Self-oligomerization of NACP, the precursor protein of the non-amyloid β/A4 protein (Aβ) component of Alzheimer's disease amyloid, observed in the presence of a C-terminal Aβ fragment (residues 25–35)

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Abstract NACP, the precursor protein of the non-amyloid β/A4 protein (Aβ) component of Alzheimer's disease (AD) amyloid, also known as α-synuclein, was suggested to seed amyloid plaque formation in AD by stimulating Aβ aggregation. We have demonstrated that NACP experienced self-oligomerization only in the presence of a modified Aβ fragment (Aβ25–35) by using dicyclohexylcarbodiimide. This NACP oligomerization, appearing as a discrete ladder on a Tricine SDS-PAGE, was not observed with other Aβ peptides such as the reverse peptide Aβ35–25 and Aβ1–40, indicating this process was specific not only for the C-terminal peptide sequence of the Aβ but also for its orientation. It might be, therefore, suggested that the NACP self-oligomers formed only in the presence of a N-terminally truncated Aβ peptide could act as a nucleation center for plaque formation during AD development.

Key words: Non-Aβ component precursor; α-Synuclein; Amyloid β/A4 protein fragment 25–35; Dicyclohexylcarbodiimide; Oligomerization; Alzheimer's disease

1. Introduction

Senile plaques are a neuropathological hallmark of Alzheimer's disease (AD) [1–3]. The major constituent of this proteinaceous amyloid deposit is a 39–43 amino acid amyloid β/A4 protein (Aβ) posttranslationally derived from its much longer precursor, APP. The Aβ peptide is, however, secreted in a soluble form from various cell cultures under physiological conditions and also found in cerebrospinal fluid (CSF) of AD patients as well as normal controls [4–6]. It was, therefore, suggested that other factors associated with the plaque or/and modified Aβ peptides could provide a nucleation center to which soluble Aβ is accreted to form the deposit [7]. The senile plaque contains a peptide called non-Aβ component of AD amyloid (NAC) as the second major constituent which amounts to less than 10% to the Aβ [8]. This peptide is proteolytically derived from its 140 amino acid precursor protein (NACP), also known as α-synuclein [9–11]. It has been recently shown that the occurrence of a missense mutation on this precursor gene which resulted in the substitution of threonine for alanine at position 53 was closely related to early-onset families of Parkinson's disease [12]. A possible role of NACP in amyloid plaque formation as a nucleation center has emerged from a study of protein aggregation occurring between NACP and an Aβ peptide [13]. NACP interacted with Aβ1–38 and formed an SDS-resistant 1:1 complex through specific hydrophobic interaction between the last 15 residues of the NAC region and Aβ25–35 which had been shown to exhibit neurotoxicity [14]. At a molar ratio of 1:125 (NACP/Aβ), additional Aβ peptides formed thioflavin S-stainable protein aggregates presumably by being attached to the 1:1 NACP/Aβ nucleation center [13].

In this report, we demonstrate that NACP experiences self-oligomerization only in the presence of an N-terminally truncated Aβ fragment (residues 25–35) by using a zero-length crosslinker of dicyclohexylcarbodiimide (DCCD). We suggest that this NACP self-oligomer could participate in amyloid plaque formation as a possible nucleation center.

2. Materials and methods

2.1. Preparation of NACP

Recombinant NACP was completely purified according to a procedure previously described [15]. The recombinant plasmid containing NACP was generously provided by Dr. R. Jakes. The NACP was kept in 20 mM MES, pH 6.5 at a concentration of 1.2 mg/ml. A relatively long-term storage would convert a small portion of the monomeric NACP into an SDS-resistant dimeric species as others have already pointed out [13].

2.2. NACP oligomerization in the presence of various Aβ fragments

NACP self-oligomerization was examined with either DCCD or 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) in the presence of various Aβ fragments such as Aβ1–40, Aβ25–35, and its reverse peptide Aβ35–25. 100 pmol of NACP was preincubated with the Aβ peptides at a constant molar ratio of 1:100 at 37°C for 30 min before the crosslinkers were added to 450 μM in a final volume of 20 μl with 20 mM MES, pH 6.5. After the addition of either DCCD (a stock of 4.5 mM) prepared in ethanol or EDAC in water, the reactions were further incubated for an additional 1 h at the same temperature and analyzed with the precast 10–20% Tricine gradient gels (SDS-PAGE) from Novex according to a procedure provided by the manufacturer. A discrete ladder formation due to NACP self-oligomerization was visualized on the gel with the silver staining procedure of Morrissey [16].

