ELSEVIER

Journal of Chromatography B, 716 (1998) 107–118

JOURNAL OF CHROMATOGRAPHY B

Purification by column chromatographies of beta-amyloid precursor proteins and their association with other 95 kDa protein in rat brain

Takeyuki Ohshita[a,*], Akiko Nakatani[a], Yuzo Hiroi[b]

[a]*School of Food and Nutrition, Shokei Junior College, Kuhonjii 2, Kumamoto 862-0976, Japan*
[b]*Department of Nutritional Biochemistry, Nakamura Gakuen University, Fukuoka 814-0198, Japan*

Received 27 March 1998; received in revised form 23 June 1998; accepted 23 June 1998

Abstract

Beta-amyloid precursor proteins (APPs) in the subcellular fractions of the homogenate of rat brain were detected immunologically. They were found to be localized in both the cytosol and microsome fractions in generally equal amounts. APPs were purified from the cytosol fraction of rat brain by column chromatography in a DEAE-anion-exchanger, Blue-Sepharose, Ni-charged chelating Sepharose, and Sephacryl S-300 columns. They migrated at about 400 kDa or above in a final gel filtration column with trypsin inhibitor activity. They gave two broad protein bands of 80 and 100 kDa and several other protein bands in sodium dodecyl sulfate–polyacryl amide gel electrophoresis (SDS–PAGE). The 80 and 100 kDa bands were highly concentrated during purification. They gave the same amino terminal sequence and were identified as rat APPs without an amino terminal signal sequence. These results suggest that rat brain APPs form a complex with themselves or with other proteins and contain APP isoforms including a serine protease inhibitor domain, APP770 or APP751, or both. An antibody produced by a rabbit immunized with the final preparation of APPs reacted with a 95 kDa protein band which migrated between the 80 and 100 kDa bands of APPs in SDS–PAGE, but it did not react with the bands of APPs. The 80 and 100 kDa APP bands were coprecipitated with a 95 kDa antigen protein band by reacting this antibody with the partially purified APPs. We conclude that APPs in the rat brain are associated directly or indirectly with another protein to yield the 95 kDa band demonstrated by SDS–PAGE.

Keywords: Proteins; β-Amyloid precursor protein

1. Introduction

The deposition of senile plaques in the brain is a characteristic feature in Alzheimer's disease and aging. The approximately 4 kDa amyloid beta-peptide is the main component of the central core of senile plaques [1]. It is a small internal domain of several larger isoforms of the beta-amyloid precursor proteins (APPs) [2–5]. APPs are produced by the alternative splicing of the primary transcript of a single gene [5] and are normally present in various tissues at various levels [6]. Their predominant isoforms are APP695, APP751, and APP770 [6]. Two major questions concerning APP biology remain to be elucidated. One is the cellular mechanisms by which the beta-amyloid peptide is produced from its precursor and is processed to form senile plaques. Another is the physiological roles of APPs. The present study was directed toward elucidating the latter question. The predicted primary structure

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00320-X

of rat APP695 shares 97% homology with its human homologue [7]. APPs appear to have common physiological roles between rats and humans. APPs were found to be secreted from various cells in culture after their amino terminal signal sequence and an approximately 11-kDa C-terminal fragment were proteolytically cleaved [8,9]. The secretory forms of APP751 and APP770 contain a serine protease inhibitor domain and were identified as protease nexin II [10,11], which is a protease inhibitor secreted from cells in culture. These secretory forms also make SDS-resistant complexes with epidarmal growth factor-binding protein, the gamma-subunit of nerve growth factor, and trypsin [12]. A metalloproteinase inhibitor domain is also present in the secretory forms of APP and is located in the C-terminal glycosylated region of the secretary forms of APP770 or APP751 or both [13]. Although the properties of the protease inhibitors of the secretory forms of APPs indicate that they may have a role in the regulation of certain proteases in the extra cellular environment [12,13], the physiological roles of APPs localized in the tissues remain to be determined. In this study, we attempted to purify APPs from the rat brain and to characterize their native form in the tissue.

2. Materials and methods

2.1. Reagents

An anti-Alzheimer precursor protein A4 monoclonal antibody was obtained from Boehringer Mannheim (Tokyo). A Western blotting detection system using enhanced chemiluminescence (ECL) and horseradish peroxidase-linked second antibodies (F(ab′)2) against rabbit IgG were purchased from Amersham Japan (Tokyo). DEAE-Cellulose (DE52) was obtained from Whatman Japan (Tokyo). Blue Sepharose CL-6B, Chelating Sepharose FF, Sephacryl S-300 HR, Protein A-Sepharose CL-4B, the molecular mass marker proteins for gelfiltration, and the silver stain reagent kit for staining proteins were from Pharmacia Biotec (Tokyo, Japan). Trypsin (from porcine pancreas) was obtained from Sigma-Aldrich Japan (Tokyo). Boc–Gln–Gly–Arg–NH–Mec (a substrate for trypsin) and leupeptin (a protease inhibitor) were from the Peptide Institute (Osaka, Japan).

2.2. Tissue fractionation

The cerebra (5.2 g) collected from 4 male rats with body weights of 250 g were rinsed with ice-cold 0.25 *M* sucrose, minced by passage through a metal mesh, and then homogenized in 5 volumes of 0.25 *M* sucrose with a Potter-Elvehjem-type homogenizer. The homogenate was centrifuged at $3200\times g$ (r_{Max}) for 10 min at 4°C. The post-mitochondrial supernatant (PMS) (20 ml) was collected and then centrifuged at $99\,600\times g$ (r_{Max}) for 60 min to separate the microsomes from the cytosol fraction. The supernatant (18 ml) of the cytosol fraction was collected. The sediment of microsomes was suspended in 15 ml of 0.25 *M* sucrose containing 0.5 *M* NaCl, 50 m*M* Tris–HCl, pH 7.4 and the protease inhibitor leupeptin (2 μg/ml) and was stored on ice for 30 min and then centrifuged at $99\,600\times g$ for 60 min to wash the microsomes. The supernatant (15 ml) containing proteins released from the microsomes by washing was collected. The sediment of the washed microsomes was suspended in 0.25 *M* sucrose containing 50 m*M* Tris–HCl, pH 7.4 and leupeptin (2 μg/ml) to obtain 15 ml of the microsomal suspension. These fractions (from PMS to the washed microsomal suspension) containing 1.25 mg of tissue equivalents were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analyses to compare the amount of APPs distributed in these fractions.

