Non-Aβ Component of Alzheimer's Disease Amyloid (NAC) Is Amyloidogenic†

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ABSTRACT: The non-Aβ component of Alzheimer's disease (AD) amyloid (NAC) was identified biochemically as the second major component in the amyloid purified from brain tissue of AD patients. NAC, derived from its 140 amino acid long precursor, NACP, is at least 35 amino acids long (NAC35) although its amino terminus is not definitely determined. An antiserum, anti-NAC-X1, was raised against the amino-terminal 9 amino acid sequence of NAC35 and purified on a peptide affinity column. This affinity-purified anti-NAC-X1 antibody immunostained amyloid in AD brain sections and recognized NAC35 but not NACP on Western or dot blot. In aqueous solutions, synthetic NAC35 self-aggregated in a time-, concentration-, and temperature-dependent manner. NAC35 was detected initially as a monomer with a molecular mass of 3500 Da but became aggregated as a function of time into a higher molecular mass component that could not migrate into the gel. The aggregate of NAC35 showed green-gold birefringence after Congo red staining when analyzed under polarized light and fiber-like structure when analyzed ultrastructually. These results suggest that NAC can form amyloid after it has been cleaved out of its precursor and may be a crucial factor in amyloidosis in the AD brain.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder with intracerebral and cerebrovascular amyloid deposits as a major pathological feature (Hardy & Allsop, 1991; Hardy & Higgins, 1992; Joachim & Selkoe, 1992; Price et al., 1992). A 39−43 amino acid polypeptide, Aβ, was found as a major component of amyloid in both neuritic plaques and blood vessels in AD (Glenner & Wong, 1984; Masters et al., 1985; Mori et al., 1992). Subsequent studies have revealed that several diseases, including familial and sporadic AD, Down's syndrome, sporadic cerebral amyloid angiopathy, parkinsonism−dementia complex of Guam, and hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-Dutch), share Aβ as a component of amyloid (Masters et al., 1985; Coria et al., 1987; van Duinen et al., 1987; Castaño & Frangione, 1988). Several other proteins may become amyloidogenic under pathological conditions (Glenner, 1980a,b). For example, cystatin C (Gamma trace) with the substitution of glutamine for leucine is the fibrillar protein in HCHWA, Icelandic type, and prion protein forms the amyloid in spongiform encephalopathy, such as Creutzfeldt−Jakob disease, Gerstmann−Sträussler−Scheinker syndrome, and Kuru (Abrahamson et al., 1989; Prusiner, 1991).

Recently, the non-Aβ component of AD amyloid, NAC, consisting of a peptide of at least 35 amino acids (NAC35), was found in the SDS-insoluble fraction of AD brain, and the cDNA encoding the precursor protein of NAC (NACP) was cloned (Uéda et al., 1993). Because *Achromobacter lyticus* protease, which cuts the peptide bond just after lysine, was used during NAC purification, we could not determine the amino-terminal amino acid of NAC. However, as no other fragments of NACP except NAC35 were found in the SDS-insoluble fraction, the 35 amino acids were considered to be a minimal fragment involved in AD amyloid, even though "full-length" NAC in amyloid might be longer by a few amino acids than this 35 amino acid fragment in its amino-terminal end. As NAC was copurified with Aβ in the presence of SDS, NAC seems to be an intrinsic component of amyloid. No other components in addition to Aβ and NAC have been identified biochemically in the AD amyloid. Further immunoelectron microscopic study with anti-NAC antibody confirmed that NAC is actually the component of amyloid fibrils in the AD brain (Uéda et al., 1993).

Recent studies have reported that Aβ is normally secreted from cells in a soluble form (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992), suggesting that extraneous factors may be required for amyloid formation in AD. The secondary structure predictions suggest that the NAC peptide sequence has a strong tendency to form a β-pleated sheet. Therefore, it is possible that NAC self-aggregates and might function as a seed for the aggregation of soluble Aβ. In this report, we investigated the amyloidogenic activity of NAC using synthetic NAC35 peptide.

MATERIALS AND METHODS

Hydropathy Analysis and Secondary Structure Prediction of NACP. Hydropathy and secondary structure of NACP were analyzed by a computer program, Prosis (Hitachi, Tokyo, Japan) (Chou & Fasman, 1978; Rose, 1978; Kyte & Doolittle, 1982). Hydropathy analysis was carried out with window size 9.

