Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11282–11286, December 1993
Neurobiology

Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease

(neurodegeneration/chaperone/amyloid β/A4 protein/neuritic plaque)

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Communicated by Dominick P. Purpura, August 2, 1993 (received for review March 30, 1993)

ABSTRACT **A neuropathological hallmark of Alzheimer disease (AD) is a widespread amyloid deposition. We analyzed the entire amino acid sequences in an amyloid preparation and found, in addition to the major β/A4-protein (Aβ) fragment, two unknown peptides. We raised antibodies against synthetic peptides using subsequences of these peptides. These antibodies immunostained amyloid in neuritic and diffuse plaques as well as vascular amyloid. Electron microscopic analysis demonstrated that the immunostaining was localized on amyloid fibrils. We have isolated an apparently full-length cDNA encoding a 140-amino-acid protein within which two previously unreported amyloid sequences are encoded in tandem in the most hydrophobic domain. We tentatively named this 35-amino acid peptide NAC (non-Aβ component of AD amyloid) and its precursor NACP. NAC is the second component, after Aβ, identified chemically in the purified AD amyloid preparation. Secondary structure predictions indicate that the NAC peptide sequence has a strong tendency to form β-structures consistent with its association with amyloid. NACP is detected as a M_r 19,000 protein in the cytosolic fraction of brain homogenates and comigrates on immunoblots with NACP synthesized in *Escherichia coli* from NACP cDNA. NACP mRNA is expressed principally in brain but is also expressed in low concentrations in all tissues examined except in liver, suggesting its ubiquitous and brain-specific functions. The availability of the cDNA encoding full-length NACP should help to elucidate the mechanisms of amyloidosis in AD.**

Amyloid deposition in the neuritic plaque and blood vessels is the most consistent neuropathology in Alzheimer disease (AD) (1, 2). The major constituent of amyloid has been found to be a 39- to 43-amino acid amyloid β/A4-protein (Aβ) (3, 4) derived from its precursor, APP (5–8). The isolation of APP cDNA prompted a burst of research in AD, culminating in the identification of APP mutations in several familial types of AD (9–12). Thus, APP and Aβ have been proposed to play a key role in the pathogenesis of this disease (13, 14). Additionally, heparan sulfate proteoglycan, ferritin, immunoglobulins, and many acute-phase proteins, such as α_1-antichymotrypsin (ACT), apolipoprotein E, complements, serum amyloid P, and trace peptides were also reported to be associated with plaque core amyloid (15–29), although supportive biochemical data demonstrating their presence in amyloid preparations are not yet available, raising the possibility that those might not be the intrinsic components of amyloid. We have further pursued the biochemical examination of the intrinsic constituents of AD amyloid by purification in SDS and sequencing, and we detected a previously unrecognized component that we tentatively call NAC (non-Aβ component of AD amyloid) in this communication.

We report isolation of an apparently full-length cDNA encoding the 140-amino-acid-long precursor of NAC, NACP.‖ Hydropathy analysis of the predicted amino acids of NACP revealed the NAC sequence to be the most hydrophobic. As Aβ is apparently a soluble physiological product (30–33), there might be another factor that alters the soluble Aβ into insoluble amyloid in AD. NAC might be such a factor to promote this process. The availability of a full-length cDNA for NACP makes it possible to test this hypothesis.

MATERIALS AND METHODS

Amyloid Preparation. Amyloid was purified by the protocol as described (34) with modifications. Briefly, AD cortex was homogenized in 2% SDS/1% 2-mercaptoethanol/50 mM Tris·HCl, pH 7.6, heated to 95°C for 10 min, and then centrifuged at 100,000 × *g* for 60 min. The pellets were suspended in 50 mM Tris·HCl, pH 7.6/1% SDS (SDS buffer) and were centrifuged at 100,000 × *g* for 60 min. The pellets were resuspended in 0.5 M sucrose in SDS buffer and subjected to 1.0/2.0 M sucrose step-gradient centrifugation at 245,000 × *g* for 2 hr. The interfaces were collected and centrifuged at 150,000 × *g* for 60 min after 1:5 dilution with SDS buffer. The pellets were sonicated in SDS buffer, dialyzed against 70% formic acid, cleaved with CNBr, and digested with *Achromobacter lyticus* protease I in 5 M urea at 30°C for 5 hr. The cleaved peptides were separated by HPLC on a C_4 column with a linear gradient (0–80%) of acetonitrile/isopropanol, 3:7 (vol/vol), in 0.1% CF_3COOH. All of the HPLC peaks were analyzed for amino acid sequences, which could be attributed to Aβ, τ, ubiquitin, ferritin, and collagen, except for two peptides named "X" and "Y."