2.3. Purification of APPs from the rat brain

The cerebra (150 g) collected from 100 male rats with body weights of 250 g were rinsed with ice-cold 0.25 *M* sucrose, minced by passage through a metal mesh, and then homogenized in 5 vol of 0.25 *M* sucrose containing 50 m*M* Tris–HCl, pH 7.5, the metallo-protease inhibitor EDTA (1 m*M*) and the serine and cysteine protease inhibitor leupeptin (1 μg/ml) with a Potter-Elvehjem-type homogenizer. The homogenate was centrifuged at $3200\times g$ (r_{Max}) for 10 min at 4°C. The PMS (800 ml) was collected and then centrifuged at $69\,600\times g$ (r_{Max}) for 60 min

at 4°C. Almost all of the microsomes were sedimented, and a fairly clear supernatant (600 ml) was collected. The supernatant was dissolved with 70% saturated ammonium sulfate and stored at −30°C until use. Proteins of the supernatant were precipitated by centrifuging at 18 000×*g* for 20 min, suspended in 100 ml of 50 m*M* Tris–HCl, pH 7.5, containing 0.15 *M* NaCl, 1 m*M* EDTA and leupeptin (1 μg/ml) and then dialized against the same buffer solution. The dialized sample was centrifuged at 69 600×*g* for 30 min to remove the insoluble materials.

2.4. DE52 anion-exchange column chromatography

The supernatant of the dialized sample was collected and mixed with 100 ml of DE52 gel, which had been equilibrated with 50 m*M* Tris–HCl, pH 7.5, containing 0.2 *M* NaCl, 1 m*M* EDTA and leupeptin (1 μg/ml). The DE52 gel was washed with the same buffer solution to remove the unadsorbed proteins, and was then packed in a column and further washed with the same buffer until the absorbance of the eluate at 280 nm decreased to below 0.02. Proteins containing APPs were then eluted with the buffer supplemented with 0.3 *M* NaCl. The eluate (160 ml) was collected, dissolved with ammonium sulfate to give a final concentration of 70% saturation, and stored at −30°C until used.

2.5. Blue-Sepharose CL-6B column chromatography

Partially purified APPs from the DE52 column were collected from 300 rats by repeating the above experiments and pooled. The proteins were sedimented by centrifugation, suspended in 25 ml of 50 m*M* Tris–HCl, pH 7.0, containing 0.2 *M* NaCl and leupeptin (1 μg/ml), and dialized against the same buffer solution. The dialized sample (30 ml) was applied to a Blue-Sepharose CL-6B column (bed volume, 40 ml) which had been equilibrated with 50 m*M* Tris–HCl, pH 7.0, containing 0.2 *M* NaCl and leupeptin (1 μg/ml). After the column was washed with the same buffer solution, proteins containing APPs bound to the column under these conditions were eluted with the buffer supplemented with 1.5 *M* NaCl.

2.6. Chelating-Sepharose FF column chromatography

The chelating-Sepharose FF column (gel bed volume, 10 ml), washed with the starting buffer (50 m*M* Tris–HCl, pH 7.0, containing 1 *M* NaCl), was charged with 250 μmol Ni^{2+} by loading 2.5 ml of 100 m*M* $NiCl_2$, followed by washing with the starting buffer. The column was loaded with the sample from the Blue-Sepharose column, following by washing with 50 m*M* sodium acetate, pH 6.0, containing 1.0 *M* NaCl.The proteins bound to the column under these conditions were eluted with 100 m*M* sodium acetate, pH 5.0, containing 1.0 *M* NaCl.

2.7. Sephacryl S-300 gelfiltration

The Sephacryl S-300 HR column (2.5 cm×92 cm) was equilibrated with 20 m*M* sodium phosphate, pH 7.4, containing 0.1 *M* NaCl. The sample from the chlating-Sepharose column was concentrated in an ultrafiltration membrane (YM-10, Amicon, Grace Japan, Tokyo) and applied to the column, and fractions of 3.4 ml were collected. Fractions containing APPs were pooled, concentrated, and subjected to re-chromatography. Fractions containing APPs were collected and used for various analyses.

2.8. Preparation of antibodies

The final preparation of APPs (0.2 mg) was emulsified with an equal volume of Freund's complete adjuvant and then injected subcutaneously into a rabbit. One month after the first immunization, the rabbit was further immunized twice with the same amount of antigen at a 2 week interval. Serum was collected one week after the last injection. For the affinity purification of the antibody, antiserum was applied to an antigen-coupled Sepharose 4 B column, which had been prepared according to the instructions given by Pharmacia, and the column was washed with 0.5 *M* NaCl/20 m*M* sodium phosphate buffer, pH 7.4. Adsorbed IgG was eluted with 4 *M* $MgCl_2$/20 m*M* sodium phosphate buffer, pH 7.4. The eluate was diluted to decrease its viscosity, concentrated and applied to a Sephacryl S-200 column equilibrated with 0.15 *M* NaCl/20 m*M*

sodium phosphate buffer, pH 7.4. The eluate containing IgG was collected and concentrated.

2.9. SDS–PAGE and Western blotting analysis

The samples were mixed with an equal volume of 125 m*M* Tris–HCl, pH 6.8, containing 3% SDS, 10% 2-mercaptoethanol, 8 *M* urea and 0.04% bromophenol blue, and then boiled for 5 min. SDS–PAGE was performed using 7.5 or 10% polyacrylamide slab gels according to the method reported by Laemmli [14]. The resolved protein bands were detected by staining with Coomassie brilliant blue R-250 or by a silver staining procedure, according to the instructions given by Pharmacia. For immunological detection, the resolved proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Japan Millipore, Tokyo). The blotted PVDF membrane was treated with blocking medium (2.9% skimmed milk containing 1% milk protein, 0.15 *M* NaCl and 20 m*M* sodium phosphate, pH 7.4) for 2 h at room temperature. It was then incubated with 30 μl antiserum or 1 μg antibody dissolved in 3 ml of blocking medium for 3 h at room temperature. After the membrane was washed with the washing medium (20 m*M* sodium phosphate, pH 7.4, containing 0.5 *M* NaCl), it was reacted with horseradish peroxidase-linked second antibodies (Amersham) (3 μl dissolved in 3 ml of blocking buffer) at 4°C overnight. After washing, the protein bands were visualized by incubation with 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) in 50 m*M* Tris–HCl, pH 7.4, supplemented with 0.01% H_2O_2 at room temperature or by the ECL method according to the instructions given by Amersham.

