ELSEVIER

FEBS Letters 338 (1994) 161–166

FEBS 13585

FEBS LETTERS

Identification of glutamine and lysine residues in Alzheimer amyloid βA4 peptide responsible for transglutaminase-catalysed homopolymerization and cross-linking to α_2M receptor

Lone K. Rasmussen[a], Esben S. Sørensen[a], Torben E. Petersen[a], Jørgen Gliemann[b], Poul Henning Jensen[b,*]

[a]*Protein Chemistry Laboratory, University of Aarhus, The Science Park, DK-8000 Aarhus, Denmark*
[b]*Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*

Received 3 November 1993; revised version received 12 December 1993

Abstract

The β-amyloid peptide (βA4), derived from a larger amyloid precursor protein, is the principal component of senile plaques in Alzheimer's disease. Here we report that the full-length (1–40) synthetic βA4 peptide, containing one glutamine and two lysine residues, is able to form homopolymers in a transglutaminase-mediated reaction. Moreover, transglutaminase catalysed the formation of heteropolymers in reactions of βA4 with α_2M receptor, a constituent of amyloid plaques, and with extracellular matrix proteins. Incorporation of site-specific probes followed by enzymatic digestion and sequencing of tracer-containing fractions demonstrated that both Lys^{16}, Lys^{28} and Gln^{15} in βA4 were susceptible to cross-linking by transglutaminase.

Key words: Alzheimer; β-Amyloid peptide; Transglutaminase; Glutamine; Lysine; α_2M receptor

1. Introduction

A prominent feature of Alzheimer's disease (AD) is the presence of amyloid-bearing senile plaques in the brain. The principal protein component of the amyloid deposits has been characterized as a 39–42 residues peptide, known as βA4 [1,2]. The βA4 peptide is derived from a larger membrane-spanning glycoprotein, the amyloid precursor protein (APP), which is expressed as multiple different mRNA splicing products (for review see [3]). Two main APP processing pathways have been described, one secretory [4–6] and one endocytic [7–9]. APP processing by the secretory pathway involves a cleavage in the βA4 region, releasing the soluble extramembranous portion and retaining a small fragment in the membrane. The endocytic pathway, in contrast, generates C-terminal fragments containing intact βA4. βA4 is a normal proteolytic product of APP and is present in the cerebrospinal fluid of AD patients and controls [10,11].

Recently, βA4 in multimeric forms as well as in aggregates with glycoproteins has been found to be constituents of AD neuritic plaques [12]. Transglutaminase activity has been detected in the brain and characterized as a tissue transglutaminase [13,14]. Transglutaminases (TG) (EC 2.3.2.13) are Ca^{2+}-dependent enzymes capable of forming covalent ε-(γ-glutamyl)lysine cross-links between proteins containing reactive lysines and glutamines (for review, see [15]). It has been proposed that TG is involved in the development of abnormal proteinaceous deposits in neurons [13] and is able to cross-link neuronal proteins [14]. To assess whether TG could take part in the amyloidogenic processes in AD, we examined full-length (1–40) βA4 peptide as a substrate for transglutaminases. While this work was in progress, Ikura et al. [26] reported that a partial-length (1–28) βA4 peptide formed homopolymers in the presence of TG. In this report, we show that βA4 in a TG-catalysed reaction is able to homopolymerize and to form heteropolymers with extracellular matrix proteins as well as α_2M receptor, a recently discovered protein in neurons [16] and in AD plaques [17]. Furthermore, TG-reactive glutamine and lysine residues in βA4 were localized using site-specific probes.

*Corresponding author. Fax: (45) (86) 131 160.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; βA4, β-amyloid peptide; Dns-peptide, dansyl conjugated to Pro-Gly-Gly-Gln-Gln-Ile-Val; PTH, phenylthiohydantoin; TG, transglutaminase; α_2M receptor, α_2-macroglobulin receptor.

