α-Synuclein Binds to Tau and Stimulates the Protein Kinase A-catalyzed Tau Phosphorylation of Serine Residues 262 and 356*

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α-Synuclein has been implicated in the pathogenesis of several neurodegenerative disorders based on the direct linking of missense mutations in α-synuclein to autosomal dominant Parkinson’s disease and its presence in Lewy-like lesions. To gain insight into α-synuclein functions, we have investigated whether it binds neuronal proteins and modulates their functional state. The microtubule-associated protein tau was identified as a ligand by α-synuclein affinity chromatography of human brain cytosol. Direct binding assays using 125I-labeled human tau demonstrated a reversible binding with a IC₅₀ about 50 pm. The interacting domains were localized to the C terminus of α-synuclein and the microtubule binding region of tau as determined by protein fragmentation and the use of recombinant peptides. High concentrations of tubulin inhibited the binding between tau and α-synuclein. Functionally, α-synuclein stimulated the protein kinase A-catalyzed phosphorylation of tau serine residues 262 and 356 as determined using a phospho-epitope-specific antibody. We propose that α-synuclein modulates the phosphorylation of soluble axonal tau and thereby indirectly affects the stability of axonal microtubules.

Filamentous nerve cell inclusions are shared characteristics of the common neurodegenerative disorders Alzheimer’s disease, Parkinson’s disease, and dementia with Lewy bodies. The inclusions of Alzheimer’s disease, the neuritic tangles, are localized to the neurites and consist of abnormally phosphorylated protein tau (1). In Parkinson’s disease and dementia with Lewy bodies, the inclusions, designated Lewy bodies and Lewy neurites, are localized to the cell body and neurites, respectively. The inclusions in the Lewy lesions contain α-synuclein (2, 3). The pathogenesis of idiopathic Parkinson’s disease is unknown. However, two independent missense mutations in α-synuclein have been shown to cause autosomal heritable early-onset Parkinson’s disease. Thus, abnormal α-synuclein metabolism is linked both to the development of rare cases of heritable Parkinson’s disease and to the common lesions in idiopathic Parkinson’s disease (4, 5).

α-Synuclein is a member of the conserved synuclein gene family of which at least three species, α-, β- and γ-synuclein are expressed in the human nervous system (for recent reviews, see Refs. 6 and 7). Synucleins have been identified in various species, in Torpedo by an antiserum raised against synaptic vesicles (8), in bovine brain by their acidic nature (9), in zebra finches as a gene product of highly regulated expression (10), in rats due to selective expression in dorsal root ganglia (11). In man, γ-synuclein was recognized as a protein that is up-regulated in various carcinomas (12–14), and β-synuclein was identified as a protein cross-reacting with an antibody against phosphorylated tau (15). Human α-synuclein was originally identified as the precursor of a peptide, non-Aβ component of Alzheimer’s disease amyloid tightly associated to Alzheimer’s disease amyloid (16) and later purified as an inhibitor of phospholipase D2 (17).

α-Synuclein is a small acidic protein of 140 amino acid residues. The N-terminal part has 7 imperfect repeats containing the consensus core sequence Lys-Thr-Lys-Glu-Gly-Val, whereas the C-terminal part has no recognized structural elements. α-Synuclein displays an extended unfolded structure and thus belongs to the group of natively unfolded proteins also comprising protein tau (18). α-Synuclein partitions between the soluble cytosolic phase and a vesicle-bound fraction (19, 20). The binding to vesicles is mediated through determinants in the N-terminal repeat region (20) and might induce an increased helical content in α-synuclein as demonstrated after binding to liposomes (21). The Parkinson’s disease causing Ala30 → Thr constructs and the deletion mutants α-synuclein(1–87), α-synuclein(30–140) and α-synuclein(55–140) were also produced by polymerase chain reaction (20). All constructs were verified by DNA sequencing and subcloned into expression plasmid pET-3d (Stratagene). Expression and purification of the recombinant proteins were done as described for α-synuclein (15). The identity of the mutant proteins were verified by matrix-assisted laser desorption ionization–mass spectrometry and N-terminal Edman degradation (22). The following human tau proteins were expressed in E. coli and purified as described previously (23, 24); tau40 and tau24, containing 4 microtu-
binding (MT-binding tandem repeats and 2 and 0 N-terminal insertions, named Cys-Gly-Ser-Leu-Gly-Asn-Ile-His-His-Lys-Pro-Gly-Gly-Gln-Val-Glu-Ile-Lys-Tyr-Glu-Lys, corresponding to the consensus sequence for a single MT binding tandem repeat in tau40), was synthesized at Kem-En-Tech, Copenhagen, Denmark. Bovine tubulin was from Cytokeleton. Bovine serum albumin was from Sigma.