2.10. Protein sequencing

Proteins were separated in SDS–PAGE and electrophoretically blotted onto a PVDF membrane. The membrane was extensively rinsed with water, stained for 1 min with 0.1% Coomassie brilliant blue R-250 dissolved in 50% methanol containing 10% acetic acid, and destained with 50% methanol and dryed. The stained bands were separately cut out to determine their amino terminal sequences with a Protein sequencer (Applied Biosystems, USA) as described by Matsudaira [15].

2.11. Immuno-precipitation analysis

For resolving the immuno-precipitated proteins by SDS–PAGE with or without decreased contamination of the heavy and light chains of IgG, an immunoaffinity matrix was prepared by cross-linking the antibody to protein A-Sepharose as described by Schneider et al. [16]. The immunoaffinity matrix prepared showed a greatly decreased release of the heavy and light chains of IgG in SDS–PAGE (Fig. 9). It was mixed with the samples in 50 m*M* sodium phosphate, pH 7.4, containing 0.15 *M* NaCl and then incubated overnight at 4°C with gently mixing. The matrix was then washed three times with the same buffer supplemented with 0.5 *M* NaCl, followed by washing with the same buffer supplemented with 0.15 *M* NaCl. The washed matrix was transferred to a new centrifuge tube and collected by gentle centrifugation. It was then subjected to SDS–PAGE and to a Western blotting analysis.

2.12. Other analytical procedures

Trypsin-inhibiting activity was measured by determining the residual trypsin activity after trypsin was incubated with each sample. One unit of inhibitor was defined as the amount that decrease the activity of trypsin by one unit. The incubation mixture (0.2 ml) contained 100 m*M* Tris–HCl, pH 8.0, 1 m*M* $CaCl_2$, 1.0 ng trypsin supplemented with bovine serum albumin (BSA) (1 mg/ml) as a stabilizer of trypsin, 10 μl of the sample and 20 nmol of Boc–Gln–Gly–Arg–NH–Mec (a synthetic substrate for trypsin [17]), and was incubated for 5–10 min at 37°C. The reaction was stopped with 2.0 ml of 0.1 *M* sodium chloroacetate/0.1 *M* sodium acetate, pH 4.5. The fluorescence intensity of the liberated 7-amino-4-methyl coumarin was measured at excitation and emission wavelengths of 370 nm and 460 nm, respectively. The concentration of trypsin was determined using the absorbancy index of trypsin (A_{280} of 1.0 mg/ml trypsin=1.5). The concentration of other proteins was determined by the micro-Biuret method of Itzhaki and Gill [18] or by the bicinchoninic acid protein assay according to

the method of Smith et al. [19] using BSA as the standard.

3. Results

3.1. Rat brain is a favorable tissue for the purification of APPs

In order to identify the physiological role of APPs, we attempted to purify APPs from various rat tissues by detecting the APPs with an antibody. We first tested some antibodies obtained commercially from Boehringer Mannheim. A mouse monoclonal antibody for APPs (Anti-Alzheimer precursor protein A4) showed the most sensitivity and specificity for the APPs of various rat tissues. APPs are expressed in both the kidney and liver of rats and were easily detected with this antibody. In these tissues, however, almost all APPs were localized in the membrane fraction and were degraded by the treatment of the membrane fraction with detergent for the extraction of APPs. In contrast, the APPs in the brain were localized in both the cytosol and microsomal fractions (Fig. 1). The rat brain is a favorable tissue for purifying APPs because its APPs were fractionated without using detergent and because they were resistant to the detergent treatment. Although APPs localized in the membrane fraction are also of interest, we first attempted to establish a method for purifying APPs from the cytosol fraction of rat brain and to characterize their native form.

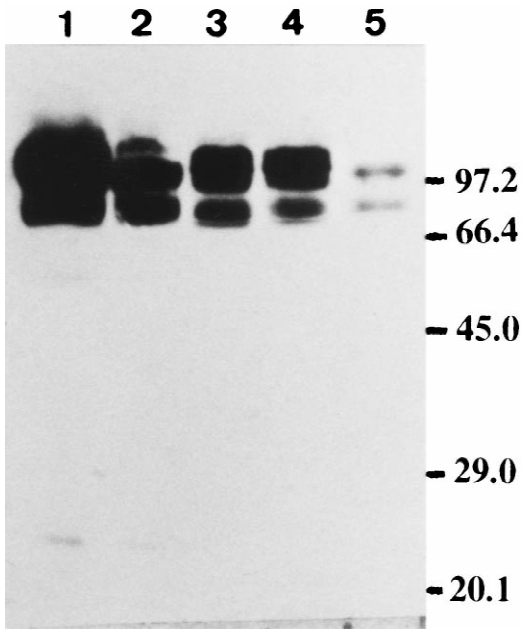

Fig. 1. Immunological detection of APPs. Rat brains were homogenized and fractionated as described in Section 2. The fractions were mixed with an equal volume of 125 m*M* Tris-HCl, pH 6.8, containing 3% SDS, 10% 2-mercaptoethanol, 8 *M* urea, and 0.04% bromophenol blue and then boiled for 5 min. The samples (10 μl each of 1.2 mg tissue equivalents) of post-mitochondrial supernatant (33.3 μg protein) (lane 1), cytosol (15.1 μg) (lane 2), microsomes (18.2 μg) (lane 3), washed microsomes (15.8 μg) (lane 4) and the washed fraction (2.4 μg) (lane 5) were resolved by SDS-PAGE on a slab gel containing 10% polyacrylamide. The resolved proteins were electrophoretically transferred onto a PVDF membrane, and the APPs were detected immunologically as described in Section 2.