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β/A4 protein; APP, Aβ precursor protein; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; NAC, non-Aβ component of AD amyloid; NACP, NAC precursor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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Peptide Synthesis. NAC35 (EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFV) was synthesized by Fujiya (Kanagawa, Japan) using the conventional *t*-Boc strategy and phenylacetamidomethyl (PAM) resin with a peptide strategy and phenylacetamidomethyl (PAM) resin with a peptide synthesizer (Applied Biosystems, Model 430A, Foster City, CA) run with the standard computer software. The peptide was cleaved from the resin by removal of the protecting group with trifluoroacetic acid (TFA) and followed by treatment for 1 h at 0 °C with anhydrous hydrogen fluoride (HF) solution composed of peptide resin/*p*-thiocresol/*m*-cresol/dimethyl sulfide/anisole/HF in ratios of 1/0.2/0.3/1/1/10. The peptide was precipitated, washed repeatedly with diethyl ether, extracted with 2 N acetic acid, and passed over ion-exchange resin, Dowex 1-X2 (AcO^- form). Peptide was purified using high-pressure liquid chromatography (HPLC) (Waters HPLC, Model 600E, Waters, Milford, MA, with YMC Pack R&D D-ODS column) with a gradient of 0−70% acetonitrile containing 0.1% TFA. The purified peptide was tested for its amino acid composition, and its purity was checked by HPLC (Waters HPLC, Model 600E/486/741, with YMC Pack R&D D-ODS column).

Antibody against NAC and NACP. To raise the specific antiserum against NAC, the N-terminal of NAC35, named "X1" (EQVTNVGGAC), was synthesized chemically with an additional cysteine at the C-terminus for conjugation to the carrier protein, keyhole limpet hemocyanin (KLH, Calbiochem) (Green et al., 1982). Ten milligrams of X1 peptide was coupled to 10 mg of KLH in 2 mL of phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl at pH 7.4) with 2.5 mg/mL *m*-maleimidobenzoyl-*N*-hydroxy-succinnimide ester (MBS) for 30 min with stirring and dialyzed overnight against PBS using 5000−6000-Da cutoff dialysis tubing to remove uncoupled peptide. Two hundred micrograms of conjugated peptide emulsified in Freund's complete adjuvant was subcutaneously injected in New Zealand rabbits, and 100 μg of peptide emulsified in Freund's incomplete adjuvant was used to boost several times at 2−3-week intervals. After serum was obtained, a specific antibody was purified using affinity chromatography on immobilized NAC35 peptide (Pierce ImmunoPure Ag/Ab immobilization kit no. 1, Pierce, Rockford, IL). Briefly, 1 mg of NAC35 peptide was coupled to agarose with an aldehyde group generated by 0.1 M sodium cyanoborohydride. Five milliliters of serum precipitated by 50% ammonium sulfate was incubated with this affinity agarose overnight at 4 °C. After several washings with PBS, the specific antibody was eluted with 0.1 M glycine hydrochloride (pH 2.8). The protein-containing fractions were immediately neutralized with 1 M Tris-HCl (pH 9.5), and the protein peaks were pooled and dialyzed against PBS using 5000−6000-Da cutoff dialysis tubing. This antibody was named "anti-NAC-X1".

The antibody, anti-NACP(131−140) recognizing the 10 amino acid sequence of the C-terminus of NACP (EGYQDYEPEA), was prepared as described previously (Iwai et al., 1995). Anti-NACP(131−140) recognized both NACP in the human brain homogenate and bacterially expressed NACP from NACP cDNA.

Specificity of Anti-NAC-X1. The immunoreactivity of anti-NAC-X1 was tested on dot blot and Western blot. The dot blotting protocol was as follows. NAC35 was solubilized in 50 mM boric buffer at pH 9.2. Peptide solutions of NAC35 (0.3, 1, 3, 10, and 30 ng) and crude NACP (0.3, 1, 3, 10, and 30 μg) were spotted on polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), blocked with PBS containing 0.3% Tween 20, and incubated with either anti-NAC-X1 or anti-NACP(131−140) at dilutions of 1:100 and 1:300, respectively. About 10% of the total protein in the cell homogenate of *Escherichia coli* harboring human NACP expression vector was NACP, and this homogenate was used as crude NACP. The PVDF membrane was then washed, incubated with 0.5 μCi/mL iodinated protein A (ICN Pharmaceuticals, Inc., Covina, CA) in PBS containing 3% BSA and 0.1% Tween 20 for 1 h, washed, and exposed to Kodak X-Omat RP film at −80 °C. Films were developed with a Konica film developer.

Western blot analysis was done as follows. One hundred nanograms of NAC35, 1.5 μg of a crude homogenate of *E. coli* expressing NACP, and 40 μg of the cytosolic and particulate fraction of human brain homogenate in Laemmli sample buffer (Laemmli, 1970) were electrophoresed on 16% tricine−SDS−polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with PBS containing 0.3% Tween 20 and incubated with either anti-NAC-X1 or anti-NACP(131−140) at dilutions of 1:100 or 1:300, respectively. The subsequent procedure was the same as described for dot blot. The specificity of anti-NAC-X1 was tested in dot and Western blots incubated with anti-NAC-X1 antibody preabsorbed with 5 μg/mL X1 peptide.

