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Membrane inserted APP fragments containing the βA4 sequence of Alzheimer's disease do not aggregate

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Previously we have shown that the COOH-terminal fragment (A4CT) of the Alzheimer amyloid protein precursor (APP), which at the NH_2-terminus carries the sequence of the amyloid βA4 protein, forms highly insoluble aggregates [EMBO J. (1988) 7, 949–957]. Here we report that aggregation is prevented if A4CT is expressed in vitro with a signal sequence at the NH_2-terminus (SPA4CT) under conditions which allow membrane insertion. Aggregates from SPA4CT are obtained after removal of membranes by chloroform/methanol extraction or heating.

A4CT; In vitro translation; Aggregation; Alzheimer's disease

1. INTRODUCTION

The histopathological features of Alzheimer's disease (AD) and the mechanisms underlying amyloid deposition in AD have been intensively investigated [2–7]. The isolation and sequence analysis of βA4 [3–6] from intracellular and extracellular amyloid in AD and Down's syndrome brains has led to the isolation and characterization of the family of amyloid protein precursors (APP's) as glycosylated, tyrosine-sulfated transmembrane proteins [8–12]. The βA4 protein is encoded within the transmembrane and extracellular domains of the APP's [8].

The normal route of APP processing involves cleavage of APP within the βA4 sequence [12–14]. This suggests that the NH_2-terminus of the amyloid βA4 protein is not produced by the normal cleavage which leads to shedding of the extracellular part of cell-surface transmembrane APP's.

The exact amyloidogenic mechanism by which βA4 is cleaved from APP and deposited in AD remains unknown, although our earlier [1] and recent studies [15] suggest that the initial step of the abnormal processing of full-length APP in Alzheimer's disease may occur at the NH_2-terminus of the βA4 sequence and generate a COOH-terminal fragment of 100 residues (A4CT), which includes the amyloid βA4 sequence, and the transmembrane and cytoplasmic domains of all known transmembrane APP forms.

To study the amyloidogenic properties of this hypothetical intermediate in the process of amyloid formation, we expressed these COOH-terminal 100 residues with (SPA4CT) and without (A4CT) the APP signal sequence.

Here we show that expression of this APP fragment in the rabbit reticulocyte lysate (RRL) resulted in an aggregating protein. Membrane insertion of A4CT obtained by expression of SPA4CT in the presence of dog pancreas membranes prevents aggregation. Post-translational removal of the membranes led to aggregation.

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2. EXPERIMENTAL

2.1. *Cloning procedures*

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Maniatis et al. [16].

2.2. *Plasmid construction*

Construct SP65/A4CT [1] was obtained by cloning the 961 bp *Bgl*II/*Hin*dIII fragment of the APP695 cDNA clone [8] into pSP65. The resulting plasmid includes methionine codon 596 of APP695 as initiation codon, the amyloid βA4 sequence (codons 597–639/640 of APP 695) and the entire COOH-terminal domain.

For construction of SP65/SPA4CT, plasmid SP65/A4CTI, which was obtained by cloning a *NCO*I oligolinker into *Sph*I/*Eco*RI-digested SP65/A4CT, was digested with *NCO*I, incubated with S1 to create blunt ends and further digested with *Hin*dIII. The resulting 960 bp fragment carrying the A4CT sequence was cloned into SP65/695, digested with Asp 718, incubated with S1 to create blunt ends and digested with *Hin*dIII. The resulting plasmid SP65/SPA4CT encodes the APP signal sequence and two additional residues from APP695 in frame with the amyloid A4 sequence and the entire COOH-terminal domain of the amyloid precursor.

2.3. *In vitro transcription [17]*

DNA templates (100 μg/ml) were transcribed in 40 mM Tris, pH

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7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, RNAsin (1 U/μl), 100 μg/ml BSA, 500 μM each of ATP, CTP and UTP, 50 μM GTP, and 500 μM $m^7G(5')ppp(5')G$. Typically, 1 unit of SP6 RNA polymerase was added per μg DNA template for a 1 h synthesis at 40°C. DTT and rNTPs stocks were prepared with diethylpyrocarbonate-treated water. The components of the transcription reaction were mixed at room temperature to prevent DNA precipitation.

Following RNA synthesis the DNA template was removed by the addition of RNAse-free DNAse, and after a 10 min incubation at 37°C, the reaction mixtures were phenol:chloroform extracted after addition of NaOAc (pH 5.5) to 0.3 M. The RNA was precipitated with ethanol and washed with 70% ethanol.

