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Ca^{2+} Binding to α-Synuclein Regulates Ligand Binding and Oligomerization*

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α-Synuclein is a protein normally involved in presynaptic vesicle homeostasis. It participates in the development of Parkinson's disease, in which the nerve cell lesions, Lewy bodies, accumulate α-synuclein filaments. The synaptic neurotransmitter release is primarily dependent on Ca^{2+}-regulated processes. A microdialysis technique was applied showing that α-synuclein binds Ca^{2+} with an IC_{50} of about 2–300 μM and in a reaction uninhibited by a 50-fold excess of Mg^{2+}. The Ca^{2+}-binding site consists of a novel C-terminally localized acidic 32-amino acid domain also present in the homologue β-synuclein, as shown by Ca^{2+} binding to truncated recombinant and synthetic α-synuclein peptides. Ca^{2+} binding affects the functional properties of α-synuclein. First, the ligand binding of ^{125}I-labeled bovine microtubule-associated protein 1A is stimulated by Ca^{2+} ions in the 1–500 μM range and is dependent on an intact Ca^{2+} binding site in α-synuclein. Second, the Ca^{2+} binding stimulates the proportion of ^{125}I-α-synuclein-containing oligomers. This suggests that Ca^{2+} ions may both participate in normal α-synuclein functions in the nerve terminal and exercise pathological effects involved in the formation of Lewy bodies.

Parkinson's disease (PD)[1] and other common neurodegenerative disorders, *e.g.* dementia with Lewy bodies and the Lewy body variant of Alzheimer's disease, are characterized by the development of the proteinaceous inclusions called Lewy bodies in the degenerating nerve cells (1). Lewy bodies comprise α-synuclein (AS)-containing filaments, and purified AS readily forms amyloid-like filaments *in vitro* (2–5). Moreover, missense mutations in the AS gene cause heritable autosomal dominant PD (6, 7). Transgenic animal models support the direct link between AS and neurodegeneration because overexpression of AS leads to neuronal loss, nerve terminal pathology, and formation of Lewy body-like inclusions (8–11). It has been proposed that the pathogenic mechanisms triggered by AS rely on structural changes occurring during the transition from the monomeric to the β-folded filamentous state (12). AS is a member of the synuclein family, which, in man, is dominated by α-, β-, and γ-synuclein (13). The synucleins are acidic proteins of about 140 amino acids that display a "natively unfolded" structure (14). The N-terminal part of the proteins is highly conserved and contains several KTKEGV consensus repeats, whereas the C-terminal portion is less well conserved and possesses no known structural elements (15). The differences in its primary structure are reflected in segregated functional domains, *e.g.* brain vesicles bind to the N-terminal part, whereas the microtubule-associated proteins tau and microtubule-associated protein 1B bind to the C-terminal part (16–18). The AS gene is dispensable for normal development and breeding as demonstrated in AS knockout mice (19). However, these mice do exhibit subtle changes in the contents of certain neurotransmitters and in synaptic transmission (19), and antisense suppression in primary nerve cell cultures causes a reduced distal pool of synaptic vesicles (20). This indicates that AS plays a role in the cellular signaling events, an observation that is in agreement with biochemical studies demonstrating that AS can affect phospholipase D2 and protein kinases and modulate phosphorylation of nerve cell proteins (17, 21, 22).

Ca^{2+} ions regulate a plethora of cellular processes. This functionality has been refined in neurons, where the propagation of action potentials over long distances and the fine-tuned neurotransmitter release from nerve terminals represent such Ca^{2+}-regulated processes (23–25).

The actions of Ca^{2+} ions are mediated by several mechanisms. The Ca^{2+}-calmodulin complex and its diverse downstream signaling pathways represent common cellular mechanisms (26). More neuron-specific Ca^{2+}-regulated proteins are represented by synaptic vesicle-associated proteins and abundant Ca^{2+}-binding neuronal proteins like parvalbumin and calbindin (27). The latter group may function as a slow buffer that modulates synaptic plasticity (28). The importance of cellular Ca^{2+} homeostasis is highlighted by the central role of Ca^{2+} ions in apoptotic processes and neuronal excitotoxicity (29, 30).

The purpose of the present study was to investigate the binding of Ca^{2+} to AS to ascertain whether Ca^{2+} can regulate normal and pathological AS functions.

