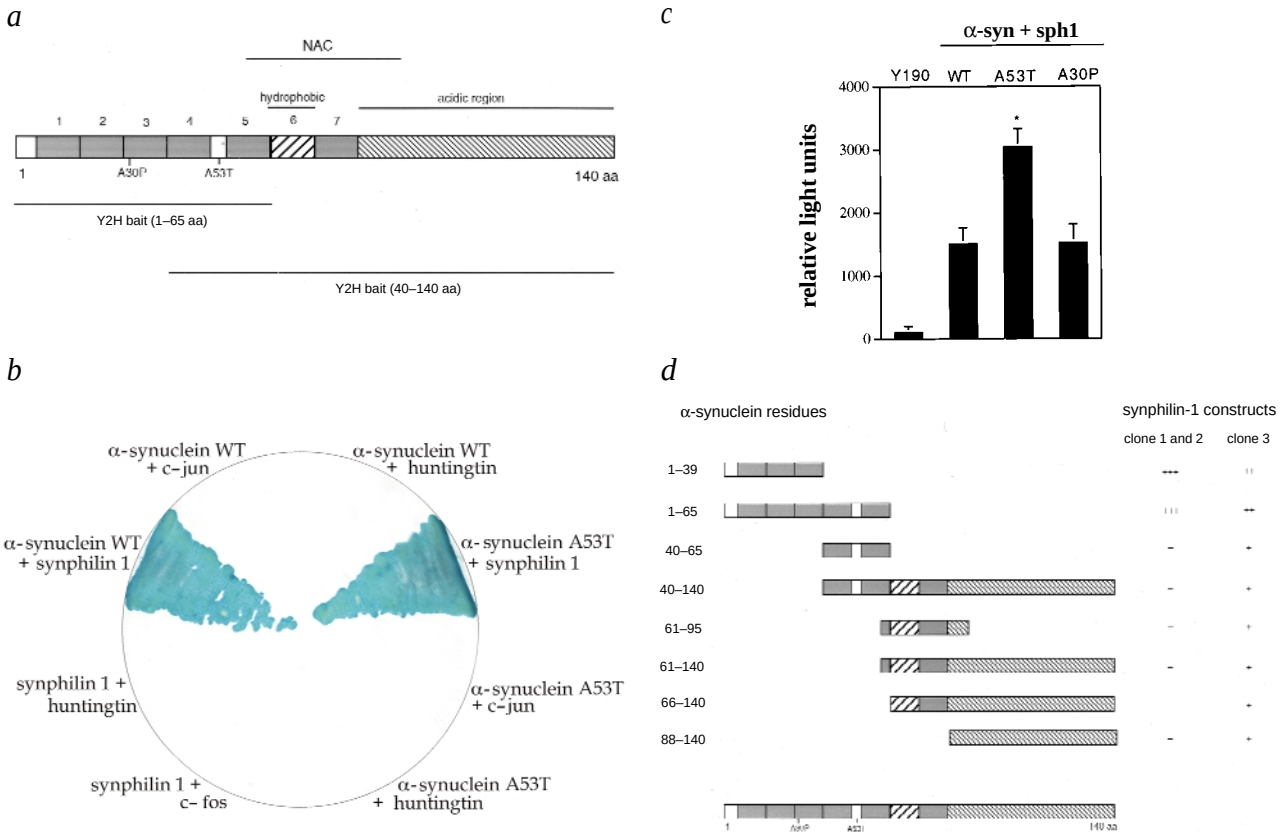

Fig. 1 Association of α-synuclein with synphilin-1 in yeast. ***a***, The box represents full-length *SNCA* cDNA with its domains[8,25]. The 11-aa repeat units are represented by grey boxes. NAC is formed by aa 61–95 of α-synuclein[25–28]. Lines represent the fragments of α-synuclein used as baits for the yeast two-hybrid screenings that yielded positive signals. ***b***, β-galactosidase filter assay showing that α-synuclein (aa 1–65) interacts specifically with synphilin-1 (clone 1; Fig. 2*a*) in yeast. There was no interaction of either α-synuclein or synphilin-1 with vector alone or different control proteins. α-synuclein (wild type or A53T) and c-fos were inserted into pPC97, whereas synphilin-1 and c-jun were inserted into pPC86 vector. Huntingtin (44Q) was inserted either in pPC97 or pPC86 vector. ***c***, β-galactosidase liquid assays showing an increase in interaction between α-synuclein A53T mutant and synphilin-1 (clone 1) when compared with wild type or A30P mutant. Error bars represent standard errors; n = 4. *Different from wild type at $P<0.01$ (Student's *t*-test). ***d***, Mapping of the synphilin-1–binding domain of α-synuclein. The results of β-galactosidase filter assays of Y190 co-transformed with different α-synuclein truncations (pPC97) and synphilin-1 clones (pPC86) are shown.