2.3. NACP oligomerizations in the presence of various amounts of Aβ25–35

The oligomerizations were induced in 20 mM MES, pH 6.5 at a final volume of 20 μl in the presence of DCCD. All reactions were carried out for 1 h with the reagent at 37°C following 30 min of preincubation between NACP and Aβ25–35. The amounts of Aβ25–35 were varied to reach molar ratios between 0 to 500 with respect to 100 pmol of NACP. For the reactions with molar ratios higher than

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Abbreviations: Aβ, amyloid β/A4 protein; AD, Alzheimer's disease; APP, Aβ precursor protein; CSF, cerebrospinal fluid; DCCD, dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NAC, non-Aβ component of AD amyloid; NACP, NAC precursor

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1:50 (NACP/Aβ25–35), the samples were centrifuged in a microfuge at 13 000 rpm for 10 min. The resulting precipitates were solubilized with the Tricine, SDS-PAGE sample buffer composed of 4% SDS, 12% glycerol, 0.0075% Coomassie blue G, and 0.0025% phenol red in 0.45 M Tris-HCl, pH 8.45 and analyzed with a precast 10–20% Tricine gradient gel from Novex.

2.4. Competition of NAC during the Aβ25–35 induced NACP oligomerization

The NACP oligomerizations were observed with 450 μM DCCD in the presence of 5 nmol of Aβ25–35 and various amounts of NAC to give molar ratios of 0, 1, 3, 5, 10, 30, 50, and 100 with respect to 0.1 nmol of NACP. The reactions were done and analyzed with the Tricine gradient gel as mentioned above.

3. Results and discussion

As has been indicated by several independent studies, NACP can exist in a dimeric form [13,15,17]. In order to examine the dimeric or possible self-oligomeric forms of NACP, we introduced zero-length crosslinkers of either the water-insoluble DCCD or EDAC as a water-soluble reagent since we expected that their accessibilities to the protein would be dependent upon their chemical natures.

Surprisingly, NACP self-oligomerization was detected as a discrete ladder formation on a 10–20% Tricine, SDS-PAGE only in the presence of Aβ25–35 with DCCD (Fig. 1). When the purified NACP was analyzed with the gel and visualized with the silver staining procedure, a small fraction of the protein appeared as a dimeric species (Fig. 1A). When the protein was reacted with 450 μM DCCD for 1 h at 37°C, the NACP band was split into a broad doublet with a slightly increased level of the dimeric form (Fig. 1B, lane 1). The doublet consisted of a band with the same mobility as NACP and another fast-mobile species which might be due to intramolecular crosslinking of the protein. When this reaction was carried out in the presence of an N-terminally truncated Aβ peptide, Aβ25–35, a distinct ladder due to NACP self-oligomerization was clearly visualized at a molar ratio of 1:100 (NACP/Aβ25–35) (Fig. 1B, lane 2). Intriguingly, however, this ladder was not formed in the presence of the reverse sequence of the modified peptide, Aβ35–25, at the same molar ratio (Fig. 1B, lane 3). The ladder was not observed even in the presence of various amounts of this reverse peptide (data not shown). This fact clearly indicates that the NACP self-oligomerization was dependent not only upon the amino acid sequence of Aβ25–35 but upon its orientation as well. When Aβ1–40 was used to determine its involvement in the oligomerization, it was not successful, indicating that additional parts of the peptide certainly interfered with the optimal interactions among NACP mediated via Aβ25–35. On the other hand, however, Aβ1–40 was self-oligomerized independently of NACP and non-specifically aggregated by itself (Fig. 1B, lane 4). This was confirmed by the smeared appearance of the peptide on the gel. When the amount of Aβ1–40 was increased to the higher concentration of 1 mM, NACP oligomerizations were still not observed. It is, therefore, pertinent to consider that an aggregate formed only within the Aβ peptides experiences a totally different mechanism of protein aggregation from that produced in the presence of NACP. It could be speculated that the presence of a large amount of physiologically soluble Aβ1–40 in the senile plaque might be caused by a secondary process of accretion following the primary formation of NACP self-oligomers induced by substances like Aβ25–35 via very tight associations with NACP. When other Aβ peptide fragments such as Aβ1–28, Aβ31–35, and Aβ1–42 were examined in terms of their abilities to induce the oligomers, however, none of them were effective (data not shown). In addition, when the water-soluble carbodiimide (EDAC) was used instead of DCCD, there were no NACP oligomerizations observed in the presence of any peptides tested (Fig. 1C). This fact indicates that hydrophobic region(s) generated upon NACP and Aβ25–35 association must be located adjacent to each other, which can be accessed and crosslinked by the relatively hydrophobic carbodiimide, DCCD.