2. Experimental

2.1. Reagents

Amyloid β-peptide (amino acids 1–40) was purchased from Bachem, Switzerland. The βA4 was iodinated by incubating 2 μg βA4 in 100 mM NaCl, 70 mM NaH_2PO_4, pH 8.0, 0.07% Triton X-100 with 0.2 mCi ^{125}I

SSDI 0014-5793(93)E1502-D

and 2.5 mg/ml chloramine-T for 1 min at 20°C. The incorporation was terminated by chromatography on a 2 ml Sephadex G-25 column equilibrated in 150 mM NaCl, 0.6 mM $CaCl_2$, 10 mM NaH_2PO_4, pH 7.4, 0.1% Triton X-100. The tracer had a specific activity of 20 mCi/mg. The dansylated peptide (dansyl-Pro-Gly-Gly-Gln-Gln-Ile-Val) (Dns-peptide) was synthesized at Kem-En-Tec, Denmark. Guinea pig liver transglutaminase and thermolysin were from Sigma, USA. Human placental factor XIII zymogen, was purified from Fibrogammin (Behringwerke, Germany, a gift from Dr. E. Schüler) and activated by brief exposure to 0.5 M $CaCl_2$ according to [18]. [1,4-^{14}C]putrescine (109 mCi/mmol), [1,4-^{3}H]putrescine (20 Ci/mmol) and ^{125}I (2.0 Ci/μmol) were from Amersham, UK. The molecular weight markers were from Boehringer Mannheim, Germany.

2.2. Transglutaminase-mediated homo- and heteropolymerization of βA4

All polymerization experiments were carried out in 50 mM Tris, pH 8.5, 0.5 mM DTE, 5 mM $CaCl_2$, and for inhibition of TG-mediated cross-linking either 5 mM EDTA or 50 mM putrescine were added. Homopolymerization was performed with 5 μM βA4 supplemented with ^{125}I-labelled βA4 (5×10^6 cpm/ml) in the presence of 0.1 μM guinea pig liver tissue TG. Incubations were terminated by denaturation in reducing SDS-sample buffer, heated at 95°C for 5 min prior to analysis by SDS-PAGE using 16% Tris-Tricine gel electrophoresis [19].

Heteropolymerization, according to Jensen et al. [20], was performed with murine basement membrane proteins (Matrigel, Collaborative Research, Inc., USA) and purified α_2M receptor stripped for the 40 kDa α_2M receptor associated protein by heparin-Sepharose chromatography [21], in a concentration of 50 μg/ml. The α_2M receptor preparation was a pure α/β chain preparation as judged by silver staining of SDS-PAGE resolved material in agreement with Moestrup and Gliemann [21]. The resulting labelled polymers were analysed by SDS-PAGE using either 16% Tris-Tricine or 8–16% gradient Tris-glycine gels [22] followed by autoradiography.

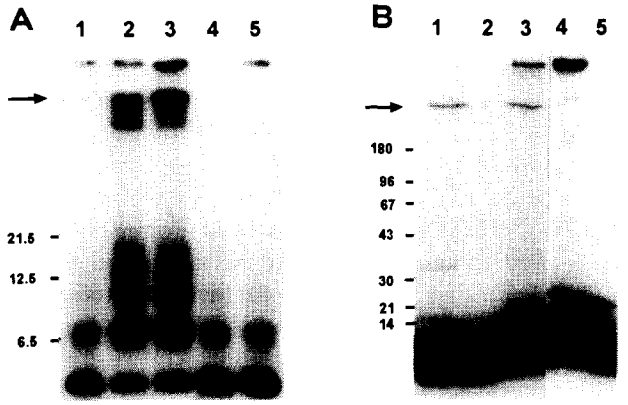

Fig. 1. Transglutaminase-catalysed formation of βA4 homopolymers and cross-linking to α_2M receptor and extracellular matrix proteins. Panel A: Homopolymerization. 5 μM βA4 supplemented with [^{125}I]βA4 (5×10^6 cpm/ml) (lane 1) were incubated with 0.1 μM guinea pig liver TG in 50 mM Tris, pH 8.5, 0.5 mM DTE, 5 mM $CaCl_2$ for 5 min (lane 2) and 60 min (lane 3). Lanes 4 and 5 show incubation with TG for 60 min in the presence of 5 mM EDTA and 50 mM putrescine, respectively. The incubations were terminated by denaturation in reducing SDS-sample buffer, heated at 95°C for 5 min prior to analysis by SDS-PAGE using 16% Tris-Tricine gel electrophoresis. The autoradiogram is presented with the molecular mass markers $\times 10^{-3}$ shown to the left. Panel B: Cross-linking to α_2M receptor and extracellular matrix proteins. ^{125}I-labelled βA4 (5×10^6 cpm/ml) was incubated with TG as in panel A, for 45 min (lane 1), supplemented with 50 μg/ml purified α_2M receptor (lane 3) or 50 μg/ml extracellular matrix proteins (lane 4). The effect of 5 mM EDTA on the polymerization in lane 1 and 4 is shown in lanes 2 and 5, respectively. The samples were analysed using 8–16% Tris-glycine gradients gels. The autoradiogram is presented with the molecular mass markers $\times 10^{-3}$ shown to the left. The arrow marks the interphase between the stacking gel and the resolving gel.

