Binding of Aβ to α- and β-synucleins: identification of segments in α-synuclein/NAC precursor that bind Aβ and NAC

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NAC, a 35-residue peptide derived from the neuronal protein α-synuclein/NAC precursor, is tightly associated with Aβ fibrils in Alzheimer's disease amyloid, and α-synuclein has recently been shown to bind Aβ *in vitro*. We have studied the interaction between Aβ and synucleins, aiming at determining segments in α-synuclein that can account for the binding, as well as identifying a possible interaction between Aβ and the β-type synuclein. We report that Aβ binds to native and recombinant α-synuclein, and to β-synuclein in an SDS-sensitive interaction (IC_{50} approx. 20 μM), as determined by chemical cross-linking and solid-phase binding assays. α-Synuclein and β-synuclein were found to stimulate Aβ-aggregation *in vitro* to the same extent. The synucleins also displayed Aβ-inhibitable binding of NAC and they were capable of forming dimers. Using proteolytic fragmentation of α-synuclein and cross-linking to ^{125}I-Aβ, we identified two consecutive binding domains (residues 1–56 and 57–97) by Edman degradation and mass spectrometric analysis, and a synthetic peptide comprising residues 32–57 possessed Aβ-binding activity. To test further the possible significance in pathology, α-synuclein was biotinylated and shown to bind specifically to amyloid plaques in a brain with Alzheimer's disease. It is proposed that the multiple Aβ-binding sites in α-synuclein are involved in the development of amyloid plaques.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder hallmarked by intracerebral and cerebrovascular amyloid deposits. The major component of AD amyloid is the 39–43-residue peptide Aβ that is derived proteolytically from the extracellular part of the transmembrane Aβ precursor protein. Increased secretion of Aβ, as observed in trisomia 21, in the presence of certain mutations of Aβ precursor protein and in a transgenic mouse model [1], leads to AD pathology (reviewed in [2,3]). Several proteins beside Aβ have been detected in senile plaques, e.g. α_1-antichymotrypsin [4], complement protein C1q [5] and apolipoprotein E [6]. However, in the SDS-insoluble AD amyloid a single significant component, designated non-Aβ component of AD amyloid (NAC), was recently identified beside Aβ [7]. NAC is a fragment (residues 61–95) of the 140-residue neuronal molecule α-synuclein, also designated the NAC precursor, which is expressed primarily in areas of the brain afflicted by AD lesions [7–9]. α-Synuclein is a member of the highly conserved synuclein gene family [10], which in man comprises the homologous α- and β-synucleins identified in the central nervous system [8]. The major difference is an approx. 11-residue deletion in β-synuclein, corresponding to the middle segment of the NAC part (residues 73–83) of α-synuclein [8]. α-Synuclein and β-synuclein are expressed predominantly in the same areas of the brain, as judged from immunohistochemical staining, with both being present in nerve terminals [8].

The normal function of the synucleins is largely unknown, but molecular experiments with song-learning in the zebra finch indicates a role for α-synuclein in neuronal plasticity [11]. Pathologically, a undefined mechanism leads to the extracellular accumulation of NAC containing fragments in AD amyloid [7,12]. At this site NAC might contribute to the amyloid formation and growth because it has been shown to stimulate Aβ aggregation *in vitro* [13,14]. In addition, NAC might be cross-linked to Aβ in a transglutaminase-catalysed reaction [15]. Thus NAC might be one of several factors responsible for the conversion of soluble Aβ to the Aβ amyloid characteristic of AD.

Recent experiments have demonstrated that the precursor molecule α-synuclein itself binds to Aβ, can promote aggregation of Aβ and can lead to the formation of complexes insoluble in SDS [12]. A possible sequence of events is therefore that α-synuclein released from damaged neurons binds to Aβ followed by proteolytic cleavage and retention of the NAC component in the amyloid. The aim of the present work was to investigate first whether parts of the α-synuclein molecule other than the NAC domain might be important for binding to Aβ, and secondly whether β-synuclein, with the deletion in the NAC segment, binds to Aβ. We demonstrate that β-synuclein, like α-synuclein, does bind to and promote aggregation of Aβ, and we identify a novel Aβ-binding domain within residues 1–56 of α-synuclein. Finally we show that biotinylated α-synuclein can be used as a probe to detect some forms of AD amyloid in tissue sections.

EXPERIMENTAL

Electrophoresis

Samples were supplemented by one-third volume of reducing SDS/PAGE buffer [100 mM Tris (pH 6.8)/5% (w/v) SDS/60 mM dithioerythritol/25% (v/v) glycerol and Bromophenol Blue] and heated to 95 °C for 3 min. Electrophoresis was performed with 10–20% (w/v) gradient polyacrylamide gels in a

Abbreviations used: AD, Alzheimer's disease; BS_3, bis(sulphosuccinimidyl)suberate; MALDI-MS, matrix-assisted laser desorption ionization MS; NAC, the non-Aβ component of AD amyloid.

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conventional Laemmli buffer system. For the analysis of smaller peptides we used the 10–20 % gels or a 10 % polyacrylamide Tris/Tricine/urea gel system [16] with 0.2 M Tris, pH 8.9, as the anode buffer and 0.1 M Tris/0.1 M Tricine (pH 8.25)/0.1 % SDS as the cathode buffer. Gels were stained with Coomassie Brilliant Blue or silver, dried and processed for autoradiography at −80 °C with Amersham MP films (Amersham, Little Chalfont, Bucks., U.K.). Molecular mass markers were from Novex (San Diego, CA, U.S.A.). Laser scanning densitometry of autoradiograms was performed with an LKB 2202 Ultroscan laser densitometer and the results were analysed by the Hoefer GS365W data logger with software for peak integration.

