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Gelsolin co-occurs with Lewy bodies *in vivo* and accelerates α-synuclein aggregation *in vitro*

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ABSTRACT

Deposition of fibrillar α-synuclein as Lewy bodies is the neuropathological hallmark of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Apart from α-synuclein, these intraneuronal inclusions contain over 250 different proteins. The actin binding protein gelsolin, has previously been suggested to be part of the Lewy body, but its potential role in α-synuclein aggregation remains unknown. Here, we studied the association between gelsolin and α-synuclein in brain tissue from PD and DLB patients as well as in a cell model for α-synuclein aggregation. Moreover, the potential effect of gelsolin on α-synuclein fibrillization was also investigated. Our data demonstrate that gelsolin co-occured with α-synuclein in Lewy bodies from affected human brain as well as with Lewy body-like inclusions in α-synuclein over expressing cells. Furthermore, in the presence of calcium chloride, gelsolin was found to enhance the aggregation rate of α-synuclein *in vitro*. Moreover, no apparent structural differences could be observed between fibrils formed in the presence or absence of gelsolin. Further studies on gelsolin and other Lewy body associated proteins are warranted to learn more about their potential role in the α-synuclein aggregation process.

1. Introduction

Fibrillar α-synuclein is the main component of Lewy bodies, intraneuronal inclusions found in brains of patients with disorders such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [1,2]. α-Synuclein consists of 140 amino acids and can be divided into three distinct regions: residues 1–60, comprising the amphipatic N-terminal region, residues 61–95, containing the hydrophobic core region and residues 96–140, corresponding to the negatively charged C-terminal region.

The physiological function of α-synuclein is still not completely understood, although a growing body of evidence suggests involvement in neurotransmitter release [3], synaptic plasticity [4] and dopamine homeostasis [5]. Point mutations and multiplications of the α-synuclein gene have implicated α-synuclein aggregation in the pathogenesis of PD and DLB. For example, two point mutations (*A30P* and *A53T*) have been associated with early-onset PD, whereas a third point mutation (*E46K*) causes a rare familial form of DLB [6–8]. In addition, patients carrying multiplications of the α-synuclein gene develop familial forms of PD or DLB [9].

However, although α-synuclein is the main fibrillar component, other proteins are also present in Lewy bodies. For example, a recent study using laser capture microdissection identified about 296 different proteins in Lewy bodies in brains of DLB patients [10]. Among others, proteins implicated in cellular signaling, apoptosis, protein synthesis, degradation and cytoskeletal plasticity were discovered. One of the identified proteins, gelsolin, is an 82 kDa actin-modulating protein regulated by Ca^{2+} and phosphoinositides [11]. Gelsolin exists in two isoforms; a secretory protein found in plasma and cerebrospinal fluid, which is believed to be a part of the actin scavenging system, and a cytosolic form regulating the actin cytoskeleton [12]. Both gelsolin isoforms contain six domains, with actin binding sites present in domains 1, 2 and 4. Secreted gelsolin differs from cytoplasmic gelsolin by a 25 amino acid

Abbreviations: Aβ, amyloid β-peptide; AFM, atomic force microscopy; BDNF, brain-derived neurotrophic factor; DLB, dementia with Lewy bodies; DMEM, Dulbecco's modified eagle medium; PD, Parkinson's disease; RA, retinoic acid; ThT, thioflavin T.

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extension at its N-terminus and the presence of a disulfide bond in domain 2 [12]. Furthermore, a point mutation at residue 187 in domain 2 of plasma gelsolin causes Familial Amyloidosis of Finnish type (FAF), a systemic amyloidogenic disease [13].

However, as gelsolin is an abundant neuronal cytosolic protein, additional techniques are needed to demonstrate that gelsolin is localized within Lewy bodies. In this study, we therefore investigated the interaction of gelsolin and α-synuclein in brain tissue from PD and DLB patients, as well as in an α-synuclein over expressing neuroblastoma cell line. Furthermore, we also investigated the effect of gelsolin on α-synuclein aggregation *in vitro*.