Antibody Production and Immunohistochemistry. The N-terminal sequences of X (X1: Glu-Gln-Val-Thr-Asn-Val-Gly-Gly-Ala) and Y (Thr-Val-Glu-Gly-Ala-Gly-Ser) peptides were used to raise rabbit antisera. Peptides with an additional C-terminal cystein were conjugated to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (35). The immunohistochemical procedures have been described (36). Specificity of the staining was demonstrated by an absorption experiment consisting of preincubation of the antibodies with the corresponding pep-

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Abbreviations: Aβ, amyloid β/A4 protein; APP, amyloid β/A4 precursor protein; AD, Alzheimer disease; ACT, α_1-antichymotrypsin; NAC, non-Aβ component of AD amyloid; NACP, NAC precursor; PHF, paired helical filaments.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L08850).

tide as described (36). To define the immunostained structures (neurofibrillary tangles, neuritic plaques, vascular amyloid, and neuropil threads) and to identify plaque subtypes (diffuse, primitive, and mature), some of the immunostained sections were further stained with a 1% aqueous thioflavin S solution and viewed with ultraviolet illumination and fluorescein filters. The immuno-EM procedures have been described (37).

Immunoblot (Western Blot) Analysis. Procedures for human brain preparation, electrophoresis, and immunoblotting detection have been reported (36). To absorb anti-X1 antibody, 0.2 mg of X1 peptide or control peptide (Glu-Gly-Tyr-Gln-Asp-Tyr-Glu-Pro-Glu-Ala-Cys) per ml was used.

PCR and Screening of cDNA Library. Sense (X1) and antisense (X2) oligonucleotides were designed based on the N- and C-terminal halves, respectively, of the X peptide amino acid sequence: X1, 5′-GARCARGTIACIAAYGTIG-GIGGIGCIGT-3′; and X2, 5′-TTYTGIGCIACIGCIGTIAC-ICCIGTIAC-3′; where I is inosine, R is A or G, and Y is C or T. One set of oligonucleotides that flanks the *Eco*RI cloning site of λgt11 was made as follows: Z (sense), 5′-ACGACTCCTGGAGCCCGTCAGTA-3′ and αZ (antisense), 5′-GTAATGGTAGCGACCGGCGCTCA-3′. PCR (38) was performed with these oligonucleotides as primers, with combinations of X1 (or X2) and Z (or αZ), and with a human fetal brain λgt11 cDNA library (39) as a template. Reaction was performed at 94°C for 1 min, 51°C for 1.5 min, and 72°C for 2 min. After 35 cycles of amplification, a PCR product of ≈280 bp from the combination of X2 and αZ was purified, digested with *Eco*RI, subcloned into the *Eco*RI-*Hinc*II site of pBluescript SK(+) (Stratagene), and sequenced. The amino acid sequence deduced from the DNA sequence thus obtained contained the N-terminal sequence of X peptide, Glu-Gln-Val-Thr-Asn-Val-Gly-Gly-Ala-Val, confirming the identity of this PCR product as the fragment of NACP cDNA. Therefore, this PCR product was used as a probe for the screening of a human brain λgt11 cDNA library (ATCC no. 37432) (40). From 5×10^5 recombinants, 22 positives were obtained. The isolated cDNAs were subcloned into pBluescript SK(+) and sequenced on both strands with the help of synthetic primers.