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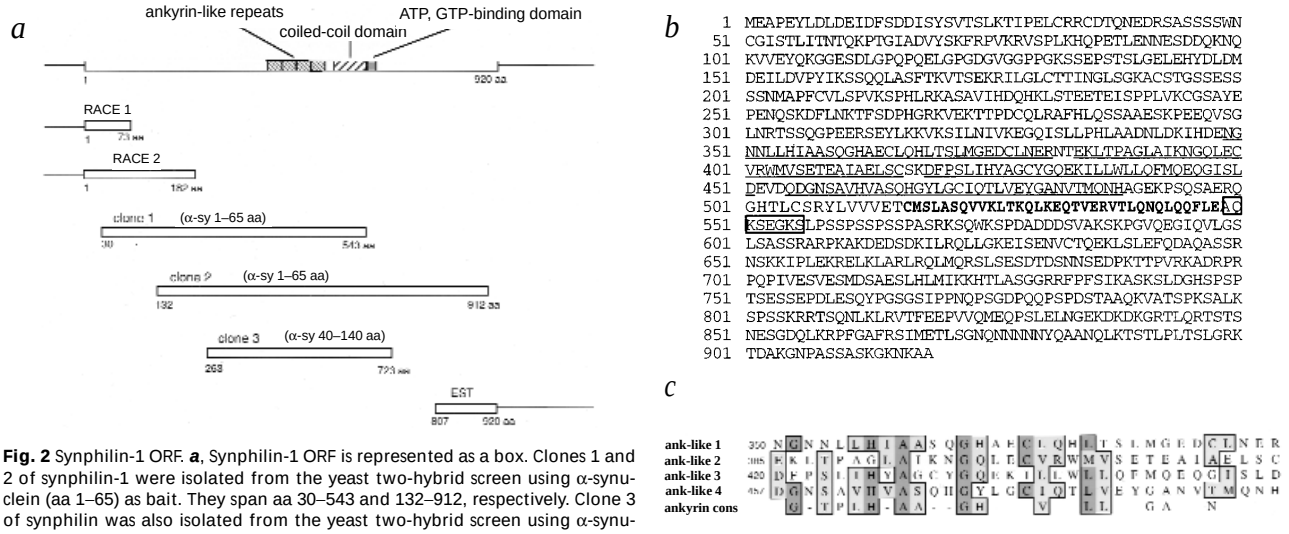


Fig. 2 Synphilin-1 ORF. ***a***, Synphilin-1 ORF is represented as a box. Clones 1 and 2 of synphilin-1 were isolated from the yeast two-hybrid screen using α-synuclein (aa 1–65) as bait. They span aa 30–543 and 132–912, respectively. Clone 3 of synphilin was also isolated from the yeast two-hybrid screen using α-synuclein (aa 40–140) as bait. RACE was used to isolate the 5′ cDNA sequence. The 3′ end of *SNCAIP* cDNA was obtained by sequencing IMAGE clone 753791. ***b***, Predicted amino acid sequence of human synphilin-1. The ankyrin-like repeats are underlined, whereas the coiled-coil domain and ATP/GTP-binding domain are bold and boxed, respectively. ***c***, Alignment of ankyrin-like repeats of synphilin-1 with the mammalian consensus for ankyrin repeats. Conserved residues (light grey) and identical residues (dark grey) are indicated.

We used several α-synuclein constructs to screen human brain libraries in the yeast two-hybrid system[11], resulting in the identification of several cDNAs encoding a single interacting protein, synphilin-1 (Fig. 1). We found no other positive clones. Three independent synphilin-1 clones were identified (clones 1, 2 and 3; Fig. 2*a*). In a liquid culture assay, the interaction of mutant α-synuclein A53T with synphilin-1 was twofold higher than with wild type or the A30P mutant (Fig. 1*c*).