2. Materials and methods

2.1. Brain tissues

Mesencephalon from one DLB case (man 67 years at death, post-mortem time 2 days) as well as mesencephalon and medulla oblongata from two PD cases (woman 83 years at death, post-mortem time 5 days and a man 83 years at death, post-mortem time 2 days) were obtained from University of Turku, Finland. All tissue samples were formalin fixed and paraffin embedded.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 5 μm thick formaldehyde fixed (4%) paraffin embedded sections. Antigen retrieval was performed by microwaving sections in preheated Bulls Eye human antigen retrieval buffer (Biocare medical, Concord, CA) followed by a 5 min incubation in 70% formic acid. The sections were permeabilized with 0.4% Triton X-100 for 20 min and blocked with Background sniper (Biocare medical) for 10 min. Primary antibody incubation using a C-terminal gelsolin antibody (ab74420, Abcam, Cambridge, MA) or a C-terminal α-synuclein antibody (4B12, Covance, Princeton, NJ) was carried out overnight at 4 °C in a humid chamber with gentle shaking. Then, the slides were incubated for 30 min with Mach 3™ mouse or rabbit polymer detection (Biocare medical). Vulcan fast red™ chromogen (Biocare medical) was used for visualizing the immune signals. Sections were counterstained with hematoxylin. The specificity of the immunostaining was determined by omitting the primary antibody.

2.3. Cell culture and differentiation

The human neuroblastoma cell line, SH-SY5Y, transfected with pcDNA 3.1 neo encoding α-syn[A53T] was used for the cell based experiments [14]. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and maintained at 37 °C with 5% CO_2. The day prior to differentiation, 10^4 cells were seeded onto 3-welled hydrophobic ADCELL slides (Thermo scientific, Waltham, MA). Next, the cells were differentiated by treatment with 10 μM retinoic acid (RA) (Sigma–Aldrich). Fresh DMEM with 10 μM RA was changed every second day and cells were maintained for six days. Finally, the cells were incubated in serum-free medium supplemented with 50 ng/ml of brain-derived neurotrophic factor (BDNF) (Millipore, Billerica, MA) and 100 μM $FeCl_2$ (Sigma–Aldrich) for an additional five days [15].

2.4. Immunocytochemistry

After 11 days of differentiation, the cells were fixed for 30 min in 4% (v/v) paraformaldehyde in PBS at room temperature. Next, the cells were washed three times in PBS and permeabilized with 0.2% Triton X-100. After blocking with Background sniper (Biocare medical), incubation at room temperature for 1 h with a gelsolin and an α-synuclein primary antibody was carried out. The monoclonal gelsolin antibody was raised against the N-terminus of gelsolin (EPR1941Y, Abcam), whereas the monoclonal α-synuclein antibody recognizes the C-terminus of α-synuclein (LB509, Santa Cruz Biotechnology, Santa Cruz, CA). Next, the cells were washed three times in PBS followed by incubation with the appropriate fluorescent secondary antibody (Alexa fluor® 594 goat anti-rabbit IgG or Alexa fluor® 488 goat anti-mouse IgG) for 30 min (Invitrogen). After washing with PBS, the sections were mounted with Vectashield® fluorescent mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA). The cells were studied using a Nikon DXM1200F™ epifluorescence microscope (Nikon, Tokyo, Japan) and a Carl Zeiss LSM 510 META™ instrument (Carl Zeiss, Jena, Germany).

2.5. Thioflavin T binding assay

Recombinant α-synuclein was expressed and purified as previously described [16]. Human and bovine plasma gelsolin were obtained from Sigma–Aldrich. α-Synuclein and human gelsolin were diluted in TBS and mixed in a 14:1 M ratio (α-synuclein:gelsolin), with a final concentration of 35 μM and 2.5 μM, respectively, in the presence or absence of 5 mM $CaCl_2$. For studies with bovine gelsolin the final concentration was 70 μM and 5 μM (α-synuclein:gelsolin). Thioflavin T (ThT) (Sigma–Aldrich) was added to a final concentration of 10 μM. The final sample volume for all studies was 100 μl. The samples were incubated in a non-binding round-bottom polystyrene microtiter plate (Greiner Bio-One, Frickenhausen, Germany) at 37 °C during agitation at 800 rpm on a Titramax 101 shaker (Heidolph Instruments, Schwabach, Germany). After 0, 15, 24, 36, 48, and 96 h of incubation, the ThT fluorescence was measured at an excitation wavelength of 435 nm and an emission wavelength of 485 nm in an Infinite M200 Pro microplate reader (TECAN, Männedorf, Switzerland). Each sample was run in duplicates.