Proteins

Human α- and β-synuclein were purified from cerebral cortex as previously described [8]. Recombinant human α- and β-synuclein, expressed in *Escherichia coli*, was purified to homogeneity by Mono Q ion-exchange chromatography (Pharmacia Biotech, Uppsala, Sweden) of the heat-soluble bacterial extract [8]. The proteins were stored at −20 °C in 50 % (v/v) glycerol/50 mM Tris buffer (pH 7.4). NAC, with an extra N-terminal tyrosine residue attached to permit iodination, and Aβ (residues 1–40) were synthesized at Schaefer-N, Town?, Denmark. Both peptides were labelled with ^{125}I (Amersham; 2.0 Ci/μmol) to a specific radioactivity of 20 mCi/mg as previously described [15,17].

Cross-linking assay

Complexes between ^{125}I-labelled Aβ or NAC and α-/β-synuclein or fragments thereof were demonstrated by chemical cross-linking in the presence of various concentrations of competitor peptides. After incubation for 16 h at 4 °C in binding buffer [150 mM NaCl/1 mM $CaCl_2$/10 mM Hepes (pH 7.4)], cross-linking was performed for 15 min at 20 °C with 1 mM bis(sulphosuccinimidyl)suberate (BS_3; Pierce, Rockford, IL, U.S.A.). The BS_3 was taken from a 50 mM stock solution in water (Sigma). The cross-linking reaction was quenched by the Tris present in the SDS/PAGE loading buffer, and the samples were processed for reducing SDS/PAGE and autoradiography.

Solid-phase binding assay

Native or recombinant α- or β-synuclein (15 μg/ml) in 200 mM $NaHCO_3$, pH 9.6, were immobilized in Polysorb microtitre plates (NUNC, Copenhagen, Denmark) for 2 h and residual protein binding sites were blocked by further incubation with 5 % (w/v) BSA for 2 h. After being rinsed, the wells were incubated with 50 pM ^{125}I-Aβ in the presence of various concentrations of unlabelled peptide for 16 h at 4 °C in binding buffer. 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 % (w/v) SDS.

Characterization of ligand-binding fragments

Recombinant α- or β-synuclein (33 μg) was digested with 0.05 μg of endoproteinase Asp-N, sequencing grade (EC 3.4.24.33; Boehringer Mannheim, Germany), in 20 mM Tris, pH 7.4, for 16 h at 20 °C; residual proteolytic activity was quenched by 10 mM EDTA. The generated peptides were separated by reverse-phase HPLC (LBK instrument assembled from a 2152 pump, a 2158 UV monitor, a 2155 column oven and a 2111 fraction collector) on a 4 mm × 250 mm Nucleosil C_{18} column by gradient elution with acetonitrile/0.1 % trifluoroacetic acid at 40 °C. Absorbance was measured at 229 nm (AUFS 0.5). The peptide-containing fractions were freeze-dried, dissolved in binding buffer and tested for ^{125}I-Aβ-binding activity with the cross-linking assay. The peptides in the ligand-binding fractions were subjected to N-terminal amino acid sequence analysis on an Knauer model 910 protein sequencer (Knauer GmbH, Berlin, Germany) with on-line analysis of the phenylthiohydantoin-amino acids. Matrix-assisted laser desorption ionization MS (MALDI-MS) was performed with a Bruker Reflex MALDI-time-of-flight instrument (Bruker-Franzen Analytic, Bremen, Germany). The mass spectrometer was equipped with a nitrogen laser operated at 337 nm and connected to a Macintosh computer for data acquisition and processing. All spectra were accumulated for 50–100 shots and samples were calibrated internally.

The following sample preparation method was used. A 0.5 μl aliquot of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in acetone containing 1 % water) was allowed to dry on the steel probe tip. A 0.5 μl portion of the sample (1–5 pmol) was applied to the matrix and the solution was left to dry at ambient temperature. All masses were measured in the linear flight mode except for peptide no. 9, which was recorded in the reflector mode. Peptide masses were fitted to the protein sequences with the GPMAW program (Lighthouse Data, Odense, Denmark).

Effect of α- and β-synuclein on Aβ aggregation

The aggregation of Aβ in the presence and absence of synucleins was evaluated by analysing the amount of peptide that could be sedimented by centrifugation on incubation. The method was essentially as described previously [12] with the modification that we used iodinated Aβ as tracer instead of measuring the amount of Aβ by antibodies. Aβ was dissolved in doubly distilled water and mixed with iodinated Aβ to a stock solution with a concentration of 1 mM and a specific radioactivity of 1000 c.p.m./μl. This stock solution was prepared immediately before use. The stock solution was diluted 10 times in 10 mM Tris/HCl, pH 7.4, to a final volume of 50 μl and incubated alone or in the presence of 10 μM α- or β-synuclein. After 24 h incubation at 37 °C, the aggregated Aβ was pelleted by centrifugation at 24000 ***g*** for 10 min in a microcentrifuge. The pellet was washed twice with 10 mM Tris/HCl, pH 7.4, and each wash was followed by the above-mentioned centrifugation. The final pellet, representing aggregated Aβ, was dissolved in 50 μl of formic acid and the radioactivity was determined with a Top Count Microplate Scintillation Counter (Packard).