3.2. Purification of APPs from the rat brain

The anti-APP antibody reacted with broad protein bands of 80 and 100 kDa in the cytosol fraction of the rat brain. The column chromatographies which were used for purifying APPs from the cytosol fraction of the rat brain are summarized in Fig. 2. The APPs which were collected from the cytosol fraction by ammonium sulfate precipitation bound to a DE52 anion-exchange column in the presence of 0.2 *M* NaCl and separated from over 90% of the total protein eluted in the flow-through fraction. They were then eluted from the column with 0.3 *M* NaCl. They bound to a Blue Sepharose column in the presence of 0.2 *M* NaCl and were then eluted with 1.5 *M* NaCl. They were separated from many of the proteins eluted in the flow-through fraction of the column (Fig. 3). They bound to a chelating column charged with Ni at pH 6 and then were eluted from the column at pH 5.0 (Fig. 4). Finally they were applied to a Sephacryl S-300 gel filtration column. They were eluted in the same fractions as about 400 kDa proteins and were separated from proteins with a higher molecular mass eluted near the void volume fractions (Fig. 5).

Proteins eluted from each column chromatography were normalized according to the amount of proteins and analyzed by SDS–PAGE and by Western blotting. In Fig. 6 the fact that protein bands of 80 and 100 kDa were concentrated by each column chroma-

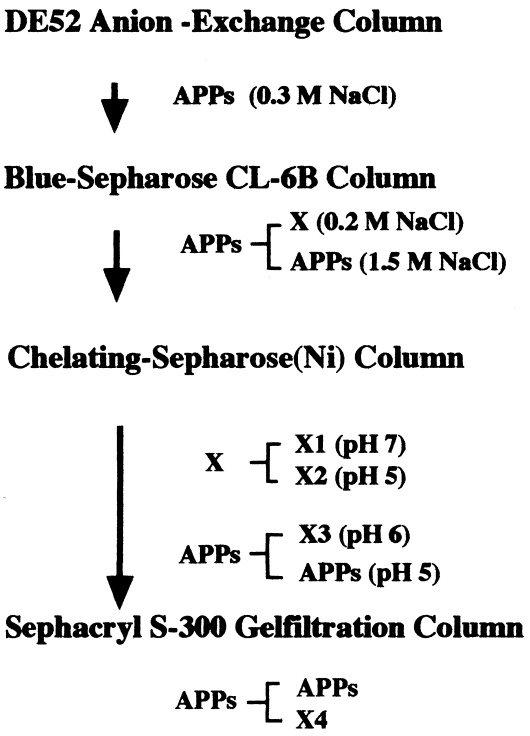

Fig. 2. Column chromatographies for purifying APPs from the cytosol fraction of the rat brain. The fraction containing APPs from the DE52 column was separated into fraction X and a fraction containing APPs with the Blue Sepharose column. Fraction X was further separated into fractions X1 and X2, and the fraction containing APPs was further separated into fraction X3 and a fraction containing APPs by Chelating Sepharose (Ni) column chromatography. The fraction containing APPs was further separated into fraction X4 and a fraction containing APPs by gelfiltration.

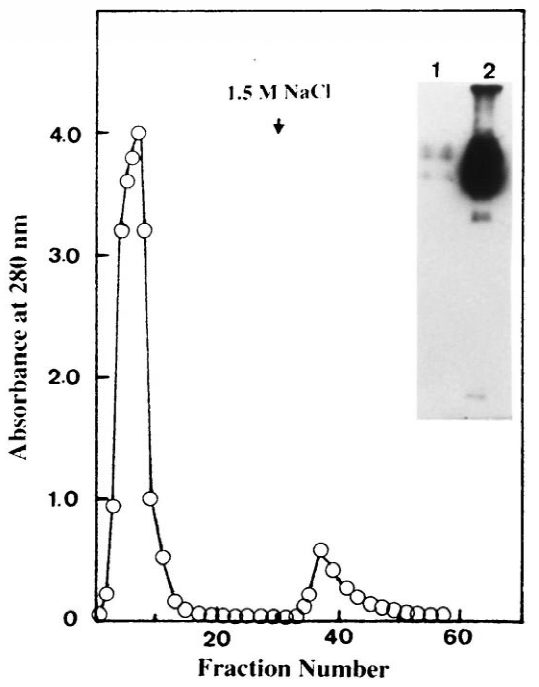

Fig. 3. Fractionation of APPs by Blue-Sepharose column chromatography. A fraction containing a APPs from DE52 column (44.3 mg protein from 278 g brain of 200 rats) was applied to a Blue-Sepharose column, and the column chromatography was performed as described in Section 2. Proteins bound to the column were eluted with the buffer supplemented with 1.5 *M* NaCl, and separated from proteins eluted in the flow-through fraction. The inset shows the immunological detection of APPs which were eluted with the starting buffer (lane 1) and with the buffer supplemented with 1.5 *M* NaCl (lane 2).

tography is shown. The relative amounts of proteins recovered by each column chromatography are summarized in Table 1. Approximately 0.2 mg of proteins were recovered in the final preparation from 100 g of brain and the specific activity of the trypsin inhibitor in the final preparation was 61 times higher than that in the fraction from the DE52 column. The 80 and 100 kDa protein bands were electrophoretically blotted onto a PVDF membrane, stained with dye and then cut out. Their amino-terminal sequences were determined with a protein sequencer. The amino-terminal amino acid sequences of the two bands were deduced by 22 and 32 residues of 100 kDa and 80 kDa band, respectively. In Fig. 7, their sequences are compared with the partial amino-terminal sequence of rat APP695 predicted from the cDNA cloned by Shivers et al. [7]. The amino-terminal sequences of the two bands were found to be identical to that of rat brain APP695 without an amino terminal signal sequence composed of 17 residues. This result is in agreement with the partial amino-terminal sequences of three protein bands of newborn rat brain APPs, reported by Takio et al. [20].

In Fig. 5 the fact that the molecular masses of the native form of APPs prepared from the cytosol fraction of rat brain are about 400 kDa and are much larger than those of their denatured form estimated by SDS–PAGE is shown. These results indicate that rat brain APPs form a complex with themselves or with other unidentified proteins.

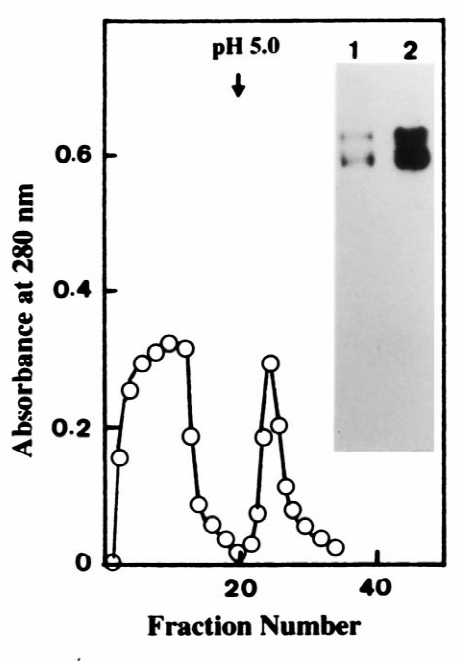

Fig. 4. Fractionation of APPs by Chelating Sepharose column chromatography. A fraction containing APPs separated on the Blue-Sepharose column was further fractionated on the chelating column as described in Section 2. The inset shows the immunological detection of APPs which were eluted with the starting buffer (lane 1) and with the pH 5.0 buffer (lane 2).