To map the synphilin-1–binding domain of α-synuclein, we co-transformed different α-synuclein truncations into yeast with synphilin-1 constructs (Fig. 2*a*). Synphilin-1 clones interacted with amino acids 1–39 and 1–65 of α-synuclein, indicating that amino acids 1–39 of α-synuclein are sufficient for synphilin-1 binding in yeast (Fig. 1*d*). Synphilin-1 (clone 3) was also able to interact with other regions of α-synuclein (Fig. 1*d*).

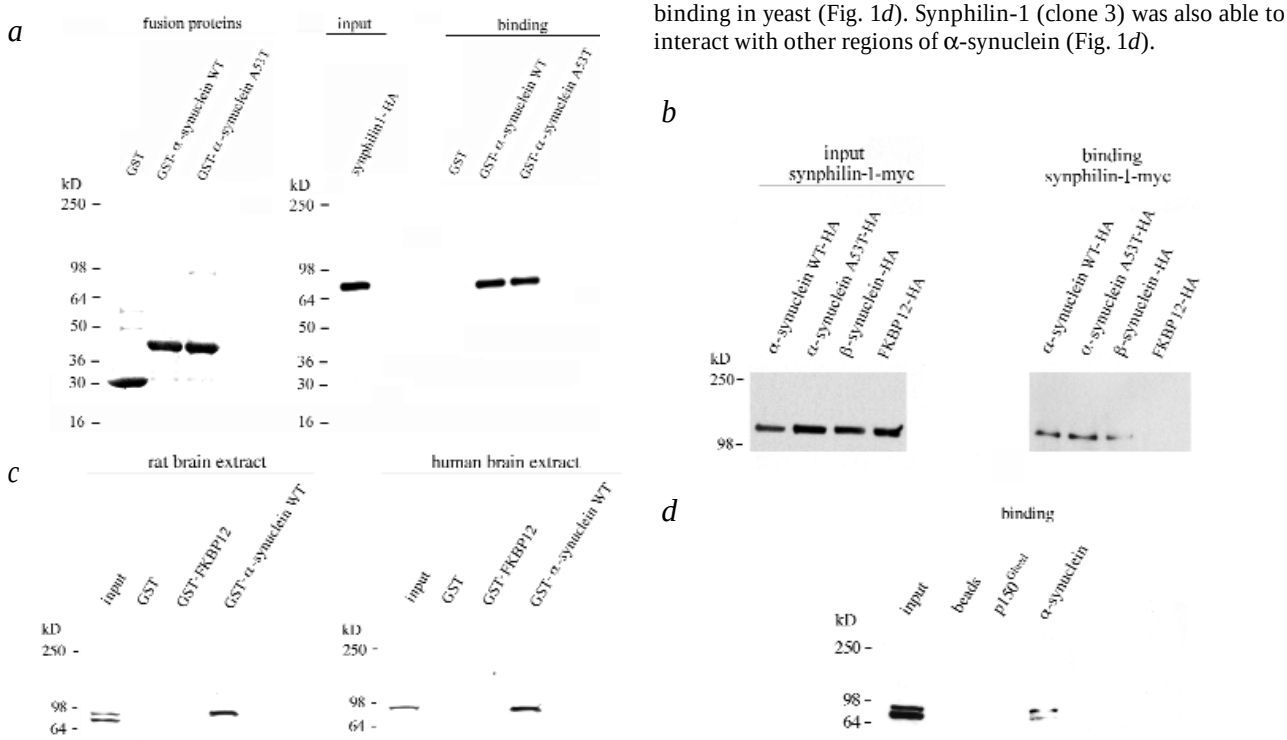


Fig. 3 Interaction of α-synuclein and synphilin-1. ***a***, Synphilin-1 binds to α-synuclein in an affinity-column experiment. Left, PAGE of GST and GST-α-synucleins. Western blot (detected with HA antibody) shows the input of HEK 293 cells transfected with synphilin-1-HA (middle) and the binding of synphilin-1-HA with GST-α-synucleins (wild type and A53T mutant), but not with GST alone (right). ***b***, Synphilin-1 co-immunoprecipitates with α-synuclein from co-transfected HEK 293 cells. The western blot was incubated with anti-myc antibody. Left, synphilin-1-myc input of co-transfected HEK 293 cells. Right, binding of synphilin-1-myc to α-synuclein-HA (wild type and A53T mutant) and β-synuclein-HA (refs 29,30), but not to FKBP12-HA. ***c***, GST-α-synuclein fusion protein binds rat and human brain synphilin-1. Rat brain or human frontal cortex lysates were incubated with GST, GST-FKBP12 or GST-α-synucleins. The incubation products were immunoblotted with anti-synphilin-1 antibodies. ***d***, Synphilin-1 co-precipitates with endogenous α-synuclein. α-synuclein was immunoprecipitated from rat brain lysate using an affinity-purified antibody to α-synuclein. The resulting immunoprecipitates were immunoblotted with antibodies to synphilin-1. Synphilin-1 was detected in both rat brain lysate (input) and in the α-synuclein-immunoprecipitate, but not in $p150^{Glued}$ immunoprecipitate or beads alone.