MATERIALS AND METHODS

Miscellaneous—^{45}Ca and ^{125}I were obtained from Amersham Pharmacia Biotech. All reagents were of analytic grade, unless stated otherwise. The synthetic peptide AS-(109–140), corresponding to amino acid residues 109–140 in human AS, was from Shaefer-N (Copenhagen, Denmark).

Proteins—The novel deletion mutants AS-(1–110) and AS-(1–125) were produced by PCR-based mutagenesis as described previously for AS-(1–95) and AS-(55–140) (18). The constructs were verified by DNA sequencing. The mutant proteins were expressed in *Escherichia coli* and purified essentially as described for wild type AS (31). The peptides were more than 95% pure as assessed by Coomassie Blue staining (Fig. 2, *middle panel, A, inset*), and their identities were verified by mass spectrometry (data not shown). Purified bovine microtubule-associated protein (MAP)-1A was kindly provided by Dr. Khalid Islam (32). It consisted essentially of the pure ~360-kDa heavy chain (Fig. 3, *top panel, inset, lane 1*). The MAP-1A was iodinated to a specific activity of

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[1] The abbreviations used are: ; PD, Parkinson's disease; BS3, bis(sulfosuccinimidyl)suberate; AS, α-synuclein; MAP, microtubule-associated protein.

about 250 mCi/mg using chloramin T as the oxidizing agent, as described previously for MAP-1B (18). The electrophoretic migration of the iodinated MAP-1A consisted of a single slow-migrating band corresponding to the nonlabeled protein (Fig. 3, *top panel, inset, lane 2*). All protein concentrations were determined using the Bio-Rad protein assay using bovine serum albumin as standard.

$^{45}Ca^{2+}$ Equilibrium Dialysis Assay—First buffers and protein stock solutions were passed through a Chelex 100 column (Bio-Rad) to remove Ca^{2+} ions to negligible levels (33). Next, solutions containing 1 mM $^{45}Ca^{2+}$ and different concentrations of unlabeled Ca^{2+} were prepared with and without a constant concentration of AS-(1–140), AS-(1–125), AS-(1–110), AS-(1–95), β-synuclein, and γ-synuclein. All experiments were performed at 4 °C in a solution containing 150 mM KCl and 20 mM HEPES, pH 7.4. The concentration of the synucleins varied from 20 to 300 μM. Binding was measured by equilibrium dialysis. For equilibrium dialysis, 30-μl plexiglass chambers were used (34). Each chamber was divided into two equal compartments by a cellulose membrane cut from dialysis tubing (Spectrum, Houston, Texas; cutoff, 3,500 Da). The left-side compartments contained 25 μl of calcium-containing samples, with or without the synucleins, and the right-side compartments contained 25 μl of buffer. Control experiments showed that equilibrium was established within 2 h (data not shown). Accordingly, the chambers were emptied after 9 h, before the samples were assayed for radioactivity and protein. The Ca^{2+} concentration was determined by liquid scintillation counting with a LKB Wallac 1209 Rackbeta counter (Turku, Finland). No quenching of the radioactivity of $^{45}Ca^{2+}$ by the synucleins was observed. The recovery of $^{45}Ca^{2+}$ was 97%, demonstrating that no significant adsorption of calcium to the cellulose membrane or dialysis chamber had occurred. The radioactivity of Ca^{2+}-containing solutions that had not been dialyzed was taken to represent the known concentration of total Ca^{2+}. In the binding experiments, the concentrations of bound and free Ca^{2+} were calculated by using the radioactivity samples taken from the synuclein-containing chambers (representing bound plus free Ca^{2+}) and the corresponding synuclein-free chambers (representing free Ca^{2+}). The concentrations of the synucleins were measured by spectroscopy at 280 nm using the extinction coefficient calculated for each of them. The protein content in the isolated samples was determined by SDS-polyacrylamide gel electrophoresis and silver staining to assure the absence of degradation and leakage through the membrane.