2.6. SDS–PAGE and Western blot

α-Synuclein fibrils formed in the presence or absence of $CaCl_2$, and human plasma gelsolin, were taken at the 96 h incubation endpoint. The samples were centrifuged at 18,000*g* for 5 min and the resulting supernatant (100 μl) was saved. Next, the pellet was washed three times with TBS and after each wash centrifuged at 18,000*g* for 5 min. The final pellet was dissolved in 100 μl TBS. 3 μl of the supernatant or pellet were mixed with 22 μl sample buffer containing 0.02 M dithiotreitol and 3% SDS. Next, the reaction mixture was boiled at 95 °C for 5 min, followed by separation on a NuPAGE® 4–12% Bis–Tris gel (Invitrogen) and transfer to an immobilon FL polyvinylidene fluoride membrane (Millipore). Finally, α-synuclein and gelsolin were visualized in an Odyssey imaging system (Li-Cor, Lincoln, NE) using an α-synuclein polyclonal antibody O1 (Innovagen, Lund, Sweden), raised against 4-oxo-2-nonenal-induced α-synuclein oligomers [16], and a monoclonal C-terminal gelsolin antibody (GS-2C4, Abcam) followed by an anti-rabbit (DyLight® 800 conjugate, Thermo scientific) or an anti-mouse (DyLight® 680 conjugate, Thermo scientific) secondary antibody.

2.7. Atomic force microscopy

For atomic force microscopy (AFM) analysis, an XE-150 large-sample AFM system (Park systems, Santa Clara, CA) equipped with a 150 mm × 150 mm XY scanner, was used. Silicon-based AFM tips were used for measurements (ACTA, Applied NanoStructures, Santa Clara, CA). Briefly, 10 μl of α-synuclein fibrils formed in the

presence or absence of bovine plasma gelsolin, and $CaCl_2$, as described above, were dialyzed against distilled water and subsequently added to a freshly cleaved mica surface (Veeco, Cambridge, UK) before overnight incubation at room temperature. Next, the substrate was washed with distilled water and the remaining water was removed by vacuum suction. All samples were analyzed at ambient temperature in true non-contact mode.

3. Results

3.1. Gelsolin co-occurs with Lewy bodies in vivo and with α-synuclein inclusions in α-synuclein over expressing SH-SY5Y cells

To study the potential co-occurrence of gelsolin in Lewy bodies, immunohistochemistry was performed on consecutive sections from the mesencephalon of one DLB case as well as the mesencephalon and the medulla oblongata of two PD cases. In all three cases, the α-synuclein pathology was prominent and α-synuclein positive Lewy bodies were readily observed at routine diagnostics. In each of the studied cases, a subset of the Lewy bodies was also immunoreactive for gelsolin (Fig. 1A–F, as indicated by arrows). Both α-synuclein and gelsolin immunostaining of the DLB mesencephalon revealed classical Lewy bodies with a uniform labeling (Fig. 1A and B), whereas the Lewy bodies in the PD case had a more laminar appearance (Fig 1C and D). The medulla oblongata of the other PD case revealed a more globular staining for both α-synuclein and gelsolin (Fig 1E and F).

To further investigate the co-occurrence between gelsolin and α-synuclein, SH-SY5Y cells, stably over expressing α-synuclein with the A53T mutation were utilized. α-Synuclein positive Lewy body-like inclusions were visible in the majority of the cells after differentiation. The inclusions were often perinuclear and differed in size. Notably, larger inclusions were limited to one or two inclusions per cell. In a subset of cells, the α-synuclein-positive Lewy body-like inclusions also stained positively for gelsolin (Fig. 2).