Human brain tissue (cerebral cortex) was homogenized in 10 volumes of homogenization buffer (5 mM HEPES, pH 8.0, 0.32 M sucrose, 5 mM benzamidine, 2 mM β-mercaptoethanol, 3 mM EGTA, 0.5 mM $MgSO_4$, and 0.05% NaN_3) containing protease inhibitors (10 μg/mL leupeptin, 5 μg/mL pepstatin A, 10 μg/mL aprotinin, and 10 mM phenylmethanesulfonyl fluoride) and phosphatase inhibitors (10 μM sodium orthovanadate, 2 mM KF, and 1 μM okadaic acid), using a Teflon/glass homogenizer at 4 °C. Homogenized samples were centrifuged at 100000*g* for 1 h at 4 °C. Supernatant was used as the cytosolic fraction, and the rehomogenized pellet in the original volume of homogenizing buffer was used as the particulate fraction. Protein concentration was determined by Bradford assay (Bio-Rad protein assay). Bacterially expressed human NACP was prepared as follows. Recombinant DNA expression construct was prepared as described previously from a cDNA clone containing the entire coding region and the 3′ nontranslated region of the NACP gene (HBS6-1) (Uéda et al., 1993). The bacteria harboring the NACP expression vector, pSENACP, were cultured in LB medium to mid log phase, and isopropyl 1-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM and cultured for 4 h. Cells were harvested by centrifugation and disrupted by sonication. The lysate was centrifuged at 100000*g* at 4 °C for 30 min to recover the supernatant that contained NACP protein.

Immunostaining of Amyloid by Anti-NAC-X1. Midfrontal cerebral cortex from AD cases was fixed in 2% buffered paraformaldehyde and cut at 40 μm thickness with a vibratome 2000 (Technical Products International, St. Louis, MO). Sections were stored in cryoprotectant (30% glycerol, 30% ethylene glycol, and 0.1 M phosphate buffer) at −20 °C until immunolabeling. Sections were blocked with normal goat serum (5%) and incubated overnight at 4 °C with anti-NAC-X1 (1:10). The sections were washed in PBS, incubated with biotinylated goat anti-rabbit IgG fol-

lowed by avidin D-HRP (Vector ABC Elite, Burlingame, CA), and reacted with diaminobenzidine (DAB; 0.2 mg/mL) in 50 mM Tris-HCl buffer (pH 7.4) with 0.001% H_2O_2. Control sections were incubated with preabsorbed anti-NAC-X1 with X1 peptide (5 μg to 1 μL of purified antibody).

NAC35 Aggregation. A NAC35 solution at a concentration of 600 μM was made in 50 mM boric buffer at pH 9.2. The solution was centrifuged at 100000*g* for 1 h, and the supernatant was collected, neutralized by adding 1.5 N hydrochloride in 10 × PBS, and diluted in 1 × PBS (pH 7.4).

The aggregation of synthetic NAC35 was followed by measurement of turbidity (Jarrett & Lansbury, 1992; Jarrett et al., 1993). Turbidity was measured at 400 nm daily for 7 days under the following two conditions: (i) various concentrations of NAC35 (10, 30, 100, 300 μM at 37 °C) and (ii) 300 μM NAC35 at various temperatures: 4, 22, and 37 °C). The peptide solutions/suspensions were mixed gently before each absorbance measurement.

The size of the aggregated and SDS-insoluble NAC35 was estimated by Western blot using anti-NAC-X1. Six aliquots of peptide solution were prepared in boric buffer, lyophilized and dissolved in water, neutralized, and diluted with PBS to a final concentration of 300 μM. They were kept at 37 °C for 0, 1, 2, 3, 5, or 7 days, dissolved in Laemmli sample buffer, electrophoresed on 16% tricine−SDS−polyacrylamide gel, and transferred to PVDF membrane for Western blot analysis.

Congo Red Staining and Birefringence. Four hundred microliters of NAC35 solution/suspension (300 μM) at pH 7.4 was stored over 2 weeks at 37 °C to promote its aggregation. The solution/suspension was centrifuged at 16000*g* for 30 min. The pellet was collected, mixed in 1 mL of PBS solution containing 1 mM Congo red for 1 min, centrifuged at 16000*g* for 5 min, rinsed with 1 mL of distilled water for 1 min, and centrifuged at 16000*g* for 5 min. The peptide precipitate was placed on a glass microscope slide and allowed to dry. Birefringence was determined with a fluorescence microscope (model BHF, Olympus, Tokyo, Japan) equipped with a polarizing filter apparatus (model BH-POL).

Electron Microscopic Examination. One microliter of NAC35 aggregate was pipetted onto Formvar-coated grids (Electron Microscopy Sciences, Fort Washington, PA). The suspensions were allowed to dry at room temperature and then stained with 1% uranyl acetate solution. Grids were analyzed with a JEOL CX 100 electron microscope.

RESULTS

Hydropathy Analysis and Secondary Structure Prediction of NACP. Hydropathy analysis and secondary structure prediction for NACP is shown in Figure 1. The N-terminal portion of NACP is hydrophilic and is likely to form an α-helix. The middle portion of NACP is hydrophobic and is likely to form a β-pleated sheet structure. The C-terminal portion of NACP is again hydrophilic with a potential β-turn structure. This profile indicates that NAC35 is located in the most hydrophobic portion of NACP and is likely to form a β-sheet structure.