In Fig. 5 the fact that trypsin inhibitory activity was present in the fractions containing APPs is also shown, suggesting the presence of APP isoforms, App751 or APP770 or both, including the serine protease inhibitor domain [10] in the cytosol fraction of the rat brain. Several protein bands other than the 80 and 100 kDa bands were present in the final APPs preparation (Fig. 6). These protein bands were not reactable with anti-APP antibodies but they were always present in the fractions containing APPs. It is important to clarify whether they are associated with APP molecules.

3.3. Specificity of antibodies prepared from a rabbit immunized with a preparation of APPs

Although a mouse monoclonal antibody for APPs (anti-Alzheimer precursor protein A4) obtained commercially was useful for detecting APPs denatured by SDS treatment, this antibody was found to be unable to react with the native form of APPs and to be unusable for the immuno-precipitation analysis. Therefore, we immunized a rabbit with a final APPs preparation and expected to prepare an antibody to react with native APPs or with proteins which may form a complex with APPs. An antibody obtained from the rabbit was found to react with a 95 kDa protein band, but not with bands of APPs. The Western blot analysis showed that a part of a protein giving 95 kDa is present in the final preparation of APPs (lane 1 in Fig. 8B), and that the remainder is also present in the fractions separated from APPs on Blue-Sepharose, Chelating Sepharose and Sephacryl S-300 columns (lanes 3-5 in Fig. 8B), and that this antibody did not react with the bands of APPs (lane 1 in Fig. 8B).

3.4. Application of the antibody for immuno-precipitation analysis

We next prepared an immunoaffinity matrix by binding antibody to protein A-Sepharose with a cross-linker for the immuno-precipitation analysis. Although this immunoaffinity matrix allowed a slight release of the heavy and light chains of IgG in SDS–PAGE (lane 3 in Fig. 9), it was highly useful for the immuno-precipitation analysis.

A sample containing APPs from a DE52 column was applied to a Sephacryl S-300 gelfiltration column. Fractions containing APPs were collected and the pooled fraction was used for an immuno-precipitation analysis. The immunoaffinity matrix linked with IgG reactable with 95 kDa band was found to precipitate almost all APPs in this sample (Fig. 10A, lanes 2p, 2s) together with the 95 kDa protein (Fig. 10B, lane 2) but that linked with anti-lactate dehydrogenase (LDH) IgG was not (Fig. 10, lanes 3p, 3s). This result indicates that APPs form a complex directly or indirectly with another protein giving 95 kDa in SDS–PAGE.

The 95 kDa protein band which was precipitated using the immunoaffinity matrix was electrophoretically blotted onto a PVDF membrane, stained with dye, cut out and subjected to amino-terminal sequencing. Its amino terminal sequence was not deduced, probably because its amino terminal is blocked.