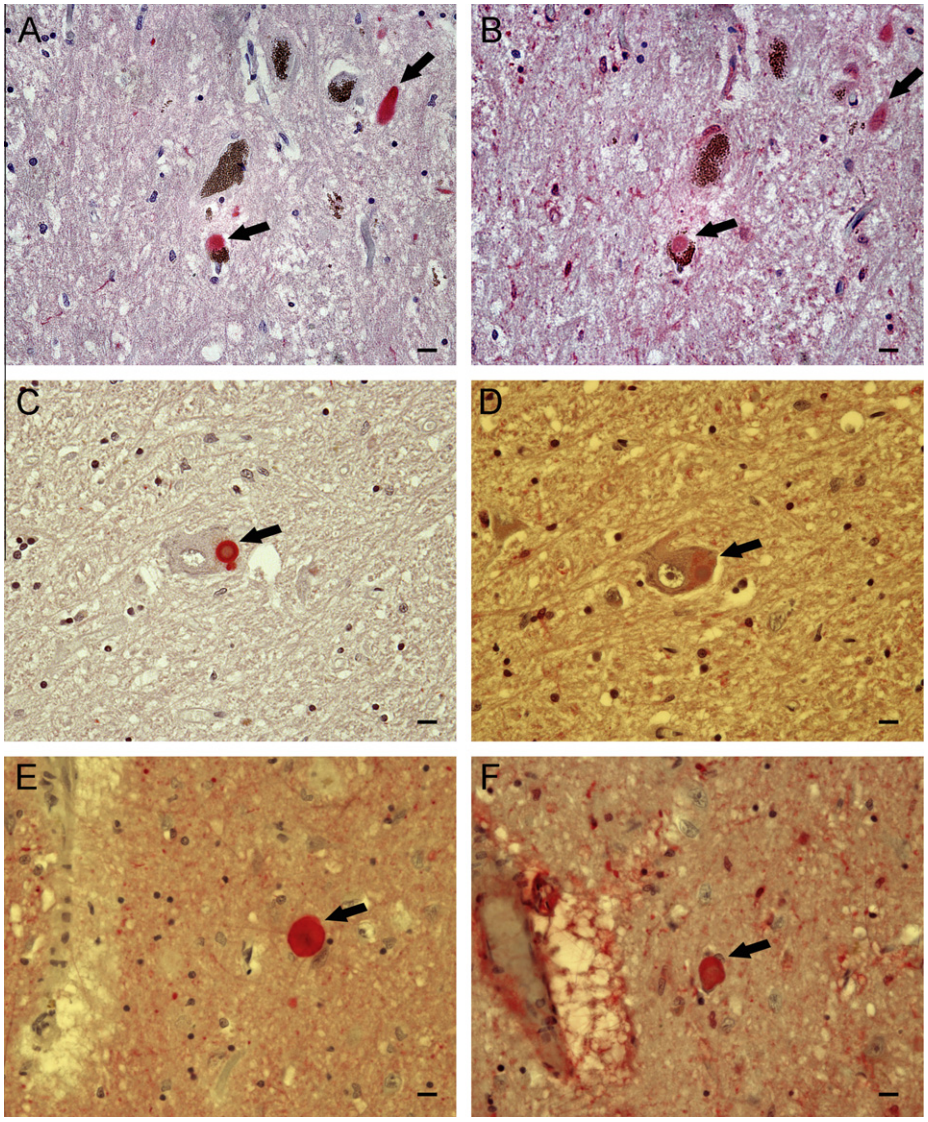

Fig. 1. Immunostaining of α-synuclein- and gelsolin-positive Lewy bodies in PD and DLB brain tissue. For each case, two consecutive sections were immunostained with an α-synuclein specific antibody and with a C-terminal specific gelsolin antibody. Black arrows indicate α-synuclein- and gelsolin-positive Lewy bodies. α-Synuclein (Fig. 1A, C, and E) and gelsolin (Fig. 1B, D, and F) positive Lewy bodies in the mesencephalon from a DLB case, and in the mesencephalon and the medulla oblongata from two PD cases, respectively. Magnification 40×, scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

