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An alternatively spliced form of rodent α-synuclein forms intracellular inclusions in vitro: role of the carboxy-terminus in α-synuclein aggregation

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Abstract

In the rat, the α-synuclein gene is alternatively spliced and exists in three forms, rat synuclein 1 (rSYN1), synuclein 2 (rSYN2) and synuclein 3. rSYN2 cDNA encodes a 149 amino acid protein that is homologous to rSYN1 and human α-synuclein for the first 100 amino acids, but is divergent for the 49 amino acid carboxy-terminal region. We demonstrate here that rSYN2 forms small aggregates throughout the cytoplasm when overexpressed in human H4 cells, whereas rSYN1 expression is diffuse. Inhibition of the proteasome promotes the formation of larger, cytoplasmic rSYN2 inclusions in transfected cells. Although a survey of the available databases suggests that there is no human splice form equivalent of rSYN2, thus arguing against a direct role in Lewy body formation and Parkinson's disease, these data nonetheless suggest that modifications of the carboxy-terminal region of α-synuclein predispose it to inclusion formation.

Keywords: Parkinson's disease; Lewy body; Proteasome; Carboxy-terminus; Aggregation

α-Synuclein has been implicated in the etiology of Parkinson's disease for two reasons: (1), two different missense mutations have been identified in the α-synuclein gene in two families with familial Parkinson's disease [7,14]; and (2), α-synuclein is one of the main components of Lewy bodies [16], the pathological hallmark of Parkinson's disease (PD). To date, the mechanism(s) involved in the aggregation of α-synuclein and its deposition into Lewy body inclusions remain to be determined.

Recently, several studies have focused on the contributions of different regions of the protein in α-synuclein aggregation. The internal non-amyloid component (NAC) peptide (amino acids 61–95) was first identified in senile plaques in the brains of Alzheimer's disease patients [18] and has the ability to nucleate the formation of Aβ fibrils in vitro [20]. Furthermore, cotransfection of NAC with synphilin-1, an α-synuclein interacting protein, results in the formation of inclusions containing both NAC and synphilin-1 in HEK293 cells [4]. Moreover, a hydrophobic stretch of 12 amino acids (71–82) within the NAC region of α-synuclein is essential for filament assembly in vitro [5]. The carboxy-terminal region of α-synuclein has also been implicated in aggregation processes, and carboxy-terminally truncated forms of α-synuclein were found to more readily form fibrils in vitro [3]. We recently demonstrated in cells in culture that the addition of 23 amino acids to the C-terminus of α-synuclein results in the aggregation of the protein into cytoplasmic inclusions [12].

Rat α-synuclein is alternatively spliced and exists in three forms [10] (Fig. 1). Rat synuclein 1 (rSYN1) is 140 amino acids in length and is the homologue of human α-synuclein. Rat synuclein 2 (rSYN2) encodes a protein that is nine amino acids longer than the human α-synuclein homologue, rSYN1, with divergent carboxy-terminal sequences (after amino acid 100; Fig. 1). Rat synuclein 3 (rSYN3) has a premature stop codon and is 42 amino acids in length. In this study, we took advantage of the naturally occurring rSYN2 splice variant to test the hypothesis that the carboxy-terminal domain of α-synuclein is important and contributes to the aggregation of the protein into cellular inclusions.