Antibodies

Monoclonal antibody H3C raised against the 15 C-terminal amino acid residues of zebra finch α-synuclein was a gift from Dr. J. George (University of Illinois, Urbana, IL) (10, 19). Tau antibodies were anti-serum BR133 raised against the 16 N-terminal amino acid residues in tau, anti-serum BR135 against amino acid residues 323–335 in tau40 (26), and rabbit anti-human tau IgG (Dako, Copenhagen, Denmark). Mouse monoclonals against neurofilament proteins 2F11 (specific for the 200-, 160-, and 70-kDa chains) and NR4 (specific for the 200-, 160-, and 70-kDa chains) were from Dako. Mouse monoclonal antibodies against α-tubulin (DM 1A) and β-tubulin Tub 2.1 were from Sigma.

Cell Culture and Immunofluorescence Microscopy

 Cultures of hippocampal cells were prepared from the brains of 18-day-old rat embryos (27) and used after 15 days of culture (stage 5 neurons). To label α-synuclein, tau, and MAP-2, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized in 0.1% Triton X-100 for 10 min and processed for immunofluorescence microscopy as described (28).

Electrophoresis and Electroblotting

Proteins and peptides were resolved by 8–16% gradient sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue (22). Immunoblotting was performed after transfer from the polyacrylamide gel onto a Immobilon polyvinyldene difluoride membrane (29).

Labeling of Synuclein and Tau Peptides

Recombinant α-synuclein was biotinylated as described previously (22). Recombinant tau40 was iodinated using chloramine T as oxidizing agent to a specific activity of 250 mCi/mg giving a molar ratio of 125I/tau40 of approximately 0.5. In brief, 6 μg of tau40 was incubated with 100 pmol of 125I in 0.2 ml phosphate, pH 8.0, containing 0.1 mg/ml chloramine T for 3 min at 20 °C, followed by filtration through a 2-ml Sephadex G25 column equilibrated in 20 mM Hepes, pH 7.4, 0.01% Tween 20. The tracer was stored at −20 °C. The iodinated tau40 CNBr fragments presented in Fig. 4B were prepared using the same procedure that yielded tracers of equal specific activities.

Affinity Purification of Human Brain Tau by α-Synuclein Affinity Chromatography

Recombinant human α-synuclein was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) at a concentration of 5 mg of α-synuclein/ml of gel according to the recommendations of the manufacturer. An equal concentration of rabbit IgG raised against human plasminogen activator inhibitor-2 was coupled to CNBr-activated Sepharose 4B as control. Human brain cytosol was prepared according to Ref. 30. The brain cytosol supernatant (200 ml) was passed through the α-synuclein-Sepharose column (2 ml bed volume) at a flow rate of 0.25 ml/min at 4 °C. The column was washed with 30 ml of phosphate-buffered saline and 1 ml EDTA, and the bound proteins were eluted by 50 mM glycine, pH 2.5, into 1 volume of 1 M Tris, pH 8.0.

RESULTS

Cytosolic Tau Binds to α-Synuclein—Affinity chromatography of human brain cytosol preparations was performed on

Microplate Assay—Recombinant α-synuclein (100 μl of 15 μg/ml in 200 mM NaHCO₃, pH 9.6, was immobilized on Maxisorb microtiter plates (Nunc) for 2 h on ice, and residual protein binding sites were blocked by further incubation with 5% bovine serum albumin (Sigma) for 2 h. After rinsing, the wells were incubated with 50 pm 125I-tau40 in the presence of various concentrations of competitors for 16 h at 4 °C in binding buffer (150 mM KCl, 2 mM MgCl₂, 0.01% bovine serum albumin, 20 mM Tris, pH 7.4). Unbound ligand was removed by rinsing three times in 200 μl of binding buffer, and bound tracer was quantified by γ-counting (Packard Cobra II) after release by incubation with 200 μl of 5% SDS.

Plasmon Surface Resonance Assay—All measurements were performed on a BIAcore 2000 instrument (Biovessor, Uppsala, Sweden) equipped with CM5 sensor chips. The carboxylated dextran matrix of the sensor chip was activated by the injection of 60 μl of a solution containing 0.2 M N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide in water. Recombinant α-synuclein was then immobilized at a concentration of 50 μg/ml in 10 mM sodium acetate, pH 3.5. A parallel channel of the sensor chip was derivatized under identical conditions in the absence of protein and was used as a control. The remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. The surface plasmon resonance signal from immobilized α-synuclein generated 714 BIAcore response units equivalent to 44.6 fmol of ligand/mm². Screening of peptides for binding to α-synuclein was performed by injecting aliquots (120 μl) of samples (approximately 100 μg of protein/ml) onto the derivatized sensor chip. The samples were 10 mM Hepes, 150 mM NaCl, 0.1% CNBr,对该肽链的表达量进行定量。

CNBr Cleavage and Peptide Purification

Lyophilized tau40 (100 μg) was dissolved in 50 μl of 70% formic acid containing 0.2 mg of CNBr (Sigma) and incubated for 24 h at 20 °C in the dark. After the cleavage reaction, the reaction was terminated by lyophilization.