Binding of biotinylated α-synuclein to AD-amyloid *in situ*

For biotinylation, 1 ml of recombinant human α-synuclein (1 mg/ml) was dialysed extensively against 0.1 M $NaHCO_3$, pH 8.0. The protein solution was then supplemented with 120 μl of 5 mM biotin-*N*-hydroxysuccinimide (Sigma) in dimethylformamide for 1 h at 20 °C. The reaction was stopped by extensive dialysis against 0.1 M Tris, pH 7.4, and biotinylated protein was stored at −20 °C in buffer containing 50 % (v/v) glycerol. The incorporation of biotin was assessed by Western blotting with streptavidin–horseradish peroxidase (Amersham). Hippocampal tissue from AD patients was obtained from The Netherlands Brain Bank, courtesy of Dr. R. Ravid. The tissue samples were cut in 10 μm sections on a rotation microtome (Micron, Walldorf, Germany). To study the same area of the tissue in adjacent sections, great care was taken to mount sections consecutively. Sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by 0.5 % H_2O_2 in methanol for 10 min at 20 °C. Sections for the biotin-α-synuclein binding experiments

were not further modified, whereas the sections for incubation with the monoclonal antibody against β-amyloid (M0872, DAKO, Copenhagen, Denmark) were treated with 100% formic acid for 1.5 min in accordance with the instructions of the manufacturer. Sections were covered for 60 min with PBS supplemented with either biotin-α-synuclein (1 μg/ml) or M0872 diluted 1:50 in PBS. The specificity of the biotin-α-synuclein binding was assessed by supplementing the biotin-α-synuclein with a 50-fold excess of non-biotinylated α-synuclein. As secondary antibody for the detection of M0872, biotinylated rabbit anti-(mouse IgG) (DAKO) was used. Tissue-bound antibody or biotin-α-synuclein was detected by incubation with a mixture of streptavidin and biotinylated horseradish peroxidase (StreptABComplex HRP kit; DAKO) for 20 min followed by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Kem-En-Tek, Copenhagen, Denmark)/0.1% H_2O_2 in PBS. The tissue was counterstained with Mayer's haematoxylin. Finally, the sections were dehydrated in graded ethanol and mounted in a hydrophobic mounting medium.

RESULTS

Aβ binding to α- and β-synuclein

The initial studies were performed to assess whether the binding of Aβ was similar to that of α- and β-synucleins purified from human brain material and to recombinant proteins produced in *E. coli*. The synuclein preparations were pure as judged from the silver-stained SDS/polyacrylamide gel (Figure 1A), and the electrophoretic mobilities of the native and recombinant proteins were identical, with the α-synucleins migrating slightly faster than the β-synucleins as previously noted [8]. The binding of Aβ was demonstrated by covalent cross-linking of ^{125}I-Aβ to α- and β-synucleins by the chemical cross-linker BS_3 (Figure 1B). There was no difference between the migrations of the tracer alone in the presence of cross-linker (lane 1) and tracer incubated with α-synuclein (lane 2) or β-synuclein (lane 5) in the absence of cross-linker, demonstrating that no SDS-resistant complexes had been formed after the 16 h incubation. However, an approx. 21 kDa labelled complex with both α- and β-synuclein appeared when the samples were supplemented with BS_3 at the end of the incubation (lanes 3 and 6). The specificity of the Aβ binding is demonstrated by the inhibition of the ^{125}I-Aβ tracer binding to both synucleins in the presence 100 μM unlabelled Aβ (lanes 4 and 7). Identical results were obtained when using recombinant α- and β-synucleins (compare Figure 3, lanes 2 and 8). Recombinant synucleins bound ^{125}I-Aβ to the same extent as the native proteins, as demonstrated by the binding of 25 pM ^{125}I-Aβ to immobilized native and recombinant α-synuclein (results not shown). Furthermore the binding was not due to the oxidation of Aβ during the iodination, because complex formation could be demonstrated between unlabelled Aβ and ^{125}I-α-synuclein (results not shown). Time-course experiments showed that the binding reached a plateau by 9 h (results not shown).

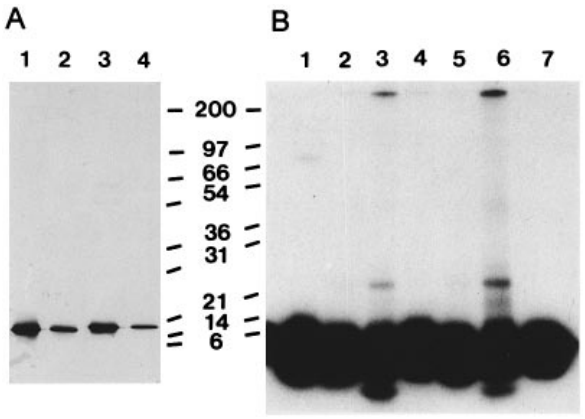

Figure 1 Aβ binds to α- and β-synucleins

(**A**) Approx. 20 ng of α- and β-synuclein purified from human brain (lanes 2 and 4) or expressed recombinant in *E. coli* (lanes 1 and 3) were resolved by SDS/PAGE [8–16% (w/v) gradient gel] under reducing conditions and detected by silver staining. (**B**) ^{125}I-Aβ (800 pM) was incubated without additions (lane 1), or with 2.5 μM native human α-synuclein (lanes 2–4) or β-synuclein (lanes 5–7). The samples shown in lanes 4 and 7 were further supplemented with 100 μM Aβ. After 16 h at 4 °C the samples shown in lanes 1, 3, 4, 6 and 7 were treated with the cross-linker BS_3 (1 mM) for 15 min. The samples were processed for autoradiography after resolution by reducing SDS/PAGE [8–16% (w/v) gradient gel]. The positions of molecular mass markers (in kDa) are indicated between the panels.