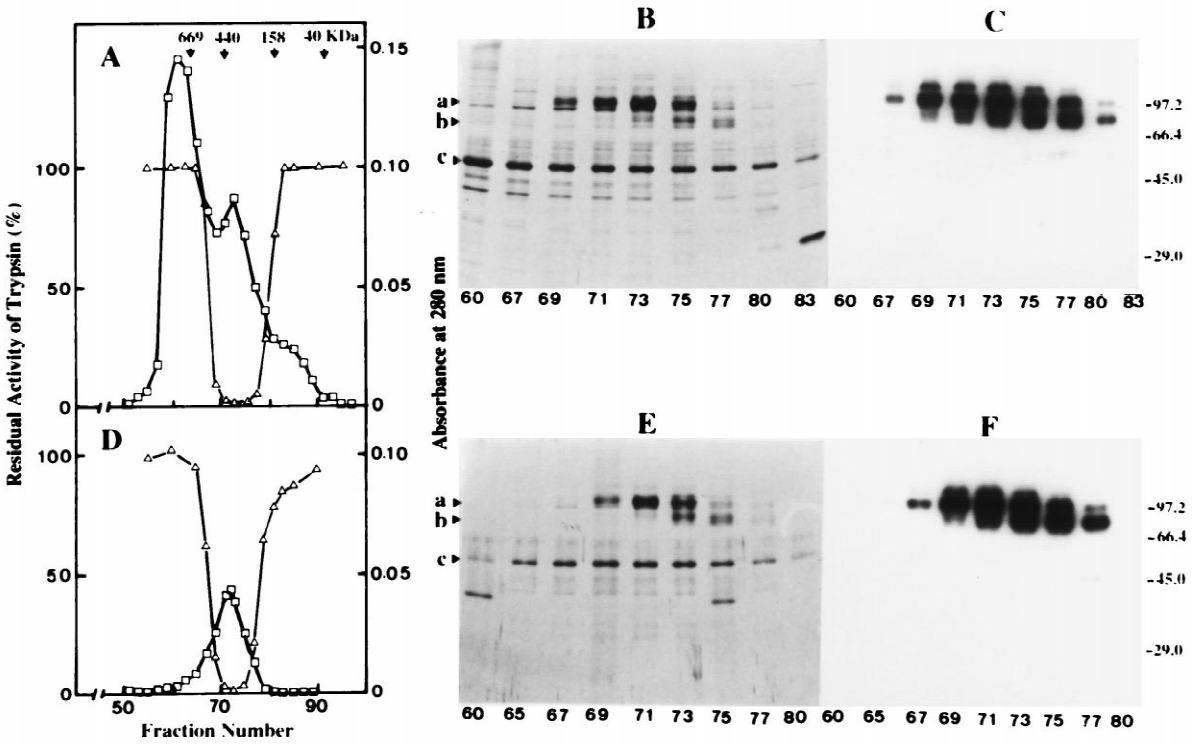

Fig. 5. Sephacryl S-300 gelfiltration column chromatography. A concentrated sample (4.5 ml, A_{280}=1.35) containing APPs eluted from a Chelating Sepharose column at pH 5.0 was applied to a Sephacryl S-300 HR column, and fractions were collected as described in the Section 2. The absorbance at 280 nm (□) and trypsin inhibitory activity (△) were measured (A). Fractions of 5 μl (10 μl of SDS-treated sample) were subjected to SDS-PAGE, and their resolved proteins were visualized by a silver staining procedure (B). The resolved proteins were electrophoretically transferred onto a PVDF membrane, and APPs were detected with anti-APP antibody (C) as described in Section 2. The fraction numbers are given at the bottom of each lane. The fractions (70-77) containing APPs shown in panel A were pooled, concentrated and subjected to re-chromatography. Fractions were collected and their absorbance at 280 nm (□) and trypsin inhibitory activity (△) (D) were measured, and SDS-PAGE (E) and Western blot (F) analyses were performed as described above. The arrowheads a, b and c indicate the positions of the 100, 80 and 52 kDa protein bands, respectively.

4. Discussion

In this study, we attempted to purify APPs from rat brains. The final preparation of APPs contained several protein bands other than APP bands. In particular, a part of a protein giving a 52 kDa band in the SDS–PAGE was always present in the fractions containing APPs. It was broadly distributed in the fractions in which 100 to 1000 kDa proteins were eluted from the gelfiltration column (Fig. 5B). We immunized a rabbit with a final preparation of APPs. The antibody obtained reacted with neither the APPs nor the 52 kDa protein band, indicating that these proteins were not antigens to the rabbit. APPs may not be recognized as foreign substances by the rabbit, because APPs are highly conserved proteins, or their epitopes may be hidden in their molecular complexes.

We deduced the amino terminal sequence of the 52 kDa protein band. It was identical to that of tubulin beta chain, which is the major constituent of microtubules [21]. Refolo et al. [22] found that 50–90% of APP in rat brain and in neural cell lines is bound to the detergent-insoluble cytoskeleton. They suggested that the function of cellular APP involves the cytoskeleton and particularly microtubules. We found that APPs are localized in both the soluble cytosol and microsome fractions in generally equal amounts (Fig. 1). APPs localized in the soluble cytosol fraction of rat brain may also be associated

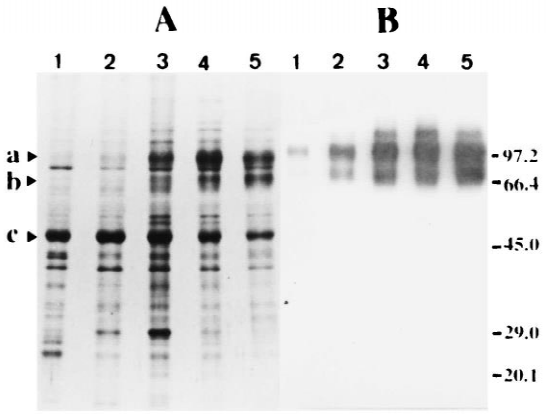

Fig. 6. Concentration of APPs by column chromatography. Proteins (0.5 μg) containing APPs which were fractionated by various forms of column chromatographies, resolved by SDS-PAGE on 10% gels and visualized by a silver staining procedure (A). The resolved proteins were transferred electrophoretically onto a PVDF membrane, and APPs were detected with an anti-APP antibody (B) as described in Section 2. The samples were obtained from DE52 (lane 1), Blue-Sepharose(lane 2), Chelating Sepharose (Ni) (lane 3), first Sephacryl S-300 HR column (lane 4) and re-chromatography of Sephacryl S-300 (lane 5). The molecular mass markers were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white albumin (45.0 kDa), bovine carbonic anhydrase (29.0 kDa) and soybean trypsin inhibitor (20.1 kDa), obtained from APRO Science, Inc. (Tokushima, Japan). The arrowheads a, b and c indicate the positions of the 100, 80 and 52 kDa protein bands, respectively.

	1 5 10
100 kDa	LEVPTDGNAG
80 kDa	LEVPTDGNAG
Rat brain APP 695	MLPSLALLLLAAWTVRALEVPTDGNAG

	11 15 20 25 30
100 kDa	LLAEPQIAMFxGK
80 kDa	LLAEPQIAMFxGKLNMHMNVQNG
Rat brain APP 695	LLAEPQIAMFCGKLNMHMNVQNG

Fig. 7. The amino-terminal sequences of 80 and 100 kDa bands of APPs purified from rat brain. The final preparation of APPs obtained by the re-chromatography of Sephacryl S-300 were resolved by SDS-PAGE and electrophoretically blotted onto a PVDF membrane. The amino-terminal sequences of the resolved protein bands with 80 and 100 kDa were determined as described in Section 2. They were compared with the amino-terminal sequence of rat brain APP695 predicted by Shivers et al. [[7]]. The unidentified amino acid is indicated by x.

with tubulin beta chain. In this study, relatively large amounts of APPs bound to the detergent-insoluble cytoskeleton may have been lost into a nuclear fraction because the brain homogenate contained large amounts of membranous or lipid-like materials that formed a sediment of a nuclear fraction.

In this study, we first noted that the size of the native form of APPs estimated by gelfiltration is much larger than that of their denatured form or their molecular size predicted from their mRNA. We confirmed that APPs form a complex with another protein giving a 95 kDa band in SDS–PAGE, by an immuno-precipitation analysis using the antibody newly prepared in this study. We found that this antibody precipitated not only its antigen protein giving an 95 kDa band but also APPs. This finding indicates that a 95 kDa protein forms a complex with APPs. The amino-terminal sequence of this 95 kDa protein band was not determined, probably because its amino terminal is blocked. The physiological roles of this protein are as yet unknown; their identification is necessary for estimating the physiological roles of APPs. It may be noteworthy that the native form of the 95 kDa protein was broadly distributed between 100 to 1000 kDa. If the 1000 kDa form is a final mature high-molecular-mass

Table 1
Protein recovery and concentration of trypsin inhibitor activity at each stage of purifying APP from rat brain

Purification step	Protein recovery mg/100 g brain	Trypsin inhibitor activity mU/mg protein
Homogenate	11 300	–
69 600 g supernatant	1430	–
DE52	15.94	15.1
Blue-Sepharose	4.18	56.4
Chelating Sepharose (Ni)	0.683	292.8
Sephacryl S-300	0.208	920.4

