p25α Stimulates α-Synuclein Aggregation and Is Co-localized with Aggregated α-Synuclein in α-Synucleinopathies*

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Aggregation of the nerve cell protein α-synuclein is a characteristic of the common neurodegenerative α-synucleinopathies like Parkinson’s disease and Lewy body dementia and it plays a direct pathogenic role as demonstrated by early onset diseases caused by missense mutations and multiplication of the α-synuclein gene. We investigated the existence of α-synuclein pro-aggregatory brain proteins whose dysregulation may contribute to disease progression, and we identified the brain-specific p25α as a candidate that preferentially binds to α-synuclein in its aggregated state. Functionally, purified recombinant human p25α strongly stimulates the aggregation of α-synuclein in vitro as demonstrated by thioflavin-T fluorescence and quantitative electron microscopy. p25α is normally only expressed in oligodendrocytes in contrast to α-synuclein, which is normally only expressed in neurons. This expression pattern is changed in α-synucleinopathies. In multiple systems atrophy, degenerating oligodendrocytes displayed accumulation of p25α and dystopically expressed α-synuclein in the glial cytoplasmic inclusions. In Parkinson’s disease and Lewy body dementia, p25α was detectable in the neuronal Lewy body inclusions along with α-synuclein. The localization in α-synuclein-containing inclusions was verified biochemically by immunological detection in Lewy body inclusions purified from Lewy body dementia tissue and glial cytoplasmic inclusions purified from tissue from multiple systems atrophy. We suggest that p25α plays a pro-aggregatory role in the common neurodegenerative disorders hallmark by α-synuclein aggregates.

The group of α-synucleinopathies is dominated by the frequent neurodegenerative disorders Parkinson’s disease (PD),

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† The abbreviations used are: PD, Parkinson’s disease; AS, α-synuclein; CD, circular dichroism; LBD, Lewy body dementia; MSA, multiple systems atrophy; NEPHGE, nonequilibrium pH-gradient gel electrophoresis, PBS, phosphate-buffered saline; SASD, sulfosuccinimidyl-2-[(pyridinedithio)ethyl]-1,3-dithiopropionate.

The mechanisms governing AS aggregation in the sporadic α-synucleinopathies remain unexplained, as is the case for tau and Aβ in sporadic Alzheimer’s disease, but they are likely to involve perturbations in age-dependent, genetic, and environmental balances of pro- and anti-aggregative factors. We identified p25α as an AS filament-binding protein, which in stoichiometric amounts stimulates AS aggregation. p25α was originally co-purified with a tau kinase preparation from bovine brain (25) as a protein localized to oligodendrocytes (26). Functionally, p25α is subject to phosphorylation by several kinases (25, 27, 28), and it acts as a microtubule-associated protein that causes the formation of aberrant bundles of microtubules.
**Materials and Methods**

**Miscellaneous and Proteins**—Recombinant human full-length AS (1–140), AS (1–125), AS (1–110), AS (1–95), and p25-synuclein were expressed in *Escherichia coli* and purified as described previously (30–32). This p25-synuclein, was followed by an additional reverse phase-high pressure liquid chromatography purification step on a Jupiter C18 column (Phenomenex, Torrance, CA) in 0.1% trifluoroacetic acid with an acetonitrile gradient. The proteins were subsequently aliquoted, lyophilized, and stored at −80 °C. The synthetic peptide AS (109–140) was originally described by Nielsen et al. (32). The affinity-purified sheep anti-α-synuclein antibody has been described previously (33). The rabbit IgG p25-synuclein was raised against aggregated AS as described previously (34). The anti-bovine p25-synuclein was raised against Aβ1-42, Aβ1-40, AS-(1–140), AS-(1–125), AS-(1–110), AS-(1–95), and Aβ1-40) as formaldehyde-fixed cross-linked gels. The identity of human AS in Lewy bodies, Lewy neurites, and glial cytoplasmic inclusions. This suggests that by bringing the pro-aggregative p25 into contact with AS, abnormal p25-synuclein expression in neurons and AS in oligodendrocytes may actively contribute to the degenerative process. This would seem to represent a prodigenerative factor in the group of α-synucleinopathies.

**Phoboaaffinity Cross-linking of α-Synuclein Aggregate-bound Proteins**—A chemical cross-linking assay was established in order to investigate protein-protein interactions involving aggregate-bound AS. For this purpose, we used the cross-linker sulfo-SMCC (Pierce). SADS is a heterobifunctional cross-linking reagent that contains a photoreactive cross-linking group and an amine-reactive group and produces a 18.9-Å bridge between conjugated molecules (38). The bridge of SADS contains a disulfide bridge that provides covalently an aggregation site. Its photosensitive part can be radiolabeled with 125I prior to the conjugation reaction. After cleavage by a reducing agent, the radioactive label will remain attached to the protein conjugated by photoinitiation and thus allow identification of the labeled protein. SADS radiolabeling was performed by using the oxidizing agent, IODO-GEN (1,3,4,6-tetrachloro-3,6-diphenylglycouril, Pierce), which was plated on the surface of the reaction mixtures prior to iodination. The conditions and cross-linking were as follows. 20 μg of IODO-GEN was dissolved in 100 μl of chloroform and added to a glass tube. The chloroform was slowly evaporated under N2. SADS was dissolved in dimethyl sulfoxide to 50 μM and further diluted in PBS, pH 7.4, to a final concentration of 0.5 μM. Next, 200 μl of the SADS solution and 100 μg of Na125I were added to the IODO-GEN-coated tube and incubated for 30 s at room temperature prior to iodination. The photooxidation and cross-linking were exposed to UV irradiation (Desega Uvis lamp, Copenhagen, Denmark) at 254 nm and at 10 cm from the source for 10 min at room temperature to activate the photoreactive cross-linker. The 125I-SADS was subsequently incubated with the purified AS aggregates (200 μg) in PBS, pH 7.4, for 1 h at room temperature to create the 125I-SADS-protein complex. The 125I-labeled aggregates were then plated on a 4% sucrose cushion and centrifuged at 15,000 rpm for 30 min to remove free radioactive iodine which was not bound. The TCA-precipitated SADS-AS aggregate tracer, with a specific activity of 1 × 106 Ci/mmol AS monomer in the aggregate, was resuspended from the pellet in PBS and used immediately. The SADS-modified AS aggregate tracer was then incubated with p25-synuclein for 30 min at 37 °C during continuous shaking. All the above procedures were carried out in the dark, and all reaction vessels were covered with aluminum foil. After incubation, the reaction mixtures were exposed to UV irradiation (Desega Uvis lamp, Copenhagen, Denmark) at 254 nm and at 2 cm from the source for 10 min at room temperature to activate the photoreactive cross-linker. The disulfide bridge in SADS was cleaved by 2% (v/v) β-mercaptoethanol (Applichem, Darmstadt, Germany), and samples were analyzed by SDSPAGE and autoradiography.