Bacterial Expression of NACP Protein. NACP protein was expressed in *Escherichia coli* by using the pSENACP expression vector. Plasmid pHBS6-1 was digested with *Afl* II, treated with Klenow polymerase to generate a blunt end, and then digested with *Nco* I to release the coding region. This 1.2-kb *Nco* I–*Afl* II NACP cDNA fragment containing the entire coding sequence and 3′ nontranslated region was ligated into a bacterial expression vector, pSE380 (Invitrogen), previously linearized by digestion with *Nco* I and *Sma* I. Resultant pSENACP expresses NACP protein under the control of a *trp/lac* fusion promoter that is inducible with isopropyl β-D-thiogalactoside.

RNA (Northern) Blot-Hybridization Analysis. Procedures for RNA preparation, electrophoresis, and hybridization have been described (41). Briefly, total RNA was isolated (42) from different human tissues. RNA (10 μg) was electrophoresed on a 1% formaldehyde/agarose gel and blotted to a nitrocellulose membrane. Hybridization was carried out in 50% formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin/0.5% SDS/100 μg of denatured salmon sperm DNA per ml/10% dextran sulfate at 42°C for 15 hr with ^{32}P-labeled 1.1-kb NACP cDNA that includes the X and Y sequences with a 3′ noncoding sequence. The membrane was washed with 0.1× SSPE/0.1% SDS at 42°C and was exposed for 3 days at −80°C. A higher stringency wash of 0.1× SSC/0.1% SDS at 65°C was also used. A Northern blot was obtained from Clontech (no. 7760-1, lot 32409).

RESULTS AND DISCUSSION

Identification of NAC in Amyloid. Amyloid from the frontal cortex of patients with typical clinical and neuropathological features of AD was purified essentially as described by Kondo *et al.* (34) in the presence of SDS, was solubilized in formic acid, and was digested sequentially with CNBr and endopeptidase. Peptides thus produced were purified by HPLC. Analysis of the amino acid sequence of all HPLC peaks showed, in addition to the major Aβ sequence (31.1 nmol), two peptides, X (2.0 nmol) and Y (2.3 nmol), with unknown sequences (X: Glu-Gln-Val-Thr-Asn-Val-Gly-Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val-Ala-Gln-Lys and Y: Thr-Val-Glu-Gly-Ala-Gly-Ser-Ile-Ala-Ala-Xaa-Xaa-Gly-Phe-Val). Since two sequences were recovered in essentially the same concentration, we hypothesized that they were derived from a single larger peptide that we tentatively called NAC, signifying a non-Aβ component of AD amyloid. However, because our amyloid fraction contained sequences of τ and ubiquitin, known components of paired helical filaments (PHF), the X and Y peptides might have derived from potentially contaminating PHF. To exclude this possibility, we set out to localize X and Y with immunological probes.

Immunohistochemistry of Anti-NAC Antibodies. As the synthesized X peptide was not soluble in any aqueous solution examined, the two peptides (X1: Glu-Gln-Val-Thr-Asn-Val-Gly-Gly-Ala-Cys and Y: Thr-Val-Glu-Gly-Ala-Gly-Ser-Cys) were used to raise rabbit antisera. Immunohistochemical analyses of AD brain sections with these two antibodies revealed immunostaining of amyloid in diffuse, primitive, and mature plaques (Fig. 1 *A* and *B*) as well as in cerebral vessel walls (not shown), all of which were identified by double staining with thioflavin S. Neither preimmune sera nor antisera preabsorbed with the corresponding peptide stained amyloid in AD brain tissue (Fig. 1*C*). The anti-Y antibody stained not only amyloid in plaques but also nuclei of small, possibly glial cells, cytoplasm of some small cells, and neuropil threads. The staining of these structures other than amyloid was not observed with the anti-X1 antibody. There are two potential explanations for the difference in staining properties between the anti-X1 and -Y peptide an-