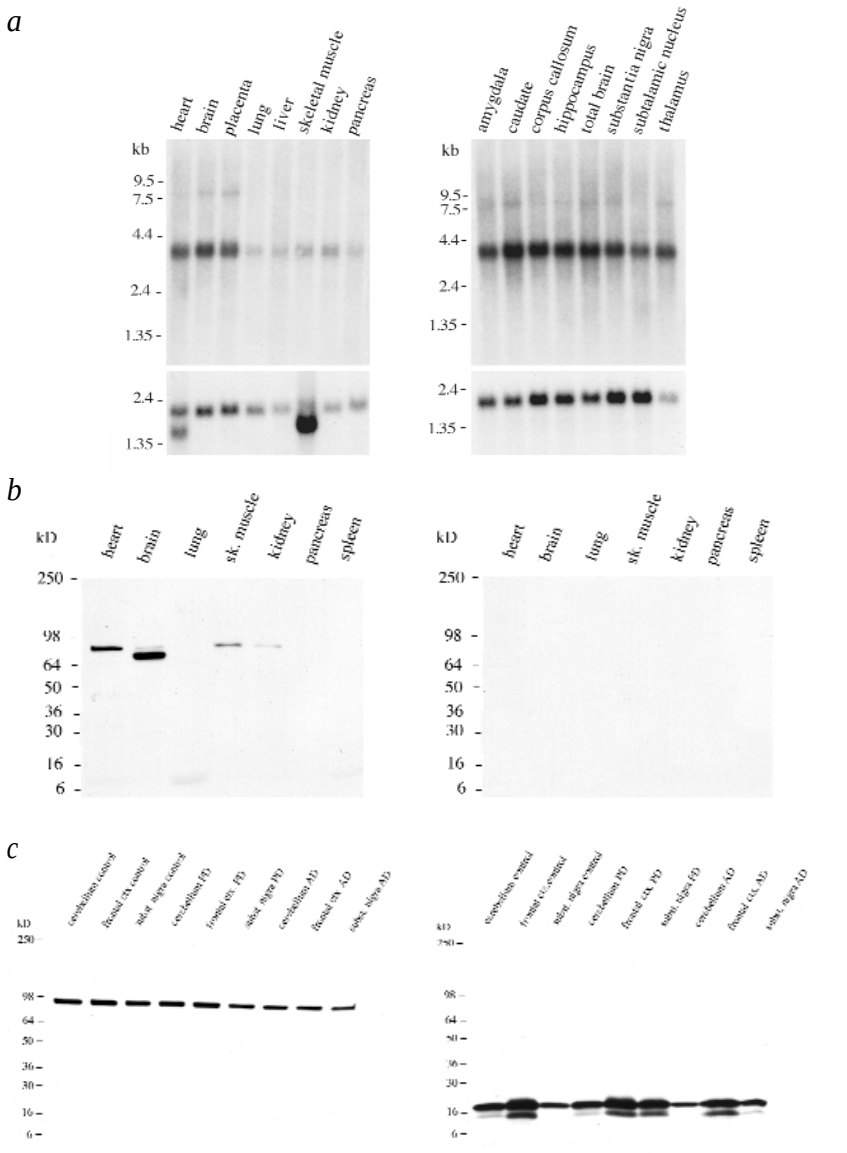


Fig. 4 Expression pattern of synphilin-1 and co-localization with α-synuclein. ***a***, Northern blots of human poly(A)$^+$ RNA from various tissues (left) or brain subregions (right) were hybridized with ^{32}P-labelled *SNCAIP* cDNA (clone 1). Bottom, the same blots hybridized with a β-actin probe to confirm mRNA integrity. ***b***, Synphilin-1 protein expression in rat tissues. Immunoblot of rat protein lysate (50 μg) from various tissues detected with synphilin-1 antibody (left) is shown. To determine the specificity of synphilin-1 antibody, an identical blot of rat protein lysate (50 μg) from various tissues was incubated with synphilin-1 antibodies preabsorbed with antigen (right). For this, synphilin-1 antibody (1 μg/ml) was incubated with excess (10×) GST-synphilin-1 (aa 30–543). ***c***, Synphilin-1 protein distribution in human brain regions. Immunoblot of protein lysate (50 μg) from various human brain tissues (normal, PD and AD) detected with synphilin-1 antibody (left) and α-synuclein antibodies (right). ***d***, Co-localization of α-synuclein and synphilin-1 in primary cortical neurons. Indirect immunofluorescence of primary cortical neuronal culture (12 DIV) labelled with anti-α-synuclein (top) and anti-synphilin-1 antibodies (bottom) is shown. Scale bar, 50 μm.