2.3. Labelling of βA4 with site-specific probes

In the glutamine-labelling experiment, βA4 (100 μg at a concentration of 5 μM) was labelled with 13 μCi/ml [^{14}C]putrescine and 20 μCi/ml [^{3}H]putrescine, at a final putrescine concentration of 100 μM, by guinea pig liver transglutaminase (50 nM) in 50 mM Tris, pH 8.5, 5 mM $CaCl_2$, 0.5 mM DTE at 37°C for 24 h. The reaction was stopped by precipitation in 20% trichloroacetic acid. The precipitate was washed with acetone in order to remove unincorporated radiolabelled putrescine. Incorporation of Dns-peptide was performed in 0.1 M Tris, 2.5 mM $CaCl_2$, 20 mM DTE, pH 8.5 using 1.74 μM Dns-peptide to 33 nmol βA4 (final concentrations). Guinea pig liver transglutaminase (2.3 nmol) was added and the mixture was incubated at 37°C for 18 h.

2.4. Purification and characterization of radiolabelled-putrescine- and Dns-peptide-labelled βA4 peptides

DNS-peptide-labeled βA4 was purified by HPLC to remove unincorporated DNS-peptides. Fractions were analysed by SDS-PAGE using a 16% Tris-Tricine gel under reducing conditions. The gel was photographed under UV-light and stained with Coomassie brilliant blue. Radiolabelled putrescine- and Dns-peptide-labelled βA4 were subjected to thermolysin digestion using an enzyme/substrate ratio of 1:25 (w/w) in 0.1 M pyridine-acetate pH 6.5, 5 mM $CaCl_2$ at 55°C for 18 h. Separation of peptides was carried out by reverse-phase HPLC on a Pharmacia LKB HPLC system. Incorporation of radiolabelled putrescine was determined by liquid scintillation counting (Beckman LS 1801). Fluorescently labelled βA4 and peptides were dissolved in Eppendorf tubes, visualized in UV-light and photographed. Automated Edman degradation was carried out on an ABI 477A/120A protein sequencer (Applied Biosystems Inc., Foster City, USA) using standard programs. The small volume of the phenylthiohydantoin derivate sample left over after on-line injection was used for radioactivity detection as described above. Peptides were hydrolyzed in 6 M HCl at 110°C for 16 h in evacuated sealed tubes. The amino acid composition was determined essentially as described by Barkholt and Jensen [23].

3. Results

3.1. Homopolymerization of βA4 and cross-linking to α_2M receptor and extracellular matrix proteins

In Fig. 1 panel A, the ability of [^{125}I]βA4 to homopolymerize in the presence of TG is shown by SDS-PAGE. The reaction was Ca^{2+}-dependent (lane 4) and abrogated by the competitive inhibitor putrescine (lane 5). The 4.2 kDa βA4 polymerized to multimeric forms of 7.9 kDa, 11.9 kDa, 15 kDa and 18.8 kDa. Unlabelled βA4 polymerized to a similar extent as judged by silver staining of the gel. The polymerization was performed at a TG/substrate ratio of 1:50 but could be carried out to a similar extent with a ratio of 1:200 (data not shown).

Fig. 1, panel B demonstrates that the presence of α_2M receptor (lane 3), a recently discovered constituent of amyloid plaques [17], and a mixture of extracellular matrix proteins (lane 4) enhanced the formation of TG-cross-linked ^{125}I-labelled βA4 in a reaction sensitive to EDTA (lane 2 and lane 5). The results presented above were obtained using guinea pig liver transglutaminase. Furthermore, purified activated human FXIII catalysed the incorporation of radiolabelled-putrescine in βA4 in a Ca^{2+}-dependent reaction (data not shown).