Polymerase chain reaction (PCR) was used to amplify the

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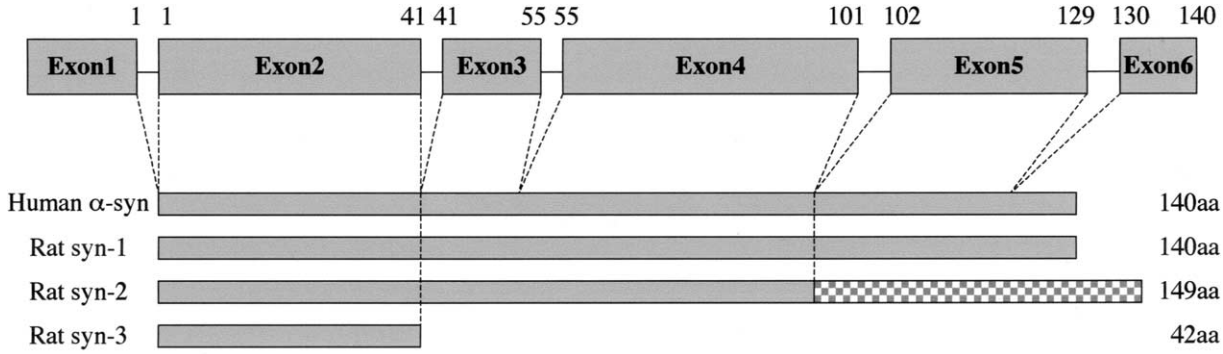

Fig. 1. Gene structure of human and rodent synuclein. The gene encoding human α-synuclein spans ~112 kb and is organized into six exons. Intron sizes range from 1270 bp (intron 1) to 93,050 bp (intron 4). Based on comparisons with mouse α-synuclein [17], rat α-synuclein most likely has a similar exon/intron organization. Three alternatively spliced forms of rat α-synuclein have been identified. rSYN1 is 140 amino acids long and the rat homologue of human α-synuclein. rSYN2 is 149 amino acids long and is divergent from rSYN1 and human α-synuclein after amino acid 100. rSYN3 is 42 amino acids long. Checkered pattern indicates the region of rSYN2 that is divergent from rSYN1.

cDNAs for rSYN1 and rSYN2 from rat brain cDNAs using oligonucleotide primer pairs rSYN12SP/rSYN1AS and rSYN12SP/rSYN2AS derived from the published cDNA sequences (GenBank accession numbers, S73007 (rSYN1) and S73008 (rSYN2)). The sequences of the primers were: 5′-AAG CCT AGA GAG CCG TGT GGA GCA-3′ (rSYN12SP); 5′-TGC AAC GAC ATT CTT AGG CTT CAG-3′ (rSYN1AS); 5′-AGT CTC TCT TTA CAG GCT CCG TAA-3′ (rSYN2AS). Resulting PCR products (476 and 529 bp; Fig. 2) were cloned into the pcDNA3.1/V5-His-TOPO expression vector, and sequenced to confirm identity and sequence integrity. The fact that rSYN2 could be amplified from rat brain cDNAs confirms the presence of the rSYN2 mRNA in adult rat brain. Interestingly, rSYN2 appears to be expressed at significantly lower levels than rSYN1, which was isolated concurrently from the same rat brain cDNAs (Fig. 2, lanes 1 and 2).

We have previously used an in vitro cell culture model to examine the expression of human α-synuclein and the two

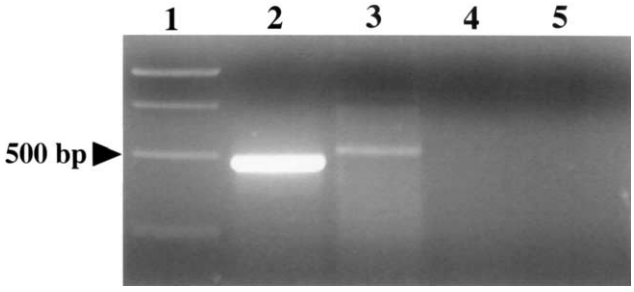

Fig. 2. rSYN2 mRNA is expressed in adult rat brain. PCR was used to isolate rSYN1 and rSYN2 from random primed cDNAs. PCR products of 476 and 529 bp were obtained for rSYN1 and rSYN2, respectively, cloned into mammalian expression vector and sequenced in their entirety. mRNA for rSYN2 (lane 3) appears to be less abundant than rSYN1 mRNA (lane 2). Molecular weight markers are in lane 1. Negative controls in which template cDNAs were omitted did not yield any PCR products (lane 4 and 5).

PD-associated mutant forms of α-synuclein [11]. Here, we performed transient transfections of rSYN1 and rSYN2 expression constructs into human H4 neuroglioma cells, maintained in OPTI-MEM supplemented with 10% fetal bovine serum, using Superfect™ transfection reagent.