NAC35 Aggregation. NAC35 was soluble in 50 mM boric buffer (pH 9.2), 70% formic acid, and 6 M guanidine thiocyanate. NAC35 aggregated in distilled water and PBS. When soluble NAC35 in boric buffer was diluted in PBS, turbidity at 400 nm of NAC35 solution increased with time.

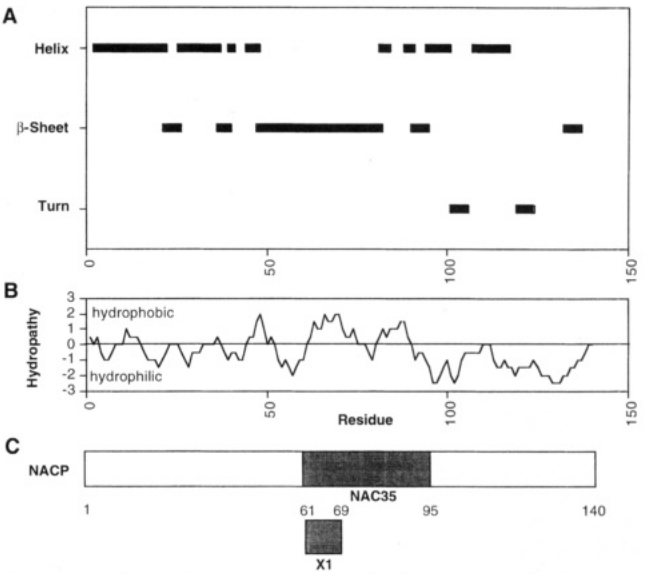

FIGURE 1: Secondary structure prediction (A) and hydropathy analysis (B) of NACP (C). NAC35 was originally purified from the SDS-insoluble fraction of AD brain. It is located in a hydrophobic portion in NACP (B) and shows a tendency to form a β-sheet structure (A). The X1 portion of NAC35 was used to raise specific antiserum that was further purified on a peptide affinity column.

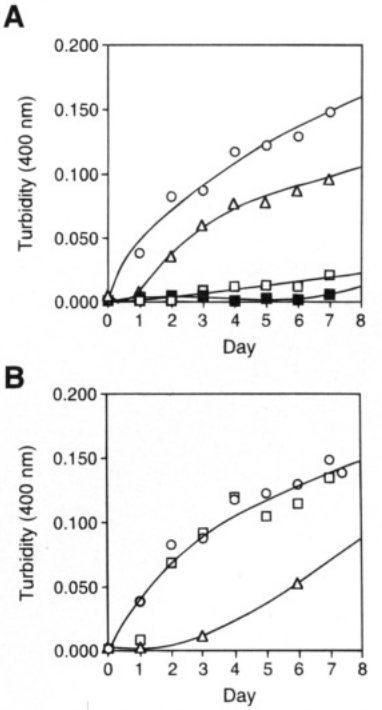

FIGURE 2: NAC35 self-aggregation measured by turbidity increase at OD 400 nm. Turbidity increase was dependent on time, concentration (A), and temperature (B). The NAC aggregation was studied in PBS (pH 7.4) solution at 37 °C with various concentrations of NAC (A: ○, 300 μM; △, 100 μM; □, 30 μM; ■, 10 μM) and at various temperatures at the NAC concentration of 300 μM (B: ○, 37 °C; □, 22 °C; △, 4 °C).

This increase was dependent on both the concentration of NAC35 (Figure 2A) and the temperature (Figure 2B). At day 1, 300 μM was required for NAC35 to aggregate whereas at day 2, 100 μM NAC35 started to aggregate. Aggregation of NAC35 at less than 30 μM was not remarkable within our experimental conditions. Aggregation of NAC35 at 22 °C was comparable to that at 37 °C although at 4 °C aggregation was markedly delayed. This partial temperature sensitivity of the aggregation is compatible with the idea that

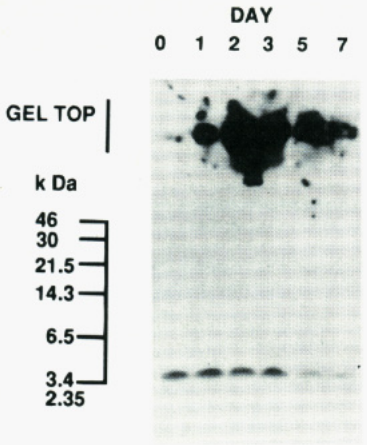

FIGURE 3: Western blot analysis of NAC35 aggregation. NAC35 monomer migrated at an apparent molecular mass of 3500 Da. The signal intensity of the 3500-Da band was significantly decreased on days 5 and 7. On the other hand, aggregated NAC35 was found at the top of the gel. This signal increased to a maximum by day 3. This aggregated NAC35 band diminished after 5 days, suggesting the formation of larger aggregates that did not enter the gel. No intermediate-size bands were observed. Note that the signal on the gel top is disproportionately strong as compared to monomeric NAC. This is due to the extreme preference of anti-NAC-X1 antibody toward aggregated NAC over soluble monomeric NAC. The same differential staining is also seen in Figure 5.

there are two steps in this reaction, one thermodynamically favorable and the other unfavorable. Recent work by Han et al. (1995) identified the thermodynamically favorable step as the addition of NAC monomers to the nucleus whereas the step of nucleation was identified as thermodynamically unfavorable. As was seen in the case of Aβ (Barrow et al., 1992), it is likely that there is a conformational change in NAC as a function of temperature.