2.4. *In vitro translation*

Translation of mRNA in a cell-free rabbit reticulocyte lysate or a wheat germ expression system was done following the procedures as described in the supplier's manuals (Promega, Germany). The reactions were carried out for 60 min at 30°C in the presence of 50 μCi of [^{35}S]methionine and 0.5–1.0 μg mRNA.

Then the translation mixture was diluted with 2× Laemmli sample buffer. After heating for 15 min at 70°C, labeled proteins were analyzed by SDS-PAGE and fluorography with EN^{3}HANCE (Du Pont).

2.5. *Immunoprecipitation [18]*

For immunoprecipitation, 50–100 μl of the cell lysate was diluted with 500 ml Sol buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM PMSF) and incubated for 30 min with 5 μl pre-immunserum and 30 μl (3 mg) protein A-Sepharose (Pharmacia, Freiburg, Germany). The samples were briefly centrifuged and the supernatants were incubated with 5 μl of antiserum for 60 min at room temperature. Following incubation, 30 μl (3 mg) protein A-Sepharose was added for an additional 30 min at room temperature. The insoluble complexes were washed once with wash A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40, 2 mM EDTA), once with wash B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% NP-40, 2 mM EDTA) and once with wash C (10 mM Tris-HCl, pH 7.5). The pellet was resuspended in 2× Laemmli sample buffer. After boiling for 5 min at 100°C, labelled proteins were analyzed by SDS-PAGE and fluorography with EN^{3}HANCE (Du Pont).

2.6. *Membrane sedimentation and carbonate extraction [19]*

To selectively isolate integral membrane proteins, the translation products (20 μl) obtained in the presence of microsomal membranes were diluted with 100 μl ice-cold 0.1 M Na_2CO_3 pH 11 and incubated at 0°C for 15 min. Then the samples were layered over a sucrose cushion (50 μl 0.25 M sucrose/0.1 M Na_2CO_3, pH 11) and centrifuged for 10 min at 30 psi in a Beckman airfuge using the A-100/30 rotor and cellulose propionate centrifuge tubes. After centrifugation the entire supernatant including the sucrose cushion was removed and precipitated with an equal volume of 20% trichloroacetic acid. The pellet was resuspended in 10 μl 2% SDS. Samples used for protein sequence analysis were dialysed against 0.01 M NH_4HCO_3 for 36 h using membranes with a cut-off value of 5,000.

2.7. *Protein sequence analysis*

Amino-terminal sequence analysis was performed on an Applied Biosystems model 470A gas/liquid-phase sequencer. For radiosequencing the PTH-amino acids were collected, dried and dissolved in 0.15 ml of methanol. After addition of 10 ml of Quickszint (Zinsser), the radioactivity released at each cycle was determined in a Kontron liquid scintillation counter.

2.8. *Lipid extraction: chloroform/methanol extraction [20]*

To 1 vol. of cell lysates or translation mixture were added 3.75 vols. of methanol–chloroform (2:1, v/v). The mixture was shaken intermittently for 1–2 h, centrifuged, and the supernatant extract was transferred to another tube. The remainder was then extracted again with 4.75 vols. of methanol–chloroform–water (2:1:0.8, v/v), and the mixture was centrifuged. The combined extracts were diluted with 2.5 vols., each of chloroform and water and then centrifuged. The lower chloroform phase was withdrawn and the upper water phase was used for TCA precipitation.

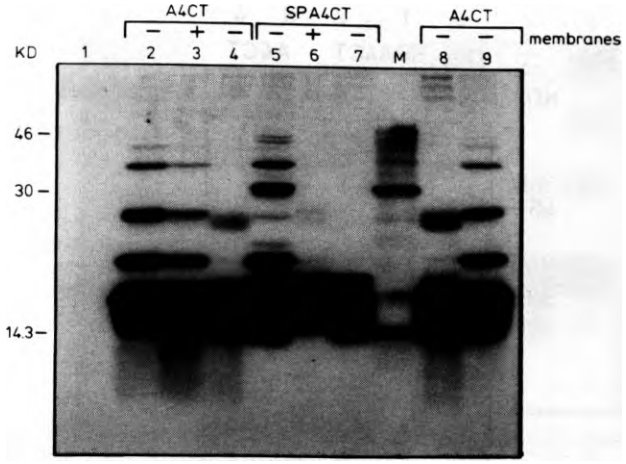

Fig. 1. In vitro expression of A4CT and SPA4CT. mRNA was transcribed from the corresponding SP6 vectors, translated in the RRL in the presence of [^{35}S]methionine, immunoprecipitated and analyzed by SDS-PAGE (12.5%). Lanes 1 and 9, in vitro translation of A4CT in the RRL; lane 2, same as lane 1, but translation in the presence of microsomal membranes; lanes 3 and 8, same as lane 1, but post-translational incubation with 0.2% Triton X-100; lane 4, in vitro translation of SPA4CT in the RRL; lane 5, same as lane 4, but translation in the presence of microsomal membranes; lane 6, same as lane 4, but post-translational incubation with 0.2% Triton X-100; M, molecular mass marker.