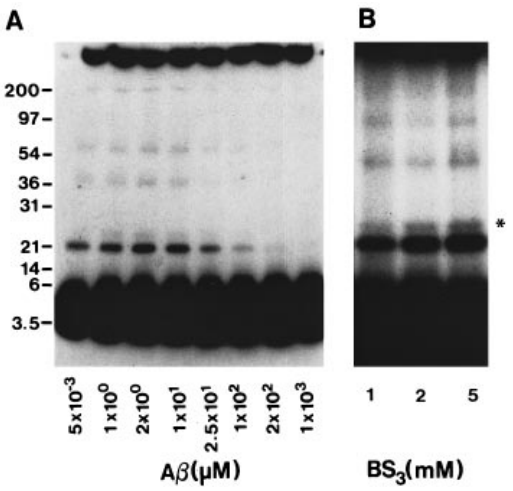

Figure 2 IC_{50} of the ^{125}I-Aβ binding to α-synuclein and assessment of binding stoichiometry

(**A**) ^{125}I-Aβ (5 nM) was incubated with 2.5 μM recombinant human α-synuclein in the presence of unlabelled Aβ as indicated for 16 h at 4 °C. After cross-linking, the samples were resolved by reducing SDS/PAGE [10–20% (w/v) gradient gel] and analysed by autoradiography. The positions of molecular mass markers (in kDa) are indicated at the left. (**B**) ^{125}I-Aβ (5 nM) was incubated as above with 2.5 μM recombinant human α-synuclein in the presence of 2 μM unlabelled Aβ, subjected to cross-linking by 1, 2 and 5 mM BS_3 and analysed as above. The asterisk indicates the appearance of a labelled approx. 25 kDa band compatible with a ^{125}I-labelled $(Aβ)_2$/α-synuclein complex.

The affinity of the Aβ binding to α-synuclein was analysed by fluid-phase cross-linking assay. Figure 2(A) demonstrates the gradual inhibition of the complex formation between 5 nM ^{125}I-Aβ and 2.5 μM recombinant α-synuclein by increasing the concentration of unlabelled Aβ to 1 mM. The IC_{50} was approx. 20 μM as determined by laser scanning densitometry. This figure is comparable to the IC_{50} obtained when using the solid-phase binding assay with immobilized α-synuclein (results not shown). The similar affinity revealed by the two assays supports the validity of the other results obtained with the solid-phase binding assay. The IC_{50} for the binding of Aβ to β-synuclein was indistinguishable from that for the binding to α-synuclein (results not shown).

The inclusion of 1–10 μM unlabelled Aβ when incubating labelled ^{125}I-Aβ and recombinant α-synuclein caused the appearance of a faint band of approx. 25 kDa (Figure 2A). This band disappeared in parallel with the inhibition of the

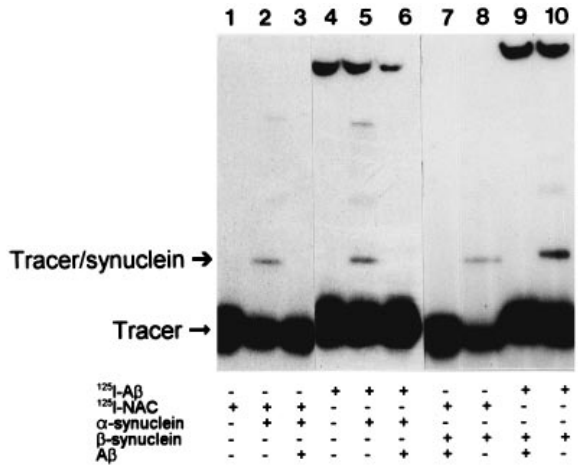

Figure 3 NAC binds to Aβ-saturable sites on α-synuclein

(**A**) ^{125}I-labelled NAC (800 pM, lanes 1–3, 7 and 8) or Aβ (lanes 4–6, 9 and 10) were incubated with 2.5 μM recombinant α-synuclein (lanes 2, 3, 5 and 6) or β-synuclein (lanes 7–10) in the absence (lanes 1, 2, 4, 5, 8 and 10) or presence (lanes 3, 6, 7 and 9) of 100 μM Aβ for 16 h at 4 °C, followed by chemical cross-linking. The samples were analysed by reducing SDS/PAGE [10–20% (w/v) gradient gel] and autoradiography.

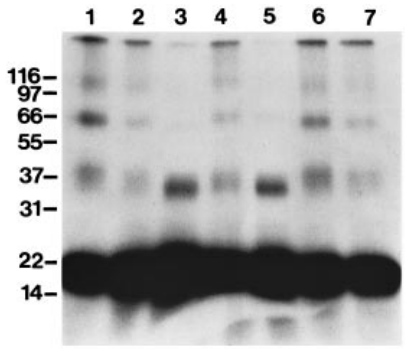

Figure 4 Synucleins form dimers

^{125}I-α-synuclein (5 nM) was incubated without additions (lane 1), with 10 nM or 1 μM α-synuclein (lanes 2 and 3), β-synuclein (lanes 4 and 5) or BSA (lanes 6 and 7) before chemical cross-linking, reducing SDS/PAGE [10–20% (w/v) gradient gel] and analysis by autoradiography. The positions of molecular mass markers (in kDa) are indicated at the left.