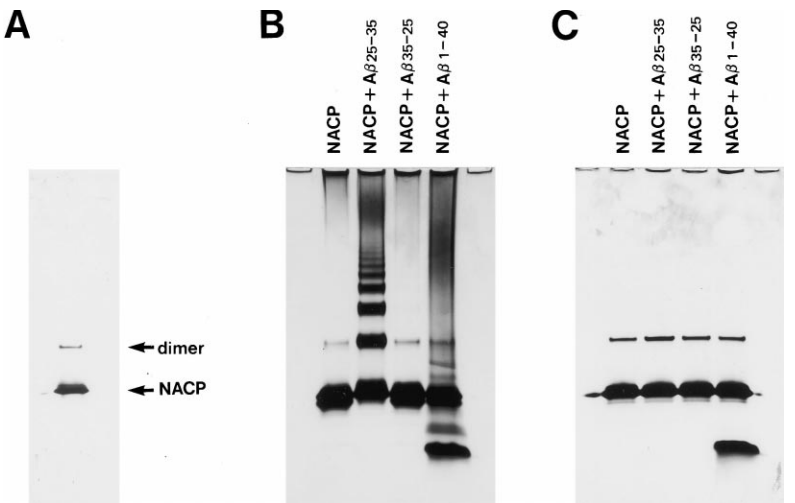

Fig. 1. NACP and its self-oligomerization in the presence of Aβ-derived peptides accessed with the zero-length crosslinkers DCCD (B) and EDAC (C). A: The completely purified recombinant NACP (100 pmol) was analyzed on the gel. B: DCCD treatment of NACP (100 pmol) in the presence of various Aβ fragments at a constant molar ratio of 1:100 (NACP/Aβ peptides). Self-oligomerization of NACP was observed with 450 μM DCCD only in the presence of 10 nmol of Aβ25–35 (lane 2). Lane 1 contains 100 pmol of NACP reacted with DCCD in the absence of Aβ peptides. Lane 3 is NACP in the presence of the reverse sequence of Aβ25–35, Aβ35–25. Lane 4 exhibits the effect of Aβ1–40 on NACP. C: EDAC treatment to examine the effects of Aβ peptides on NACP in the same reaction conditions as B. Lane 1, NACP reacted with 450 μM EDAC in the absence of Aβ peptides; lane 2, NACP with Aβ25–35; lane 3, NACP with Aβ35–25; lane 4, NACP with Aβ1–40.

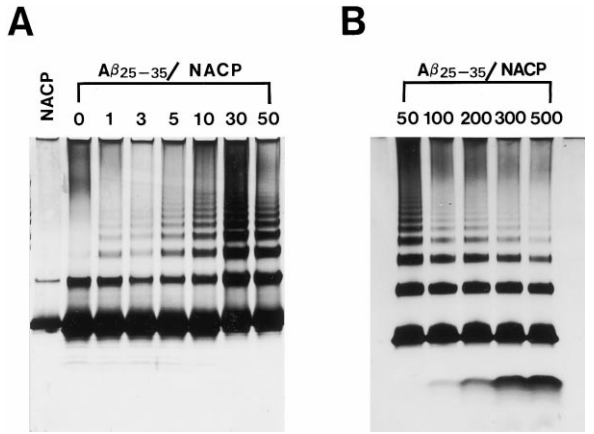

Fig. 2. NACP self-oligomerization in the presence of various amounts of Aβ25–35. All reactions were carried out in 20 mM MES, pH 6.5 at 37°C for 1 h with DCCD following 30 min of preincubation at the same temperature between NACP and the peptide prior to the addition of the crosslinker. Stock solutions of NACP and Aβ25–35 were prepared in 20 mM MES, pH 6.5 at final concentrations of 1.2 mg/ml and 1 nM, respectively. A: The oligomerization was induced with 225 μM DCCD in a total volume of 20 μl in the presence of various amounts of the Aβ25–35 in molar ratios of 0, 1, 3, 5, 10, 30, and 50–100 pmol of NACP (lanes 2–8). Lane 1 shows NACP silver-stained in the absence of DCCD. B: The same reactions were performed with 450 μM DCCD in a total volume of 60 μl with increasing amounts of the Aβ peptide of 5, 10, 30, 40, and 50 nmol (lanes 1–5) in the presence of 100 pmol of NACP, which led to the ratios (Aβ25–35/NACP) of 50, 100, 200, 300, and 500 as indicated. After the reactions were completed, the samples were centrifuged in a microfuge at 13 000 rpm for 10 min. The resulting precipitates were solubilized with the Tricine, SDS-PAGE sample buffer.