2.9. *SDS-PAGE*

Analysis of in vitro translation products by SDS-PAGE was performed according to Laemmli [21]. Prior to SDS-PAGE the samples were heated for 5 min at 100°C in Laemmli sample buffer (0.0625 M Tris/HCl, pH 6.8, 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.005% Bromophenol blue). The gels were analyzed by flourography with EN^{3}HANCE (Du Pont, Boston).

3. RESULTS

3.1. *Expression of amyloid βA4-containing sequences in rabbit reticulocyte lysates*

Previously we reported the in vitro expression of the APP fragment termed A4CT in the rabbit reticulocyte lysate (RRL). A4CT has the methionine residue preceding the amyloid βA4 sequence at the NH_2-terminus and includes the complete βA4 sequence, the transmembrane domain and the cytoplasmic domain of APP. In Fig. 1, lanes 2 and 3 show that translation of A4CT, in the presence and absence of membranes, and subsequent analysis by immunoprecipitation and SDS-PAGE did not result in a single band on SDS-PAGE but gave rise to a broad band at 17 kDa, and additional bands at approximately 20, 27, 40 kDa and higher molecular weights. This is in agreement with our previous finding that A4CT has a high tendency for aggregation [1].

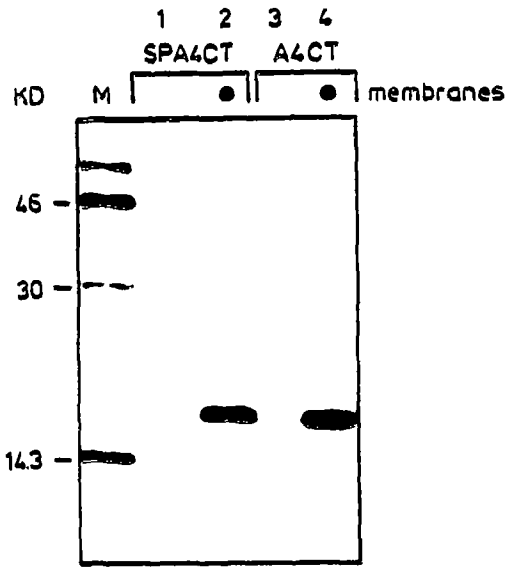

Fig. 2. Analysis of membrane insertion of A4CT and SPA4CT. Translation as in Fig. 1. The translation products were analysed with the sedimentation assay for integral membrane proteins. Lanes 1 and 2, sedimentation of SPA4CT translated in the absence (lane 1) or presence (lane 2) of membranes. Lanes 3 and 4, sedimentation of A4CT translated in the absence (lane 3) or presence (lane 4) of membranes. M, lane with molecular mass marker proteins.

As shown recently, post-translational incubation of the A4CT translation products with 0.2% Triton X-100 prior to immunoprecipitation and aggregation analysis by SDS-PAGE influenced the aggregation pattern (Fig. 1, lanes 4 and 8)[22]. The complex band pattern was reduced into one consisting of only two prominent bands at 17 and 26 kDa. Since A4CT carries the single transmembrane domain of APP, and since we previously demonstrated that APP is indeed a transmembrane protein, we analysed the properties of A4CT when inserted into membranes. For this we expressed plasmid SP65/SPA4CT, which encodes A4CT with the authentic signal sequence of APP. This protein is referred to as SPA4CT. Expression of SPA4CT in the RRL should give rise to two different proteins depending on the presence or absence of dog pancreas microsomal membranes. In Fig. 1, lane 5 shows that in the absence of membranes, SPA4CT-expression resulted in an aggregation pattern similar to that of A4CT-expression. Only one additional band at 30 kDa was visible. In the presence of microsomal membranes, expression of the same construct SP65/SPA4CT resulted in one prominent broad band at 18 kDa on SDS-PAGE (Fig. 1, lane 6). Unlike expression in the absence of membranes, no additional bands at higher molecular mass were detectable. This suggests that membrane insertion prevented aggregation of A4CT.