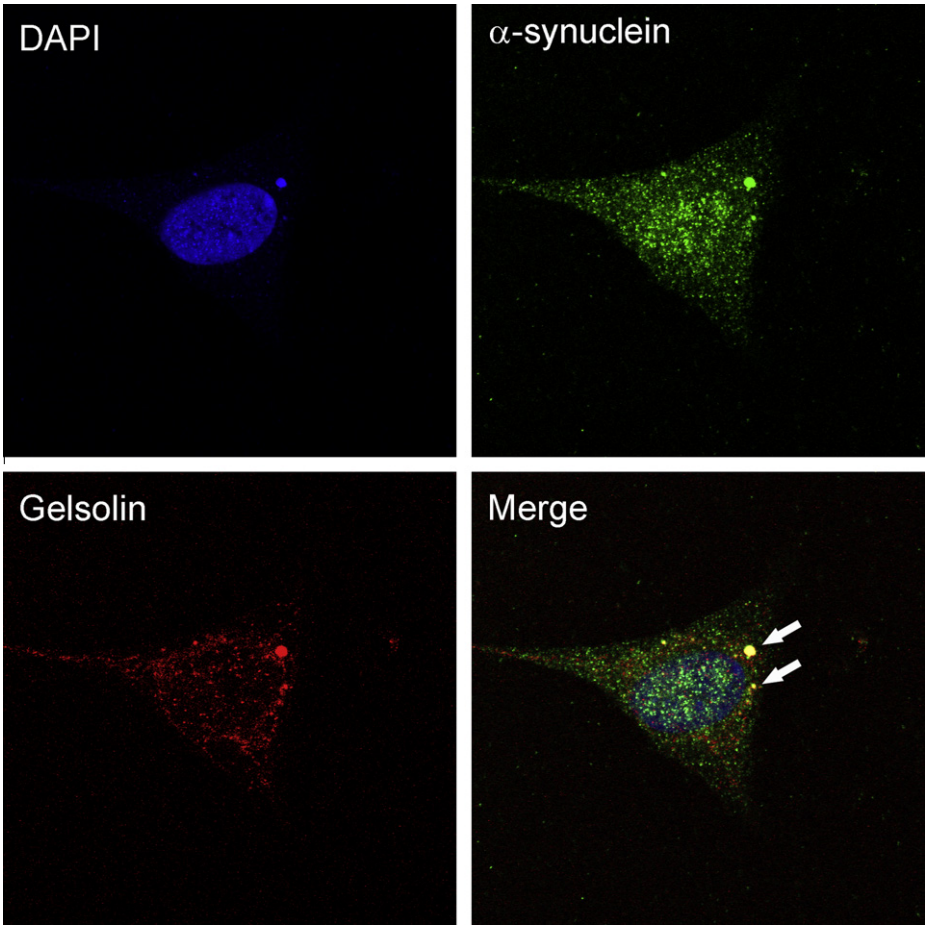

Fig. 2. α-Synuclein and gelsolin co-occur in intracytoplasmic inclusions in differentiated SH-SY5Y cells over expressing α-synuclein. Confocal microscopy image of differentiated A53T SH-SY5Y cells immunostained with an α-synuclein antibody (green) and an N-terminal specific gelsolin antibody (red). DAPI was used for counter staining the nuclei (blue). White arrows indicate inclusions positive for both α-synuclein and gelsolin. Magnification 63×. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