3.2. Identification of transglutaminase acceptor and donor sites in βA4

Radiolabelled putrescine and Dns-peptide were used

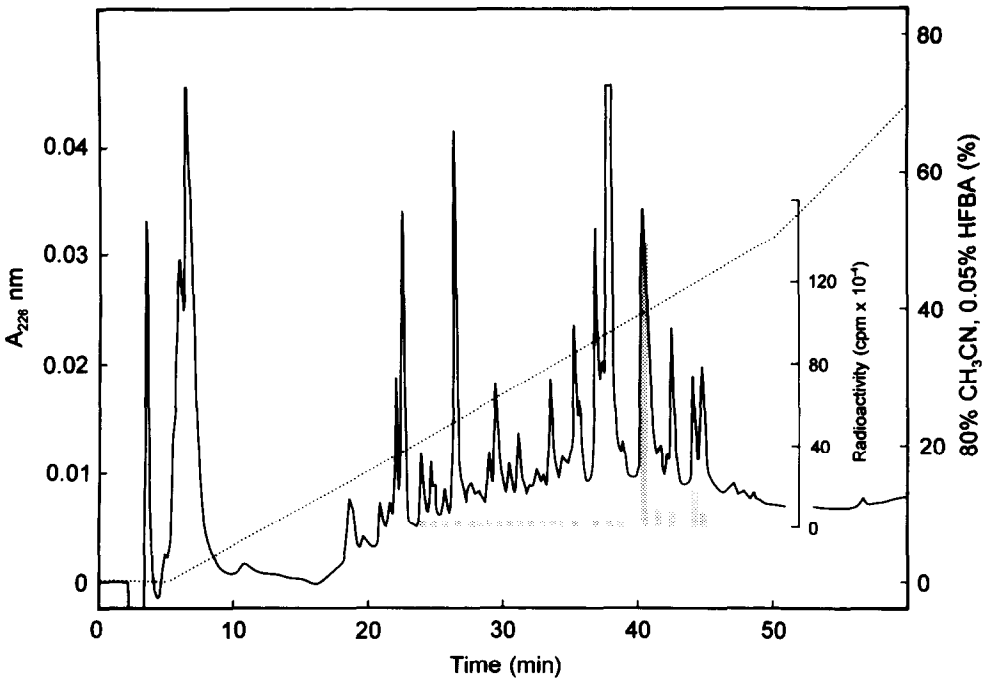

Fig. 2. Separation of a thermolysin digest of [^{14}C]putrescine-labelled βA4 was digested with thermolysin. The resulting peptides were separated on a Vydac C_{18} (10 μm) reverse-phase HPLC column using 0.05% heptafluoroacetic acid (solvent A) and 0.05% heptafluoroacetic acid in 80% acetonitrile (solvent B) with a stepwise linear gradient (...). The flow rate was 0.5 ml/min and the column temperature was 40°C. Peptides were detected in the effluent by recording the absorbance at 226 nm (——) and collected manually. Hatched bars indicate total amount of radioactivity in the respective peaks as determined by liquid scintillation counting.

as site-specific substrates for the TG-catalysed incorporation of radioactive putrescine on the γ-glutaminyl residues [24], and of the fluorescently dansylated peptide on the ε-amino groups of lysines [25].

Only one major radioactively labelled peak was found in the thermolysin digest of [^{14}C]putrescine-labelled βA4 (Fig. 2). Sequence analysis of this peak showed one major sequence: Val-His-His-Gln-Lys corresponding to residues 12–16 in βA4. In the sequence analysis a low yield of PTH-Gln was observed in cycle 4, indicating a putrescine-linked glutamine. Scintillation counting of the PTH-amino acids showed radioactivity in cycle 4, thus confirming incorporation of putrescine at Gln^{15} (data not shown).

The Dns-peptide-labelled βA4 was purified by reverse-phase HPLC (Fig. 3A). The fractions were monitored by SDS-PAGE followed by fluorescent detection of the gel in UV-light (Fig. 3B, left) and subsequent staining with Coomassie brilliant blue (Fig. 3B, right). Coomassie staining of the last eluting fraction (peak 3) resulted in a ~4 kDa band (lane 4 right) coinciding with a fluorescent band (lane 4 left), thus showing that this fraction contained the Dns-peptide labelled βA4. The major fractions eluting at approximately 40–45 min consisted of unincorporated Dns-peptide, since only fluorescent bands were observed (lane 3, left compared with lane 3, right). Lack of detectable bands in the Coomassie stained gel is due to the inability of the Dns-peptide to bind the dye. No fluorescent labelling of βA4 occurred in a control experiment in which 50 mM EDTA was included (data not shown).