The peptides were purified on a μRPC C2/C18 PC 3.2/3 reversed phase column (Amersham Pharmacia Biotech) using a SMART chromatography system (Amersham Pharmacia Biotech). The peptides were loaded on the column in 0.1% trifluoroacetic acid and eluted using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The eluate was monitored using absorbances of 212 and 280 nm (μPeak Monitor, Amersham Pharmacia Biotech) and collected on a μFraction collector (Amersham Pharmacia Biotech) in the peak fractionation mode. The samples specified in Fig. 4 were lyophilized and dissolved in Binding Buffer, and their purity was analyzed by SDS-PAGE. The protein concentration of the samples was analyzed by laser scanning densitometry of the Coomassie Brilliant Blue-stained bands by comparing the integrated intensities of the bands with a serial dilution from 5 to 0.25 μg of bovine serum albumin. The synuclein binding activity of the samples was further analyzed by surface plasmon resonance analysis and, after iodination, by binding to immobilized recombinant synuclein (see above).

Tau Phosphorylation Assay

Prior to phosphorylation, tau isoforms and α-synuclein were dialyzed against water. The phosphorylation assay was carried out in 40 mM Tris-HCl, pH 7.4, 20 mM Mg acetate at 37 °C. First, tau (1 μmol) was incubated with different concentrations of α-synuclein for different time periods to facilitate their interaction. Subsequently, phosphorylation was initiated by the addition of ATP (2 mM) and the catalytic subunit of protein kinase A (PKA; Promega) (0.5 units/ml) and the incubation was allowed to proceed another 2 h. The reaction was terminated by addition of 1/3 volume of SDS-gel electrophoresis sample buffer followed by heating to 95 °C for 3 min. The samples were processed by SDS-PAGE and immunoblotted using the mouse monoclonal antibody 12E8 at a dilution of 1/50,000 for the detection of phosphorylated serines 262 and 266 (31). For the detection of all tau, irrespective of its phosphorylation state, the monoclonal antibody 12E8 was stripped of the membrane by heating to 95 °C for 3 min and residual protein binding sites were blocked with 100 μg of Protein A/ml of gel according to the recommendations of the manufacturer. An equal concentration of rabbit IgG raised against human plasminogen activator inhibitor-2 was coupled to CNBr-activated Sepharose 4B as control. Human brain cytosol was prepared according to Ref. 30. The brain cytosol supernatant (200 ml) was passed through the α-synuclein-Sepharose column (2 ml bed volume) at a flow rate of 0.25 ml/min at 4 °C. The column was washed with 30 ml of phosphate-buffered saline and 1 ml EDTA, and the bound proteins were eluted by 50 mM glycine, pH 2.5, into 1 volume of 1 M Tris, pH 8.0.

The abbreviations used are: MT, microtubule; MT-BD, microtubule-binding domain; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A.
immobilized recombinant human α-synuclein to identify α-synuclein binding proteins. Fig. 1 shows that proteinaceous material only eluted from the α-synuclein-matrix but not from the control matrix. The proteins in fraction 4 from the α-synuclein affinity column comprised a complex mixture of proteins in the molecular range from 40 to 250 kDa as determined by silver staining of SDS-polyacrylamide gels (data not shown). We have previously demonstrated specific binding of biotinylated α-synuclein to human hippocampal tissue (22). Initial experiments showed that preincubation of the tissue sections with some antibodies against the neuronal cytoskeletal elements α- or β-tubulin, neurofilament chains, and tau inhibited the binding of biotin-α-synuclein, whereas antibodies against actin, neuronal enolase and synaptophysin had no effect (data not shown). Accordingly, we searched for candidate α-synuclein ligands among proteins associated with the cytoskeleton. Only tau was identified as two BR135 anti-tau binding polyanionic glycosaminoglycan, heparin, also inhibited the tau binding to α-synuclein, indicating that charge interactions are important (data not shown).

We expressed and purified the two α-synuclein mutants Ala50 → Pro and Ala53 → Thr since they are associated with rare cases of early-onset Parkinson’s disease (4, 5). However, the mutations did not perturb the tau binding activity as determined by the direct 125I-tau40 binding assays (data not shown). Immobilized recombinant human β-synuclein and γ-synuclein bound 125I-tau40 to about the same level as α-synuclein (data not shown).