MAP-1A Binding Assay—The ^{125}I-MAP-1A binding to AS peptides immobilized in Polysorb microtiter plates (Nunc, Copenhagen, Denmark) was performed essentially as described previously for tau (17). The binding buffer consisted of 150 mM KCl, 20 mM HEPES, pH 7.4, 0.01% extensively dialyzed bovine serum albumin, 0.1 mM EDTA, and 0.1 mM EGTA supplemented with various concentrations of $CaCl_2$ and $MgCl_2$. The even immobilization of the C-terminally truncated AS peptides was verified by their similar specific binding of a ^{125}I-labeled affinity-purified antibody (ASY-3) raised against a synthetic peptide corresponding to the N-terminal 31 residues of AS (data not shown).

Chemical Cross-linking of AS Oligomers—AS and C-terminal-truncated peptides (1 μM) supplemented with 500 pM of the corresponding ^{125}I-labeled AS were incubated for 2 h at 20 °C in 150 mM KCl, 20 mM 4-morpholinepropanesulfonic acid, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, and 0.5 mM dithioerythreitol in the absence and presence of Ca^{2+}. The distribution of monomers, oligomers, and higher aggregates was subsequently stabilized by the addition of a short-length hydrophilic chemical cross-linker, bis(sulfosuccinimidyl)suberate (BS3) (1 mM), for 15 min, and then the cross-linker was quenched by the addition of an equal volume of Tris-containing SDS, dithioerythreitol loading buffer. The samples were subsequently resolved by reducing gradient SDS-polyacrylamide gel electrophoresis followed by visualization by autoradiography.

RESULTS

AS Contains a Novel Ca^{2+}-binding Motif—$^{45}Ca^{2+}$ equilibrium dialysis was performed to determine whether AS is a Ca^{2+}-binding protein. Fig. 1 demonstrates that human recombinant AS binds Ca^{2+} with a half-saturation of about 300 μM. The saturation of the binding approaches 0.5 mol Ca^{2+}/mol AS at 1 mM Ca^{2+}, which indicates the presence of a single binding site. Mg^{2+} (8 mM) fails to inhibit the $^{45}Ca^{2+}$ tracer binding (1 μM) significantly as compared with the ~85% inhibition obtained by 1.5 mM unlabeled Ca^{2+}. Hence, the binding site displays a Ca^{2+} selectivity among the dominating intracellular divalent cations (Fig. 2, *middle panel, A*).

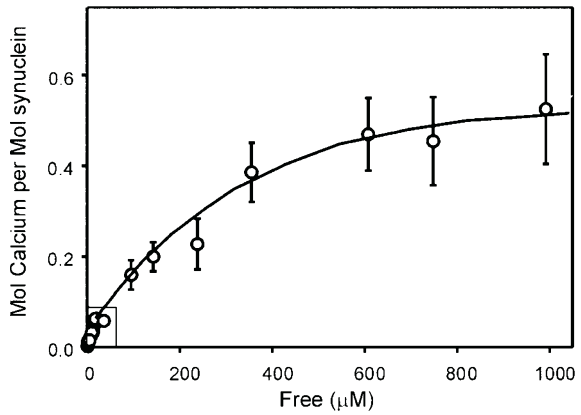

FIG. 1. **Ca^{2+} binding isotherm for AS-(1–140).** Recombinant human AS (~100 μM) was incubated with 1 μM $^{45}Ca^{2+}$and increasing concentrations of unlabeled Ca^{2+} in a microdialysis apparatus for 9 h at 4 °C, and then the $^{45}Ca^{2+}$ concentration was measured on each side of the dialysis membrane. The *abscissa* shows the free Ca^{2+} concentration, and the *ordinate* shows mol Ca^{2+} bound/mol α-synuclein. The *points* represent the mean ± 1 S.D. of five experiments. The *square* in the *bottom left corner* of the graph represents the data demonstrated in the *bottom panel* of Fig. 2.