The purified Dns-peptide-labelled βA4 was digested with thermolysin and the resulting peptides were separated by reverse-phase HPLC (Fig. 4A). The fractions were evaporated, dissolved in 0.1% trifluoroacetic acid and the Dns-peptide containing fractions were visualized in UV-light (Fig. 4B). As seen, only peaks 3 and 6 were found to contain the fluorescent probe. Sequencing of peak 3 gave the sequence: Val-Gly-Ser-Asn-Xxx-Gly-Ala, where Xxx denotes that no PTH-amino acid was detected in that cycle. This sequence aligns with residues 24–30 in βA4, assuming that the missing PTH-amino acid in cycle 5 is a Dns-peptide-linked lysine residue. Sequence analysis of peak 6 showed the sequence: Val-His-His-Gln-Xxx, indicating that this corresponds to residues 12–16 in βA4 with a C-terminal Dns-peptide-linked lysine residue. Amino acid analysis verified the identities of the fluorescently labelled peptides, and further showed that the C-terminal Val residue of the Dns-peptide was cleaved off by thermolysin as would be expected. Thus, we conclude that Gln^{15} as well as both Lys^{16} and Lys^{28} in βA4 act as amine acceptor and donor sites, respectively, in a TG-mediated incorporation of site-specific probes.

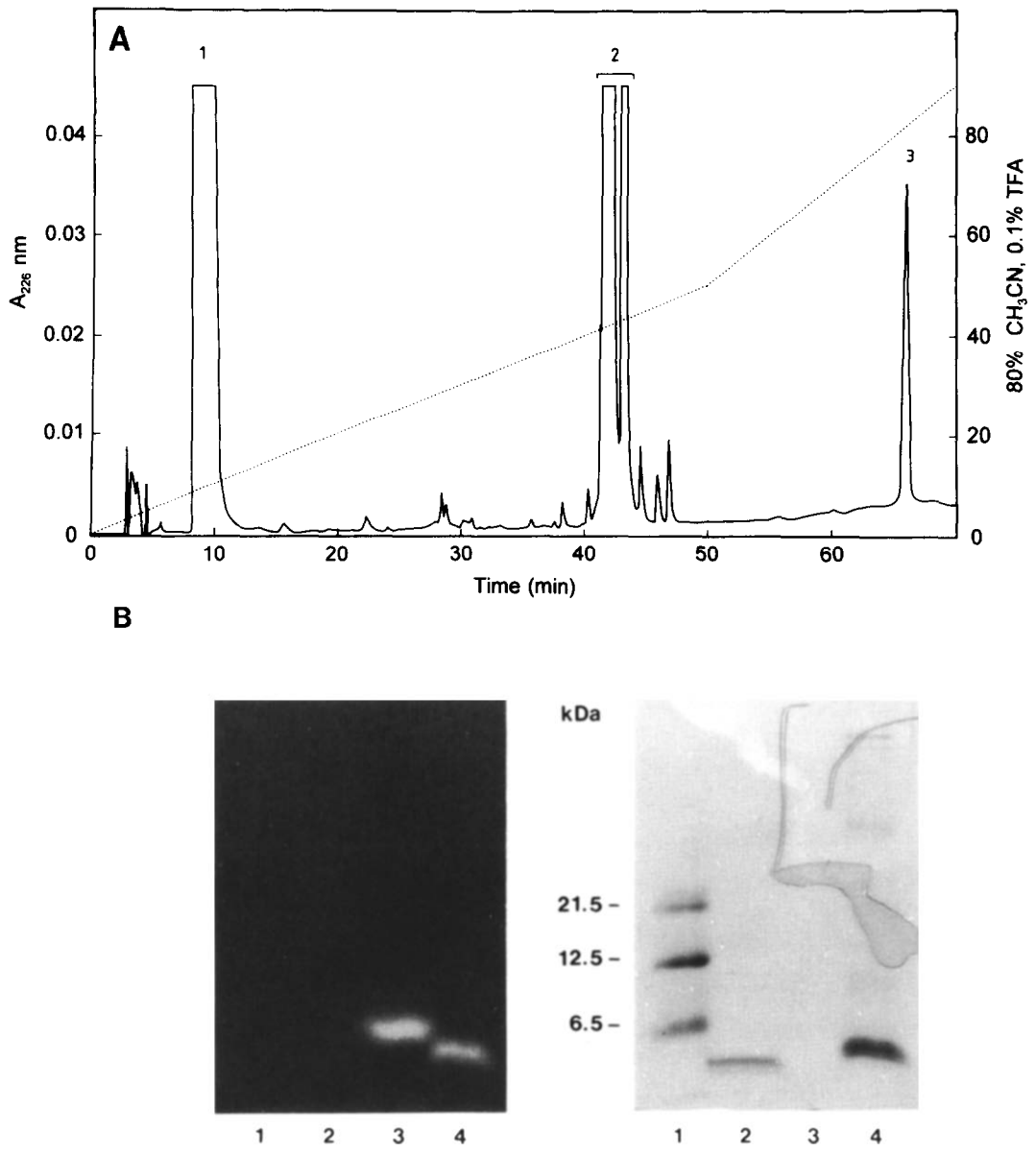

Fig. 3. (A) Purification of Dns-peptide-labelled βA4 by HPLC. βA4 labelled with Dns-peptide was purified on a Vydac C_{18} (10 μm) reverse-phase HPLC column eluted with a stepwise linear gradient (...) of 80% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min