The clones used to assemble full-length cDNA encoding human synphilin-1 are shown (Fig. 2*a*). Synphilin-1 is a novel protein with little similarity to other cDNAs in the database. It contains several protein-protein interaction domains, such as ankyrin-like repeats[12] and a coiled-coil domain[13,14] (Fig. 2). Synphilin-1 may act as an adaptor molecule that anchors α-synuclein to intracellular proteins involved in vesicle transport and cytoskeletal function. Synphilin-1 also contains a predicted ATP/GTP-binding domain (Fig. 2*a*,*b*). Synphilin-1 was localized to human chromosome 5q23.1–23.3 by radiation hybrid mapping (unpublished data).

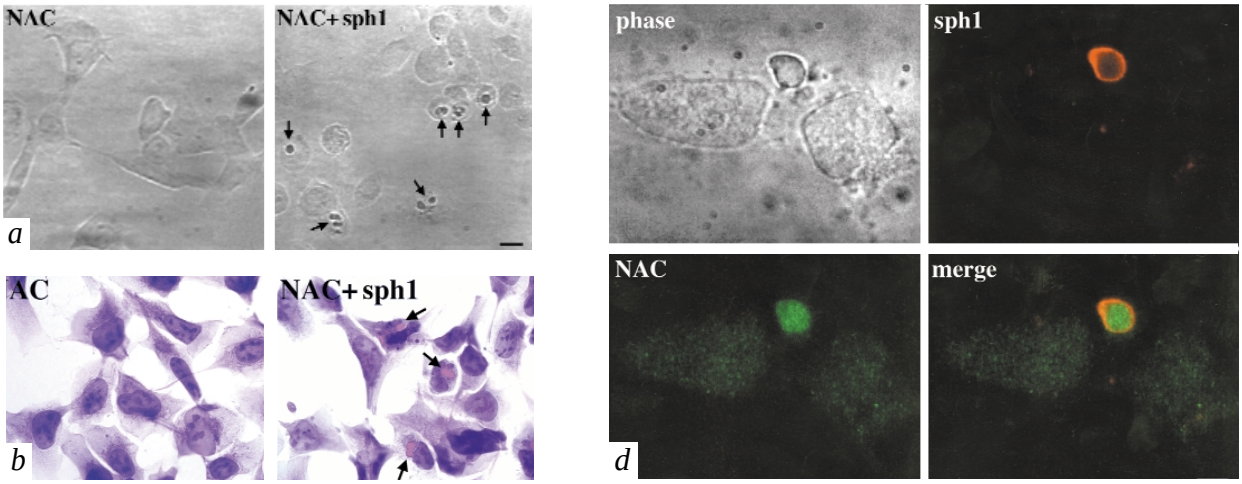
To confirm the interaction between synuclein and synphilin-1, we incubated α-synuclein GST fusion proteins with protein extracts from HEK 293 cells transfected with synphilin-1 fused to a haemaglutinin (HA) tag (Fig. 3*a*), or with rat or human brain protein extracts (Fig. 3*c*). In both cases, synphilin-1 interacted with α-synuclein (wild type or mutants) but not with glutathione S-transferase (GST) alone or GST-FKBP12 (FK506-binding protein 12) as controls (Fig. 3*a*,*c*). Wild-type, A53T and A30P mutant α-synuclein proteins specifically co-precipitated synphilin-1 from co-transfected HEK 293 cells (Fig. 3*b*). We found that synphilin-1 specifically co-immunoprecipitated with endogenous α-synuclein from brain tissue (Fig. 3*d*), indicating that these two proteins interact *in vivo*.