Identification of the Synuclein Binding Domain in Tau—Our initial strategy for identifying the α-synuclein binding domains in tau was to fragment the protein and characterize the α-synuclein binding peptides. Recombinant human tau40 was fragmented by CNBr, and the digest was subsequently resolved by reversed phase chromatography (Fig. 4A). The two peaks, A and B, were resolved to near base-line levels upon elution of the C18 column with a gradient of acetonitrile. The eluates corresponding to the dark bars were collected, lyophilized, and dissolved in equal volumes of binding buffer. Peaks A and B each contained one predominating peptide as judged by a Coomassie Blue stained SDS-polyacrylamide gel (Fig. 4B, lanes 1 and 2). Matrix-assisted laser desorption ionization-mass spectrometry of samples from peaks A and B detected only a single peptide in each peak, their masses being compatible with the recombinant tau40 CNBr cleavage products, amino acid residues 128–250 and 251–419, respectively (data not shown). Their identity was further confirmed by immunoblotting with the epitope-specific

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**Fig. 1. Purification of tau by α-synuclein affinity chromatography.** A human brain cytosol preparation was passed through 2-ml columns containing either α-synuclein-Sepharose (5 mg/ml) or anti-plasminogen activator inhibitor-2 rabbit IgG-Sepharose (5 mg/ml) and eluted by 50 mM glycine, pH 2.5. The ordinate shows the protein concentration as expressed by absorbance at 280 nm. The presence of tau in the samples was analyzed by anti-tau immunoblotting (inset). Samples (20 μl) from fractions 1–8 were supplemented with sample buffer, resolved by 8–16% gradient SDS-polyacrylamide gel electrophoresis, electroblotted onto a polyvinylidene difluoride membrane and probed by the anti-tau rabbit serum BR135 in a dilution of 1/10000. Bound antibody was detected by chemiluminescence. The two tau immunoreactive bands migrated corresponding to molecular masses of 51 and 54 kDa, respectively. Silver staining of a SDS-polyacrylamide gel corresponding to the above blotted gel revealed that bands corresponding to the molecular mass of tau represented a significant, but not major, fraction of the proteins eluted from the affinity column.
Fig. 2. α-Synuclein and tau colocalize in axons. The localization of α-synuclein, tau, and the somatodendritic marker MAP-2 was investigated in fetal rat hippocampal neurons cultured for 15 days. α-Synuclein was demonstrated by the mouse monoclonal antibody H3C diluted 1/1000 (panels A, D, and F), and MAP-2 by the polyclonal antibody 514, diluted 1/2000 (panel B). Tau was demonstrated by the rabbit polyclonal antibody BR134 diluted 1/3000 (panels C, E, and G). Fluorescein isothiocyanate - and rhodamine-conjugated secondary antibodies were used for the detection of the mouse and rabbit IgG, respectively. The bar below panel A represents 20 μm and applies to the three top panels, and the bar below panel D represents 10 μm and applies to the four lower panels.

Fig. 3. Direct binding of tau40 to immobilized α-synuclein. Recombinant human α-synuclein, immobilized in microtiter wells, was incubated with 125I-labeled recombinant human tau40 (50 pM) in the presence of increasing concentrations of unlabeled tau40. The ordinate shows the ratio of bound versus free tau40 (B/F), and the abscissa shows the total concentration of free tau40. The points show the mean ± 1 S.D. of four replicates. One of three similar experiments are presented.

Anti-tau sera BR133 and BR135, respectively. Only peptide B bound BR135, which is raised against amino acid residues 323–335 in the MT-binding domain of tau40 (Fig. 4B, lane 7), whereas none of the peptides were recognized by the N-terminal specific BR133 (data not shown). The small amounts of Coomassie Blue-stained higher molecular weight peptides in peak B (Fig. 4B, lane 2) also contained the epitope for BR135 (Fig. 4F, lane 7).

Peptides A and B were iodinated by the procedure described for tau40 yielding the tracers displayed in Fig. 4B (lanes 4 and 5). In the synuclein binding assay, 125I-peptide B bound to α-synuclein whereas 125I-peptide A did not bind (Fig. 4C). We next tested the binding activity of the unlabeled peptides A and B by surface plasmon resonance technique, which measures binding of peptide mass to immobilized α-synuclein (Fig. 4D).

Application of 500 nM peptide B resulted in the binding corresponding to approximately 600 response units as compared with approximately 1100 response units for the positive control tau40 applied at the same molar concentration, and binding of both ligands was reversible upon removal of the unbound ligand (Fig. 4D). The application of a similar concentration of peptide A to the α-synuclein chip generated a small response of about 20% of that of peptide B. Peptide A did not contain any contaminating peptide B as determined by immunoblotting using the BR135 antiserum or matrix-assisted laser desorption ionization-mass spectrometry (data not shown). The small response might reflect nonspecific adsorption or a low affinity binding not detected by the microtiter plate assay (Fig. 4C). Hence, the major α-synuclein binding site in tau resides in the C-terminal segment, which also contains the MT-BD (Fig. 5).