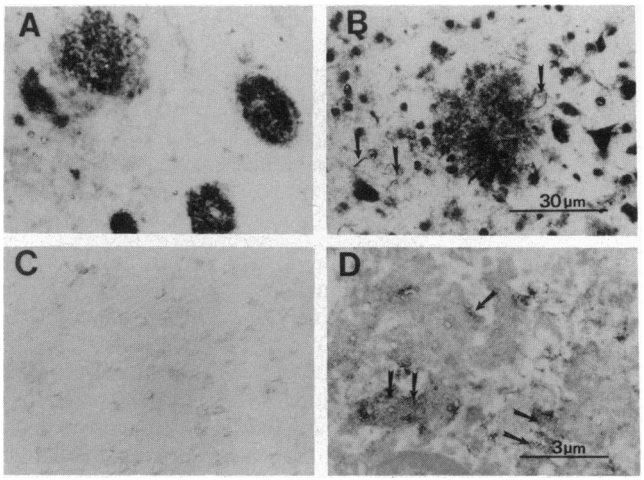

FIG. 1. Immunodetection of NAC in AD brain sections. (*A* and *B*) AD hippocampal sections were stained with anti-NAC antibodies, anti-X1 (*A*) and anti-Y (*B*). Both antibodies stained neuritic plaque amyloid that was thioflavin S-positive. Occasional staining of neuropil threads (arrows in *B*) was detected with anti-Y antibody. Absorption with the corresponding peptide eliminated the staining. The brain section in *C* was stained with preabsorbed anti-X1. The magnification is the same for micrographs *A–C*. (*D*) Electron micrograph showing the anti-X1 staining of amyloid fibrils (arrows). Amyloid fibrils were also stained with anti-Y antibody.

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   1    G CTC TCG GAG TGG CCA TTC GAC GAC AGT GTG GTG TAA AGG AAT TCA   46
                                                  *
  47  TTA GCC ATG GAT GTA TTC ATG AAA GGA CTT TCA AAG GCC AAG GAG GGA   94
   1        M   D   V   F   M   K   G   L   S   K   A   K   E   G    14

  95  GTT GTG GCT GCT GCT GAG AAA ACC AAA CAG GGT GTG GCA GAA GCA GCA  142
  15   V   V   A   A   A   E   K   T   K   Q   G   V   A   E   A   A   30

 143  GGA AAG ACA AAA GAG GGT GTT CTC TAT GTA GGC TCC AAA ACC AAG GAG  190
  31   G   K   T   K   E   G   V   L   Y   V   G   S   K   T   K   E   46

 191  GGA GTG GTG CAT GGT GTG GCA ACA GTG GCT GAG AAG ACC AAA GAG CAA  238
  47   G   V   V   H   G   V   A   T   V   A   E   K   T   K   E   Q   62

 239  GTG ACA AAT GTT GGA GGA GCA GTG GTG ACG GGT GTG ACA GCA GTA GCC  286
  63   V   T   N   V   G   G   A   V   V   T   G   V   T   A   V   A   78
                                       X

 287  CAG AAG ACA GTG GAG GGA GCA GGG AGC ATT GCA GCA GCC ACT GGC TTT  334
  79   Q   K   T   V   E   G   A   G   S   I   A   A   A   T   G   F   94
                                       Y

 335  GTC AAA AAG GAC CAG TTG GGC AAG AAT GAA GAA GGA GCC CCA CAG GAA  382
  95   V   K   K   D   Q   L   G   K   N   E   E   G   A   P   Q   E  110

 383  GGA ATT CTG GAA GAT ATG CCT GTG GAT CCT GAC AAT GAG GCT TAT GAA  430
 111   G   I   L   E   D   M   P   V   D   P   D   N   E   A   Y   E  126

 431  ATG CCT TCT GAG GAA GGG TAT CAA GAC TAC GAA CCT GAA GCC TAA GAA  478
 127   M   P   S   E   E   G   Y   Q   D   Y   E   P   E   A   **     140