Immunocytochemistry was performed 24 h after transfection to detect rSYN1 and rSYN2 expression. Cultures were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with 1.5% normal donkey serum for 1 h. rSYN1 and rSYN2 expression was detected with the antibodies Sc7012 (1:100), synuclein 1 (1:500), or AB5464 (1:1000), all of which recognize both rSYN1 and rSYN2, for 1–2 h at room temperature or overnight at 4 °C, followed by washing three times with 0.05 M Tris-buffered saline (TBS; pH 7.4) and the application of a fluorochrome-linked secondary antibody (anti-mouse, -goat, or -rabbit Cy3 or fluorescein (Fl)) for 1 h at room temperature. Cultures were washed with TBS and either coverslipped using aqueous mounting solution or stored in TBS at 4 °C. In all immunostaining experiments, secondary antibodies were applied in the absence of primary antibody to control for cross-reaction with elements in the cells in culture. Confocal scanning microscopy revealed a widespread, diffuse distribution of the rSYN1 protein throughout the cell body and nucleus, essentially identical to that of overexpressed human α-synuclein or endogenous α-synuclein [11] (Fig. 3A). By contrast, rSYN2 expression is predominantly concentrated in distinct, punctate, accumulations throughout the cell body (Fig. 3C).

Lewy bodies contain abundant ubiquitin [8] and it has been reported that α-synuclein is degraded by the proteasome [2]. Moreover, we have demonstrated that inhibition of the proteasome leads to an increase in cellular inclusions containing C-terminally modified α-synuclein [12]. To determine the effect of proteasomal inhibition on rSYN2 expression, we treated H4 cells with 5 μg/ml *n*-acetyl-leu-leu-norleucinal (ALLN) for 15 h following transfection of