Different tau isoforms are expressed in the human central nervous system during development. They differ with regard to the presence of zero, one or two N-terminal insertions and the presence of 3 or 4 repeats in the MT-BD. The longest isoform of 440 amino acid residues, tau40 (Fig. 5) contains 2 N-terminal insertions and 4 MT binding repeats. As a further approach to identify the ligand binding segment in tau, we determined the inhibitory effects of truncated recombinant tau peptides on the binding of 125I-tau40 to immobilized α-synuclein. Fig. 6 confirms that a binding site resides in the C-terminal part of tau as demonstrated by the inhibition by the tau40-(192–383) peptide as compared with the lack of inhibitory activity of the N-terminal tau40-(1–192). The MT-BD peptide, representing 3 repeats of the MT-BD, possessed full inhibitory activity like tau40 (Fig. 6). We next tested the inhibitory activity of MAP2C, which is an isoform of the MAP-2 gene product expressed in the fetal brain. Its only structural homology to tau is in the 3 terminal tau40-(1–192). The MT-BD peptide, representing 3 repeats of the MT-BD, possessed full inhibitory activity like tau40 (Fig. 6). We next tested the inhibitory activity of MAP2C, which is an isoform of the MAP-2 gene product expressed in the fetal brain. Its only structural homology to tau is in the 3 terminal tau40-(1–192). The MT-BD peptide, representing 3 repeats of the MT-BD, possessed full inhibitory activity like tau40 (Fig. 6). We next tested the inhibitory activity of MAP2C, which is an isoform of the MAP-2 gene product expressed in the fetal brain. Its only structural homology to tau is in the 3 terminal tau40-(1–192). The MT-BD peptide, representing 3 repeats of the MT-BD, possessed full inhibitory activity like tau40 (Fig. 6). We next tested the inhibitory activity of MAP2C, which is an isoform of the MAP-2 gene product expressed in the fetal brain. Its only structural homology to tau is in the 3

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2 to 1 molar (data not shown). The results suggest that two or three of the repeat units in the positively charged MT-BD are needed for full binding activity.

Identification of the Tau Binding Segment in α-Synuclein—

The primary structure of α-synuclein is characterized by the presence of 7 imperfect Lys-Thr-Lys-Glu-Gly-Val consensus core repeats and a positive net charge within the N-terminal two thirds as compared with the remaining C-terminal part, which carries a strong negative charge and no known structural or repeated elements (Fig. 5). To determine the tau binding segment, we expressed and purified 3 deletion mutants, α-synuclein-(30–140), α-synuclein-(55–140) and α-synuclein-(1–87), which lack the N-terminal 2 and 4 Lys-Thr-Lys-Glu-Gly-Val repeats and the acidic C terminus, respectively. Fig. 7 (upper panel) shows that the acknowledged slow migration of wild type α-synuclein (lane 1) is accentuated for the more acidic N-terminal deletion mutants. The identity of all peptides were verified by mass spectrometry and N-terminal amino acid sequencing. When tested for tau binding activity in the microtiter plate assay, it was evident that the N-terminally truncated proteins retained their binding activity, whereas removal of the C-terminal residues 88–140 reduced the binding by more than 90% (Fig. 7, lower panel). As a positive control for the correct immobilization of α-synuclein-(1–87), we measured the binding of 125I-αβ to the same peptides. The 125I-αβ binding to α-synuclein-(1–87) corresponded to 70% of the binding to the wild type α-synuclein. This is compatible with the known presence of several αβ-binding sites, some of which are located within residues 1–87 (22, 33).

The identification of the MT-BD of tau as the α-synuclein binding site made us investigate whether tubulin inhibits the interaction. Fig. 8 shows that tubulin indeed inhibits the binding of 50 pM 125I-tau40 to immobilized α-synuclein (IC50 about 500 nM) although less effectively than tau40 itself (Figs. 8 and 3). The inhibition of the binding was not due to tubulin binding to α-synuclein (data not shown). The competition between
FIG. 5. Schematic representation of the domain structure of tau40 and α-synuclein and their interacting segments. Tau40 is shown with its 2 N-terminal insertions (hatched), the 4 tandem repeats in the microtubule binding domain (shaded), and the positions of the phosphoserines 262 and 356 recognized by the monoclonal 12E8 antibody. The scale bar above tau40 shows the spacing of the 440 amino acid residues. Different tau isoforms vary by the presence of N-terminal insertions and the number of tandem repeats in the microtubule binding domain (see “Experimental Procedures”). The 140-amino acid α-synuclein is shown with the 7 consensus Lys-Thr-Lys-Glu-Gly-Val core repeats, and the positions of residues 30, 55, and 87, which represent the borders of the truncated peptides used for identifying the tau binding segment. The borders of the interacting segments in the two proteins are shown by arrows.