Changes in the NACP oligomerization in the presence of various amounts of Aβ25–35 were studied to demonstrate the oligomer's possible role as a nucleation center during amyloid development. Firstly, the oligomerization began to be generated even at a 1:1 molar ratio between the two molecules (Fig. 2A), indicating that the NACP self-oligomerization can be induced even in the presence of a small amount of Aβ25–35 with high affinity. This can rationalize our approach to investigate the effects of Aβ25–35 on NACP rather than observing NACP-induced phenomena such as Aβ25–35 aggregation. Secondly, the extents of oligomerization became biphasic as Aβ25–35 concentrations were increased (Fig. 2). The oligomerization was enhanced until the molar ratio between NACP and Aβ25–35 reached between 1:30 and 1:50 (Fig. 2A) and decreased thereafter (Fig. 2B). Interestingly, this disappearance of the oligomers did not augment the NACP band. In addition, these oligomers were found exclusively in the precipitates following the oligomerization reactions. Hence, these facts possibly indicate that SDS-PAGE-impermeable protein aggregates were formed at high Aβ25–35 concentrations (Fig. 2B).

Competitive effects of NAC on the Aβ25–35 interaction with NACP were investigated to confirm whether the hydrophobic NAC region in NACP is responsible for the Aβ25–35 mediated oligomerization (Fig. 3). NAC protected the oligomerization from a molar ratio of 1:30 between NACP and NAC, indicating that the interactions between Aβ25–35 and the NAC region could be critical during this process. However, the lack of complete protection even in the presence of 100-fold molar excess of NAC to its precursor cannot exclude the possibility that additional sequences of NACP besides the hydrophobic NAC region may have certain functions during the oligomerization [17]. This result also implies that protein aggregations mediated by an amyloidogenic NAC and the NACP self-oligomerization should be differentiated from each other in terms of their underlying biochemical mechanisms [18,19].

It was originally demonstrated that NAC was immunologically found not only in mature neuritic plaques but also in diffuse, primitive amyloid deposits [8]. The NAC is an intrinsic constituent of AD amyloid because its isolation required a series of extensive treatments such as sonication in SDS buffer, CNBr cleavage in 70% formic acid, and *Achromobacter lyticus* protease I digestion in 5 M urea and its immunological localization was observed electron microscopically unlike other amyloid-associated factors. Unfortunately, however, the definitive structures of the N- and C-termini of NAC in the plaque could not be identified because of the extensive chemical and proteolytic digestions. In fact, the N-terminal portion of NAC in the precursor contains 11 lysine residues which can be cleaved by the protease out of 60 amino acid residues. In addition, the C-terminal acidic region of NACP with three lysines and one methionine, a target for CNBr cleavage, might be difficult to thoroughly analyze with the C4 reversed-phase HPLC because of its hydrophilic property [8]. Therefore, the actual involvement of NACP in plaque formation has never been completely understood. Based on these facts together with our observations, we suggest that the NACP self-oligomers generated in the presence of a modified Aβ peptide like Aβ25–35 could be a reasonable candidate to seed plaque formation although the actual presence of any Aβ peptide fragments in physiological conditions, which are capable of causing NACP oligomerization, needs to be examined. Moreover, in order to unravel the significant role of NACP self-oligomers, it should be examined where the oligomers are initially generated and whether they are susceptible to various intracellular or extracellular proteases. Although the exact nature of NACP during senile plaque formation in AD remains to be determined, the molecule and the phenomenon of self-oligo-

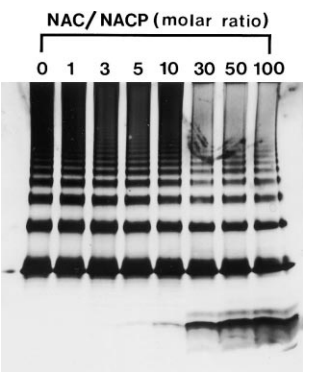

Fig. 3. NAC effect on NACP self-oligomerization in the presence of Aβ25–35. The amounts of NAC were 0, 0.1, 0.3, 0.5, 1, 3, 5, and 10 nmol (lanes 1–8) with respect to 0.1 and 5 nmol of NACP and Aβ25–35, respectively. The reactions were done with 450 μM of DCCD at 37°C for 1 h in a total volume of 20 μl following 30 min of preincubation among NACP, Aβ25–35, and NAC. NAC stock was prepared at 1 nM in 20 mM MES, pH 6.5. The Tricine gradient gel (10–20%) from Novex was used and the oligomers were visualized with silver stain.

merization are certainly valuable tools to approach the abnormal protein depositions. Finally, we expect that this visualization method of the NACP oligomers with DCCD in the presence of Aβ25–35 can provide a powerful assay system not only to approach the structure-function relationship of NACP during amyloid formation but also to evaluate various physiological or pharmacological effectors with respect to developing therapeutic or preventive strategies for both Alzheimer's and Parkinson's diseases.

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