**Biological Characterization**—Urea denaturation experiments were carried out at p25-synuclein concentrations of 2.5 μM in 2 mM dithierythritol, PBS, pH 7.4, at 25 °C. Fresh 10× urea stock solutions were prepared on a daily basis. All CD studies were performed on a Jasco J-715 spectropolarimeter (Jasco Spectroscopy Co., Tokyo, Japan) with a Jasco PTC-345W temperature control unit. Spectra were recorded in a 0.1-cm path length cuvette with resolution at 0.2 nm, bandwidth at 1.0 nm, sensitivity at 50 millidegree, response at 2.0 s, and speed of 20 nm/min at 25 °C. Three scans were averaged to yield the final spectrum. Protein concentrations were 20 (far-UV CD, 250–205 nm) and 200 μM (near-UV CD, 320–250 nm).

**p25-synuclein Antibody Production**—Rabbits were immunized with a fusion protein of a hexahistidine tag linked to the N terminus of p25-synuclein, and serum was affinity-purified on CNBr-activated Sepharose (Amersham Biosciences) followed by protein A chromatography. The resulting rabbit IgG p25-synuclein was dialyzed against 1 mM EDTA, PBS, pH 7.4, and stored at −20 °C.

**Iodination of p25-synuclein**—Purified recombinant human p25-synuclein (6 μg) was iodinated essentially as described previously for tau (36), yielding a tracer with a specific activity of 4.8 × 106 Ci/mmol.

**α-Synuclein Aggregate Analyses—Aggregates of recombinant AS (1–140), AS (1–125), AS (1–110), AS (1–95), and Aβ1-40 were formed as described for AS (1–140), AS (1–95), and Aβ1-40 and quantified by Coomassie Blue-stained SDSPAGE after their isolation from the pellet following density gradient centrifugation (34). Aggregate-binding proteins, isolated after aggregate co-sedimentation, were identified after nonequilibrium pH-gradient gel electrophoresis (NEPHGE) for the resolution of basic protein (37) followed by mass spectrometric tryptic peptide mapping of individual protein spots (34).**
LBD \((n = 3)\), and control subjects \((n = 4)\) were obtained from the Netherlands Brain Bank and the National Health and Medical Research Council South Australian Brain Bank. Samples of the cerebral cortex, temporal cortex, and basal ganglia were obtained from MSA cases \((n = 3)\) from the NHMRC South Australian Brain Bank. The brain samples from this bank were bisected, and one-half was fixed with 4% formaldehyde and 2% picric acid, and the other was sliced, fresh frozen, and stored at \(-80^\circ\)C. Control samples were obtained from patients without previous neurological disease before death and no pathological changes in sections of the substantia nigra pars compacta. In the human autopsies, the PD diagnosis was based on loss of Lewy bodies in the remaining substantia nigra pars compacta neurons in eosin-stained sections as described previously in detail (see Refs. 39 and 40). To verify the distribution of p25 in optimally treated brain tissue, material from four brains were collected from adult male Wistar rats processed for immunocytochemistry (40).

**Immunohistochemistry of Paraffin Sections**—Paraffin sections \((6 \mu m)\) were obtained from pathologically confirmed PD \((n = 4)\), LBD \((n = 4)\), MSA \((n = 4)\), and age-matched control cases \((n = 3)\) without significant brain pathology. The sections were deparaffinized, treated with antigen retrieval (boiling in 1x EDTA, pH 8.0, for 10 min), and placed on 10 min in 3% \(\text{H}_2\text{O}_2\) to bleach the endogenous peroxidase reactivity. The sections were incubated with 20% normal horse serum for 60 min and then incubated overnight at 4°C with affinity-purified rabbit p25\(^\text{AA} \text{IgG} (1:1000)\) and biotinylated donkey anti-rabbit IgG (1:200, The Jackson Laboratory) for 2 h. Following incubation with streptavidin-biotin-peroxidase complex for 1 h, the sections were developed using 3,3-diaminobenzidine tetrahydrochloride as chromogen. Control staining was done by omitting primary antibodies. The rat brains were cut at 40 \(\mu m\) and processed for immunofluorescence. All sections were incubated overnight at 4°C with a polyclonal rabbit anti-p25\(^\text{AA} \text{IgG} (1:200)\) sheep anti-human \(\alpha\)-synuclein antibody (Abcam, Cambridge, UK) diluted 1:200. Specific binding was verified by using either biotinylated swine anti-rabbit antibody diluted 1:500 (Dakopatts, Copenhagen, Denmark) or biotinylated rabbit anti-sheep antibody diluted 1:500 (Abcam, Cambridge, UK), followed by amplification with biotinylated tyramide peroxidase conjugate (PerkinElmer Life Sciences) and incubated diluted 1:100 for 5 min followed by horseradish peroxidase-conjugated streptavidin-biotin complex for 30 min. The sections were developed as described for the human specimens.

**Immunofluorescence Double Staining of Paraffin Sections**—To achieve double labeling, human brain sections were pretreated as described above and simultaneously incubated overnight with affinity-purified sheep anti-p25\(^\text{AA} \text{IgG} (1:300)\) in combination with affinity-purified rabbit p25\(^\text{AA} \text{IgG} (1:200)\) and subsequently for 2 h in fluorescent dye-tagged secondary antibodies. Secondary antibodies were Cy3-conjugated anti-sheep IgG and Cy2 conjugated anti-rabbit IgG (1:100, The Jackson Laboratory). Control staining was performed by omitting the primary antibodies. Following the completion of fluorescence immunostaining, sections were mounted on gelatin-coated slides. 20% buffered glycerol was used as coverslips. Sections were examined by using a Bio-Rad confocal laser-scanning microscope and software package (MRC 1024, Bio-Rad). Lewy bodies were identified in relevant brain regions of the section (50 \(\mu m\) and its equatorial plane. This plane was defined at the z axis coordinate where it exhibited the clearest central halo or largest circumference. Images were captured at \(\times 2400\) magnification.

**Immunolabeling of Isolated Lewy Bodies**—Lewy body and Lewy neurites were isolated essentially as described previously (33, 41). The pellet, enriched in Lewy bodies and Lewy neurites along with other cellular components, was smeared on slides and air-dried. Following a 10 min fixation with 4% formaldehyde and 2% picric acid in phosphate buffer, pH 7.4, the smears were immunostained for AS (sheep anti-AS, 1:300) and p25 antibody (rabbit p25\(^{\text{AA}} \text{IgG}, 1:400)\). Following labeling with Cy3-conjugated anti-sheep IgG and Cy2-conjugated anti-rabbit IgG (1:100, The Jackson Laboratory, West Grove, PA), the slides were incubated with avidin-biotin complex and were examined by using a Bio-Rad confocal laser-scanning microscope as described above. Control staining was done by omitting the primary antibodies.