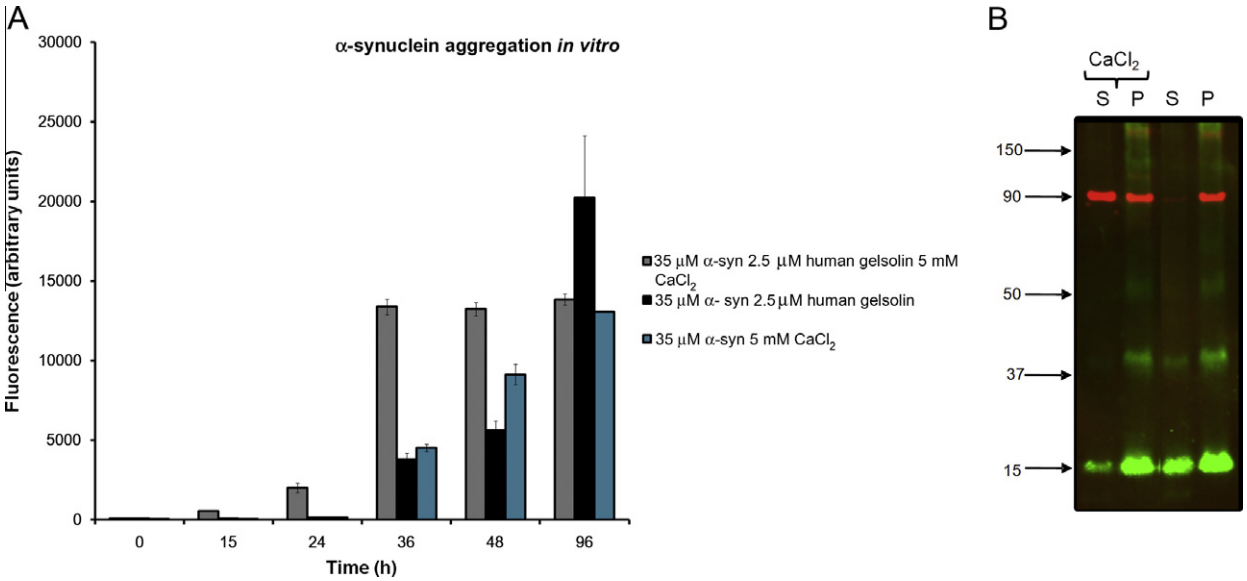

Fig. 3. ThT binding study of the effect of human gelsolin on α-synuclein aggregation. In the presence of $CaCl_2$, gelsolin promoted α-synuclein aggregation (A). The onset of aggregation occurred between 15 h and 24 h of incubation and the ThT signal reached its maximum level by 36 h of incubation. In contrast, samples containing only α-synuclein and $CaCl_2$, or α-synuclein and gelsolin, started to fibrillate between 24 h and 36 h of incubation and the ThT signal increased up to 96 h of incubation (A). Each bar represents the mean ± SD of a duplicate run from a representative experiment. Western blot of the supernatant (S) and pellet (P) of α-synuclein fibrillar samples formed in the presence (lane 1 and 2) and absence (lane 3 and 4) of 5 mM $CaCl_2$, and gelsolin, after 96 h of incubation (B). α-synuclein was predominantly found in the pellet of both samples. In the presence of 5 mM $CaCl_2$ gelsolin was present both in the supernatant and the pellet, although the band was more strongly labeled in the supernatant. In the absence of $CaCl_2$, gelsolin was mainly present in the pellet fraction.

3.2. Gelsolin enhances α-synuclein aggregation in vitro in the presence of Ca^{2+}

The aggregation propensity of α-synuclein in the absence or presence of gelsolin was evaluated by a ThT binding assay. In the presence of human gelsolin and 5 mM $CaCl_2$, α-synuclein started to fibrillate between 15 h and 24 h and the ThT signal reached its maximum by 36 h of incubation (Fig. 3A). In contrast, α-synuclein samples only containing $CaCl_2$ started to fibrillate between 24 h and 36 h of incubation and the ThT signal increased up to 96 h of incubation (Fig. 3A). Thus, the onset and the rate of fibrillation was much faster for α-synuclein samples with gelsolin present. When $CaCl_2$ was omitted, no seeding effect could be observed for human gelsolin (Fig. 3A). We also investigated the seeding effect on α-synuclein by bovine plasma gelsolin, since it has 99% homology with human plasma gelsolin (http://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). As for human gelsolin, an increased seeding effect could be seen for bovine plasma gelsolin, but only in the presence of 5 mM $CaCl_2$ (data not shown). No increase in ThT signal could be observed when incubating either form of gelsolin alone, neither in the presence nor in the absence of 5 mM $CaCl_2$ (data not shown). Furthermore, $CaCl_2$ did not affect the fibril formation capacity of α-synuclein alone, as similar aggregation kinetics were observed with α-synuclein with or without 5 mM $CaCl_2$ (data not shown).

Next, we investigated whether gelsolin was part of the formed α-synuclein fibrils. α-Synuclein samples, with human plasma gelsolin in the presence or absence of $CaCl_2$, were collected at the end point of the ThT experiment, centrifuged and analyzed by Western blot (Fig. 3B). In both cases, α-synuclein was predominantly found in the pellet. For the samples incubated with $CaCl_2$, gelsolin was found both in the pellet and supernatant, although the gelsolin band was more strongly labeled in the supernatant. In contrast, for samples incubated in the absence of $CaCl_2$, where no seeding effect could be observed, gelsolin was mainly located in the pellet.

Finally, AFM analysis was performed on α-synuclein fibrils formed in the presence of $CaCl_2$ or in the presence of $CaCl_2$ and bovine plasma gelsolin. α-Synuclein fibrils formed in the presence of $CaCl_2$ were ~60–80 nm in width and 4 nm in height (Fig. 4A). The fibrils formed in the presence of $CaCl_2$ and gelsolin had a similar size with a width of ~60–80 nm and a height of 4–6 nm (Fig. 4B).