Truncated recombinant AS peptides with deletions of the N-terminal 29- and 54-amino acid residues and the C-terminal 45-amino acid residues were used for initial localization of the Ca^{2+}-binding site. Fig. 2, *middle panel, B* demonstrates that only the C-terminal truncation inhibited the binding, whereas the N-terminal truncation has no effect. No inhibition of the Ca^{2+} binding is observed when testing the mutations causing PD (A30P and A53T) (Fig. 2, *middle panel, A*). Acidic amino acid residues often participate in the binding of Ca^{2+} ions as noted in the EF-hand, C_2-domain and the low-affinity Ca^{2+}-binding sites in S100 proteins (35–37), and such residues account for 33% of the C-terminal 45 residues. Fig. 2, *top panel,* demonstrates a striking identity in the spacing of 10 of the 12 acidic residues in the C-terminal 32 residues of α- and β-synuclein. γ-Synuclein, however, shows no such similarity. The similarity in the spacing of acidic residues is reflected at the functional level, where α- and β-synuclein, but not γ-synuclein, bind Ca^{2+} (Fig. 2, *middle panel, A*). The acidic residues in the C terminus of α- and β-synuclein are organized as a tandem repeat of 16 amino acids (Fig. 2, *top panel*), and the integrity of this structure may be required for the binding of Ca^{2+} ions. This hypothesis was explored by examining the expression and purification of recombinant truncated AS peptides lacking (i) the C-terminal repeat AS-(1–125) and (ii) both repeats AS-(1–110) and AS-(1–95) (Fig. 2, *middle panel, B, inset*). Fig. 2, *middle panel, B* shows that removal of the single C-terminal repeat in AS-(1–125) inhibits the Ca^{2+} binding to the level of the peptides lacking both repeats or the entire 45 C-terminal residues. Moreover, a synthetic peptide corresponding to the tandem repeat structure, AS-(109–140), binds $^{45}Ca^{2+}$ to the same extent as wild type AS (Fig. 2, *middle panel, B*), and its binding isotherms reveal indistinguishable affinities for Ca^{2+} (Fig. 2, *bottom panel*). Accordingly, the C-terminal repeat structure in α- and β-synuclein is necessary and sufficient to bind Ca^{2+} and represents a *bona fide* Ca^{2+}-binding domain.

Ca^{2+} Binding to α-Synuclein Modulates Ligand Interactions—The propensity of Ca^{2+} ions to modulate ligand binding to AS was analyzed in terms of the effect of such binding of (i) the amyloidogenic Aβ(1–40) peptide and (ii) the microtubule-associated proteins tau and MAP-1B. Ca^{2+} ions have no significant effect on these interactions (data not shown). MAP-1A is a novel AS ligand, as demonstrated by the binding of ^{125}I-