21 kDa ^{125}I-Aβ/α-synuclein complex by higher concentrations of Aβ. The 25 kDa band suggests the presence of more than one Aβ-binding site being revealed by the cross-linking of unlabelled Aβ to α-synuclein having bound one ^{125}I-Aβ peptide. The detection of such a complex should be facilitated by increasing the cross-linking efficiency. Figure 2(B) demonstrates the progressive increase in the intensity of the 25 kDa band marked with an asterisk when the concentration of cross-linker is increased from 1 to 5 mM. This indicates a 2:1 stoichiometry of the interaction between Aβ and α-synuclein.

NAC binds to the Aβ-binding sites in α- and β-synuclein

A structural comparison of the Aβ and NAC peptides has not yet been reported. However, the Aβ and NAC peptides share several characteristics. For example, both are tightly bound to AD amyloid, form amyloid fibrils *in vitro*, are substrates for brain transglutaminase and form SDS-resistant complexes with apoE [7,14,15,17–19]. To test whether the Aβ binding to α-and β-synuclein reflects an affinity for AD-amyloidogenic peptides in general, we compared the binding of ^{125}I-labelled NAC and ^{125}I-Aβ to the synucleins. Figure 3, lanes 2, 5, 8 and 10, shows, by chemical cross-linking, that both Aβ and NAC bind to α- and β-synucleins. The binding of ^{125}I-NAC to both synucleins was completely inhibited by 100 μM unlabelled Aβ, suggesting that NAC binds to the Aβ-binding site (Figure 3, lanes 3 and 9). A quantitative comparison of the binding of Aβ and NAC to the synucleins, using the solid-phase assay, revealed that the NAC tracer bound approximately twice as much to either synuclein (results not shown). Direct measurement of the affinity of the NAC peptide binding could not be determined because the peptide is insoluble at the high concentrations needed for competition experiments.

Synucleins form homodimers

The affinity of the α-synuclein fragment NAC for its own precursor molecule raised the question of whether α-synuclein can form homodimers or a heterodimer with β-synuclein. We investigated this by incubating ^{125}I-α-synuclein with increasing concentrations of unlabelled α-synuclein, β-synuclein or BSA followed by cross-linking and reducing SDS/PAGE. BSA served as a negative control. Figure 4, lane 1, shows that more than 95% of the label migrated as the approx. 17 kDa α-synuclein. The inclusion of any of the supplements at 10 nM caused little change in the migration of the tracer (lanes 2, 4 and 6). However, the presence of 1 μM of either α-synuclein (lane 3) or β-synuclein (lane 5) in the incubation medium caused the formation of a labelled complex with a molecular mass of approx. 34 kDa, compatible with a ^{125}I-α-synuclein–synuclein dimer. No complex formation was observed between 1 μM BSA and ^{125}I-α-synuclein (lane 7). The disappearance of the higher-molecular-mass components of the ^{125}I-α-synuclein preparation in the presence of unlabelled synuclein (lanes 3 and 5) might indicate the formation of large SDS-insoluble complexes with the synucleins, these complexes then not entering the gel.

Identification of Aβ- and NAC-binding sites in α- and β-synucleins

To identify the binding sites for NAC and Aβ we fragmented the synucleins and tested the generated peptides for binding activity. Figure 5(A), lanes 2 and 6, demonstrates the fragmentation of α- and β-synuclein after endoproteinase Asp-N treatment as observed by Coomassie Brilliant Blue staining of the electrophoretically resolved digest. The binding activity of the fragments generated is shown by cross-linking to ^{125}I-Aβ followed by autoradiography (lanes 4 and 8). Similar results were obtained when the digest was incubated with ^{125}I-NAC (results not shown). Figure 5(B) shows the purification of the α-synuclein peptides by reverse-phase HPLC. The indicated peaks 1–11 were resolved to near baseline levels, except peak 10, which is expected to contain some of the peptide in peak 11 (compare Table 1). The eluates corresponding to peaks 1–11 were freeze-dried, resuspended in equal volumes, and incubated with ^{125}I-labelled Aβ or NAC before chemical cross-linking by BS_3. Binding activity, as judged by the appearance of more-slowly migrating complexes with ^{125}I-Aβ (Figure 5C) or ^{125}I-NAC (results not shown), was detected for peptides 8, 9, 10 and 11. Peptides 8 and 9, presenting the most intense signals, displayed a tendency to form polymers. To identify the ligand binding peptides, we performed N-terminal amino acid sequencing and/or MALDI-MS (Table 1 and Figure