On a Western blot, the monomer form of NAC35 migrated at the apparent molecular mass of 3500 Da (Figure 3). The signal intensity of the 3500-Da band significantly decreased only after 5 days of incubation. On the other hand, aggregated NAC35 was found at the top of the gel from the first day. This signal increased to a maximum at day 2−3. The immunoreactivity detected at the gel top is disproportionately high as compared to the decrease in the NAC monomer band. This is due to the much higher reactivity of anti-NAC-X1 antibody toward the aggregated form of NAC than that toward soluble NAC as is systematically studied later in Figure 5. The parallel experiments with dot blots demonstrated that the amount of NAC loaded on each lane of the gel was equal. No intermediate-size bands were detected by anti-NAC-X1.

Alteration of NAC35 Conformation As Detected by Congo Red Staining, Change in Antigenicity, and Fibril Formation. Aggregated proteins with β-pleated structure can be revealed by the presence of birefringence after Congo red staining. The NAC35 aggregate stained by Congo red exhibited green-gold birefringence when viewed with cross-polarization microscopy (Figure 4). The aggregated state of the peptide may also be detected as an altered antigenicity. Thus, the specificity of anti-NAC-X1 was analyzed on Western blots of NACP and NAC35. In both dot and Western blot analysis, anti-NAC-X1 recognized NAC35 (Figure 5A,B, column 2) but did not recognize NACP (Figure 5A,B, columns 3−5). Under similar conditions anti-NACP(131−140) detected readily the NACP (Figure 5A,B, columns 6−8). The anti-NAC-X1 signal was abolished when the antibody was preincubated with 5 μg/mL X1 peptide (Figure 5A,B, columns with an asterisk). Please note that 100 ng of NAC was detected on the Western blot as a weak band (Figure 5B, lane 2) after 15 h of exposure whereas 3 ng of NAC was detected on the dot blot as a strong signal (Figure 5A) after 2 h of exposure, suggesting that anti-NAC-X1 has stronger affinity to aggregated NAC. This avid binding of

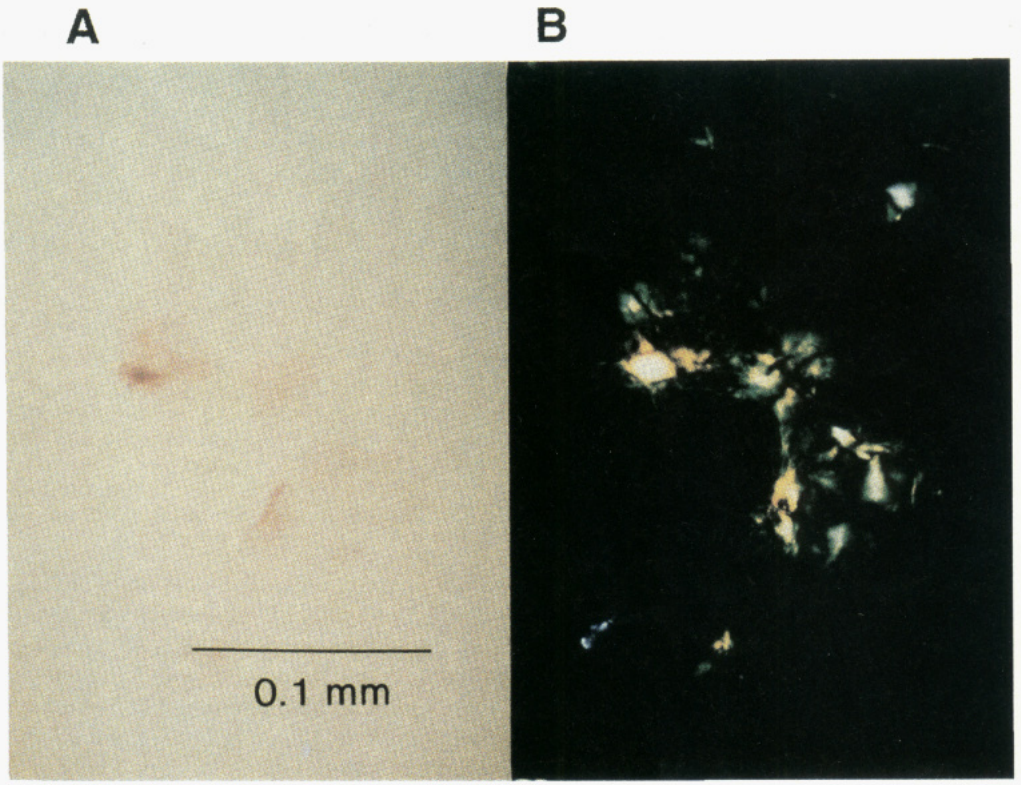

FIGURE 4: Birefringence of Congo red-stained NAC35 viewed by cross-polarization microscopy. Bright-field (A) and cross-polarized light (B) photomicrographs of the NAC35 preparation stained with Congo red are shown.