 479  ATA TCT TTG CTC CCA GTT TCT TGA GAT CTG CTG ACA GAT GTT CCA TCC  526
 527  TGT ACA AGT GCT CAG TTC CAA TGT GCC CAG TCA TGA CAT TTC TCA AAG  574
 575  TTT TTA CAG TGT ATC TCG AAG TCT TCC ATC AGC AGT GAT TGA AGT ATC  622
 623  TGT ACC TGC CCC CAC TCA GCA TTT CGG TGC TTC CCT TTC ACT GAA GTG  670
 671  AAT ACA TGG TAG CAG GGT CTT TGT GTG CTG TGG ATT TTG TGG CTT CAA  718
 719  TCT ACG ATG TTA AAA CAA ATT AAA AAC ACC TAA GTG ACT ACC ACT TAT  766
 767  TTC TAA ATC CTC ACT ATT TTT TTG TTG CTG TTG TTC AGA AGT TGT TAG  814
 815  TGA TTT GCT ATC ATA TAT TAT AAG ATT TTT AGG TGT CTT TTA ATG ATA  862
 863  CTG TCT AAG AAT AAT GAC GTA TTG TGA AAT TTG TTA ATA TAT ATA ATA  910
 911  CTT AAA AAT ATG TGA GCA TGA AAC TAT GCA CCT ATA AAT ACT AAA TAT  958
 959  GAA ATT TTA CCA TTT TGC GAT GTG TTT TAT TCA CTT GTG TTT GTA TAT 1006
1007  AAA TGG TGA GAA TTA AAA TAA AAC GTT ATC TCA TTG CAA AAA TAT TTT 1054
1055  ATT TTT ATC CCA TCT CAC TTT AAT AAT AAA AAT CAT GCT TAT AAG CAA 1102
1103  CAT GAA TTA AGA ACT GAC ACA AAG GAC AAA AAT ATA AAG TTA TTA ATA 1150
1151  GCC ATT TGA AGA AGG AGG AAT TTT AGA AGA GGT AGA GAA AAT GGA ACA 1198
1199  TTA ACC CTA CAC TCG GAA TTC CCT GAA GCA ACA CTG CCA GAA GTG TGT 1246
1247  TTT GGT ATG CAC TGG TTC CTT AAG TGG CTG TGA TTA ATT ATT GAA AGT 1294
1295  GGG GTG TTG AAG ACC CCA ACT ACT ATT GTA GAG TGG TCT ATT TCT CCC 1342
1343  TTC AAT CCT GTC AAT GTT TGC TTT ATG TAT TTT GGG GAA CTG TTG TTT 1390
1391  GAT GTG TAT GTG TTT ATA ATT GTT ATA CAT TTT TAA TTG AGC CTT TTA 1438
1439  TTA ACA TAT ATT GTT ATT TTT GTC TCG AAA TAA TTT TTT AGT TAA AAT 1486
1487  CTA TTT TGT CTG ATA TTG GTG TGA ATG CTG TAC CTT TCT GAC AAT AAA 1534
1535  TAA TAT TCG ACC ATG AAA AAA AAA AA                              1560
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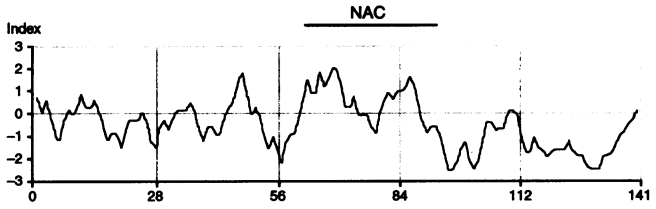

FIG. 2. (*Upper*) Nucleotide and deduced amino-acid sequence in single-letter code of clone HBS6-1 encoding NACP. The nearest in-frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. The sequence shows a 140-residue open-reading frame resulting in a calculated M_r of 14,459.46. Sequences for X and Y peptides obtained from amyloid fractions are boxed. The synthetic oligonucleotide mixtures used for PCR are indicated as lines above the corresponding cDNA. Polyadenylylation signals are underlined. (*Lower*) Hydropathy plot of NACP. The analysis was performed by the method of Kyte and Doolittle (44) with window size 9. The main hydrophobic domain of the deduced amino acid sequence (amino acids 62–90) is within the NAC sequence (amino acids 61–95).

tibodies. This different staining may represent a genuine difference in the distribution of two products of a single precursor protein or, alternatively, it may be a result of a less specific staining by the anti-Y peptide antibody. It appears likely that the latter explanation is correct because anti-Y antibody stains dozens of bands on Western blots of brain extract (data not shown), though a final conclusion must await future investigations. Although recent work has shown that 50% of intracellular neurofibrillary tangles (NFTs) and 100% of extracellular NFTs contain Aβ (43), the thioflavin-positive NFTs did not stain positively with anti-X1 or -Y antibodies.