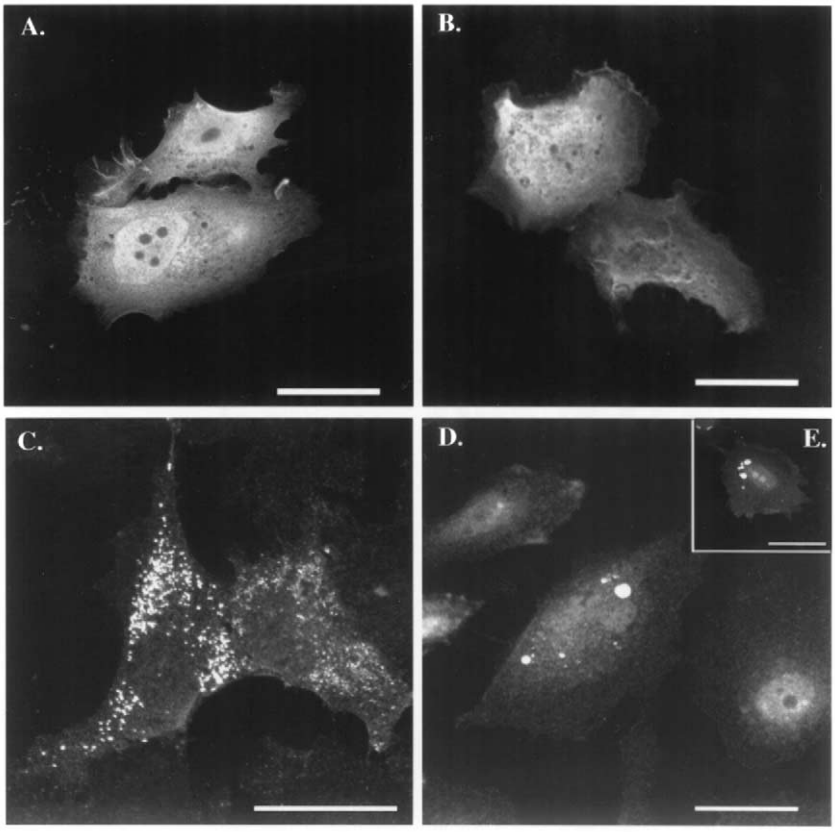

Fig. 3. rSYN2 aggregates when overexpressed in H4 cells. H4 cells were transfected with either rSYN1 or rSYN2 expression constructs and treated with 10 μg/ml ALLN for 15 h or dimethyl sulfoxide (0.1%). Transfected cells were detected immunocytochemically using antibodies that recognize both rSYN1 and rSYN2 and a Cy3-linked secondary antibody. In both DMSO (A) and ALLN (B) treated cultures, rSYN1 is expressed throughout the cell with no apparent inclusion formation. In DMSO treated cultures, rSYN2 expression is concentrated in small aggregates throughout the cytoplasm (C), whereas in ALLN treated cultures, rSYN2 forms larger cytoplasmic inclusions (D,E). Scale bars, 25 μm.

rSYN2. Immunostaining reveals that ALLN treatment leads to the formation of larger inclusions containing rSYN2 (Fig. 3D). By contrast, ALLN treatment had no effect on the expression of rSYN1 (Fig. 3B). To determine if the inclusions that form are ubiquitinated, we used anti-ubiquitin antibodies (1:500 MAB1510; 1:200 Ubiquitin) to immunostain both control dimethyl sulfoxide (DMSO) treated and ALLN treated transfected H4 cells. In both DMSO and ALLN treated cells, the inclusions that formed did not immunostain for ubiquitin (data not shown).

α-Synuclein aggregation in vitro was recently demonstrated to be a nucleation dependent event [19]. To determine if rSYN2 can act as a seed for α-synuclein aggregation, we cotransfected rSYN2 with full-length wild-type human α-synuclein. Human wild-type α-synuclein expression was distinguished from rSYN2 expression using the antibody H3C (1:5000), which recognizes amino acids 128–140 of human α-synuclein and does not recognize rSYN2. Immunostaining revealed the presence of aggregates containing rSYN2, but there was no evidence of wild-type human α-synuclein in the aggregates (data not shown).

Given the overall homology in the sequences of rodent and human α-synuclein (95.3%) [9] and the fact that rSYN2 has a propensity to aggregate when overexpressed in cells in culture, we were interested to determine if a human homologue of rSYN2 existed. To address this question, RNA was isolated using the Ambion Pure PolyA + kit, from fresh-frozen substantia nigra tissue from two subjects with a pathological diagnosis of diffuse Lewy body disease (DLB). In an effort to identify a human homologue of rSYN2, PCR was performed from random primed cDNAs (prepared from DLB tissue as described), as well as from commercially available Quick-Clone cDNAs, using the primers rSYN12SP and rSYN2AS. Additional primers, based on the rSYN2 cDNA sequence, as well as degenerate PCR primers, were also implemented for PCR. In addition, PCR was used to amplify human α-synuclein from random primed cDNAs as a positive control for the integrity of cDNA samples. Despite the variety of conditions, primer pairs, and RNA/cDNA, we were unable to identify a human homologue of rSYN2 (data not shown).

When the C-terminal sequence of rSYN2 is used in a BLAST search [1], a region of identity is identified within

intron 4 of both the mouse and rat α-synuclein genes, suggesting that the divergent C-terminal sequences are spliced from within the ~82,500 base pair intron [17]. The sequence of the human α-synuclein gene, SNCA, and the equivalent 93,050 base pair intron 4, is known in its entirety but does not contain sequences with any similarity to the C-terminal rSYN2 sequences [17]. Moreover, BLAST searches of the human genome sequence to date (first draft completed) reveal no identity to the rSYN2 C-terminal sequences. Taken together with the fact that efforts to amplify human SYN2 from normal control human brain tissue as well as DLB brain tissue failed to identify a human SYN2, we conclude that a homologue of rSYN2 may not be present in humans.