Treatment of SPA4CT translated in the absence of membranes with Triton X-100 reduced the complex aggregation pattern into only the monomeric band at 18 kDa (Fig. 1, lane 7). Thus the aggregational behavior of SPA4CT in the presence of Triton differs from that of A4CT, which we propose to be due to the retained signal sequence in the absence of membranes.

3.2. *Analysis of membrane insertion*

Because SPA4CT translated in the presence of membranes did not aggregate, we analysed whether this molecule indeed becomes inserted into the membrane and thus protected from aggregation. Evidence for a tight membrane association of SPA4CT was obtained with the sedimentation assay for integral membrane proteins [23]. SPA4CT translated in the presence of microsomes could be selectively sedimentated with the sodium carbonate technique (Fig. 2, lanes 1 and 2), as expected for proteins inserted into the lipid bilayer. A4CT translated in the presence of membranes could also be sedimentated with the sodium carbonate technique (Fig. 2, lanes 3 and 4). This shows that even in the absence of a signal sequence, A4CT strongly interacts with the microsomal vesicles. We attribute this effect to the presence of the APP transmembrane domain.

To analyse whether the artificial signal sequence of SP65/SPA4CT was cleaved after membrane insertion, we sequenced [^{35}S]Met-labeled SPA4CT protein synthesized in the cell-free system. For sequencing, membrane-inserted SPA4CT was enriched by the sodium carbonate technique. Radiosequence analysis revealed release of radioactivity at degradation step 37, which corresponds to methionine residue 54 of SPA4CT (Fig. 3). This result was compatible with the expected removal of the signal sequence of APP, which consists of 17 residues [1]. This cleavage generated an A4CT variant with two additional residues at the NH_2-terminus. The two extra amino acids were introduced by the cloning procedure. The sequencing showed that if cloned in front of the βA4 sequence, the signal sequence of the amyloid βA4 precursor protein functioned as a membrane insertion signal.

3.3. *Membrane removal*

To study the effects of membrane damage on the aggregational properties of SPA4CT, we removed the membranes prior to the addition of SDS sample buffer. SPA4CT was sedimentated with the sodium carbonate technique, and the extraction of the membrane lipids was done with chloroform/methanol. The APP fragment was found in the initial methanol/chloroform phase. After further chloroform extractions, SPA4CT partitioned with the water phase. From there it was precipitated with trichloroacetic acid. This treatment resulted in SPA4CT aggregates (Fig. 4, lane 2). Furthermore, heating of this extracted APP fragment enhanced the aggregation process (Fig. 4, lane 3). Heating for 10 min at 100°C without prior membrane extraction also led to aggregation of SPA4CT (data not shown).

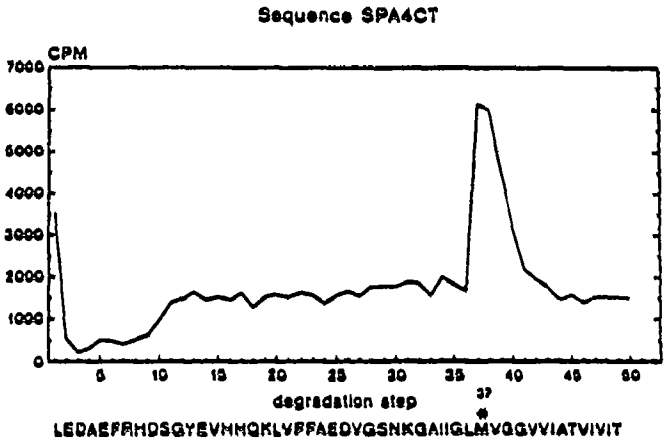

Fig. 3. Radiosequence analysis of SPA4CT translated in the presence of membranes. Translation was as described for Fig. 1. The peak of radioactivity at degradation step 37 is compatible with the expected removal of the 17 residues of the signal sequence of APP encoded by SPA4CT. The amino acid sequence given is that for mature SPA4CT.

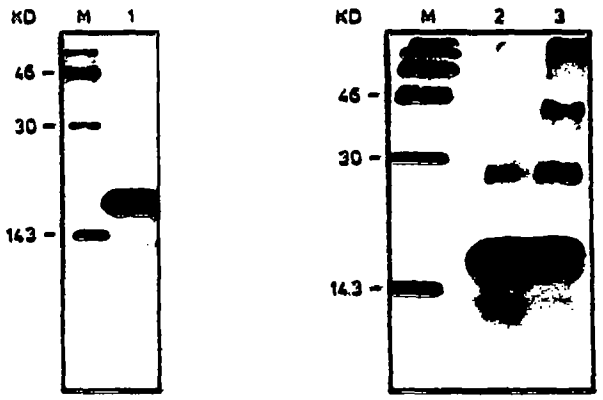

Fig. 4. Chloroform/methanol extraction of membrane-inserted SPA4CT. SPA4CT was translated and sedimentated as described in Fig. 2. Lane 1, sedimentation of SPA4CT translated in the presence of membranes. Lane 2, chloroform/methanol extraction of the same translation product. Lane 3, heating of the extracted SPA4CT for 10 min at 100°C. M, lane with molecular mass marker proteins.