Analysis by immuno-EM of OsO_4-intensified diaminobenzidine staining by anti-X1 antibody showed specific localization on amyloid fibrils (Fig. 1*D*). Anti-Y antibody also stained amyloid fibrils (not shown). These results indicate that both X and Y peptides are tightly associated with the amyloid fibrils. At this point, the available data are compatible with

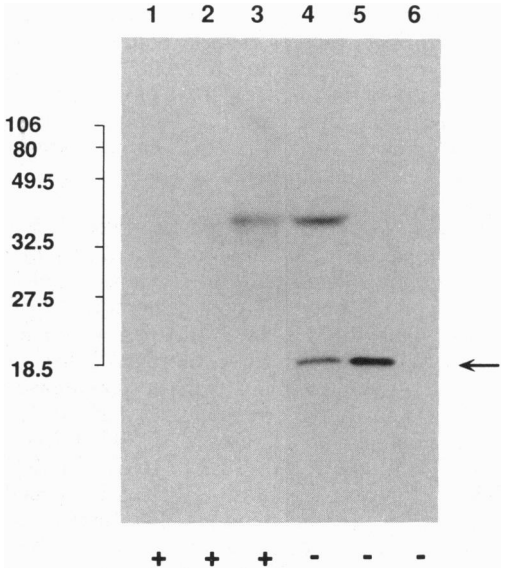

FIG. 3. Western blot analysis of NACP. Human cortex cytosolic fractions and bacterially expressed NACP in soluble fractions were detected with anti-X1. Lanes: 1 and 6, *E. coli* transfected with pSE380 vector control; 2 and 5, *E. coli* transfected with pSENACP expressing NACP; 3 and 4, normal human brain. Antibody was preabsorbed either with X1 peptide (lanes +) or with control peptide (lanes −). The arrow indicates NACP detected as a M_r 19,000 protein whose staining was blocked by preabsorption of the antibody with X1 peptide, showing its specificity. NACP was not detected in particulate fractions from human cortex or from NACP-expressing *E. coli*. A shorter exposure time was employed for lanes 1–3 compared with lanes 4–6 because of its high background. The M_r 19,000 band was not observed in lanes 2 and 3 even after a 4-times longer exposure. Size is shown to the left in $M_r \times 10^{-3}$.

the hypothesis that NAC is actually an unrecognized amyloid component and not a component of contaminants such as PHF. As shown later, cDNA cloning revealed that NAC contains both X and Y sequences in tandem in a single polypeptide.

Molecular Cloning of NACP cDNA. Next, we sought a precursor for the NAC peptide. To identify the putative precursor NACP, we set out to isolate the cDNA. We amplified a piece of cDNA encoding the NAC peptide by PCR (38) and used this PCR product as a probe for the screening of cDNA libraries. Clone HBS6-1 contained the entire coding sequence of NACP. Sequence analysis of the clone revealed a 420-bp open-reading frame encoding 140-amino-acid residues with a calculated M_r of 14,459 (Fig. 2 *Upper*). The sequence surrounding the predicted initiator methionine codon (GCCATGG) agrees with the Kozak consensus sequence (45). The nearest in-frame stop codon was found 18 bp upstream of this ATG. The deduced amino-acid sequence shows that X- and Y-peptide sequences are localized immediately next to each other in the middle of the precursor. Neither an apparent signal peptide sequence nor canonical N-linked glycosylation sites were found. Secondary structure predictions (46–48) indicate that the NAC peptide sequence has a strong tendency to form a β-sheet configuration like Aβ, consistent with its association with amyloid. The hydropathy profile (Fig. 2 *Lower*) shows this region to be hydrophobic, possibly a membrane-associated domain of the protein.