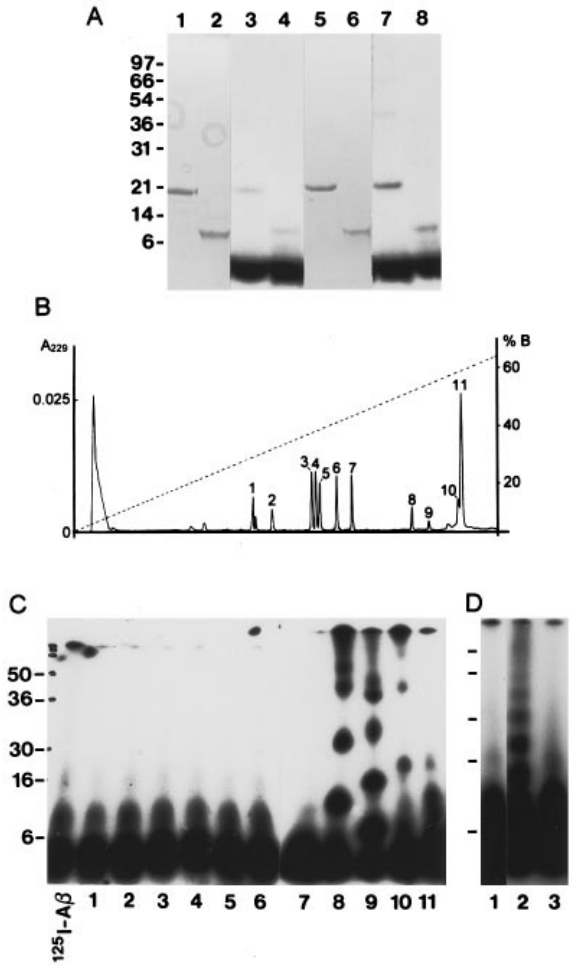

Figure 5 Purification of Aβ- and NAC-binding synuclein peptides

(**A**) α- and β-synuclein were treated with endoproteinase Asp-N in the absence or presence of 5 mM EDTA to inactivate the enzyme (control). The samples (approx. 3 μg of protein) were then incubated with 800 pM ^{125}I-Aβ, subjected to chemical cross-linking and resolved by SDS/PAGE [10–20% (w/v) gradient gel]. Lanes 1 and 2 (α-synuclein) and 5 and 6 (β-synuclein) show Coomassie Brilliant Blue staining of the digested (lanes 2 and 6) and control (lanes 1 and 5) samples. Lanes 3 and 4, and 7 and 8 show autoradiograms of the stained gel presented to the left of each autoradiogram. The positions of molecular mass markers (in kDa) are indicated at the left. (**B**) A 30 μg sample of the α-synuclein digest presented in (**A**), lane 2, was loaded on a Nucleosil C_{18} column (4 mm × 250 mm). The peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (dotted line). The flow rate was 0.8 ml/min and the column temperature 40 °C. Peptides were detected in the effluent by recording the absorbance at 226 nm. Fractions were collected at 20 s intervals and the eluates corresponding to the numbered peaks were pooled; this was followed by analysis of ^{125}I-Aβ-binding activity, N-terminal amino acid sequencing and mass spectrometry. (**C**) Aliquots (30 μl) from peaks 1–11 in (**B**) were freeze-dried, reconstituted in 50 μl of binding buffer containing 800 pM ^{125}I-Aβ and incubated for 16 h at 4 °C before chemical cross-linking and analysis by reducing SDS/PAGE [10% (w/v) gel] with the Tris/Tricine/urea buffer system. The lane at the left shows the migration of the ^{125}I-Aβ tracer alone and lanes 1–11 represent incubations with samples from peaks 1–11. (**D**) ^{125}I-Aβ (800 pM) was incubated without (lane 1) or with (lanes 2 and 3) the synthetic peptide α-synuclein 32-57 at 3.8 μM in the absence (lanes 1 and 2) or presence (lane 3) of 100 μM Aβ. The positions of molecular mass markers (in kDa) are indicated at the left.

6). The N-terminal amino acid sequencing shows that the generated sequences agreed with the specificity of endoproteinase Asp-N, cleaving N-terminally to Asp and Glu. Considering the low enzyme-to-substrate ratio, no contaminating endoproteinase Asp-N peptides would be expected, and none of the investigated peptides could have been generated from autodigestion of the proteinase. The observed masses for peptides 3–9 correspond to the expected masses within the precision of the instrument (0.1%) and do not fit with other potential α-synuclein fragments. The masses of peptides 10 and 11 could be explained by assuming that Met-1 and Met-5 in peptide 11, and Met-5 in peptide 10, had been oxidized, as this gives a difference between the observed and the expected mass of 32 and 16 Da respectively. Why these methionine residues were not oxidized in peptide 8 is not clear. Peptide 8, corresponding to residues 1–56, and peptide 9, corresponding to residues 57–97, were purified to baseline separation on reverse-phase HPLC (Figure 5B) and were pure as judged by mass spectroscopy (Figure 6). Both possessed ligand-binding activity (Figure 5C). This demonstrates that α-synuclein, on fragmentation with endoproteinase Asp-N, contains two independent binding segments for the amyloidogenic peptides Aβ or NAC.

During the characterization of ligand-binding β-synuclein fragments, we identified an active peptide with the NH_2-terminal EKTKEG and a size of the ^{125}I-Aβ/peptide complex of approx. 6 kDa (results not shown). The N-terminal amino acid sequence of the peptide corresponds to residues 25–30 in β-synuclein and represents an endoproteinase Asp-N cleavage product unique for β-synuclein owing to the presence of Glu-31, compared with Gly-31 in α-synuclein. The peptide presumably represents residues 31–56 in β-synuclein, as the following endoproteinase Asp-N cleavage site is after residue 56, and the size estimate from the SDS/PAGE indicates the binding of a peptide of approx. 2 kDa to the ^{125}I-Aβ. This fragment supports the presence of an Aβ-binding site between residues 31 and 56 in synuclein, and indeed we demonstrate a synthetic peptide corresponding to residues 32–57 in α-synuclein-bound labelled Aβ (Figure 5D) and NAC (results not shown).