Our studies of rSYN2 have led to the following novel observations: (1), the mRNA for the alternatively spliced form of α-synuclein, rSYN2, is present in adult rat brain, although at lower levels than rSYN1; (2), overexpression of rSYN2 in H4 neuroglioma cells results in the formation of small cytoplasmic inclusions; (3), inhibition of the proteasome leads to the formation of larger inclusions containing rSYN2.

Although the absence of SYN2 from humans negates the possibility that this alternatively spliced form promotes the aggregation of α-synuclein in Parkinson's disease, we believe that it may still provide important information supporting the observation that C-terminal manipulations of the α-synuclein protein predispose it to aggregation. This work also supports our previous observation where the presence of a C-terminal tag (enhanced green fluorescent protein (EGFP), truncated EGFP or V5/6xHistidine) on α-synuclein induced the aggregation of the protein in an in vitro cell culture system and indicates that modifications of the C-terminal region of α-synuclein are fundamental to its aggregation. However, because the C-terminal one-third of rSYN2 is distinct from that of α-synuclein, yet both form inclusions, we conclude that the exact amino acid sequence of the C-terminal domain of the α-synuclein molecule is not necessary for aggregates to form. Instead, we propose a model in which the normal conformation of the C-terminal domain conceals or otherwise inhibits a potentially self-aggregating domain within the molecule, probably within the NAC region of the protein [5] (Fig. 4). We suggest that the amino acid changes produced by extending or altering the C-terminal domain lead to an altered conformation, potentially exposing the self-aggregating domain. This formulation predicts that wild-type α-synuclein would maintain a normal folding structure, and is not available to co-precipitate in cellular inclusions; our experimental results confirm this prediction.

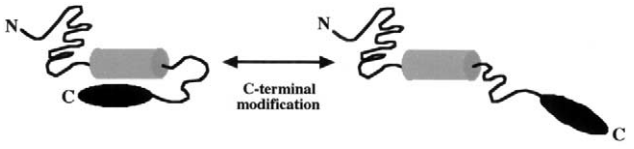

Fig. 4. Schematic model of mechanism predisposing α-synuclein aggregation. The normal conformation of α-synuclein is such that the carboxy-terminal domain of the protein obscures a self-aggregating domain (gray shading). Carboxy-terminal modification(s) alters the conformation of α-synuclein to expose the self-aggregating domain.

This model also implies that other modifications of the carboxyl-terminus of α-synuclein might predispose it to aggregation. We postulate that post-translational modifications of the C-terminus of α-synuclein, such as phosphorylation or glycosylation, could predispose the aggregation of α-synuclein in vivo. Recently, Shimura et al. [15] identified a glycosylated form of α-synuclein containing *O*-linked sugars that is a specific target for ubiquitination by *parkin*, however, it has not been determined where α-synuclein is glycosylated. *O*-linked sugars occur on serine or threonine residues, therefore serine 129 at the C-terminus of α-synuclein is a potential target for glycosylation. Similarly, α-synuclein has been found to be a substrate for the protein tyrosine kinase p72syk (Syk), which phosphorylates three tyrosyl residues in its C-terminal domain (Y-125, Y-133, and Y-136) [13] and affects the aggregation properties of the protein. In addition, α-synuclein has been determined to undergo phosphorylation at Serine 129 [6]. Furthermore, the fact that inhibition of the proteasome leads to the formation of larger intracellular inclusions containing rSYN2 suggests that the ubiquitin–proteasome system is critical and, by extension, that perturbations of this pathway may be central to the aggregation of α-synuclein in Parkinson's disease.