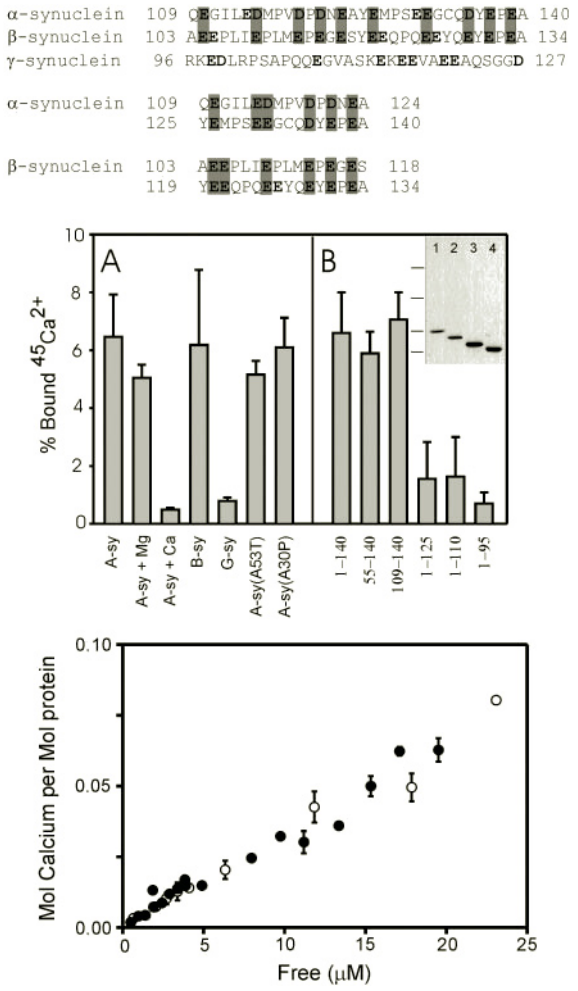

FIG. 2. **Both α- and β-synuclein contain a C-terminal Ca^{2+} binding site.** *Top part* of the *top panel,* alignment of the C-terminal 32 amino acid residues in human α-, β-, and γ-synuclein. Acidic residues are shown in *bold*, and those positions where acidic residues are identical to AS are marked by *gray boxes*. *Bottom part* of the *top panel,* the acidic amino acids in the C terminus of α- and β-synuclein represent a 16-residue acidic tandem repeat. Residues 109–124 are aligned with residues 125–140 in AS, and residues 103–118 are aligned with residues 119–134 in β-synuclein. Repeated acidic residues are indicated by *gray boxes*. *Middle panel,* binding of $^{45}Ca^{2+}$ to recombinant synucleins and truncated recombinant synuclein peptides. The $^{45}Ca^{2+}$ binding experiments were performed as described in the Fig. 1 legend. The *ordinate* demonstrates the percentage of tracer binding as the mean ± 1 S.D. of three independent experiments. *A,* Ca^{2+} binding to human recombinant wild type AS, AS containing the Parkinson's disease-causing point mutations A30P and A53T, β-synuclein, and γ-synuclein. Supplementing the buffer with 8 mM unlabeled Mg^{2+} did not significantly inhibit the tracer binding as compared with 1.5 mM Ca^{2+}. *B,* Ca^{2+} binding to AS, truncated AS proteins, and a synthetic peptide corresponding to C-terminal residues 109–140. The *columns* represent the mean ± 1 S.D. of three independent experiments. The *numbers below* the columns correspond to the amino acid residues in the peptides, *e.g.* 1–140 for full-length AS. The *inset* demonstrates the purity of the recombinant proteins by a Coomassie Blue-stained SDS-polyacrylamide gel with the molecular size markers in kDa (60, 36, 22, and 6) indicated to the *left*. *Bottom panel,* comparison of Ca^{2+} binding to recombinant AS and the synthetic peptide AS-(109–140). Both peptides (100 μM) were incubated with 1 μM $^{45}Ca^{2+}$ and increasing concentrations of Ca^{2+} as described in the Fig. 1 legend. The *abscissa* shows the free Ca^{2+} concentration, and the *ordinate* shows mol Ca^{2+} bound/mol peptide. The *points* represent the mean ± 1 S.D. of three experiments. *Open circles* represent AS, *closed circles* represent AS-(109–140).