4. Discussion

To date, it is not known if and how Lewy body associated proteins affect the fibril formation capacity of α-synuclein, and thus contribute to the pathogenesis of PD and DLB. In the present study, we investigated the co-occurrence of gelsolin in Lewy bodies *in vivo*, as well as in α-synuclein inclusions of α-synuclein over expressing neuroblastoma cells. We also studied the effect of gelsolin on α-synuclein aggregation *in vitro*.

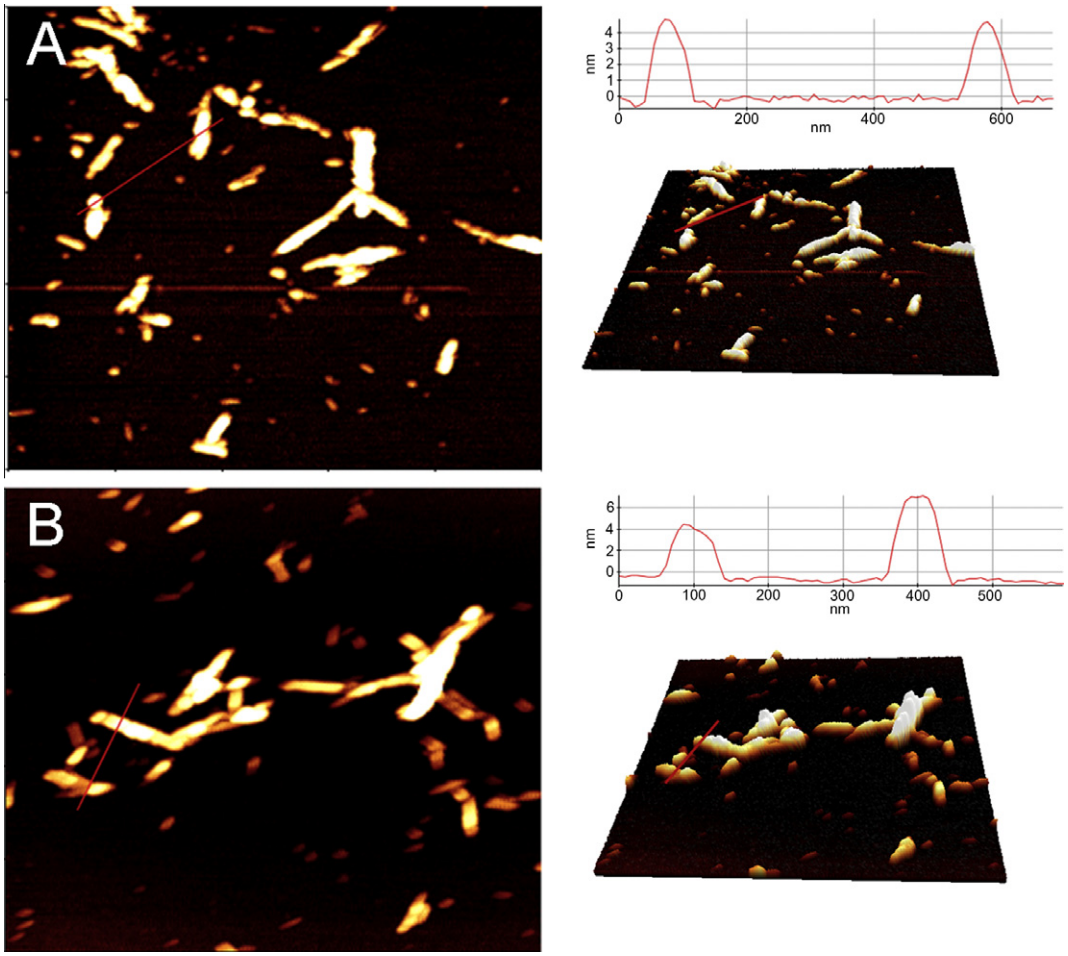

Fig. 4. Atomic force microscopy of α-synuclein fibrils formed in the presence of bovine gelsolin and $CaCl_2$ (A) or $CaCl_2$ (B). No structural difference could be detected between the two fibril types, which were ~100 nm in width and 4 nm in height for native α-synuclein and ~100 nm in width and 4–6 nm in height for gelsolin modified α-synuclein. The diagram demonstrates line-profiles of the measured areas (red line). Scan size 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As expected, α-synuclein positive Lewy bodies were detected in the investigated PD and DLB brains. Moreover, gelsolin-positive staining could be seen in a subset of the Lewy bodies. There was no obvious relationship between the shape or size of the Lewy bodies and whether they stained positive for gelsolin or not. Hence, gelsolin reactivity could be seen both in those Lewy bodies with a uniform labeling, in those with a characteristic laminarity as well as in those with a globular staining (Fig. 1). Interestingly, an earlier immunohistochemical study has also shown that gelsolin is associated with both nigral and cortical Lewy bodies in PD and DLB patients [17]. However, in that study an anti FAF antiserum, raised against amyloid fibrils consisting of N-terminal peptides of gelsolin, was utilized, and the results could not be verified using a monoclonal antibody raised against the C-terminus of gelsolin. Since our findings indicate the presence of the C-terminal epitope of gelsolin, the present study, together with the earlier study by Haltia and colleagues [17], suggests that full length gelsolin indeed is present in Lewy bodies.

Next, we studied the relation of gelsolin and α-synuclein in SH-SY5Y cells, over expressing α-synuclein with the A53T mutation. Previous studies have shown that differentiation of SH-SY5Y cells over expressing α-synuclein by RA and BDNF, in the presence of $FeCl_2$, leads to the formation of α-synuclein positive intracytoplasmic Lewy body-like inclusions [18]. Indeed, α-synuclein positive inclusions could be observed and in a subset of the cells the inclusions also stained positively for gelsolin (Fig. 2).