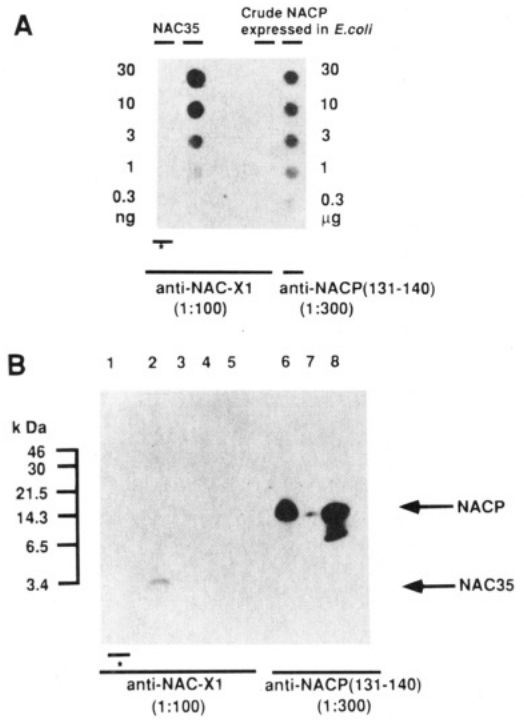

FIGURE 5: Conformational alteration of NAC detected by anti-NAC-X1 antibody. (A) Dot blot; (B) Western blot, lanes 1 and 2, NAC35 (100 ng); lanes 3 and 6, cytosolic fraction of human brain (40 μg); lanes 4 and 7, the particulate fraction of human brain (40 μg); lanes 5 and 8, homogenate of *E. coli* cells (1.5 μg) expressing NACP. Signals of anti-NAC-X1 were abolished by preincubation of antibody with X1 peptide (5 μg to 1 μL of antibody), indicated by an asterisk. The anti-NAC-X1 antibody specifically recognized NAC35 but not its precursor, NACP, in human brain and *E. coli* homogenate containing NACP. NACP was readily recognized by anti-NACP(131−140). In this figure, the dot blot (A) was exposed for 2 h whereas the Western blot was exposed for 15 h. Anti-NACP(131−140) detected NACP in both dot blot and Western blot equally well whereas anti-NAC-X1 detected aggregated NAC35 on dot blots far better than the soluble monomeric form of NAC35 on Western blot.

anti-NAC-X1 to aggregated NAC was also shown in the experiments in Figure 3. The specificity of anti-NAC-X1 toward aggregated NAC was further confirmed by the immunostaining of amyloid by this antibody. The amyloid deposited as the core of senile plaques in AD brain tissue, which was thioflavin-S positive (Figure 6B), was stained by anti-NAC-X1 (Figure 6A). This staining was abolished by antibody preincubation with the X1 peptide. In the photograph, anti-NAC-X1 staining of plaque amyloid is uneven whereas thioflavin-S staining is even, which may be due to the photographic reproduction of thioflavin-S staining. The denser staining of the core portion of amyloid by anti-NAC-X1 than of the peripheral portion was confirmed by laser confocal microscopic analysis of sections double-labeled with anti-NAC-X1 and anti-Aβ (manuscript in preparation).

Finally, the appearance of structures by aggregated peptides may be revealed by electron microscopic examination. Electron microscopic analysis of NAC35 aggregates revealed clusters of loosely assembled fibrils ranging in diameter from 9 to 11 nm (Figure 6C).

DISCUSSION

Hydropathy analysis and secondary structure prediction for NACP showed that NAC35 was hydrophobic and would form a β-sheet structure. Thus, NAC might form an amyloid. The following characteristics were used as the definition of amyloid: (1) green birefringence after Congo red staining when viewed under a polarized light; (2) a typical structure under the electron microscope (fine, rigid, nonbranching fibrils); (3) insolubility in aqueous solution (Haan & Roos, 1990). These properties could be attributed to the β-pleated sheet structure, and it was therefore required that all three criteria had to be fulfilled before a substance is called "amyloid". The experiments were designed to test the possibility that NAC35 is actually the amyloid.