α-synuclein and tubulin for the MT-BD in tau was also demonstrated in assays using immobilized tau and radiolabeled tubulin (data not shown). Accordingly, α-synuclein is a ligand for soluble tau whereas MT-bound tau is unable to interact with α-synuclein. This explains why we were unable to demonstrate ternary complexes between MT-tau and α-synuclein in a classical MT spin-down assay (data not shown) and is in agreement with previous studies of MT-associated proteins where α-synuclein has not been recognized.

α-Synuclein Enhances the Protein Kinase A-catalyzed Phosphorylation of Serines 262 and 356 in Tau—Stimulation of tubulin assembly and stabilization of microtubules are recognized functions of tau caused by its interaction with monomeric tubulins. The interaction between tubulins and tau is known to be regulated by tau phosphorylation. The interaction between α-synuclein and the MT-BD of tau made us investigate whether α-synuclein is a candidate neuronal modulator of tau phosphorylation. The phosphorylation of tau is complex with multiple Ser/Thr residues being recognized by several kinases. As a model system for the study of the α-synuclein effect on tau phosphorylation we chose to study the phosphorylation of Ser262/356 in tau by PKA. The reasons were: (i) PKA phosphorylation of tau generates phospho-epitopes recognized by the monoclonal antibody 12E8 in neuritic tangles, (ii) the PKA-catalyzed phosphorylation of Ser262/356 is specifically recognized by 12E8 (31), and (iii) Ser262/356 are within the proposed α-synuclein binding domain.

FIG. 6. The microtubule binding repeat region of tau binds α-synuclein. [125]I-Tau40 (50 pM) was incubated with immobilized α-synuclein in the absence (left column) and presence of 1 μM each of various recombinant tau fragments, full-length tau40, tau40-(1–192), tau40-(192–383), MT-BD corresponding to the three-repeat MT-binding domain in tau23, and the microtubule-associated protein MAP-2C. The columns show the tracer binding presented as the mean ± 1 S.D. of four replicates. One of three similar experiments is demonstrated.

α-Synuclein in the absence (left column) and presence of 1 μM each of various recombinant tau fragments, full-length tau40, tau40-(1–192), tau40-(192–383), MT-BD corresponding to the three-repeat MT-binding domain in tau23, and the microtubule-associated protein MAP-2C. The columns show the tracer binding presented as the mean ± 1 S.D. of four replicates. One of three similar experiments is demonstrated.

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of monoclonal antibody 12E8 as demonstrated by immunoblotting (Fig. 9 A, lane 1). Inclusion of α-synuclein in the reaction mixture caused a dose-dependent increase in the 12E8 binding to tau when used at α-synuclein/tau ratios of 3, 6, 12, and 24 (Fig. 9 A, lanes 2–5). α-Synuclein stimulated phosphorylation of more than one site, as demonstrated by the change in the electrophoretic migration of the phosphorylated tau from a major 66-kDa 12E8 immuno-reactive band to a predominance of a slightly slower migrating species. Laser scanning densitometry of the tau bands in Fig. 9 A shows the probing of the membrane with the monoclonal 12E8 antibody specific for tau phosphorylated on serines 262 and 356. B, to ascertain the tau loading of the gel, the same membrane was stripped of the 12E8 antibody and probed with the phosphorylation independent anti-tau serum BR133. The binding of each primary antibody was visualized using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. The molecular size markers × 10−2 are indicated to the left. One of four similar experiments are shown. C, dose response of the protein kinase A-catalyzed phosphorylation of α-synuclein (24 μM) was incubated with increasing concentrations of protein kinase A (lanes 2 and 3, 1.5 units/ml; lane 4, 2 units/ml; and lanes 5 and 6, 2.5 units/ml). Lanes 3 and 6 were supplemented with recombinant α-synuclein (24 μM). Tau phosphorylation was detected using the 12E8 antibody as shown in panel A. Laser scanning densitometry of the 12E8-labeled tau double band demonstrated an almost linear phosphorylation response to the kinase concentrations employed in the absence of α-synuclein. At protein kinase A concentrations of 1.5 and 2.5 units/ml, α-synuclein caused approximately 66% and 22% increase in the 12E8 immunoreactivity as compared with the samples without α-synuclein. One of three similar experiments is shown. D, α-synuclein does not induce novel 12E8 immunoreactive epitopes in tau43. The phosphorylation of 1 μM tau43 by protein kinase A (1 unit/ml) (lane 1) is increased by the presence of 24 μM α-synuclein (lane 2). By contrast, no 12E8 immunoreactivity is generated when the double mutant tau43(Ser262→Ala/Ser356→Ala) is incubated with protein kinase A in the absence or presence of 24 μM α-synuclein (lanes 3 versus 4). The loading of tau43 in lanes 1–4 is demonstrated by the reprobing of the membrane with the anti-tau serum BR133 (lanes 5–8). One of two similar experiments is shown.