**Immunoelectron Microscopy**—Immunoelectron microscopic localization of recombinant p25\(^\text{AA} \text{as an } \alpha\text{-Synuclein Filament-binding Protein} \text{Cellular proteins binding to aggregated AS may represent targets or modifiers of the toxic effect of the aggregates (34) or modulators of the aggregative process. We used in vitro formed AS aggregates to search for such proteins in rat brain cytosol, deploying an AS aggregate sedimentation technique (34). A range of proteins co-sedimented with the AS aggregates upon cytosol incubation with the AS aggregates, and two-dimensional NIEFHE was used to resolve basic proteins. Fig. 1A (right panel) presents a part of a silver-stained gel that demonstrates a strong protein spot with a weaker, slightly more acidic isoform that was only present when the cytosol was incubated with the AS aggregates. This protein was absent in the cytosol control (Fig. 1A, left panel) and the AS aggregate control (data not shown). This spot was subjected to mass spectrometric peptide mapping (Fig. 1C), and the spectrum identified the protein as the rat homologue of human p25\(^\text{AA} \text{peptide IgG (Fig. 1D), which was raised against a peptide in bovine p25\(^\text{AA} \text{peptide (25). The AS aggregate-binding property was not restricted to rat p25\(^\text{AA}, as incubation of a human brain extract with AS aggregates enabled the co-sedimentation of human p25\(^\text{AA} \text{Fig. 1B), p25 is a brain phosphoprotein (see Ref. 25; data not shown) and rat brain cytosol comprises multiple pI isoforms of p25 as compared with the single immunoreactive spot for recombinant human hexahistidine-p25\(^\text{AA} \text{Fig. 1D, upper versus lower panel). All these isoforms could associate with AS aggregates as demonstrated by co-sedimentation (Fig. 1D, middle panel). AS aggregates display amyloid-type characteristics that were accounted for by structural determinants in the N-terminal repeat region (42, 43). This allowed the assembly of protein aggregates from full-length AS (1-140) and C terminally truncated AS (1-95), which lacks the entire acidic C terminus, as demonstrated in Fig. 1E. p25\(^\text{AA} \text{was bound only by the aggregate...
FIG. 1. Identification of p25α as an α-synuclein filament-binding protein. A, rat brain cytosol was incubated in the absence and presence of 35 μg of α-synuclein (AS) aggregates, whereupon the samples were subjected to density gradient centrifugation. Pellets were resolved by nonequilibrium pH-gradient two-dimensional gel electrophoresis and subjected to silver staining. Electrophoresis resolved the basic proteins in the pH range 7–11, which left the acidic AS out of the gel. Left panel, part of the gel containing the cytosolic sample with some insoluble proteins stained at the bottom. Right panel, similar part of the gel containing AS aggregate cytosol sample. Arrow indicates AS aggregate-associated protein subjected to mass spectrometric peptide mapping. Arrowhead marks putative, more acidic isoform of the same protein. Anode and cathode localizations are presented on top of the gels. The fractionation corresponding to molecular weight (MW) is displayed to the left.

B, immunoblot demonstration of the binding of human brain p25α to AS aggregates. Human brain detergent extract was co-incubated with AS aggregates as in A. Pellets were subjected to reducing SDS-PAGE and immunoblotting using rabbit anti-p25α antibody p25α-1. Lane 1, 10% of inputs of brain extract. Lane 2, pellet from extract without aggregates. Lane 3, pellet from extract plus AS aggregates containing associated human brain proteins. Molecular size markers in kDa are indicated to the left. C, mass spectrum of tryptic digests of protein spot indicated by arrow in A. The gel spot was reduced and alkylated with iodoacetamide prior to digestion. The abscissa shows the mass divided by charge (m/z) of individual ions, and the ordinate shows the signal intensity corresponding to the ions. The peptide pattern was searched against the NCBI
gates of full-length AS but not by the C terminally truncated AS (Fig. 1E). The lack of a role of the amyloid structure generated by the N-terminal part of AS was corroborated by the absence of binding to aggregates formed from the amyloidogenic peptide Aβ(1–40) (Fig. 1E). p25α bound preferentially to AS in its aggregated state as demonstrated by the inability of p25α to bind AS aggregates in the presence of a 32-fold excess of monomeric AS competing with the binding of p25α (Fig. 1F). Accordingly, p25α binds to aggregate-selective determinants within the C terminal segment of AS.

**Characterization of Recombinant Human p25α—** The p25α protein was first identified as a bovine brain-specific phosphoprotein (25), and it has recently been attributed to tubulin-assembling properties (29). Sequence analysis demonstrates that p25α belongs to the highly conserved p25 gene family present in mammals, flies, nematodes, and even tetrhymaneae (44). The human genome contains at least three p25-like genes, here designated as p25α, p25β, and p25γ, but previously named 25-kDa brain-specific protein (Swiss-Prot accession number O94811), brain-specific protein (Swiss-Prot accession number P59282), and protein CGI-38 (45, 46) (Swiss-Prot accession number Q9BW30), among which only the p25α protein has been detected. These three gene products display a high degree of sequence identity in their C termini, but their N termini differ, with the α-form containing a unique 43-amino-acid insertion (Fig. 2A).

We cloned the human p25α from a human fetal brain cDNA library and made a deletion mutant p25α-(Δ3–43) truncated for the specific N-terminal insertion. Both proteins were expressed in E. coli and purified to more than 95% purity by a protocol based on their heat stability, ion exchange chromatography, and gel filtration (Fig. 2B, left panel). The recombinant proteins displayed a tendency to form diulfide-bridged dimers (Fig. 2B, right panel), and subsequent analyses were performed under reducing conditions. Rabbits were immunized with recombinant human hexahistidine-tagged p25α purified by immobilized metal affinity chromatography. The purification protocol for the hexahistidine p25α differed from the protocol for purifying the untagged p25α, which reduced the risk for raising antibodies against possibly contaminating proteins in the untagged p25α preparation. The immune serum was subsequently affinity purified onto immobilized recombinant untagged human p25α. The resulting rabbit IgG, p25α-1 was specific, as demonstrated by the binding to a single band of 27 kDa in PC12 cells transfected with a human p25α vector, but not the empty vector (data not shown), to human brain p25α (Fig. 1B) and to the 27-kDa recombinant p25α band, which could be inhibited by preincubation of the antibody with recombinant human p25α (Fig. 2C, lanes 1 and 2). The p25α-1 antibody also bound to a less abundant ~55-kDa species in the reduced recombinant p25α preparation (Fig. 2C, lanes 1–4). However, this immunoreactive species is of very low abundance as no such bands were detectable by sensitive silver staining methods (Fig. 2B). These bands may represent dimeric p25α species bonded by a reduction-insensitive bond, which may bind the p25α-1 antibody avidly. A similar band was detected in rat brain cytosol (Fig. 2C, lanes 5–8) but was absent in human brain extract (Fig. 1B). The antibody also bound to the N-terminally truncated recombinant p25α-(Δ3–43) peptide (data not shown).