and the column temperature was 40°C. The effluent was monitored at 226 nm (——). (B) Fractions were analysed by SDS-PAGE using a 16% Tris-Tricine gel under reducing conditions. The gel was photographed under UV-light (left) and stained with Coomassie brilliant blue (right). Lane 1, molecular mass markers; lane 2, βA4 peptide; lane 3, fraction 2; lane 4, fraction 3.

4. Discussion

The present results are in general agreement with the recent finding that a partial-length (1–28) βA4 peptide is a substrate for TG [26]. Whether Lys^{16} and/or Lys^{28} participate in the cross-linking of βA4 remains to be elucidated. By using two modified versions of the partial-length βA4 peptide (Lys^{16} or Lys^{28} replaced by L-norleucine) Ikura et al. [26] found that Lys^{16} was involved exclusively in the polymerization. However, exclusion of Lys^{28} as a potential donor site is questionable, since this amino acid is positioned C-terminal in the partial-length peptide. βA4 being a TG-substrate might explain the presence of βA4 in large proteinaceous aggregates isolated from plaques [12].

TG-mediated cross-linking of βA4 may be relevant to amyloid deposition in AD. Cell membrane damage may be an early event in the disease [27] and certainly occurs as the lesions develop. This might cause an increase in the intracellular Ca^{2+} concentration and activation of TG, as well as a leak of cytosolic TG to the extracellular environment. Normal processing of APP occurs within