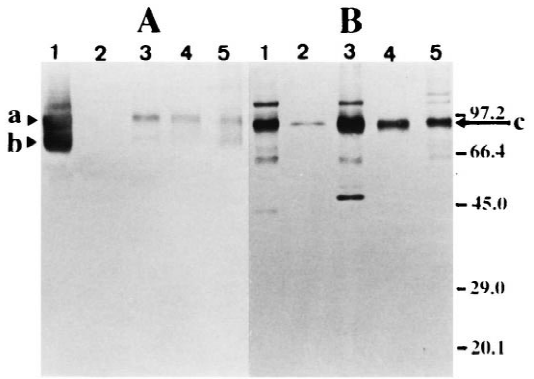

Fig. 8. Specificity of antibodies prepared from a rabbit immunized with a final preparation of APPs. Various fractions were separated by the method summarized in Fig. 2. Their proteins (2.5 μg each) were resolved by SDS-PAGE on 10% gels, providing final preparations of APPs (lane 1), X1 (lane 2), X2 (lane 3), X3 (lane 4) and X4 (lane 5). The resolved proteins were transferred onto PVDF membranes, and their specific protein bands were detected with anti-APP monoclonal antibody obtained commercially (A) or with antiserum (30 μl/3 ml) prepared from a rabbit immunized with the final preparation of APPs (B). The arrowheads a and b and arrow c indicate the positions of the 100 and 80 kDa bands of APPs and the 95 kDa protein band, respectively.

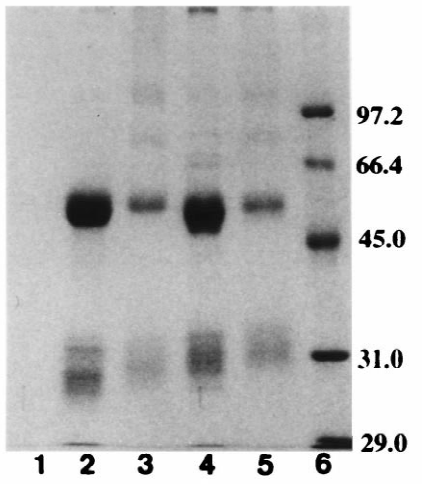

Fig. 9. Preparation of immunoaffinity matrix. An immunoaffinity matrix was prepared by cross-linking IgG to Protein A-Sepharose [[16]]. Proteins of the various fractions were resolved by SDS-PAGE on a 10% gel as described in Section 2: Protein A-Sepharose containing 0.5 ml gel (lane 1), Protein A-Sepharose bound with IgG reactable with 95 kDa protein, before (lane 2) or after (lane 3) the cross-linking, Protein A-Sepharose bound with anti-LDH IgG, before (lane 4) or after (lane 5) the cross-linking, and molecular mass markers (lane 6) as shown in Fig. 6.

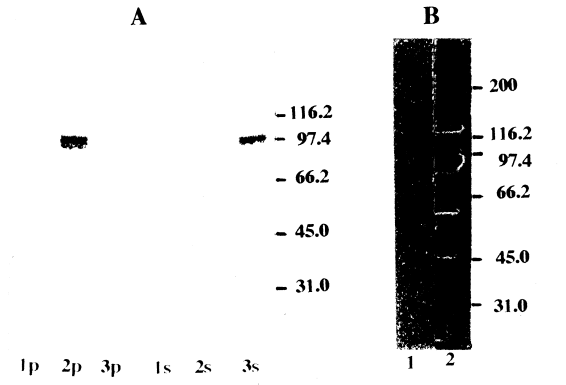

Fig. 10. Immuno-precipitation analysis. (A) IgG reactable with the 95 kDa protein band was purified using a Sepharose 4B affinity column coupled with proteins of the X2 fraction. The immuno-affinity matrix was prepared as described in Section 2. The reaction mixtures (0.2 ml) contained immunoaffinity matrix (10 μl gel), the crude sample of APPs (30 μg proteins) prepared with DE52 and Sephacryl S-200 columns and 50 m*M* phosphate buffer, pH 7.4, containing 0.15 *M* NaCl. The immuno-precipitates and their supernatants were prepared as described in Section 2. Samples of the immuno-precipitates (lanes 1p, 2p and 3p) and the supernatants (lanes 1s, 2s, 3s) were obtained using protein A-Sepharose (as a negative control) (lanes 1p and 1s), or using the immunoaffinity matrix coupled with newly prepared polyclonal IgG that reacts with 95 kDa protein band (lanes 2p an 2s) or coupled with anti-LDH IgG (as a negative control) (lanes 3p and 3s). They were resolved by SDS–PAGE on a 7.5% gel. The resolved protein bands on a gel were transferred onto a PVDF membrane, and their specific protein bands were detected with anti-APP monoclonal antibody obtained commercially. (B) An SDS-treated sample of the immuno-precipitate obtained using the immunoaffinity matrix coupled with a newly prepared polyclonal IgG that reacts with the 95 kDa protein band was applied to a well with width 1 cm containing 7.5% polyacrylamide gel and the proteins were resolved by SDS–PAGE. The resolved protein bands on the gel were transferred to a PVDF membrane. The sample lane was cut into two sections. One section was reacted with anti-APP monoclonal antibody obtained commercially to detect APP (lane 1) and the other was reacted with the newly prepared polyclonal IgG that reacts with the 95 kDa protein to detect the 95 kDa protein band (lane 2).

complex, APPs may play a role similar to a molecular chaperone in the course of the maturation of the high molecular mass complex of the 95 kDa protein. Conversely, the 95 kDa protein may be a molecular chaperone for various proteins including APPs.

An interesting feature of APPs in the rat brain is that the APPs were localized in both the cytosol and microsomal fraction (Fig. 1). To obtain further

information concerning the physiological roles of APPs, the native form of the membrane-bound APPs must be characterized. Our preliminary information suggests that the membrane-bound APPs are also associated with other proteins and form higher molecular mass complexes. Shioi et al. [23] reported that APP is a component of the proteoglycans. Smith et al. [24] reported that the microtubule-associated protein, tau, which is a component of neurofibrillary tangles (NFT), directly interacts with APP in vitro. Okamoto et al. [25] reported that the membrane-bound APP has a receptor function whereby it associates with G protein. Recent reports show that APP forms a complex with a 59 kDa novel protein [26], collagen [27], presenilin 2 [28,29] and apolipoprotein E [30]. These reports suggest that APPs are associated with several other proteins which may play a role in each other's physiological activities. APPs are probably multi-functional proteins [31], and their cellular functions may require various high molecular mass complexes composed of APPs and other proteins. An abnormality of these complexes may lead to the loss of various cellular functions, abnormal proteolytic processing and/or the production of amyloidogenic fragments of APPs in Alzheimer's disease.