Human *SNCAIP* mRNA (~4 kb) was present in many tissues, and enriched in brain, heart and placenta (Fig. 4*a*, left). It was present in many regions in brain, including substantia nigra (Fig. 4*a*, right). Purified synphilin-1 antibodies recognized a protein of approximately 80–90 kD in rat protein extracts from different tissues (Fig. 4*b*, left). In rat brain, the antibody recognized two bands, both of which disappeared when the antibodies were preincubated with antigen (Fig. 4*b*, right).

In human brain, synphilin-1 appeared as a single band of approximately 90 kD in several brain regions, with no differences in the level of expression in controls, patients with PD or patients with Alzheimer disease (AD; Fig. 4*c*).

Immunofluorescence of rat cortical neurons grown in primary culture showed that both proteins were found in cell bodies and neurites of the same cells (Fig. 4*d*; and consistent with a previous study of α-synuclein[15]). α-synphilin-1 antibody yielded little or no signal in immunohistochemical studies of rat tissues and no signal from human post-mortem brain material.

When we co-transfected HEK 293 cells with vectors encoding synphilin-1 and full-length α-synuclein (wild type or A53T), or an α-synuclein fragment (aa 30–120), we did not observe any morphological change. When constructs encoding synphilin-1 and the non-Aβ component AD amyloid (NAC) portion of α-synuclein



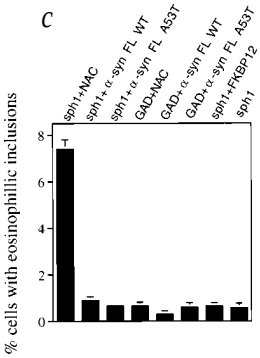


Fig. 5 Formation of cytoplasmic inclusions by co-transfection of constructs encoding NAC and synphilin-1. ***a***, HEK 293 cells co-transfected with constructs encoding NAC (aa 61–95 of α-synuclein) and full-length synphilin-1 develop cytosolic phase-dense inclusions (right). The inclusions are not present in cells transfected with constructs encoding NAC plus vector (left) or synphilin-1 plus vector (data not shown). Scale bar, 50 μm. ***b***, HEK 293 cells co-transfected with constructs encoding NAC and full-length synphilin-1 develop cytoplasmic eosinophilic inclusions when stained with H&E. Scale bar, 20 μm. ***c***, Quantification of eosinophilic inclusion formation in HEK 293 cells transfected with various constructs. The number of cells containing eosinophilic inclusions is relative to the number of cells (transfected and untransfected). Approximately 7.4% of cells co-transfected with constructs encoding NAC and synphilin-1 contained eosinophilic inclusions, whereas co-transfection of constructs encoding full-length α-synuclein (wild type or A53T mutant) and synphilin-1 resulted in the formation of less than 1% of eosinophilic inclusions. Co-transfection of constructs encoding FKBP12 and GAD 65 with synphilin-1 and NAC, respectively, also led to the formation of inclusions in less than 1% of cells. Error bars represent standard deviation; n=4. ***d***, Phase-dense inclusions are stained for both NAC and synphilin-1. HEK 293 cells co-transfected with constructs encoding NAC and full-length synphilin-1 were double-labelled for myc (red, synphilin-1) and HA (green, NAC). The figure represents a confocal section and shows that the inclusion is stained for both NAC and synphilin-1. Scale bar, 10 μm.