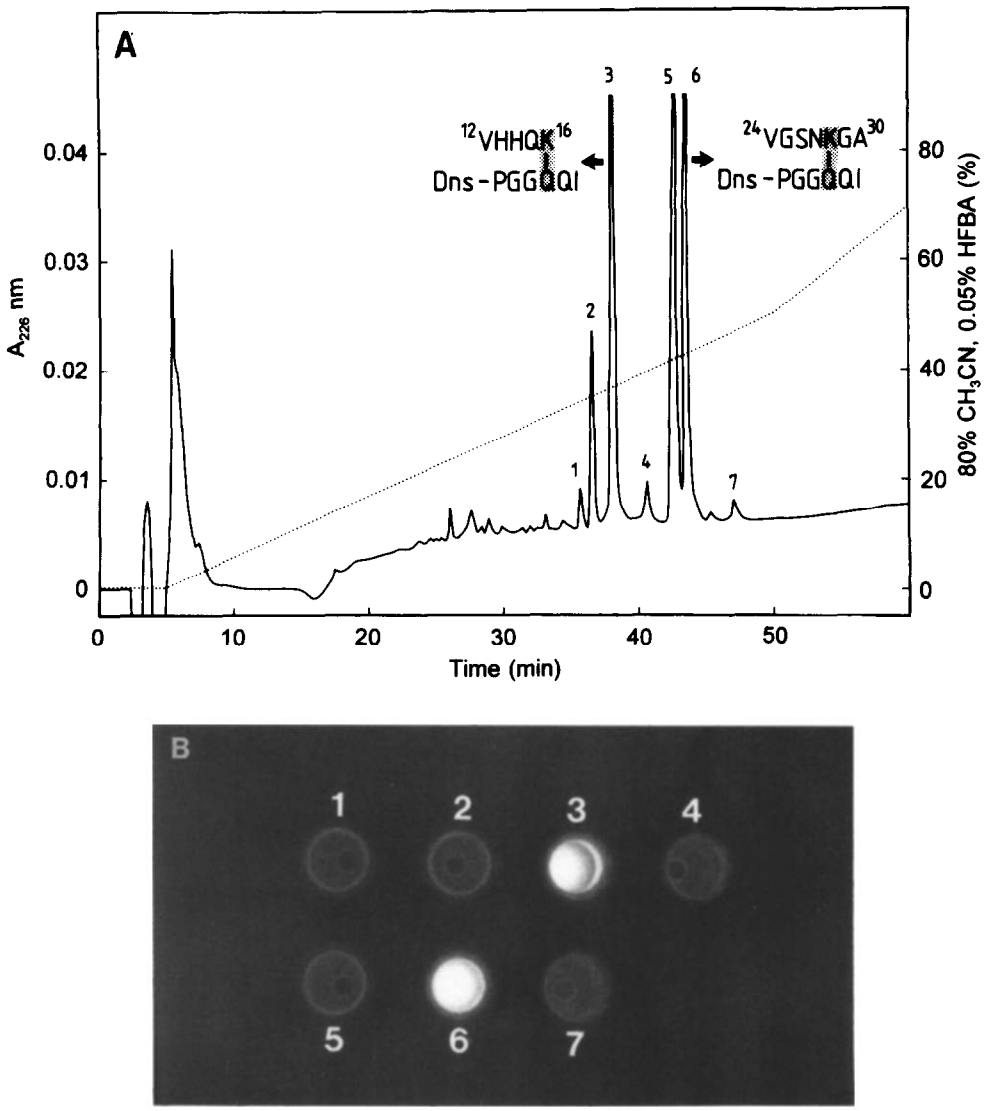

Fig. 4. (A) Separation of a thermolysin digest of Dns-peptide-labeled βA4. βA4 labelled with the Dns-peptide was digested with thermolysin and the resulting peptides were separated by reverse-phase HPLC using the same conditions as in Fig. 2. (B) Fluorescent detection. The isolated peptides from the thermolysin digest were evaporated, dissolved in 0.1% trifluoroacetic acid and photographed under UV-light. Fluorescent-containing peaks, 3 and 6 were subjected to amino acid composition and sequence analysis.

the βA4 sequence between Gln^{15} and Lys^{16} or Lys^{16} and Leu^{17} [4–6]. Assuming APP like βA4 is a TG-substrate, cross-linking of Gln^{15} and Lys^{16} might impair the normal secretase cleavage. Based on our results and integrating recent studies, we propose a working hypothesis for the formation and growth of senile plaques. In normal cells, normal processing of APP results in slow secretion of βA4 which is matched by an unknown clearance mechanism resulting in low extracellular βA4. The initial steps of the abnormal processing of APP are generally unknown. However, extracellular βA4 accumulation causes (i) significant binding of βA4 to complement factor 1 and activation of the complement cascade [28] which damages the cell, and (ii) precipitation of βA4 with components of plaques, e.g. ApoE4, a βA4-binding protein [29,30] and α_2M [31]. Cross-links between some βA4 molecules and other TG-substrates e.g. α_2M [32] are formed by activated TG. The cross-link-stabilized precipitate presents several epitopes for binding to the multi-ligand and multi-site receptor α_2M receptor which is overexpressed in this state [17]. The endocytosis is thought to be partially futile because the βA4 moieties are poorly degraded in lysosomes [33]. Finally, the cell dies, and debris including βA4 is embedded in senile plaques together with α_2M receptor, apoE4, α_2M and several other constituents.

Future studies should show whether the multimeric forms of βA4 and aggregates found in AD brain are in fact the result of such transglutaminase reactions.

Acknowledgements: We thank H.A. Due and N. Jørgensen for technical assistance. This work was supported by the Danish Cancer Society.