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[1] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res., 25 (1997) 3389–3402.
[2] Bennett, M.C., Bishop, J.F., Leng, Y., Chock, P.B., Chase, T.N. and Mouradian, M.M., Degradation of α-synuclein by the proteasome, J. Biol. Chem., 274 (1999) 33855–33858.
[3] Crowther, R.A., Jakes, R., Spillantini, M.G. and Goedert, M., Synthetic filaments assembled from C-terminally truncated α-synuclein, FEBS Lett., 436 (1998) 309–312.
[4] Engelender, S., Kaminsky, Z., Guo, X., Sharp, A.H., Amaravi, R.K., Kleiderlein, J.J., Margolis, R.L., Troncoso, J.C., Lanahan, A.A., Worley, P.F., Dawson, V.L., Dawson, T.M. and Ross, C.A., Synphilin-1 associates with α-synuclein and promotes the formation of cytosolic inclusions, Nat. Genet., 22 (1999) 110–114.
[5] Giasson, B.I., Murray, I.V.J., Trojanowski, J.Q. and Lee, V.M.-Y., A hydrophobic stretch of 12 amino acid residues in the middle of α-synuclein is essential for filament assembly, J. Biol. Chem., 276 (2001) 2380–2386.
[6] Iwatsubo, T., Fujiwara, H., Kawashima, A., Dohmae, N.,

Takio, K. and Hasegawa, M., α-Synuclein is phosphorylated in synucleinopathy lesions, Nat. Cell Biol., 4 (2002) 160–164.
[7] Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and Riess, O., Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease, Nat. Genet., 18 (1998) 106–108.
[8] Kuzuhara, S., Mori, H., Izumiyama, N., Yoshimura, M. and Ihara, Y., Lewy bodies are ubiquitinated: a light and electron microscopic immunocytochemical study, Acta Neuropathol., 75 (1988) 345–353.
[9] Lavedan, C., The synuclein family, Genome Res., 8 (1998) 871–880.
[10] Maroteaux, L. and Scheller, R.H., The rat brain synucleins; family of proteins transiently associated with neuronal membrane, Mol. Brain Res., 11 (1991) 335–343.
[11] McLean, P.J., Kawamata, H., Ribich, S. and Hyman, B.T., Membrane association and protein conformation of α-synuclein in intact neurons: effect of Parkinson disease linked mutations, J. Biol. Chem., 275 (2000) 8812–8816.
[12] McLean, P.J., Kawamata, H. and Hyman, B.T., α-Synuclein-enhanced green fluorescent protein fusion proteins form proteasomal sensitive inclusions in primary neurons, Neuroscience, 104 (2001) 901–912.
[13] Negro, A., Brunati, A.M., Donella-Deana, A., Massimino, M.L. and Pinna, L.A., Multiple phosphorylation of α-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation, FASEB J., 16 (2002) 210–212.
[14] Polymeropoulos, M.H., Lavedan, C., Leroy, E., Idle, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papaetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Iorio, G.D., Golbe, L.I. and Nussbaum, R.L., Mutation in the α-synuclein gene identified in families with Parkinson's disease, Science, 276 (1997) 2045–2047.
[15] Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J., Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease, Science, 293 (2001) 263–269.
[16] Spillantini, M.G., Schmidt, M.L., Lee, M.-Y., Trowjanowski, J.Q., Jakes, R. and Goedert, M., α-Synuclein in Lewy bodies, Nature, 388 (1997) 839–840.
[17] Touchman, J.W., Dehejia, A., Chiba-Falek, O., Cabin, D.E., Schwartz, R.R., Orrison, B.M., Polymeropoulos, M.H. and Nussbaum, R.L., Human and mouse α-synuclein genes: comparative genomic sequence analysis and identification of a novel gene regulatory element, Genome Res., 11 (2001) 78–86.
[18] Uéda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D., Kondo, J., Ihara, Y. and Saitoh, T., Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease, Proc. Natl. Acad. Sci. USA, 90 (1993) 11282–11286.
[19] Wood, S.J., Wypych, J., Steavenson, S., Louis, J.-C., Citron, M. and Biere, A.L., α-Synuclein fibrillogenesis is nucleation-dependent: implications for the pathogenesis of Parkinson's disease, J. Biol. Chem., 274 (1999) 19509–19512.
[20] Yoshimoto, M., Iwai, A., Kang, D., Otero, D., Xia, Y. and Saitoh, T., NACP, the precursor of the non-amyloid β/A4 protein (Aβ) component of Alzheimer disease amyloid, binds Aβ and stimulates Aβ aggregation, Proc. Natl. Acad. Sci. USA, 92 (1995) 9141–9145.