In this study, APPs were fractionated with the use of various columns. Their elution behaviors from the various columns were probably influenced by characteristics of the proteins with which they are associated.

5. Abbreviations

APPs: beta-amyloid precursor proteins
Boc: t-Butyloxycarbonyl
Mec: 4-methylcoumaryl
PVDF: polyvinylidene difluoride

Acknowledgements

We thank Dr. H. Kido, The Institute for Enzyme Research, Tokushima University, Japan, for providing the opportunity to begin this study. This study was supported in part by a grant from the Sasakawa Health Science Foundation.

References

[1] G.G. Glenner, G.W. Wong, Biochem. Biophys. Res. Commun. 120 (1984) 885–890.
[2] J. Kang, H.G. Lemaire, A. Underbeck, J.M. Salbaum, C.L. Masters, K.H. Grzeschik, G. Multhaup, K. Beyreuther, B. Muller-Hill, Nature 325 (1987) 733–736.
[3] P. Ponte, P. Gonzalez-DeWhitt, J. Schilling, J. Miller, D. Hsu, B. Greenberg, K. Davis, W. Wallace, I. Lieberbung, F. Fuller, B. Cordel, Nature 331 (1988) 525–527.
[4] R.E. Tanzi, A.I. McClatchy, E.D. Lamperti, L. Villa-Komaroff, J.F. Gusella, R.L. Neve, Nature 331 (1988) 528–530.
[5] N. Kitaguchi, Y. Takahashi, Y. Tokushima, S. Shiojiri, H. Ito, Nature 331 (1988) 530–532.
[6] S. Tanaka, S. Shiojiri, Y. Takahashi, N. Kitaguchi, H. Ito, M. Kameyama, J. Kimura, S. Nakamura, K. Ueda, Biochem. Biophys. Res. Commun. 165 (1989) 1406–1414.
[7] B.D. Shivers, C. Hilbich, G. Multhup, M. Salbaum, K. Beyreuther, P.H. Seeburg, EMBO J. 7 (1988) 1365–1370.
[8] F.S. Esch, P.S. Keim, E.C. Beattie, R.W. Blacher, A.R. Culwell, T. Oltersdore, D. McClure, P.J. Ward, Science 248 (1990) 1122–1124.
[9] J.P. Anderson, F.S. Esch, P.S. Keim, K. Sambamurti, I. Lieberburg, N.K. Robakis, Neurosci Lett. 128 (1991) 126–128.
[10] T. Oltersdorf, L.C. Friz, D.B. Schenk, I. Lieberburk, K.L. Johnson-Wood, E.C. Beattie, P.J. Ward, R.W. Blacher, H.F. Dovey, S. Sinha, Nature 341 (1989) 144–147.
[11] W.E. Van Nostrand, S.L. Wagner, M. Suzuki, B.M. Choi, J.F. Farrow, J.W. Geddes, C.W. Cotman, D.D. Cunningham, Nature 341 (1989) 546–549.
[12] W.E. Van Nostrand, D.D. Cunningham, J. Biol. Chem. 262 (1987) 8508–8514.
[13] K. Miyazaki, M. Hasegawa, K. Funahashi, M. Umeda, Nature 362 (1993) 839–841.
[14] U.K. Laemmli, Nature 227 (1970) 680–685.
[15] P. Matsudaira, J. Biol. Chem. 262 (1987) 10035–10038.
[16] C. Schneider, R.A. Newman, D.R. Sutherland, U. Asser, F.G. Melvyn, J. Biol. Chem. 257 (1982) 10766–10769.
[17] S. Kawabata, T. Miura, T. Morita, H. Kato, K. Fijikawa, S. Iwanaga, K. Takada, T. Kimura, S. Sakakibara, Eur. J. Biochem. 172 (1988) 17–25.
[18] R.F. Itzhaki, M.D. Gill, Anal. Biochem. 9 (1964) 401–410.
[19] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[20] K. Takio, M. Hasegawa, K. Titani, Y. Ihara, Biochem. Biophys, Res. Commun. 160 (1989) 1296–1301.
[21] I. Ginzburg, A. Teichman, H.J. Dodemont, L. Behar, U.Z. Littaure, EMBO J. 4 (1985) 3667–3673.

[22] L.M. Refolo, I.S. Wittenberg, V.L. Friedrich Jr., N.K. Robakis, J. Neurosci. 11 (1991) 3888–3897.
[23] J. Shioi, M. N. Pangalos, A. Wu, N. K. Robakis, Trends Glycosci. Glycotechnol. 8 (1996) 253–263.
[24] M.A. Smith, S.L. Siedlak, P.L. Richey, P. Mulvihill, J. Ghiso, B. Frangione, F. Tagliavini, G. Giaccine, O. Bugiani, D. Praprotnik, R.N. Kalaria, G. Perry, Nat. Med. 1 (1995) 365–369.
[25] T. Okamoto, S. Takeda, Y. Murayama, E. Ogata, I. Nishimoto, J. Biol. Chem. 270 (1995) 4205–4208.
[26] N. Chow, R.L. Neve, J.R. Korenberg, X.-N. Chen, J. Biol. Chem. 271 (1996) 11339–11346.
[27] D. Beher, L. Hesse, C.L. Masters, G. Multhaup, J. Biol. Chem. 271 (1996) 1613–1620.
[28] A. Weidemann, K. Paliga, U. Durrwang, C. Czech, G. Evin, C.L. Masters, K. Beyreuther, Nat. Med. 3 (1997) 328–332.
[29] M. Waragai, I. Imafuku, S. Takeuchi, I. Kanazawa, F. Oyama, Y. Udakawa, M. Kawabata, H. Okazawa, Biochem. Biophys. Res. Commun. 239 (1997) 480–482.
[30] C. Haas, P. Cazorla, C.D. Miguel, F. Valdivieso, J. Vazquez, Biochem. J. 325 (1997) 169–175.
[31] M.P. Mattson, Physiol. Rev. 77 (1997) 1081–1132.