4. DISCUSSION

Previously we showed that expression of the COOH-terminal 100 residues of APP, termed A4CT, in the RRL resulted in an aggregating protein [1]. In this report we provide experimental evidence that membrane-inserted A4CT does not aggregate.

As shown recently, the addition of detergent resolved the complex aggregation pattern of A4CT into two prominent bands at 17 and 26 kDa. We proposed that without detergent, A4CT undergoes a hydrophobic interaction with components of the RRL, and that the two prominent bands in the presence of detergent represent A4CT monomers and -dimers [22].

To analyse the role of membrane damage, which we propose is the prerequisite for amyloid formation [1,8], in A4CT aggregation, we expressed A4CT together with a signal sequence (SPA4CT), which allowed us to synthesize membrane-inserted A4CT proteins.

Expression of SPA4CT without membranes resulted in an aggregation pattern similar to the aggregation pattern of A4CT. The slightly higher molecular mass of the SPA4CT aggregates at approximately 30 kDa is proposed to be due to the retention of the signal sequence, which was not cleaved in the absence of membranes and which accounts for the additional 2 kDa. In the presence of detergent only monomerics could be detected, which indicates that in the absence of membranes, all observed interactions of SPA4CT are through hydrophobic residues. Since A4CT forms dimers under the same conditions, the different behavior of SPA4CT may be due to the additional hydrophobic signal sequence.

By membrane sedimentation and subsequent radiosequencing, we could show that the signal sequence of APP, if cloned in front of the βA4 sequence, is recognized by the signal peptidase and allows membrane insertion of SPA4CT. Also, A4CT expressed without a signal sequence did selectively sediment with membranes. These data suggest that both proteins, A4CT and SPA4CT, were tightly associated with the membrane: SPA4CT due to the signal sequence and co-translational membrane insertion, and A4CT probably due to post-translational hydrophobic interactions of the transmembrane sequence with the membrane lipids. Although both SPA4CT and A4CT can associate with membranes, their aggregational behavior is different.

Expression of SPA4CT in the presence of membranes resulted in only the monomeric molecule. This is not the case for membrane associated A4CT, which is still capable of aggregating. This showed that membrane-insertion driven by the signal sequence of APP interfered with the process of aggregation.

Post-translational removal of the membrane lipids or heating restored the aggregational properties of membrane-inserted SPA4CT. Both procedures, heating and lipid extraction, can lead to membrane disruption. Thus SPA4CT may come in direct contact with the content of the RRL. In the context of our novel findings that the metal-catalyzed oxidation systems contained in the RRL in the form of hemoglobin, hemin and iron transformed non-aggregated A4CT into aggregated A4CT by radical attack and protein crosslinking [22], we would propose that membrane insertion protects the part of SPA4CT relevant for aggregation from direct contact with the radical generation system and thus prevents the aggregation.

From our results we assume that two steps of the process leading to amyloid formation in Alzheimer's disease require membrane damage. First, the COOH-terminal part of the βA4 sequence is contained within the transmembrane domain of APP and is therefore

protected by the lipid bilayer from aberrant proteolytic processing which generates the COOH-terminus of the amyloidogenic βA4 sequence. Thus cleavage at the COOH-terminus of βA4 requires membrane damage.

Second, membrane insertion protects the amyloidogenic fragment A4CT from protein crosslinking through radical attack. As we have shown recently, radical attack transformed non-aggregated βA4-bearing APP fragments into stable aggregated and amyloidogenic molecules [22]. Also membrane damage is required for this amyloidogenic transformation induced by metal-catalyzed oxidation systems.

Because iron is considered to be the most probable agent responsible for lipid peroxidative damage in the brain, radical attack may be involved in several steps in amyloidogenesis in Alzheimer's disease [23]. Radical attack may release βA4-bearing APP-fragments from the lipid bilayer by means of peroxidative damage of the membrane lipids, and subsequent radicals may also transform the released non-aggregated βA4 bearing APP fragments into stable aggregated, and thus amyloidogenic, molecules.

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