Synthetic NAC35 was found to be insoluble in distilled water and PBS but soluble in formic acid and 6 M guanidine thiocyanate, both of which were found to solubilize amyloid in AD brain (Masters et al., 1985). NAC35 was also soluble in the basic boric buffer (pH 9.2). Solubilized NAC35 in boric buffer aggregated when diluted in PBS, increasing the turbidity for at least 7 days. This aggregation step was dependent on the concentration of peptide and temperature. Western blot analysis revealed that NAC35 peptide solution/suspension contained both monomers and large polymers of NAC35. The monomers migrated into the gel with an apparent molecular mass of 3500, and the large polymers remained on the gel top. The results of the Western blot and turbidity measurement suggest that the aggregation may proceed through three aggregation stages: SDS-soluble aggregation, SDS-insoluble small aggregation, and SDS-insoluble large aggregation. In the first stage (day 1), the NAC35 aggregates (as detected by turbidity increase), can be dissolved in the SDS sample buffer, and behaves on Western blot as the soluble monomer. Here, please note that, in spite of substantial aggregation detected by turbidity change (Figure 2), the amount of NAC35 monomer detected on Western blots did not change, indicating that NAC35 aggregates are largely SDS soluble. In the second stage (days 2 and 3), although the majority of aggregates are SDS soluble as detected by a Western blot, some aggregates mature and become SDS insoluble. However, the aggregates are not so large and migrate into the soft stacking gel. Therefore, the second-stage aggregate can be detected as the gel top smear. It seems that NAC35 changes conformation at this stage. Our anti-NAC-X1 antibody detects the aggregated form of NAC35 much more strongly than the soluble form (Figure 3). Please note in Figure 3 that the SDS-soluble NAC35 concentration did not change noticeably during the 3-day period although the NAC35 aggregates detected by anti-NAC-X1 increased markedly. The preferential detection of the aggregated conformation by anti-NAC-X1 was also noticed when the same amount of NAC35 was immunostained on dot blots and Western blots: anti-NAC-X1 antibody detected NAC35 extremely well on dot blots (Figure 5), probably due to its aggregation, whereas the same amount of nonaggregated NAC35 on Western blot provided only a poor signal when probed with the same anti-NAC-X1 antibody. This was not because of the loss of protein during electroblotting. The comparable amount of NAC35 was detected by protein staining of the Western blot. The inability of our peptide-purified anti-NAC-X1 to stain the full-length NACP is also likely to be due to the conformational difference. In the third stage, NAC35 aggregates mature further and cannot enter the stacking gel. Such aggregates cannot be detected in the Western blot. Interestingly, no smear was observed between the gel top and the 3500-Da band, which indicates that no intermediate-size NAC35 polymers were present. The synthetic Aβ aggrega-

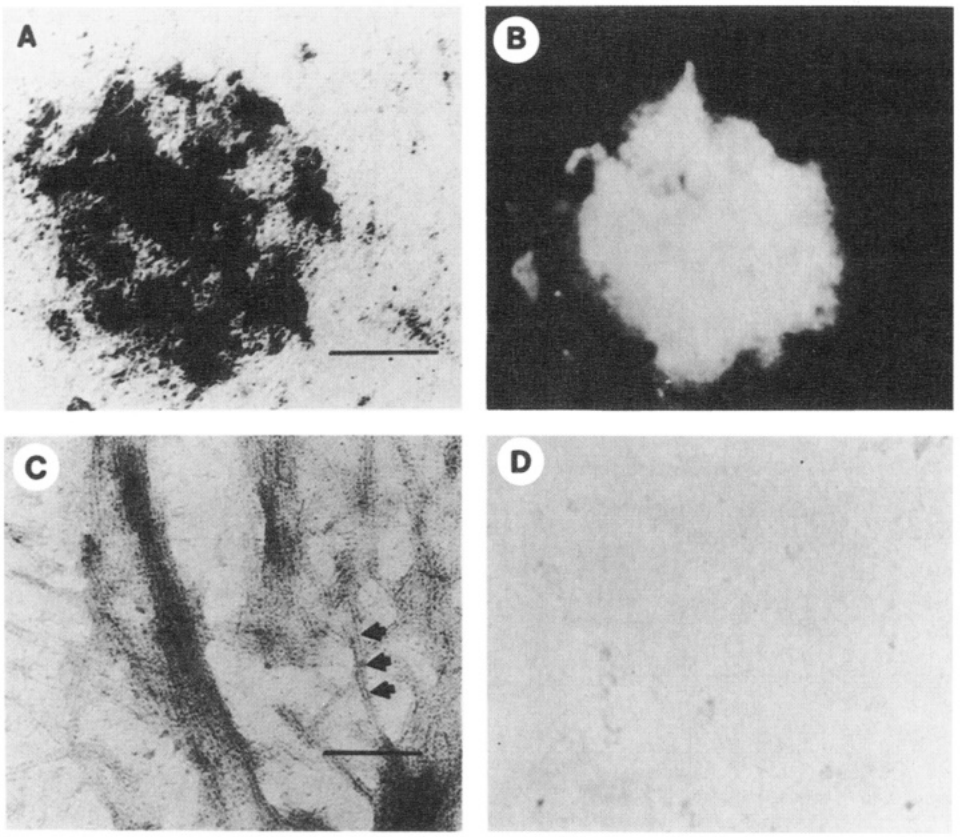

FIGURE 6: Morphologic evidence for NAC to be amyloid. (A) Immunostaining of NAC in amyloid found in the brain tissue of an AD patient. Anti-NAC-X1 antibody stained amyloid in the core of senile plaques in midfrontal cerebral cortex of AD patients. This staining was abolished by preincubation of antibody with X1 peptide (5 μg to 1 μL of antibody) (not shown). The scale bar is 15 μm. (B) The same amyloid is positive with thioflavin-S staining. (C) Electron micrograph of aggregated NAC35. Sample preparation is described under Materials and Methods. In (D), the same protocol was repeated as (C) except NAC35 was omitted. Arrows show amyloid fibrils. The scale bar is 90 μm.