The interaction of α-synuclein and cytoskeletal components, such as actin and tubulin, has been reported earlier [19,20]. For example, α-synuclein has been shown to accelerate actin depolymerization by sequestrating actin monomers [21]. Moreover, tubulin has been shown to promote α-synuclein aggregation both *in vivo* and *in vitro* [20]. Although the effect of gelsolin on α-synuclein aggregation has not been investigated, a previous *in vitro* study showed that plasma gelsolin bound to the amyloid beta-peptide (Aβ) and inhibited fibril formation [22]. Furthermore, peripherally administrated plasma gelsolin reduced Aβ levels in the brain of a transgenic Alzheimer's disease mouse model [23].

To investigate the effect of gelsolin on α-synuclein aggregation *in vitro*, we used a ThT binding assay. In the presence of gelsolin and Ca^{2+}, α-synuclein started to fibrillate earlier and exhibited a much shorter growth phase as compared to α-synuclein samples containing only Ca^{2+} (Fig. 3). A similar seeding effect could also be observed with bovine gelsolin (data not shown). The finding that gelsolin to a lesser degree was part of the formed fibrils in the α-synuclein samples where a seeding effect was observed (i.e. in the presence of Ca^{2+}), compared to the gelsolin-containing α-synuclein samples where no seeding effect was observed (i.e. in the absence of Ca^{2+}), is noteworthy (Fig. 3). This indicates that gelsolin does not need to be directly incorporated into α-synuclein fibrils in order to promote aggregation. These data also fit nicely with the observation that α-synuclein fibrils formed in the presence of gelsolin and $CaCl_2$ did not differ ultrastructurally from α-synuclein fibrils formed only in the presence of $CaCl_2$ (Fig. 4).

So how does gelsolin induce α-synuclein aggregation *in vitro*? A seeding effect was only observed at high Ca^{2+} concentrations and it did not require a direct incorporation of gelsolin into the fibrillar structure. Furthermore, gelsolin did not under any conditions form amyloid fibrils on its own (data not shown). Taken together, our data indicate that gelsolin in the presence of high Ca^{2+} concentrations changes its conformation and acts as a catalyst; promoting α-synuclein aggregation. However, it should be noted that the Ca^{2+} concentrations used in this study (5 mM) are higher than levels normally found within the cell, where the resting cytosolic free Ca^{2+} levels are maintained at ~100 nM, but can reach levels up 500 μM in certain compartments (e.g. the endoplasmatic reticulum and the mitochondria) [24]. Hence, it is unclear if gelsolin could have a similar effect on α-synuclein aggregation also *in vivo*.

In summary, our *in vivo* data demonstrate that gelsolin and α-synuclein co-occur in Lewy bodies in human brain as well as in an α-synuclein over expressing cell system. Together with the *in vitro* data, we suggest that gelsolin could be involved in the aggregation process of α-synuclein and thus might affect Lewy body formation.

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