Western Blot Analysis of NACP Protein. Western blot analyses of brain homogenate with anti-X1 antibody detected NACP as a M_r 19,000 protein mostly in the cytosolic fractions (Fig. 3, lane 4). Anti-Y antibody stained dozens of bands in addition to the M_r 19,000 band (data not shown)—the reason why we mainly used anti-X1 to study NAC. The M_r 19,000

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A
   10
N---KAKEGV--
   21
  --KTKQGV--
   32
  --KTKEGV--
   43
  --KTKEGV--
   58
  --KTKEQV--
   80
  --KTVEGA--
  102
  --KNEEGA---C

B
   48
N---VVHGVATVA--
   70
  --VVTGVTAVA---C

C
NACP  1       10        20        30        40        50
      MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKT••KEGVVHGVATV
      :::: :: : :::::: : :::::::: ::: :::::: ::: :      ::
EST   MDVFKKGFSIAKEGVVGAVEKTKQGVTEAAEKTKEGVMYVGLHFFFKERIN*.....
      1       10        20        30        40        50
```

FIG. 4. (*A* and *B*) Repetitive motifs of NACP. The Lys-Thr-Lys-Glu-Gly-Val (KTKEGV) motif is repeated seven times (*A*). Two 9-amino acid homologous sequences are also found (*B*). Bold letters indicate the common amino acids among the repeat. (*C*) Amino acid sequence comparison of NACP with a human-expressed sequence tag (EST01420). Computer homology search (FASTA program) revealed that the EST01420 sequence has some homology to the N-terminal region of NACP. EST01420 is a 223-bp human-expressed sequence obtained by random sequencing of human brain cDNAs (49).

protein staining was abolished when the antibodies were preabsorbed with the corresponding peptide (Fig. 3, lane 3). The anti-X1-positive M_r 40,000 band was not blocked by preabsorption and was not detected with anti-Y antibody, indicating that this is a nonspecific band. Furthermore, bacterially expressed NACP protein comigrated with this M_r 19,000 protein (Fig. 3, lane 5), confirming the identity of the M_r 19,000 protein as NACP.

Structural Features of NACP Protein. A unique feature of the NACP sequence is found in the presence of repeated motifs (Fig. 4 *A* and *B*). There are seven Lys-Thr-Lys-Glu-Gly-Val sequence motifs, although amino acid positions 2–6 are sometimes substituted. In addition, the homologous sequence to amino acids 48–56 is found at positions 70–78. The threonine residue in the Lys-Thr-Lys-Glu-Gly-Val motif seems to constitute a favorable protein kinase C target (50). A computer search of the DNA sequence data base (European Molecular Biology Laboratory/GenBank Libraries) revealed that EST01420, recently identified by random sequencing of human brain cDNAs (49), is homologous to NACP. Comparison of the DNA sequence showed 74% identity in 124 nucleotides resulting in 80% identity in a 41-amino acid residue from the initiation methionine (Fig. 4*C*). These two cDNAs are substantially homologous in the N-terminal region, but this reported sequence has the termination codon at base pair 206 and, therefore, could encode only 51 amino acid residues. The presence of homologous cDNA suggests that NACP may be a member of a gene family.

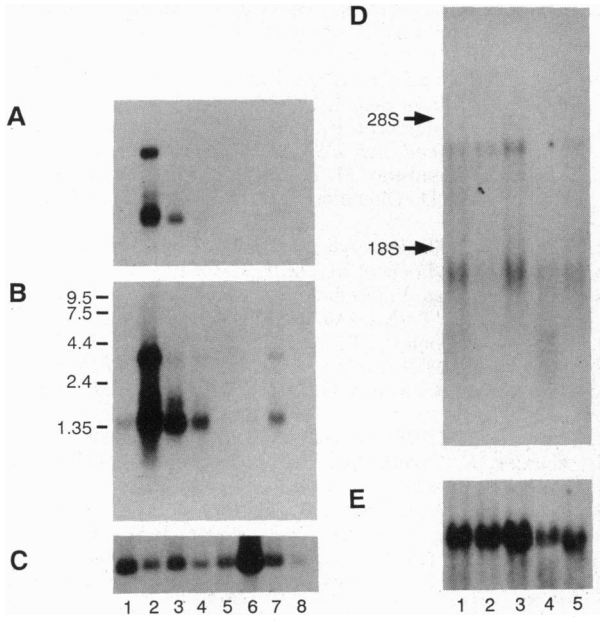

FIG. 5. Northern blot analysis of NACP mRNA. (*A–C*) Normal human tissues. Lanes: 1, heart (male, 43 yr old); 2, brain (male, 57); 3, placenta (female, 28); 4, lung (male, 75); 5, liver (male, 40); 6, skeletal muscle (male, 45); 7, kidney (female, 55); 8, pancreas (female, 24). *A* and *B* were exposed at −80°C for 1.5 hr and 2 days, respectively; *C* was exposed at −80°C for 2 days. (*D* and *E*) Normal, AD, and fetal brain. Lanes: 1, normal adult midfrontal cortex (female, 88 yr old); 2, cerebellum from the same individual as in lane 1; 3, fetal whole brain (female, 24-week fetus); 4, midfrontal cortex from AD individual (female, 83); 5, cerebellum from the same individual as in lane 4. *A*, *B*, and *D* were probed with NACP cDNA, and *C* and *E* were probed with glyceraldehyde-3-phosphate dehydrogenates (GAPD) cDNA.