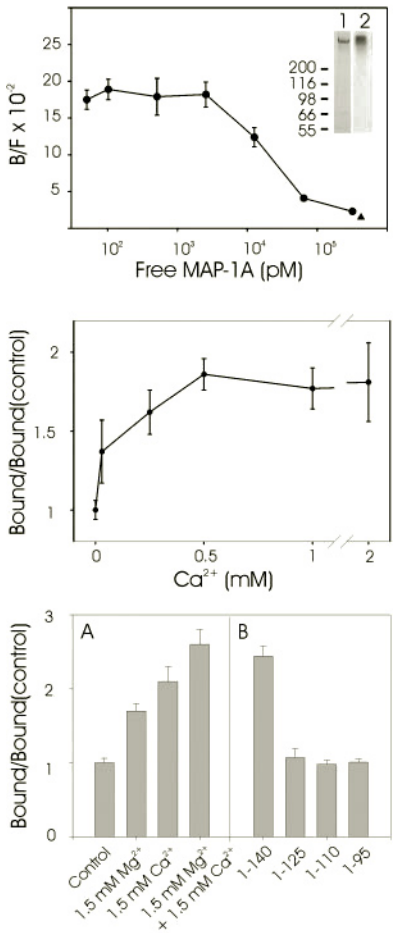

FIG. 3. **Ca^{2+} binding to AS regulates the interaction with MAP-1A.** *Top panel,* recombinant AS, immobilized in microtiter plates, was incubated with 50 pM ^{125}I-MAP-1A and increasing concentrations of unlabeled MAP-1A (●) or AS (▲). The *ordinate* represents the percentage of bound/free (*B*/*F*) ligand, and the *abcissa* represents the concentration of free ligand. The *points* represent the mean ± 1 S.D. of four replicates in one of four similar experiments. *Inset, lane 1,* purified bovine MAP-1A (4 μg) was mixed with 10,000 cpm ^{125}I-MAP-1A, resolved by 8–16% reducing SDS-polyacrylamide gel, and stained with Coomassie Blue. *Lane 2,* autoradiogram of the same gel. The molecular size markers (in kDa) are shown on the *left*. *Middle panel,* the Ca^{2+} dependence of ^{125}I-MAP-1A binding to AS. The binding (as described in the *top panel*) was determined in the presence of increasing concentrations of Ca^{2+} ions. The *ordinate* represents the ratio between bound MAP-1A and the control binding of MAP-1A in the absence of Ca^{2+} ions, and the *abscissa* represents the Ca^{2+} concentration. The *points* represent the mean ± 1 S.D. of four replicates in one of three similar experiments. *Bottom panel, A,* effect of Ca^{2+} and Mg^{2+} on the binding of ^{125}I-MAP-1A to AS. The binding was determined in the absence (*Control*) and presence of the indicated concentrations of divalent cations. *Bottom panel, B,* the binding of ^{125}I-MAP-1A to immobilized full-length AS-(1–140) and the C-terminal-truncated AS peptides 1–125, 1–110, and 1–95 was determined in the presence of 1.5 mM Ca^{2+}. The *columns* in *A* and *B* represent the mean ± 1 S.D. of four replicates in one of three similar experiments. The equal immobilization of the different AS peptides was verified by their similar binding of the ^{125}I-labeled ASY-3 antibody that recognizes the N terminus of AS.

labeled bovine MAP-1A to immobilized AS (Fig. 3, *top panel*). The association of 50 pM ^{125}I-MAP-1A reaches a plateau within 9 h at 4 °C (data not shown), and all incubations are therefore performed for 16 h. The interaction is specific, as demonstrated by the inhibition of ^{125}I-MAP-1A binding by both unlabeled MAP-1A and AS, and it exhibits a high affinity (IC_{50} ~ 30 nM; Fig. 3, *top panel*).

The binding of MAP-1A to AS is enhanced by Ca^{2+} ions (Fig. 3, *middle panel*), and a maximal stimulatory effect of about 90% is obtained at concentrations greater than 0.5 mM (Fig. 3, *middle panel*), with a half-maximal stimulation at about 0.3 mM Ca^{2+} (Fig. 3, *middle panel*). Both Mg^{2+} and Ca^{2+} (1.5 mM) stimulate MAP-1A binding to AS, but the effect of Ca^{2+} alone is about 60% greater than that for Mg^{2+} ions alone; when com-

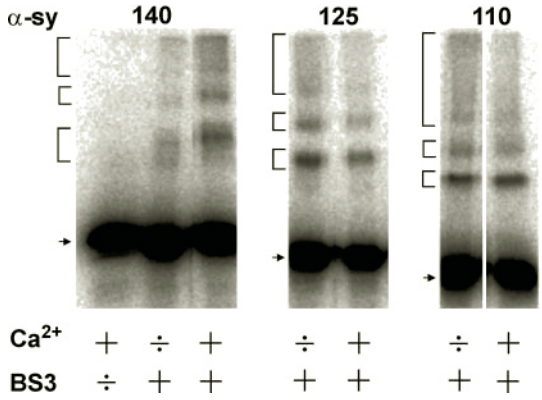

FIG. 4. **Ca^{2+} regulates AS oligomerization.** AS and the AS peptides (1 μM) with truncations in the Ca^{2+}-binding domain were supplemented with 500 pM of the corresponding ^{125}I-labeled AS peptide and incubated in the absence and presence of 1.5 mM Ca^{2+} for 2 h at 20 °C. The incubates were subsequently cross-linked with BS3 (1 mM), resolved by SDS-polyacrylamide gel electrophoresis, and processed for autoradiography. The panels represent the autoradiographic image of AS-(1–140) and the C-terminal-truncated peptides AS-(1–125) and AS-(1–110). The presence of Ca^{2+} and BS3 is indicated *below* the panels. *Brackets* to the *left* indicate the localization of dimers, trimers, and larger oligomers. The monomer is indicated by an *arrow*.

bined, their effect is synergistic (Fig. 3, *bottom panel, A*). Disruption of the Ca^{2+}-binding domain in AS obtained by removal of the C-terminal 15, 30, and 45 amino acid residues completely abrogates the Ca^{2+}-stimulatory effect on MAP-1A binding (Fig. 3, *bottom panel, B*) and demonstrates that the Ca^{2+} effect was indeed based on the AS moiety. Removal of the Ca^{2+}-binding site in AS increases the binding of MAP-1A to the truncated AS peptide (data not shown), but it abrogates the stimulatory Ca^{2+} effect (Fig. 3, *bottom panel, B*). This indicates a negative regulatory effect of the C-terminal segment of AS on the MAP-1B interaction that is alleviated by binding of Ca^{2+} ions. The stimulatory effect of Mg^{2+} ions on MAP-1A binding may thus be mediated via the MAP-1A moiety. Many Ca^{2+} effects are mediated through the binding of Ca^{2+} to calmodulin, but AS does not bind to calmodulin-Sepharose in either the absence or presence of Ca^2 (data not shown).