were co-transfected, however, we observed the formation of cytosolic phase-dense inclusions (Fig. 5*a*, right). Control cells co-transfected with a construct encoding NAC and vector alone (pRK5) had few or no inclusions (Fig. 5*a*, left). Cytosolic inclusions were eosinophilic when stained with haematoxylin and eosin (H&E; Fig. 5*b*). Approximately 7% of cells had cytosolic eosinophilic inclusions when co-transfected with constructs encoding synphilin-1 and NAC (Fig. 5*c*). As the transfection efficiency was 30–40%, the percentage of cells expressing synphilin-1 and NAC that develop inclusions is probably 15–20%. In contrast, when constructs encoding synphilin-1 or NAC were transfected alone or with control proteins, only approximately 1% of cells had eosinophilic inclusions. Even when more NAC cDNA (2× or 4×) was used, alone or with glutamate decarboxylase 65 (GAD 65) as control, we did not observe inclusions (data not shown). Co-transfection of a construct encoding an α-synuclein truncation (aa 40–95, wild type or A53T mutant) with one encoding synphilin-1 also led to inclusion body formation, at approximately one-half the rate seen with NAC (data not shown).

Immunofluorescence of HEK 293 cells co-transfected with constructs encoding synphilin-1 and NAC showed that both polypeptides were found in these inclusions (Fig. 5*d*). Synphilin-1 usually had a ring-like appearance, whereas α-synuclein reactivity sometimes had a homogeneous appearance and sometimes a ring-like appearance. Penetration of the antibody into the inclusion may be variable[16].

In this study, we have identified synphilin-1, a novel protein that interacts with α-synuclein *in vivo* and co-localizes with α-synuclein in neurons. When constructs encoding portions of α-synuclein and synphilin-1 are co-transfected in mammalian cells, we observe the formation of eosinophilic cytosolic inclusions resembling the Lewy bodies of PD. In PD, it is possible that proteolysis of α-synuclein, leading to an NAC-related polypeptide, may facilitate its aggregation. Both amino and carboxy termini of α-synuclein are present in Lewy bodies[6,7], but partially truncated forms of α-synuclein have also been detected in Lewy bodies[17], suggesting that proteolytic processing of α-synuclein may have a role in the pathogenesis of PD. This is similar to several other neurodegenerative diseases in which proteolysis of gene products has been implicated, including APP, PS1 and PS2 in AD and huntingtin in Huntington disease[18–20].

We find that synphilin-1 interacts more strongly in yeast with A53T than with wild type or the A30P mutant. It is possible that this increase in binding is related to the pathogenesis of this familial form of PD, but it has also been shown that the A53T mutant of α-synuclein is more prone to self-aggregate *in vitro* than the wild-type form[21,22]. Although α-synuclein aggregates by itself *in vitro*, it may be that aggregation *in vivo* is facilitated by an associated protein such as synphilin-1. Apparently, the A53T mutation does not enhance the number of inclusions in HEK 293-transfected cells. This is in agreement with the fact that fragments of wild-type α-synuclein are found in Lewy bodies[17].

Our data suggest that synphilin-1 modulates α-synuclein aggregation. It is possible that the interaction between α-synuclein and synphilin-1 has a role in inclusion body formation in PD. Our cell model may prove to be useful in studying the pathogenesis of α-synuclein aggregation, and correspondingly, of PD.

Methods

Yeast two-hybrid screening and β-galactosidase assays. We fused α-synuclein clones into a yeast two-hybrid vector containing the GAL4 DNA-binding domain[11] (pPC97). Yeast two-hybrid screenings were performed by transforming a human fetal brain cDNA library fused to pPC86 (GAL4-activating domain) into Y190 yeast containing pPC97-α-synuclein truncations (wild type or A53T mutant). We performed β-galactosidase filter lift assays for all grown colonies. To confirm the interaction, cDNA clones in pPC86 were rescued and co-transformed with pPC97-α-synuclein. We used Y190 co-transformed with c-Jun (aa 246–335) and c-Fos (aa 117–197) as positive controls. Liquid β-galactosidase assays were performed by

inoculating yeast scrapes (~200 yeast colonies/scrape) into Trp$^-$, Leu$^-$, His$^-$ medium. We determined β-galactosidase activity by luminescence according to the manufacturer's instructions (Clontech).

Cell culture and transfection. HEK 293 cells were grown in DMEM containing 10% FBS in a 5% CO_2 atmosphere. We transiently transfected cells by the calcium phosphate precipitation method using 10 μg of plasmid DNA per 10-cm plate or 0.5 μg of each plasmid DNA per well[14] (4-well chamber slides). Full-length synphilin-1 and GAD 65 were inserted into a vector containing a myc tag. The rest of the constructs were inserted into a vector containing an HA tag. We processed cells 48 h after transfection.