Synucleins stimulate Aβ(1-40) polymerization

Several proteins have been demonstrated to enhance the rate of Aβ polymerization, e.g. α_1-antichymotrypsin, apolipoprotein E, heparan sulphate proteoglycans [20]; recently α-synuclein was shown to possess this property [12]. As both α- and β-synuclein bind Aβ, we speculated that both synucleins were able to stimulate the polymerization of Aβ. Using a polymerization assay based on a determination of the amount of ^{125}I-Aβ(1–40) that became sedimentable after 24 h, we found that β-synuclein increased the Aβ polymerization to the same extent as α-synuclein (161 ± 28% compared with 170 ± 34%, means ± S. D.). Both were increased significantly ($P < 0.05$) compared with the spontaneous Aβ aggregation of 100%, when evaluated by the Mann–Whitney test for difference in medians. Considering the conservation of the synucleins between species one would expect this to be a general property of this class of neuronal proteins.

Binding of α-synuclein to AD amyloid

α-Synuclein was biotinylated to probe binding to AD amyloid *in situ*. Figure 7 shows neighbouring sections from the hippocampus of a patient deceased from AD. Figure 7(A) demonstrates an Aβ-positive amyloid plaque situated in the granule cells layer. Incubation of the adjacent tissue section with 1 μg/ml biotinylated α-synuclein resulted in the binding of the probe to the amyloid plaque (Figure 7B). Most amyloid plaques and perivascular Aβ-positive deposits bound biotinylated α-synuclein, although some amyloid plaques were negative (results not shown). This suggests that the binding might be affected by the physical state of the Aβ in the amyloid, e.g. condensed compared with non-condensed aggregates, or content of longer

Table 1 Characterization of the endoproteinase Asp-N-generated peptides from α-synuclein

Abbreviation: N.D., not determined.

Peptide*	N-terminus	Mass (Da)	Binding activity	Identified peptide	
				Theoretical mass† (Da)	Amino acid residues
1	DYEPEA	ND	No	722.7	135–140
2	EMPSEE..	ND	No	1069.1	126–134
3	DMPVD..	1265.4	No	1265.3	115–125
4	EMPSEE..	1774.3	No	1773.8	126–140
5	DQLGKN..	1827.9	No	1826.9	98–114
6	DMPVDP..	2319.5	No	2316.4	115–134
7	DMPV..	3024.2	No	3021.1	115–140
8	MDVFM..	5664.6	Yes	5662.6	1–56
9	EKT..	4003.2	Yes	4003.6	57–97
10	MDVF..	9674.9‡	Yes	9648.1	1–97
	DVF..	9529.9§		9516.9	2–97
11	MDVFM..	9681.9‡	Yes	9648.1	1–97

* Peptide numbering corresponds to that shown in Figures 5(B) and 5(C).
† The theoretical masses were calculated from the published aa sequence of α-synuclein [7].
‡ Contains two oxidized methionine residues (experimentally determined mass 9680.1).
§ Contains one oxidized methionine residue (experimentally determined mass 9532.9).

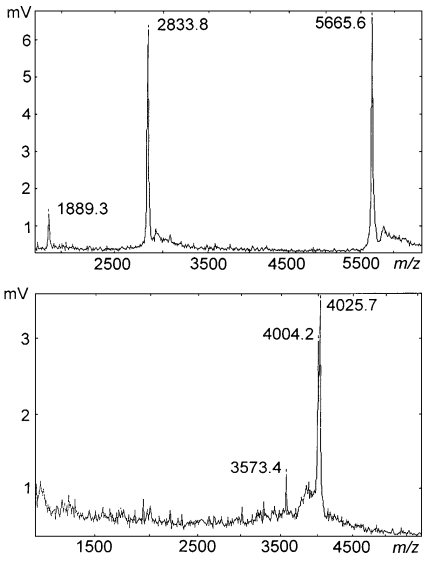

Figure 6 MALDI mass spectrum of the Aβ-binding peptides in peaks 8 and 9

Upper panel: mass spectrum of peptide 8 showing the MH^+ (m/z 5665.6), MH_2^{2+} (m/z 2833.8) and MH_3^{3+} (m/z 1889.3) ions. Lower panel: mass spectrum of peptide 9 showing the MH^+ (m/z 4004.2) and MNa^+ (m/z 4025.7) ions. The peak at m/z 3573.4 could not be related to any part of α-synuclein.

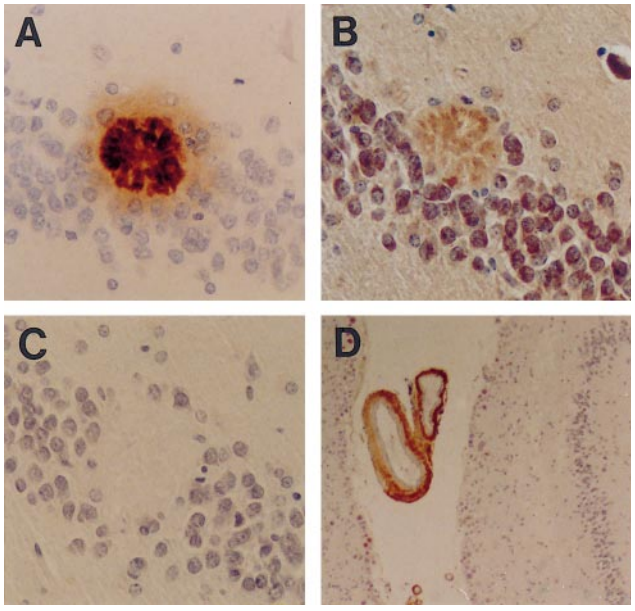

Figure 7 Binding of biotinylated α-synuclein to AD amyloid *in situ*

Adjacent tissue sections from a paraformaldehyde-fixed and paraffin-embedded hippocampus of an AD patient were incubated with monoclonal antibody against Aβ (**A**); biotinylated α-synuclein (1 μg/ml) (**B**); biotinylated α-synuclein plus 50 μg/ml unlabelled α-synuclein (**C**) (bound antibody and synuclein were revealed by a streptavidin/horseradish peroxidase complex, with 3,3′-diaminobenzidine tetrahydrochloride as chromogen). (**D**) As (**C**) but presenting a part of the section with the pia and subpial tissue.

compared with shorter Aβ-forms. All the parenchymal binding of biotin-α-synuclein to hippocampal tissue sections was specific because the binding was completely inhibited by a 50-fold excess of unlabelled α-synuclein (Figure 7C). In contrast, the pia mater, which covers the brain tissue and pial vessels, binds biotinylated α-synuclein non-specifically in the presence of an excess of unlabelled α-synuclein (Figure 7D).