Northern Blot Analysis of NACP mRNA. Northern blot analysis with a NACP cDNA probe showed two principal transcripts of 3.6 and 1.5 kb most enriched in brain, with lower concentrations in all tissues examined except in liver (Fig. 5 *A* and *B*), suggesting its important role in brain. Comparable mRNA patterns were observed in both normal and AD neocortex and cerebellum (Fig. 5*D*). HBS6-1 is 1560 nucleotides long and probably corresponds to the shorter transcript. A minor 1-kb band in Fig. 5*D* might represent a transcript with a shorter 3′ noncoding region, resulting from the use of polyadenylylation signals at 1023 bp or 1079 bp. The presence of the 3.6-kb NACP transcript may be explained by the alternative splicing that is under developmental and tissue-specific regulation. The ratio of the different-size transcripts varies depending on the age and origins of the tissue used (Fig. 5).

CONCLUSION

The current study demonstrated the tight association of a previously unrecognized peptide, NAC, with the amyloid of AD. NAC is at least 35 amino acids long, although we cannot determine the definite length in the current study because of the use of enzymatic digestion. NAC is an intrinsic component of amyloid based on copurification with amyloid in the presence of SDS and immunological localization on amyloid fibrils with EM, which contrasts with many other components associated with plaque core amyloid detected only by immunological probes at light microscopy levels (16–29). Although ACT was reported to be localized on amyloid fibrils at the EM level (15), no amino acid sequences corresponding to ACT were detected in our amyloid preparation, suggesting that the amount of ACT in amyloid may be too small to detect in our preparation or, alternatively, that the association of ACT with amyloid may be less tight than that of NAC and, therefore, ACT may be lost during our amyloid preparation. Based on the yield of X, Y, and Aβ sequences, the concentration of NAC in amyloid seems to be <10% of that of Aβ. It is unlikely that the entire M_r 19,000 protein (NACP) is associated with amyloid because, in spite of our sequencing all peaks eluted from HPLC, no other sequences of NACP besides X and Y were detected in the amyloid preparation.

Recent studies showed that Aβ is generated and secreted from various types of cells under physiological conditions (30–33). Thus, an important question for the elucidation of mechanisms of amyloidosis in AD is what causes the conformational alteration of soluble Aβ into insoluble amyloid. A recent study by Roses and his associates showed that apolipoprotein E binds Aβ (29). Thus, apolipoprotein E may

act as a molecular chaperone that mediates the β-pleated amyloid formation of Aβ as suggested by Wisniewski and Frangione (28). Because of its strong hydrophobic character and of its tendency to form β-structures, NAC might be a factor to promote the process of amyloid formation by serving as a seed or a core.

We thank Dr. R. L. Neve for a gift of the cDNA library, I. Jansen for assistance with making antibodies, Drs. S. Shimasaki (The Whittier Institute), R. D. Terry, R. Katzman, G. Cole, and K. Horsburgh for discussions and comments, the AD research center in San Diego for brain tissue, and I. Hafner and R. W. Davignon for editorial help. This work was supported by grants from National Institutes of Health (AG05131), the American Health Assistance Foundation, and the Sigma Kappa Foundation.

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