tion, on the other hand, showed intermediate-size polymers on the Western blot (Burdick et al., 1992). These data suggest that polymerization of NAC35 is initiated at a nucleus where the addition of monomers occurs at multiple sites, resulting in rapid polymerization without having any stable intermediate dimers, tetramers, etc. (Jarrett & Lansbury, 1993).

The aggregate of NAC35 showed a green-gold birefringence in polarizing light when stained with Congo red (Figure 4), a yellow fluorescent structure when stained with thioflavin-S (data not shown), and a fibril structure when viewed with an electron microscope (Figure 6C). Since these features satisfy the definition of "amyloid", NAC35 can be called "amyloidogenic". In other amyloidogenic proteins, such as Aβ, prion protein, and OsmB protein, at least three repeats of the GXXX sequence (X is G, A, V, I, L, F, W, Y, T, S, or M) were found to be important for its amyloidogenic activity (Jarrett & Lansbury, 1992). Another peptide sequence, AGAAAAGA, was also suggested to be highly amyloidogenic in prion protein (Gasset et al., 1992). NAC does not have these sequences, but it has many valines and threonines (NAC35 has eight valines and five threonines). Therefore, these β-branched residues may contribute to the formation of a β-sheet structure.

The rabbit antibody against NAC, named anti-NAC-X1, recognized NAC35 well without recognizing its precursor, NACP, in both dot and Western blot analysis. Previously, we used crude anti-NAC-X1 antiserum to detect both amyloid and NACP (Uéda et al., 1993). The fraction of anti-NAC-X1 antiserum which recognizes NAC was likely to be preferentially purified by the peptide affinity purification. The peptide affinity-purified anti-NAC-X1 antibody seems to recognize both the sequence and the conformation, allowing the detection of only NAC35 but not NACP that contains the NAC35 sequence. However, anti-NAC-X1 is not simply the conformational antibody because it did not detect Aβ blotted under the comparable conditions (data not shown). This affinity-purified anti-NAC-X1 stained amyloid cores in AD senile plaques. These data suggest that NAC fragments when cleaved from NACP accumulate as amyloid and are detected by anti-NAC-X1.

Recent studies have reported that Aβ is physiologically secreted as a soluble form (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). However, the mechanism by which Aβ becomes an amyloid aggregate is largely unknown except for rare familial AD cases with APP mutations where altered Aβ length and concentration provide conditions that favor amyloidogenesis (Citron et al., 1992; Cai et al., 1993; Suzuki et al., 1994). It is probable that the Aβ conformation changes under certain conditions that may be met in AD. For example, Aβ may be posttranslationally modified in order to fold into a β-pleated structure. Compatible with those possibilities, it is reported that low pH (Barrow et al., 1992), structural rearrangements caused by the conversion of aspartyl residues to the L-isoaspartyl form or racemization (Roher et al., 1993; Tamiyama et al., 1994), or amino acid oxidation and protein cross-linking accelerates the Aβ aggregation (Dyrks et al., 1993; Fabian et al., 1994). On the other hand, it is possible that additional molecules are required for amyloid formation. Oxidized apolipoproteins

E3 and E4 form a complex with Aβ that resists dissociation by boiling in SDS (Strittmatter et al., 1993). It is reported that α_1-antichymotrypsin and transthyretin bind to Aβ (Fraser et al., 1993; Schwarzman et al., 1994). Further binding of zinc, aluminum, α_1-antichymotrypsin, and apolipoprotein E to Aβ seems to promote its process of aggregation (Bush et al., 1994; Kawahara et al., 1994; Ma et al., 1994). Our recent investigation has demonstrated that NACP binds to Aβ with the concomitant promotion of Aβ aggregation, raising the possibility that NACP may also serve as an exogenous factor for amyloid formation from Aβ (manuscript in preparation). In addition, a recent study demonstrated that NAC35 can form amyloid fibrils that seed amyloid formation from Aβ (Han et al., 1995). Immunohistochemically, the central portion of amyloid in senile plaques is more strongly stained by anti-NAC antibodies than is the peripheral portion whereas anti-Aβ antibodies stain amyloid evenly in AD brain tissues (manuscript in preparation). Biochemically, as shown in this report, NAC aggregates and forms amyloid readily. However, since the major component of amyloid found in the brain tissue of patients with AD is Aβ, it is likely that NAC cannot explain the global process of amyloid formation in AD. Rather, it is speculated that NAC is involved in only the initial process of amyloid formation or condensation of the major amyloidogenic peptide Aβ. Thus, NAC may serve as a seed to form compact amyloid from Aβ in the brain tissue of AD patients.

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