Ca^{2+} Ions Regulate the Oligomeric Distribution of AS Molecules—Abnormal filamentous AS is a characteristic of diseased brain tissue, and AS aggregation represents a nucleation-dependent process, where the nucleation by oligomeric AS species may represent a rate-limiting step. We used the short-length hydrophilic chemical cross-linker BS3 to covalently stabilize AS oligomers in the absence and presence of Ca^2. This analysis is likely to underestimate the oligomeric content because the cross-linking efficiency is <100%. However, the method has the advantage of visualizing molecules associated through low-affinity interactions. Gel filtration methods and other time-consuming procedures for separating oligomerized and monomeric species may not be able to reveal such interactions due to dissociation during the procedures. No significant AS-(1–140) oligomers are present without cross-linking (Fig. 4). The same applies to AS-(1–125) and AS-(1–110) (data not shown). Supplementing the AS solution with 1 mM BS3 for 15 min before reducing SDS-polyacrylamide gel electrophoresis causes the formation of ^{125}I-labeled bands compatible with AS dimers, trimers, and higher oligomers with a higher oligomeric content among the C-terminal-truncated peptides (Fig. 4). Saturation of the Ca^{2+} binding site (1.5 mM) increases the oligomeric content of AS-(1–140) 2-fold for dimers and 2.5-fold for trimers, and higher aggregates, whereas no Ca^2 effect is observed for the truncated peptides. The oligomers are not an artifact of the iodination of AS because the distinct oligomeric pattern is absent without the presence of 1 μM unlabeled AS. Accordingly, Ca^{2+} binding to the C-terminal tandem repeat domain favors the formation of AS oligomers.

DISCUSSION

The present study identifies a novel Ca^{2+}-binding motif in the C terminus of AS. The binding of Ca^{2+} alters the interactions between AS molecules in the process of oligomerization and between AS and certain nerve cell proteins as exemplified by MAP-1A. AS binds Ca^{2+} ions selectively as compared with the predominant cytosolic divalent cation Mg^{2+}, which suggests that AS functions can be regulated by Ca^{2+} ions in a cellular context.

Several synuclein genes are expressed in man; the most predominant of these are AS, β-synuclein, and γ-synuclein (15). The localization of α- and β-synuclein in normal nervous tissue is restricted to the nerve terminals (38, 39), in contrast to γ-synuclein, which is localized in the somatodendritic compartment (40). The nerve terminal localization parallels the Ca^{2+} binding properties of the proteins because α- and β-synuclein, but not γ-synuclein, bind Ca^{2+}. We therefore wish to suggest a functional significance of Ca^{2+} binding to AS in the nerve terminals where high local Ca^{2+} concentrations are reached (41) and AS regulates complex nerve terminal processes related to neurotransmitter homeostasis and maintenance of the distal pool of synaptic vesicles (19, 20).