p25α is a heat-stable protein, which would suggest that the protein is either (a) folded but very thermostable, or (b) natively unfolded. To gain structural insight into recombinant human p25α, the purified protein was analyzed by far-UV CD spectroscopy (Fig. 2D). Deconvolution of the spectrum by the k2d program (kal-el.ugr.es/k2d/k2d.html) predicted 15% α-helix, 30% β-helix, and 55% random coil, but the fit was rather poor (data not shown), suggesting unusual features in its protein structure. However, p25α clearly has some degree of organized structure, because incubation of the protein in 5 μm urea (well above the midpoint of denaturation, which is around 3.7 μm urea, data not shown) caused a marked loss of spectral intensity (Fig. 2D). This was corroborated by the fluorescence spectrum, where the emission intensity peaks at 330 nm and undergoes a dramatic red shift to around 355 nm in the presence of 5 μm urea that is typical of a buried Trp residue (Fig. 2E). The above data clearly indicate that human p25α possesses both a secondary and a tertiary structure, which are lost at high denaturant concentrations.

**Characterization of the Interaction between α-Synuclein and p25α—** The binding of brain p25α to AS aggregates (Fig. 1, A and B) could in principle be mediated via unidentified cytosolic linker proteins or require specific, yet concealed post-translational modifications of p25α. Different experimental approaches were used to demonstrate a direct binding between purified recombinant human p25α and purified AS aggregates as follows: first, co-sedimentation analysis (Fig. 3A); second, immunoelectron microscopic demonstration of recombinant

| Identified peaks, m/z | Predicted mass MH+ | Rat p25α peptide position |
|-----------------------|--------------------|---------------------------|
| 902.4                 | 902.4              | 123–130                   |
| 1436.7               | 1436.8             | 155–167                   |
| 1896.9               | 1897.0             | 175–190                   |
| 2300.0               | 2300.1             | 172–190                   |
| 2632.3               | 2632.3             | 96–121                    |
| 2789.0               | 2788.4             | 96–122                    |
| 2944.4               | 2944.5             | 95–122                    |

nr protein data base using the Mascot search program (Matrix Science, UK) and showed a significant correlation with rat p25α (peak assignment in Table 1). p25α was identified in three independent experiments using different rats. D. rat brain cytosol and AS aggregate-binding proteins from cytosol were isolated by centrifugation, and the pellets were resolved by two-dimensional gel electrophoretic analysis as in A, but subjected to immunoblotting with the p25α-1 antibody. Input demonstrates multiple p1 isoforms in cytosol. The sample to be incubated with the aggregates was supplemented with 50 ng of hexahistidine-tagged recombinant human p25α fusion protein. The lower part demonstrates immunoreactivity of unmodified recombinant hexahistidine-tagged protein as detected with hexahistidine-binding antibody. The middle part demonstrates immunoreactivity of the same blot as the lower part upon stripping of hexahistidine-binding antibody and membrane reprobing with p25α-1 antibody. This demonstrates that both the native and the acidic isoforms can bind AS aggregates. One of two similar experiments is shown. E. aggregates, assembled from full-length AS-(1–140) (A-140) and C terminally truncated AS-(1–95) (A-95), were incubated with rat brain cytosol (C), and their p25α binding was analyzed by sedimentation analysis. Input of cytosol and AS-(1–140) aggregates and pellet fractions of individual samples were analyzed for their p25α immunoreactivity (upper panel) and silver-stainable proteins in 12–24-kDa range (lower panel). Molecular size markers in kDa are shown to the left in the lower panel. Aggregates assembled from AS lacking the C-terminal 45 residues cannot bind p25α. One of three similar experiments is shown. F. rat brain p25α (p25) binds preferentially to aggregated AS. Negligible p25α amounts were present in the pellet upon sedimentation without aggregates (lane 1) and in the presence of monomeric AS (lane 9). In contrast, p25α with aggregates was present in lanes 2–8, and the amount of aggregate-associated p25α was not significantly inhibited upon co-incubation with increasing amounts of monomeric AS. Ratio of monomeric to aggregated AS (M/A) is indicated. One of three similar experiments is shown.
Cloning and expression of recombinant human p25α. A, alignment of the amino acid sequence of the three human p25 gene products p25α (p25a), p25β (p25b), and p25γ (p25g). α-Specific N-terminal segment is marked by a line. This segment is deleted in the recombinant human p25α (Δ3–43) protein used in this study. Amino acid sequence identity marked by grey boxes and insets are shown by vertical lines. Number of last amino acid in each line is shown to the right. B, left panel, purity of 5 μg of recombinant human p25α and deletion mutant p25α (Δ3–43) assessed by Coomassie Blue staining after reducing SDS-PAGE. Right panel, p25α (2 μg) heated in SDS-loading buffer in the absence (Ox.) and presence of 20 mM dithioerythritol (Red.) and subjected to SDS-PAGE and silver staining. Molecular size markers in kDa indicated to the left. C, characterization of p25α-1 antibody. Dilutions of recombinant p25α (lanes 1 and 2, 2 μg; lane 3, 0.4 μg; lane 4, 0.1 μg) and rat brain cytosol (lanes 5 and 6, 26 μg; lane 7, 13 μg; lane 8, 6 μg) resolved by reducing SDS-PAGE and by subjecting dilutions to immunoblotting using rabbit p25α-1 IgG. Effect of pre-absorbing p25α-1 IgG (9 μg/ml) with recombinant p25α (450 μg/ml) demonstrated in lanes 2 and 6. Molecular size markers in kDa indicated to the left. D, purified recombinant human p25α analyzed by far-UV CD spectroscopy under native (solid line) and denaturing conditions (dotted line). Abscissa represents the wavelength and the ordinate the corresponding molar ellipticity. E, fluorescence emission spectra of p25α under native (solid line) and denaturing conditions in 5 M urea (dotted line). The abscissa shows wavelength and the ordinate the corresponding fluorescent emission. Experiments in D and E were performed on two preparations of p25α.
Characterization of the direct binding of p25α to α-synuclein. A, purified recombinant human p25α (p25α) was incubated in the absence and presence of 10 μg of purified AS aggregates (A) and analyzed for co-sedimentation. p25α content in 10% of input and total pellet fractions was analyzed by immunoblotting. B, electron microscopic demonstration of p25α binding to AS filaments. AS filaments were incubated with recombinant p25α, whereupon the p25α–AS filament complexes were isolated by density gradient centrifugation. Purified filaments were incubated with p25α-IgG (panel 1) and nonimmune IgG (panel 2), and subsequently anti-rabbit IgG was conjugated to 5-nm gold particles and analyzed by negative staining electron microscopy. A bar representing 100 nm is presented to the right. C, characterization of 125I-SASD/AS aggregate tracer by SDS-PAGE and autoradiography. 125I-SASD-AS aggregate (1,000,000 cpm) was either incubated in SDS-loading buffer without heating (lane 1); depolymerized by incubation in 4 M urea and 2% SDS overnight prior to addition of SDS-loading buffer and heating (lane 2); subjected to activation of photoreactive cross-linker prior to treatment as in lane 2 (lane 3); or subjected to activation of photoreactive cross-linker followed by reduction prior to treatment as in lane 2 (lane 4). UV designation photoinactivation, red sample reduction. Molecular size markers in kDa presented to the left. D, specificity of photoaffinity labeling of ligands by 125I-SASD-AS aggregate tracer (1,000,000 cpm) demonstrated by incubating with FITA-1 IgG (6 μg), which binds AS aggregates, and nonimmune (N.I.) IgG (6 μg) as negative control. Samples were resolved by reducing SDS-PAGE. Left panel demonstrates protein stain of the gel by Coomassie Blue to ensure equal IgG. Right panel represents the 125I-labeling in the gel shown in the left panel as an autoradiograph. Cross-linker activation by UV light (UV) demonstrated below. Molecular size markers in kDa are presented to the left. E, specificity of photoaffinity labeling of ligands by 125I-SASD-AS aggregate tracer (1,000,000 cpm) demonstrated by incubating with FITA-1 IgG (6 μg), which binds AS aggregates, and nonimmune (N.I.) IgG (6 μg) as negative control. Samples were resolved by reducing SDS-PAGE. Left panel demonstrates protein stain of the gel by Coomassie Blue to ensure equal IgG. Right panel represents the 125I-labeling in the gel shown in the left panel as an autoradiograph. Cross-linker activation by UV light (UV) demonstrated below. Molecular size markers in kDa are presented to the left. F, quantitative analysis of 125I-p25α binding to immobilized AS-(1–140) aggregates. Purified AS aggregates were immobilized in microtiter plates and incubated with 50 μl 125I-p25α in the absence and presence of competitors; purified aggregates of AS-(1–140) (filled circle), AS-(1–125) (open circle), AS-(1–110) (filled triangle); monomeric AS-(1–140) (open triangle), β-synuclein (filled diamond), a synthetic peptide corresponding to AS-(109–140) (open diamond), p25α (open square), and p25α-(Δ3–43) (closed square). Ordinate displays percentage of bound/free tracer and the abscissa the concentration of competitors. Points display mean ± 1 S.D. of triplicates from one of three representative experiments. Inset, p25α (3 μg) mixed with 10,000 cpm 125I-p25α and resolved by SDS-PAGE. Gel stained by Coomassie Blue (lane 1) and subjected to autoradiography (lane 2). Molecular size markers in kDa indicated to the left.
both p25α and the deletion mutant p25α-(Δ3–43) were photoaffinity-labeled upon incubation with the 125I-SASD conjugated AS aggregates (Fig. 3E, lanes 5 and 7).