DISCUSSION

The present results show that Aβ can bind independently to the two purified consecutive segments of α-synuclein (residues 1–56 and 57–97), and a synthetic peptide corresponding to residues 32–57 of α-synuclein exhibits ^{125}I-Aβ binding activity (Figures

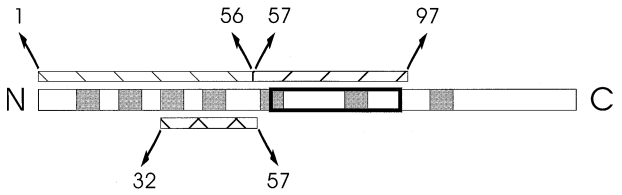

Figure 8 Localization of Aβ-binding peptides in α-synuclein

The seven KTKEGV consensus motifs are shown as shaded boxes and the sequence corresponding to the NAC fragment (residues 61–95) is shown in bold. The two Aβ-binding segments identified in the α-synuclein digest are indicated above the model, and the synthetic peptide (residues 32–57) containing Aβ-binding activity is shown below.

5C, 5D and 8). The C-terminal domain corresponds largely to the domain recently identified as being between residues 58 and 100 by using deletion mutants [12]. Whether both binding domains are active in intact α-synuclein, or only the C-terminal domain is active owing to the trapping of the N-terminal domain in an inactive conformation, is less clear. However, the appearance of a second labelled complex on incubation of α-synuclein with increasing concentrations of Aβ (Figure 2B) supports the idea of at least two Aβ-binding sites. The reported absence of binding of ^{125}I-Aβ to an N-terminal segment of α-synuclein comprising residues 1–58 might be due to the use of a solid-phase binding assay [12] as compared with our fluid-phase assay. It remains to be established whether β-synuclein also possesses more sites for Aβ. However, because the apparent affinities are similar for binding Aβ to α- and β-synuclein, we suggest that the binding modes are similar for the two synucleins.

Surprisingly, the NAC peptide was bound to its precursor molecule α-synuclein as well as to β-synuclein. Because Aβ competed for the binding, the sites for binding of the two peptides might be overlapping. The previous more detailed mapping of the Aβ-binding site to residues 81–95 was based on the inhibition of ^{125}I-Aβ binding to immobilized α-synuclein by a peptide comprising this segment of NAC [12]. It follows from the present results that the inhibition might be due to saturation of the Aβ-binding site in α-synuclein by the peptide rather than binding of the peptide to Aβ.

The interaction between Aβ and synucleins might be important in the pathogenesis of AD because NAC constitutes up to 10% of the SDS-insoluble protein in AD amyloid plaques, and because at least α-synuclein is expressed particularly in regions of the brain where AD lesions are abundant. The complex sequence of events leading to the formation of amyloid plaques is uncertain. Concerning the involvement of synucleins, the following working hypothesis can be proposed. As a result of neuronal degradation, synucleins will be released and captured by amyloid plaques owing to their affinity for amyloid plaques as shown for α-synuclein (Figure 7). The presence of two Aβ-binding sites might make synucleins function as templates that stimulate Aβ aggregation and facilitate the conversion of monomeric Aβ to the neurotoxic fibrillar form. The transglutaminase-reactive Gln and Lys residues, previously demonstrated in Aβ and the NAC segment [17,25] might, when brought into proximity, facilitate cross-linking catalysed by brain transglutaminase and thus further stabilize insoluble forms. Concomitantly, released synucleins are subject to degradation. It should be noted that in contrast with the NAC segment of α-synuclein, fragments of β-synuclein have not been detected in the SDS-insoluble fraction of AD amyloid plaques. It can be speculated that the 11 residues present in the NAC segment of α-synuclein, but absent from β-synuclein, are necessary to facilitate the conversion of initially SDS-sensitive complexes (Figure 1B) to an SDS-resistant form. This would imply that β-synuclein fragments are lost in the purification procedure for the amyloid.

Irrespective of the proposed role of synucleins in the pathogenesis of AD, the identification of peptides that bind Aβ might open new possibilities for preventing the formation of AD plaques. The strategy is to identify small peptides that inhibit Aβ self-aggregation and the formation of complexes between Aβ and synucleins or their fragments. Thus a peptide derived from Aβ was recently shown to inhibit Aβ self-aggregation [26]. Future studies should show whether peptides derived from synucleins might prevent aggregations and whether they might be used as lead substances for the construction of drugs.

In conclusion, we find that both α- and β-synucleins bind Aβ and NAC. α-Synuclein has two binding sites within residues 1–56 and 57–97 respectively, and a peptide corresponding to residues 32–57 binds Aβ. The presence of multiple binding sites in synucleins might be important for the Aβ-fibril-promoting activity.

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