The Ca^{2+}-binding motif is localized to the C-terminal 32 residues of AS and comprises an acidic tandem repeat rich in proline residues. This structure is sufficient and necessary to confer Ca^{2+} binding activity and requires the presence of both repeats as demonstrated both by the full binding activity of the synthetic peptide AS-(109–140) and the absence of binding to AS-(1–125). The Ca^{2+}-binding domain does not resemble any of the hitherto recognized Ca^{2+}-binding structures such as the EF-hand, the C_2-domain, or the less defined low-affinity binding sites in the SH-100 class of proteins, with the exception of the clustering of negatively charged residues (35–37). AS is natively unfolded, and circular dichroism spectroscopy does not reveal any structural changes in the absence or presence of Ca^{2+} (14). However, it is not always necessary for Ca^{2+} ions to cause gross structural changes for functional effects to arise, as shown for the C_2A domain in synaptotagmin I, where Ca^{2+} works as an electrostatic switch that facilitates binding to syntaxin I and acidic phospholipids (42, 43). The IC_{50} for the Ca^{2+} binding to AS is about 300 μM, and Ca^{2+} concentrations close to this magnitude are only encountered in normal nerve cells close to Ca^{2+} channels at the plasma membrane during propagation of action potentials and at neurotransmitter release (41). However, cofactors may increase the Ca^{2+} affinity and thus increase the potential significance of the Ca^{2+} binding in analogy with the approximate 1000-fold increase in the apparent Ca^{2+} affinity of the synaptotagmin C_2A domain upon phospholipid binding (43). Candidate cofactors are the kinases casein kinase-1, casein kinase-2, src, and fyn that have been implicated in the phosphorylation of Ser^{129} and Tyr^{125} (44–46). Tyr^{125} is conserved from fish and birds to man. Such phosphorylation will increase the negative charge of the Ca^{2+}-binding domain and thereby potentially increase the Ca^{2+} affinity.

α-Synuclein and β-synuclein are soluble proteins with vesicle-binding properties that are localized to nerve terminals. Their local concentration is very high because they constitute about 0.1% of the total protein in rat brain extracts (47), and this may make them suited to be presynaptic Ca^{2+} buffers.

The Ca^{2+} binding to the C-terminal domain in AS stimulates binding of the novel ligand MAP-1A, and this domain probably plays a negative regulatory role because its removal increases MAP-1A binding (data not shown) but abrogates the stimula-

tory Ca^{2+} effect. MAP-1A belongs to the same group of microtubule-associated proteins as the AS ligands tau and microtubule-associated protein-1B (17, 18), and several characteristics favor a physiological interaction between MAP-1A and AS. First, their developmental expression profiles are parallel with a low to absent expression in the fetal period, followed by increased expression during postnatal development (48–51). Second, both proteins are predominantly carried as part of the slow component b of axonal transport, indicating subcellular contacts to the same transporting structures (52, 53). Third, a significant part of the transported proteins is incorporated into stationary axonal structures (52, 53). The functional significance of such a putative interaction remains unsolved, but AS is known to change the functional properties of its ligands (17, 21).

AS-containing filaments accumulate in Lewy bodies during the year-long process of neurodegeneration in PD. *In vitro,* filament formation is a nucleation-dependent process, as demonstrated, where preformed oligomers/filaments can seed the growth of filaments (54). Accordingly, if oligomer formation represents a rate-limiting step, then even a small increase in their rate of formation, regulated by pathogenic factors, may enhance filament growth significantly (12). Known factors with this property are: (i) AS mutations linked to familial Parkinson's disease (5), and (ii) proteolytic activities directed against the AS C-terminal because C-terminally truncated AS preparations more readily form fibrils (4), contain a higher proportion of oligomers, as revealed by chemical cross-linkers (Fig. 4), and such peptides are recovered from pathological brain tissue and isolated Lewy bodies (3, 18). Increased Ca^{2+} concentrations represent a novel fibrillogenic factor, as demonstrated by the increased oligomeric content upon binding of Ca^{2+} to the tandem repeat domain in AS. This makes AS resemble synaptotagmin VII whose oligomerization is stimulated by Ca^{2+} (55). High levels of AS filaments have been reported in preparations of recombinant protein (56). However, this study was performed with prolonged incubation, elevated temperature, and acidic pH as compared with our 2-h incubation at pH 7.4. The low oligomeric content in Ca^{2+}-stimulated wild type AS is, by contrast, in accordance with gel filtration experiments demonstrating oligomers with low solubility (<10%) even after 66 days of incubation at pH 7.4 (5). The inhibitory role of the C-terminal part of AS on fibril formation may rely on an electrostatic repulsion from these negatively charged segments. The molecular mechanism exploited by proteolysis and Ca^{2+} binding would then be similar because both remove negative charges from the C terminus.

Conclusively, our study extends our knowledge of AS functions in relation to both normal and pathological nerve cell paradigms by linking AS functions to the important cellular messenger Ca^{2+}. This may facilitate future studies on the still poorly understood mechanisms underlying the gain in toxic function by AS in neurodegenerative disorders.

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