A recently developed AS aggregate solid phase binding assay (34) allowed a detailed quantitative analysis of the binding of p25α to AS aggregates (Fig. 3F). The 125I-labeled p25α tracer migrated predominantly as a single 25-kDa band, which co-migrated with Coomassie Blue-stained recombinant p25α (Fig. 3F, inset, lane 2 versus 1). The binding of 50 pm 125I-p25α to immobilized aggregated AS was inhibited by fluid phase AS aggregates with an IC50 of about 10 nM and to the same extent as when using unlabeled p25α and p25α-(Δ3–43) (Fig. 3D). Monomeric AS displayed an ~10-fold higher IC50 than aggregated AS (Fig. 3F). However, the molar concentration of the aggregates could not be expressed precisely due to their heterogeneous homopolymeric nature, and they are accordingly expressed by the concentration of their monomeric content. The selectivity of p25α for aggregated AS on a molar basis is hence even larger, and it is likely to lie in the range of 102–103, given that the aggregates contain 20–100 monomeric subunits. Accordingly, p25α binds to monomeric AS but exhibits a higher affinity for aggregated AS.

The basis for the aggregate selectivity within the AS primary structure was investigated using aggregates prepared from AS molecules truncated for the last 30 residues AS-(1–110) and 15 residues AS-(1–125). To ensure equal concentrations of the aggregates stock solutions were depolymerized and subsequently compared by Coomassie Blue staining of SDS-PAGE (data not shown). AS-(1–110) aggregates displayed a 100-fold higher IC50 than the wild type peptide (Fig. 3F), whereas the IC50 for binding to AS-(1–125) was indistinguishable from the wild type AS. Moreover, β-synuclein, whose C terminus resembles AS in terms of content and spacing of their negatively charged residues, and a synthetic peptide AS-(109–140), corresponding to AS C terminus, were unable to inhibit the tracer binding (Fig. 3F). The high affinity binding of p25α to AS aggregates accordingly relies on the presentation of a segment within the AS C terminus close to residues 110–125 in the context of an AS aggregate.

**p25α Stimulates α-Synuclein Aggregation**—The binding of p25α to monomeric AS raised the possibility that p25α may affect the process of AS aggregation. This question was first investigated by an aggregate-sedimentation assay where AS was incubated in the absence and presence of substoichiometric concentrations of p25α followed by isolation of the AS aggregates by centrifugation (Fig. 4A). At time 0, a small amount of AS was detected in the pellet, which represents the background level of the assay when using 340 μM AS. A slight, spontaneous aggregation of AS was detected after 72 h of incubation. However, co-incubation of the AS with 3 and 10 μM recombinant human p25α produced a dose-dependent increase in the amount of aggregated AS. p25α was recovered in the pellet with the AS aggregates (Fig. 4A) but remained in the supernatant in the absence of AS (data not shown). p25α also stimulated the sedimentation of AS peptides carrying either of the two PD-causing mutations A30P and A53T (data not shown).