FIG. 9. α-Synuclein stimulates the protein kinase A-catalyzed phosphorylation of Ser262/356 of tau40. A and B, tau40 (1 μM) was incubated with the catalytic subunit of protein kinase A (0.5 units/ml) for 2 h in the absence (lane 1), and presence of recombinant α-synuclein in a concentration of 3, 6, 12, and 24 μM (lanes 2–5), respectively. Lane 6 represents a sample as in lane 5 but without ATP. The samples were resolved by reducing 8–16% gradient SDS-polyacrylamide gel electrophoresis, electroblotted, and analyzed by immunoblotting. A shows the probing of the membrane with the monoclonal 12E8 antibody specific for tau phosphorylated on serines 262 and 356. B, to ascertain the tau loading of the gel, the same membrane was stripped of the 12E8 antibody and probed with the phosphorylation independent anti-tau serum BR133. The binding of each primary antibody was visualized using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. The molecular size markers × 10−2 are indicated to the left. One of four similar experiments are shown. C, dose response of the protein kinase A-catalyzed phosphorylation of tau40. Tau40 (1 μM) was incubated with increasing concentrations of protein kinase A (lane 1, 1 unit/ml; lanes 2 and 3, 1.5 units/ml; lane 4, 2 units/ml; and lanes 5 and 6, 2.5 units/ml). Lanes 3 and 6 were supplemented with recombinant α-synuclein (24 μM). Tau phosphorylation was detected using the 12E8 antibody as shown in panel A. Laser scanning densitometry of the 12E8-labeled tau double band demonstrated an almost linear phosphorylation response to the kinase concentrations employed in the absence of α-synuclein. At protein kinase A concentrations of 1.5 and 2.5 units/ml, α-synuclein caused approximately 66% and 22% increase in the 12E8 immunoreactivity as compared with the samples without α-synuclein. One of three similar experiments is shown. D, α-synuclein does not induce novel 12E8 immunoreactive epitopes in tau43. The phosphorylation of 1 μM tau43 by protein kinase A (1 unit/ml) (lane 1) is increased by the presence of 24 μM α-synuclein (lane 2). By contrast, no 12E8 immunoreactivity is generated when the double mutant tau43(Ser262→Ala/Ser356→Ala) is incubated with protein kinase A in the absence or presence of 24 μM α-synuclein (lanes 3 versus 4). The loading of tau43 in lanes 1–4 is demonstrated by the reprobing of the membrane with the anti-tau serum BR133 (lanes 5–8). One of two similar experiments is shown.

DISCUSSION

We have identified the MT-binding protein tau as a ligand for α-synuclein by affinity chromatography of human brain cytosol and confirmed the direct interaction by in vitro binding assays using recombinant human α-synuclein and tau. α-Synuclein is recognized as a preterminal protein (34) and is, as such, not expected to colocalize with tau in the axon. However, in our study of axonal synuclein transport in the rat optic system, we find α-synuclein predominantly is moved by the slow component b of axonal transport and even with approximately 15% moved by the axoskeleton in slow component a.2 Tau is predominantly moved by slow component a and to a minor extent slow component b (35). Hence, α-synuclein and tau have ample opportunities for interactions within the axonal compartment. The α-synuclein binding domain in tau was localized to the MT binding repeat region by binding analysis of tau40 fragments and competition analysis using truncated recombinant tau peptides. We observed no difference in the affinity for α-synuclein of tau isoforms having 3 or 4 repeats in the MT-BD, whereas a synthetic peptide corresponding to a single repeat displayed a very poor affinity as determined by its

2 Jensen, P. H., Li, J.-Y., Dahlström, A., and Dotti, C. G. (1999) Eur. J. Neurosci., in press.
competition of tau40 binding to α-synuclein. Thus, a cooperative interaction of two or more of the MT-binding repeats seems to occur as also demonstrated for the interaction between tau and microtubules (36, 37). Other ligands binding to the MT binding domain in tau comprise tubulins, heparan sulfate proteoglycans, and presenilin-1 (38, 39), but α-synuclein is the first tau ligand whose expression is almost exclusively limited to the nervous system. The tau binding-site in α-synuclein resides in the acidic C-terminal 53 residues in analogy to the tau binding acidic C termini of tubulins (40). The importance of charge for the interaction is reflected in the sensitivity to increased ionic strength, the polyanion heparin, and the positive tau binding to β- and γ-synuclein, being only 36% and 17% identical in this region, but with a negative net charge. The tau binding site in α-synuclein is different from the binding sites for the previously recognized ligands, Alzheimer’s disease amyloid peptide Aβ and rat brain vesicles, which both bind within the conserved N-terminal 95 residues that contain the synuclein-specific consensus repeats (20, 22, 33). This opens the possibility that α-synuclein might have bridging functions and bring different classes of ligands together.