In vitro binding assays. We purified GST-fusion proteins (full-length α-synuclein, β-synuclein or FKBP12) on glutathione sepharose 4B beads according to the manufacturer's instructions (Pharmacia Biotech). For binding experiments, HEK 293 cells transfected with a construct encoding synphilin-1-HA (aa 30–543) were lysed in Hepes (50 mM, pH 7.4), NaCl (150 mM), EDTA (3 mM), 1% Triton X-100, 0.1% SDS and a protease inhibitors cocktail (Complete, Boehringer). We removed insoluble material by centrifugation at 14,000*g* for 5 min. Supernatant of cell lysates were incubated with GST-fusion proteins for 1 h at 4 °C. Beads were washed 5 times with lysis buffer containing NaCl (500 mM). Proteins were eluted from beads with SDS sample buffer and detected by western blot using anti-HA antibody (1:10,000; Babco). For GST 'pull down' assays, either rat or human brain homogenates were incubated with GST fusion proteins. Briefly, brain tissues were homogenized in Tris-HCl (50 mM; pH 7.4), KCl (140 mM), EDTA (3 mM) and 0.5% Triton X-100 supplemented with protease inhibitors cocktail (Complete). Homogenate was clarified by centrifugation at 5,000*g* for 10 min. Binding and washing conditions were as described above. We detected bound proteins by western blot using purified synphilin-1 antibody (1 μg/ml).

Preparation of synphilin-1 antibody. Rabbits were immunized with GST-synphilin-1 (pGEX-4T-2-synphilin-1; aa 30–543). Immune serum was filtered through a GST-Sepharose 4B column to eliminate anti-GST antibodies. To purify anti-synphilin-1 antibodies, pre-cleared serum was incubated overnight with GST-synphilin-1 immobilized on PVDF membrane strips. After extensive washings with NaCl (500 mM), antibodies were eluted from the strips with glycine (100 mM; pH 2.5) and dialysed against phosphate-buffered saline.

In vitro and *in vivo* co-immunoprecipitations. For *in vitro* co-immunoprecipitations, we co-transfected HEK 293 cells with full-length synphilin-1-myc and α-synuclein-HA (wild type or A53T mutant), β-synuclein-HA or FKBP12-HA. Co-transfected HEK 293 cells were lysed as for *in vitro* binding assays and the supernatant of cell lysates was incubated with HA affinity matrix (20 μl; Babco) for 2 h at 4 °C. Immunoprecipitates were washed with Tris-HCl (50 mM; pH 7.4), NaCl (400 mM) and 0.5% Triton X-100, and detected by western blot using anti-myc antibody (1:10,000; Calbiochem). For *in vivo* immunoprecipitations, one frozen rat brain was homogenized as described above for GST 'pull down' assays. Homogenate was preabsorbed with protein G-agarose beads for 1 h at 4 °C. Affinity-purified anti-synphilin-1 antibody or anti-p150^{Glued} antibodies were coupled with protein G-agarose beads[23]. After incubation with protein homogenate, beads were washed with lysis buffer and eluted with SDS sample buffer. We developed blots with purified synphilin-1 antibody (1 μg/ml).

Immunocytochemistry. Transfected HEK 293 cells were permeabilized with 0.1% Triton X-100 for 1 min, fixed with 4% paraformaldehyde for 15 min at RT and processed as described[14]. Primary cortical neurons were fixed with 4% paraformaldehyde and processed as described[14]. Cells were labelled with anti-HA (1:500; Babco) and anti-c-myc antibodies (1:100; Santa Cruz) or purified polyclonal synphilin-1 antibody (5 μg/ml) and anti-synuclein 1 (1:50; Transduction Laboratories).

Primary culture of rat brain cortex. Rat brain embryos were recovered at d 18 from gestating Sprague-Dawley rats and primary cultures were performed as described[24]. Cells were analysed by immunocytochemistry after 12 d of *in vitro* culture.

Quantification of eosinophilic inclusions. We performed H&E staining according to the manufacturer's instructions (Sigma). All cells were counted in fields chosen at random from four different quadrants of the tissue culture well. Counting was done by an investigator blind to the experimental condition.

GenBank accession number. *SNCAIP*, AF076929.

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