Thioflavin-T fluorescence has been used extensively to monitor the development of amyloid-type aggregates. Supplementing AS with p25α produced a time- and dose-dependent increase in the thioflavin-T fluorescence measured after incubation for 1 and 3 days (Fig. 4B). The dose dependence is clearly demonstrated after incubation for 1 day where 3% p25α, but not 1%, stimulated the aggregation of 100 and 200 μM AS. The largest relative p25α-stimulated increase in AS aggregation was observed by using lower concentrations of AS (100 and

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** p25α stimulates α-synuclein aggregation. A, AS (340 μM) was incubated in the absence and presence of p25α (3.4 μM (1%) and 10 μM (3%)) at 37 °C for 3 days. The aggregational state of AS was determined by density gradient centrifugation. Input and insoluble pellets at days 0 and 3 were analyzed by reducing SDS-PAGE. Positions of AS and p25α are indicated to the left. B, AS was incubated in the absence (filled circles) and presence of 1% (open circles) and 3% (filled triangles) p25α at 37 °C, and samples were recovered for analysis of thioflavin-T (TT) fluorescence at days 0, 1, and 3. Left panel, 100 μM AS; middle panel, 200 μM AS; and right panel, 340 μM AS. Ordinate represents TT fluorescence in arbitrary units. Note the different scale of the three panels. Points represent mean of triplicates. One of five representative experiments is presented. C, AS (340 μM) aggregation performed for 3 days in the absence and presence of 1% p25α. Filaments from five individual samples were purified by density gradient centrifugation, resuspended in PBS, applied onto grids for quantitative negative stain electron microscopy, and analyzed in systematic random measuring mode. Upper and lower left panels demonstrate representative electron micrographs obtained in the absence and presence of p25α. A 1-μm bar in the lower left panel applies to the high magnification images in the upper and lower left panels, and 5-μm bars are present in the low magnification images in the middle upper and lower images. Right panel shows grid percentage covered with filaments in the absence (unfilled histograms) and presence of 1% p25α (AS + p25α). There is a significant difference (p = 1.2 × 10−5) in area fraction of filaments between negatively stained samples of AS and AS incubated with 1% recombinant p25α as visualized by electron microscopy.

200 μM), where 1–3% p25α caused an ~5–6-fold increase as compared with the autoaggregation of AS. By comparison, the autoaggregation at 340 μM AS was so large that the p25α increase only amounted to ~2-fold. The data using 100 and 200 μM AS clearly demonstrate that supplementing with 1 and 3% p25α reduces the lag phase of the aggregation process. Quantitative electron microscopy was used to confirm that the thioflavin-T positive aggregates were of a filamentous nature like those present in Lewy bodies and Lewy neurites. For this analysis, 340 μM AS was incubated in the absence and presence of 1% p25α (n = 5) for 3 days, whereupon the insoluble material
p25α and α-synuclein are co-expressed in glial cytoplasmic inclusions in multiple system atrophy. A, p25α in human oligodendrocytes of the normal brain white matter. Note the labeling of the thin perinuclear cytoplasm (arrow). B, p25α in human oligodendrocytes of the normal brain white matter shown at high power magnification. C, omitting the primary anti-p25α antibody of the immunoreaction abolishes labeling of oligodendrocytes. D, AS in human oligodendrocytes of multiple system atrophy case. Section shows labeled oligodendrocytes (arrowhead) of white matter underlying cerebral cortex. Subcellular AS distribution within individual oligodendrocytes is heterogeneous but leaves the nucleus unlabeled. E, p25α in human oligodendrocytes of same multiple system atrophy case. Numerous labeled oligodendrocytes are seen. Subcellular p25α distribution within individual oligodendrocytes is heterogeneous but leaves the nucleus unlabeled. It varied from labeling of thin perinuclear cytoplasm in normal and apparently normal oligodendrocytes (arrow) to a robust labeling of expanded cytoplasm in degenerating cells (arrowhead). F, p25α in human oligodendrocytes of multiple system atrophy case shown at high power magnification highlighting principal difference in subcellular p25α distribution within apparently normal (arrow) and pathological (arrowhead) oligodendrocytes. G-I, double-labeling fluorescence revealing co-distribution of AS (G) and p25α (H) in glial cytoplasmic inclusions (arrowhead in D and E) of multiple system atrophy case. I, superimposing the images reveals co-localization of p25 and AS in oligodendrocytes. H and I, presumably normal oligodendrocytes without glial cytoplasmic inclusions identified as p25α-labeling of thin perinuclear cytoplasmic region (arrows in E) and devoid of AS immunoreactivity (arrows in I). Scale bars = 10 μm (A, C–E, and G–I), 2.5 μm (B and F).

was applied onto grids. The negatively stained grids were examined by systematic random sampling to determine the fraction of the grid covered by filaments. Fig. 4C (left panels) demonstrates examples of the images of AS aggregates captured in the absence and presence of 1% p25α where the high magnification images clearly show the fibrillar structure of the assemblages. No filaments were present in the absence of AS (data not shown). Quantitation of the mean area fraction covered by AS filaments demonstrated an increase from 12 to 43%, which was statistically significant (Fig. 4C, right panel). No detectable difference of the filaments formed in the absence and presence of p25α was observed. Accordingly, substoichiometric amounts of p25α can stimulate the AS aggregation via a process resembling the disease-associated aggregation.

p25α Accumulates in Pathological α-Synuclein Inclusions—in the rat brain, p25α is distributed to cells with a morphology corresponding to that of oligodendrocytes and choroid plexus epithelial cells (data not shown). This is consistent with early observations in rat (26). We had the impression that virtually all oligodendrocytes present in both gray and white matter regions expressed p25α. Similarly, in human brains the vast majority of oligodendrocytes was also immunopositive for p25α. The immunoreaction product within oligodendrocytes of rat brain and human control cases distributed predominantly to the perinuclear cytoplasm leaving their nuclei unlabeled, whereas peripheral processes displayed a more punctate pattern evident both by histochemical staining and immunofluorescence microscopy (Fig. 5, A and B). Cellular labeling of oligodendrocytes was never observed when the primary antibody was omitted from the immunoreaction (Fig. 5C). MSA cases, however, showed a population of oligodendrocytes with a p25α distribution in the perinuclear cytoplasm (Fig. 5E, arrowhead) that was more robust than the slight labeling of the perinuclear cytoplasm in the oligodendrocytes (Fig. 5E, arrows) that resembled the controls. The p25α distribution appeared to be expanded in the group of oligodendrocytes with the most intense p25α immunoreactivity. A parallel to the cellular distribution of AS in oligodendrocytes during pathological conditions was made in the brains of MSA cases in which oligodendrocytes are known to express AS in glial cytoplasmic inclusions (3, 47). AS indeed labeled several oligodendrocytes of MSA (Fig. 5D), leaving oligodendrocytes of PD, LBD, and normal cases unstained (data not shown). The use of double-labeling fluorescence and confocal microscopy revealed a clear co-distribution of p25α and AS in many oligodendrocytes (Fig. 5, G–I). Quantification of images like Fig. 5, G and H, revealed...
FIG. 6. p25α in neuronal Lewy bodies. A, p25α in neurons of supraoptic nuclei of rat brain. Inset shows labeled nucleus at low power magnification. At larger magnification, the evident labeling of both supraoptic neurons (arrowheads) and oligodendrocytes of optic tract (arrows) is shown. B, p25α labeling of pigmented neuron of human substantia nigra pars compacta is shown at high power magnification. p25α distributes to the periphery of the Lewy body. C–E, double-labeling fluorescence revealing co-distribution of AS (C) and p25α (D) in the Lewy body of human substantia nigra pars compacta. Superimposing the images reveals co-localization of p25 and AS in Lewy body (E). F–H, control of specificity of p25α immunostaining. Lewy body containing neurons was labeled as in C–E, but the primary antibody was omitted in G. Scale bars = 40 μm (A), 20 μm (B), 20 μm (C–E), and 10 μm (F–H).