α-Synuclein has not been recognized among the extensively studied group of MT-associated proteins despite its abundance in brain and its affinity for tau (for a recent review, see Ref. 41). Our data show that tubulin at high concentrations, as found locally in microtubules, inhibits the binding of α-synuclein to tau (Fig. 8). This points to α-synuclein as a ligand for the pool of soluble tau in contrast to the protein phosphatases 1 and 2A (42, 43). An increased susceptibility of soluble tau to phosphorylation could be a functional consequence of the interactions as suggested by the α-synuclein-stimulated protein kinase A-catalyzed phosphorylation (Fig. 9). The α-synuclein-stimulated phosphorylation occurred at α-synuclein concentrations below 3 μM that is lower than the synuclein concentration of about 0.1% of the total protein in rat brain homogenates (44). We show that at least two serine residues are susceptible to α-synuclein modulation of their protein kinase A-catalyzed phosphorylation by the presence of two 12E8 immunoreactive bands. However, other serines are likely to be phosphorylated, as demonstrated by the α-synuclein stimulated phosphorylation of the mutant tau24(Ser262 → Ala/Ser356 → Ala) that lacks the 12E8 epitopes (Fig. 9D, lanes 1 and 2). However, this question has to be addressed by a different experimental approach. Ser262 is localized within the MT binding repeat region, and its phosphorylation inhibits tau binding to microtubules (45). Ser262 phosphorylation is observed in neuritic tangles of Alzheimer’s disease and the filamentous deposits in the tautopathies, frontotemporal dementia, and parkinsonism linked to chromosome 17, corticobasal degeneration, and progressive supranuclear palsy (1). A direct involvement of α-synuclein in the development of Ser262 phosphorylation is possible in Alzheimer’s disease, where the α-synuclein expression is increased at early disease stages (46) and α-synuclein-positive neuritic tangles have been identified in layers 5 and 6 of hippocampus (47). Moreover, coexisting abnormalities in the metabolism of tau and α-synuclein, leading to the formation of neuritic tangles and Lewy bodies, have been demonstrated within the same amygdala neurons in Alzheimer’s disease, Parkinson’s disease, and Lewy body dementia (48, 49). The absent effect of the Parkinson’s disease mutations on the α-synuclein-stimulated tau phosphorylation suggests abnormal tau interactions are unlikely to be operating in these families.

Molecular mechanisms modulating the phosphorylation state of tau rely on an interplay between kinase and phosphatases and possibly accessory proteins like α-synuclein and presenilin 1 (39). The transmembrane endoplasmic reticulum protein presenilin 1 facilitates tau phosphorylation by assembling the kinase, glycogen synthase kinase-3β and its substrate tau (39). Like presenilin 1, α-synuclein may facilitate tau phosphorylation by altering the presentation of specific serines to protein kinase A and other Ser262-reactive kinases, e.g., Ca2+-calmodulin-dependent protein kinase II and microtubule affinity-regulating kinase (50) as well as non-Ser262-directed kinases. The structural change that alters the presentation of the specific serines may have other consequences, e.g., the facilitation of binding to hitherto unrecognized tau ligands. Hypothetically, the segregation of the binding sites for tau and brain vesicles to the C- and N-terminal parts of α-synuclein, respectively, may enable α-synuclein to function as a molecular tether that brings tau in proximity to vesicle surfaces, and other putative ligands specific for the N termini of α-synuclein. Such a vesicle-targeting of tau could be dynamically regulated by phospholipases that regulate the phospholipid composition of the vesicles. Phospholipase D2 converts the neutral phosphatidyl choline to the acidic phosphatidic acid, and this changed phospholipid ratio would favor binding of α-synuclein and its cargo (21). In the same instance, α-synuclein would inhibit the phospholipase D2 and indirectly the generation of its binding sites (17).

In conclusion, we identify soluble tau as a ligand for α-synuclein and show that α-synuclein can modulate the phosphorylation of tau. This opens the possibility that axonally transported α-synuclein, on its way to the synaptic terminal, indirectly can influence MT dynamics by decreasing the concentration of MT-binding-competent tau. The stimulatory effect of α-synuclein on tau phosphorylation, and the recently demonstrated phospholipase D2 inhibitory effect of α-synuclein (17), further demonstrate that α-synuclein has the propensity to affect diverse intracellular signaling pathways.

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α-Synuclein and Protein Tau Interactions

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