References

[1] Glenner, G.G. and Wong, C.W. (1984) Biochem. Biophys. Res. Commun. 120, 885–890.
[2] Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L. and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. USA 82, 4245–4249.
[3] Holtzmann, D.M. and Mobley, W.C. (1991 Trends Biol. Sci. 16, 140–144.
[4] Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) Science 248, 1122–1124.
[5] Sisoda, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A. and Price, D.L. (1990) Science 248, 492–495.
[6] Wang, R., Meeschia, J.F., Cotter, R.J. and Sisodia, S.S. (1991) J. Biol. Chem. 266, 16960–16964.
[7] Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. and Younkin, S.G. (1992) Science 255, 728–730.
[8] Caporaso, G.L., Gandy, S.E., Buxbaum, J.D. and Greengard, P. (1992) Proc. Natl. Acad. Sci. USA 89, 2252–2256.
[9] Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. and Selkoe, D.J. (1992) Nature 357, 500–503.
[10] Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I. and Schenk, D. (1992) Nature 359, 325–327.
[11] Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.-D., McKay, D.M., Tintner, R., Frangione, B. and Younkin, S.G. (1992) Science 258, 126–129.
[12] Roher, A.E., Lowenson, J.D., Clarke, S., Wolkow, C., Wang, R., Cotter, R.J., Reardon, I.M., Zürcher-Neely, H.A., Heinrikson, R.L., Ball, M.J. and Greenberg, B.D. (1993) J. Biol. Chem. 268, 3072–3083.
[13] Selkoe, D.J., Abraham, C. and Ihara, Y. (1982) Proc. Natl. Acad. Sci. USA 79, 6070–6074.
[14] Miller, C.C.J. and Anderton, B. (1986) J. Neurochem. 46, 1912–1922.
[15] Greenberg, C.S., Birckbichler, P.J. and Rice, R.H. (1991) FASEB J. 5, 3071–3077.
[16] Moestrup, S.K., Gliemann, J. and Pallesen, G. (1992) Cell Tiss. Res. 269, 375–382.
[17] Tooyama, I., Kawamata, T., Akiyama, H., Moestrup, S.K., Gliemann, J. and McGeer, P.L. (1993) Mol. Chem. Neuropathol. 18, 153–160.
[18] Traoré, F. and Meunier, J.-C. (1991) J. Agric. Food Chem. 39, 1892–1896.
[19] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[20] Jensen, P.H., Lorand, L., Ebbesen, P. and Gliemann, J. (1993) Eur. J. Biochem. 214, 141–146.
[21] Moestrup, S.K. and Gliemann, J. (1991) J. Biol. Chem. 266, 14011–14017.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Barkholt, V. and Jensen, A.L. (1989) Anal. Chem. 177, 318–322.
[24] Lorand, L. and Conrad, S.M. (1984) Mol. Cell. Biochem. 58, 9–35.
[25] Lorand, L., Parameswaran, K.N. and Velasco, P.T. (1991) Proc. Natl. Acad. Sci. USA 88, 82–83.
[26] Ikura, K., Takahata, K. and Sasaki, R. (1993) FEBS Lett. 326, 109–111.
[27] Dyrks, T., Dyrks, E., Masters, C. and Beyreuther, K. (1992) FEBS Lett. 309, 20–24.
[28] Rogers, J., Cooper, N.R., Webster, S., Schultz, J., McGeer, P.L., Styren, S.D., Civin, W.H., Brachova, L., Bradt, B., Ward, P. and Lieberburg, I. (1992) Proc. Natl. Acad. Sci. USA 89, 10016–10020.
[29] Wisniewksi, T. and Frangione, B. (1992) Neurosci. Lett. 135, 235–238.
[30] Strittmatter, W.J., Weisgraber, K.H., Huang, D., Dong, L.-M., Salvesen, G.S., Pericak-Vance, M., Schmechel, D., Saunders, A.M., Goldgaber, D. and Roses, A.D. (1993) Proc. Natl. Acad. Sci. USA 90, 8098–8102.
[31] van Gool, D., de Strooper, B., van Leuven, F., Triau, E. and Dom, R. (1993) Neurobiol. Aging 14, 233–237.
[32] Mortensen, S.B., Sottrup-Jensen, L., Hansen, H.F., Rider, D., Petersen, T.E. and Magnusson, S. (1981) FEBS Lett. 129, 314–317.
[33] Knauer, M.F., Soreghan, B., Burdick, D., Kosmoski, J. and Glabe, C.G. (1992) Proc. Natl. Sci. USA 89, 7437–7441.