that about 60% of the AS-positive inclusions were p25α-positive. The number of AS-positive oligodendrocytes was, however, significantly lower compared with the number of oligodendrocytes labeled with p25α.

Beside its distribution to oligodendrocytes and the choroid plexus of the adult rat brain, p25α was also confined to the small group of neurons in the supraoptic nucleus. This was the sole p25α-positive neuronal population observed during the screening of the rat brain (Fig. 6A, inset). In these neurons, p25α was distributed to the entire perikaryal cytoplasm, and the nucleus and peripheral processes were unlabeled (Fig. 6A) similar to what has been observed in human oligodendrocytes (Fig. 5E). Likewise, p25α was expressed in many neuronal cell populations during the prenatal development of the rat brain.2 This demonstrates that neurons, at least in the rat brain, have the potential for p25α expression. The human normal control brain sections examined did not contain the supraoptic nucleus, but other normal neuronal populations were not expressing p25α (data not shown). Histochemically, p25α was occasionally demonstrated in Lewy bodies of substantia nigra pars compacta of LBD (data not shown) and PD (Fig. 6D), where the periphery of the brain stem Lewy body in the melanized neuron displayed a p25α immunoreactivity with a frequency of 1–5 labeled Lewy bodies per section. With the p25α-1 antibody, the Lewy body labeling intensity was generally low but most intense in their peripheral portion (Fig. 6B). Lewy body labeling appeared irrespective of whether melanin was removed before immunostaining or not. Confocal analysis of double fluorescence-labeled sections from cortical LBD tissue demonstrated a diffuse p25α immunoreactivity within the Lewy body in addition to the p25α-stained oligodendroglial processes (Fig. 6, C–E), which were absent when the primary antibody was omitted (Fig. 6, F–H). The double immunofluorescence analysis revealed that ~5% of the AS-positive nigral and 10% of cortical Lewy bodies in PD and LBD were p25α-positive.

The emergence of procedures to isolate AS-containing inclusions from human PD, MSA, and LBD tissue have allowed detailed structural and biochemical analyses of these structures. Fig. 7, A and B, demonstrates that p25α is present in both Lewy bodies and Lewy neurites isolated from LBD brain tissue, and the p25α in the inclusions co-localizes to a large extent with the AS as demonstrated by double immunofluorescence confocal laser-scanning microscopy. The negative controls without primary antibodies showed no staining (data not shown) as demonstrated for the tissue staining with the same antibodies in Fig. 6G. 100% of the AS-positive isolated glial cytoplasmic inclusions and 80% of isolated AS-positive Lewy bodies and Lewy neurites were p25α-positive. Biochemical analysis revealed that p25α in both isolated Lewy bodies and glial cytoplasmic inclusions appeared as a single 27-kDa band with no signs of aggregation and degradation when analyzed by reducing SDS-PAGE and immunoblotting (Fig. 7C).

Accordingly, p25α and AS are co-expressed in the inclusions that develop during the degenerative process of neurons in PD and LBD and oligodendrocytes in MSA. Most interestingly, p25α and AS are not normally co-expressed in the adult nervous system. This suggests that abnormal expression of p25α in neurons and AS in oligodendrocytes may actively contribute to the degenerative process by bringing the pro-aggregative p25α into contact with AS.

DISCUSSION

The ability of mutant AS to induce autosomal, dominant PD and LBD is evident from the rare familial cases. The role of wild type AS in sporadic PD and LBD, however, is less clear, although these diseases demonstrate a strong aggregation of the wild type AS protein in intracellular Lewy body and Lewy neurite inclusions. Recent studies (8, 9) have established a direct link between the expression of the wild type AS protein, its aggregation, and the induction of early onset PD and LBD as demonstrated in families with triplication of the AS gene. The affected members among the Swedish-American kindred had an ~40% increased level of AS protein (9), and an increased level of aggregated AS was demonstrated in the Iowa kindred (10). This indicates that the level of AS in the normal brain is close to the point where AS may aggregate spontaneously, as an elevation of about 50% triggers early onset disease. This gives credit to the hypothesis that aggregate-stimulating factors are inducing and contributory players in the pathogenesis of the spontaneous α-synucleinopathies, which also comprise MSA, which displays a strong α-synuclein aggregation in oligodendrocytes.

In our search for aggregate-stimulating factors, we looked for proteins that bind preferentially to AS in its aggregated state, and we identified p25α. We demonstrated that an increased or ectopic expression of p25α may represent one mechanism in sporadic diseases that can drive the conversion of monomeric AS into filamentous aggregates. First, at substoichiometric concentrations, p25α stimulates aggregation of AS in vitro into genuine filamentous AS aggregates as demonstrated by electron microscopy. This pro-aggregatory effect is largest at lower AS concentrations as demonstrated by thioflavin-T fluoresc-
p25α Stimulates Aggregation of α-Synuclein

FIG. 7. α-Synuclein and p25α co-localize in Lewy bodies and Lewy neurites isolated from Lewy body dementia brain tissue. Lewy bodies (A) and Lewy neurites (B) isolated from LBD brain tissue were analyzed for localization of α-synuclein (α-syn) and p25α (P25) by confocal laser-scanning microscopy. AS immunoreactivity, detected with sheep anti-α-synuclein antibody, is presented in the left columns, and p25α, detected by the rabbit p25α-1 IgG, is presented in the middle columns. Merged images are presented in the right columns. 10-μm scale bars are presented. C, immunoblot analysis of p25α in isolated brain inclusions. Extracts of human brain tissue homogenate (BH) (20 μg; lane 1), purified Lewy bodies (LB) (5 μg) from brain tissue affected by LBD (lane 2), and isolated glial cytoplasmic inclusions (GCI) (5 μg) from tissue affected by MSA (lane 3) resolved by reducing SDS-PAGE, electroblotted, and probed with anti-p25α antibody p25α-1. Molecular size markers in kDa are presented to the left.

ence, where the AS tendency to auto-aggregation is less pronounced. Second, when analyzed in situ, p25α accumulates in the majority of the AS-aggregate-containing glial cytoplasmic inclusions in MSA and cortical and nigral Lewy bodies in PD and LBD. This was demonstrated in the analysis of purified inclusions from nonfixed and nonfrozen tissue, a procedure that increases the sensitivity of detection. The tau and histone proteins have also been shown to stimulate aggregation of AS at substoichiometric concentrations, but their presence in AS aggregate-containing inclusions is rarer (48, 49).

Human p25α is predominantly expressed in oligodendrocytes, which is in agreement with early observations in rat (26) where it appears to be a very good marker for these cells as it does not stain myelin but merely the cellular soma and some processes. It is therefore not surprising to find accumulations of p25α in the AS aggregate containing glial cytoplasmic inclusion in MSA as compared with the neuronal Lewy bodies, but it may play a similar AS pro-aggregatory role in this disease. However, we demonstrate a dramatic change in the p25α expression pattern in MSA, where a large accumulation of p25α takes place in the expanded cell bodies of the apparently dystrophic oligodendrocytes containing glial cytoplasmic inclusions. Neuronal p25α expression is not a common finding in rat and human brain tissue, but the neuronal phenotype is not incompatible with p25α expression as we detected neuronal expression of p25α in the nucleus supraopticus and during prenatal brain development of rat (data not shown). This suggests that an abnormal expression of the protein occurs in the affected nerve cells in PD and Lewy body dementia. Dysregulation of p25α expression could accordingly be a contributory factor in cases of sporadic α-synucleinopathies, which makes studies of the regulation of its gene highly desired along with studies of p25α in other diseased states.

The preferential p25α binding to aggregated AS is displayed by a 100–1000-fold lower IC_{50} for the binding of aggregated versus monomeric AS, assuming that the AS aggregates contain at least 20–100 monomers. The interaction relies critically on the acidic C terminus of AS, as demonstrated by the strong inhibitory effect of truncation of the last 30 residues. This suggests a role for charge interaction with basic peptide motifs in stimulators of AS aggregation as p25α, histones, and the AS-interacting microtubule-binding segments in tau are basic peptides (32, 47), and a similar charge neutralization by Ca^{2+} binding to this segment may explain the pro-aggregative effect of this ion (32). However, a simple charge interaction cannot explain the interaction as β-synuclein, which possesses an even more acidic C terminus, and a synthetic peptide corresponding to the C-terminal 30 residues of AS are unable to bind to p25α. The solid phase binding assay demonstrated that AS aggregates formed from AS-(1–125) displayed full binding activity as compared with the 100-fold higher IC_{50} for AS-(1–110) aggregates. These are the first data to demonstrate, albeit indirectly, an aggregate specific folding of the C terminus. We believe that future studies should pay attention to the structure of this segment in aggregates rather than focus entirely on the amyloid-type β-folded segment comprising the first ~100 residues detectable in both oligomers and filaments. The C-terminal segment in the aggregates may be exposing the...
toxic AS aggregate-specific determinants, which induce the specific neurodegeneration of the α-synucleinopathies in contrast to the tauopathies, for example, which display intracellular tau aggregates of the amyloid type that likely share some characteristics with the N-terminal amyloid-type part of AS. The interaction was also demonstrated by photoaffinity labeling using AS aggregates conjugated to ^125I-TASSD. This technique represents a neat way to label a pathological human protein aggregate for subsequent use as a probe to identify its putative ligands. By using this technique, we have been able to label proteins in rat brain extracts (data not shown), which suggests that the method possesses a potential that justifies its further exploitation.

p25α was originally co-purified with a tau kinase preparation from bovine brain, identified as a brain-specific phospho-protein (25), and later shown to be a substrate for phosphatidic acid-stimulated kinases (27) and protein kinase A and CDK5 (28). Accordingly, we found that rat brain p25 was present in several p1 isoforms, which indicates phosphorylated species, but all these species bind to aggregated AS like nonphosphorylated recombinant human p25α. This indicates that p25α phosphorylation is unimportant in the regulation of its binding to AS.

Bovine p25α is a microtubule-associated protein that stimulates tubulin aggregation and probably undergoes significant conformational changes upon binding to monomeric tubulin, because both far-UV CD (29) and fluorescence spectra show loss of a spectral signal upon complex formation. However, incubation of p25α with monomeric AS does not lead to spectral changes (data not shown). The minimal interpretation of this result is that p25α binds in a different manner to AS and tubulin. It is therefore likely that the mechanism used by p25α for aggregating tubulin differs from that employed on AS. The different p25 gene products display a high degree of amino acid sequence identity, apart from the N-terminal α-specific segment in p25α, which corresponds to residues 3–43. Accordingly, they may share the ability to interact with α-synuclein.

The β- and γ-p25 forms, formerly named brain-specific protein (Swiss-Prot accession number P59282) and protein CGI-38 (Swiss-Prot accession number Q9BW30) (45, 46), have never been demonstrated at the protein level. However, they may be recognized by the p25α-1 antibody, which also binds the truncated p25α-(Δ3–43) peptide and is thus likely to cross-react with the p25 β- and γ-forms. This antibody only binds to the single p25α band in human brain extracts, which suggests that the other forms are not present at detectable levels. However, we cannot exclude that the β- and γ-forms may be expressed in some parts of the brain during pathological conditions where they may contribute to AS aggregation.

The mechanism of the pathological AS aggregation remains unclear, but the process is likely to involve reversible initial steps where monomeric AS associates into smaller polymers of the amyloid type that likely share some characteristics with the N-terminal amyloid-type part of AS. The interaction was also demonstrated by photoaffinity labeling using AS aggregates conjugated to ^125I-TASSD. This technique represents a neat way to label a pathological human protein aggregate for subsequent use as a probe to identify its putative ligands. By using this technique, we have been able to label proteins in rat brain extracts (data not shown), which suggests that the method possesses a potential that justifies its further exploitation.

The decreased affinity for monomers compared with oligomers, although other possibilities such as an increased binding surface in the oligomer-p25α complex cannot be ruled out at present. Accordingly, p25α stimulates the nucleation, not the fibrilization step, which is corroborated by the lowering of the AS concentration needed to trigger aggregation. This means that an inhibition of p25α-stimulated aggregation will inhibit the build up of both oligomers and fibrils in contrast to a selective inhibition of fibrillation, where toxic oligomers may accumulate. This makes it worth investigating the mechanisms involved in inhibiting the pro-aggregatory function of p25α or its expression as novel pathways for neuroprotective strategies in the α-synucleinopathies.

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