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Secretory cleavage site of Alzheimer amyloid precursor protein is heterogeneous in Down's syndrome brain

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Abstract Aβ (β/A4) is the major constituent of brain amyloid in Alzheimer's disease (AD), Down's syndrome (DS) and normal aged persons. This protein is presumably derived by normal proteolysis from a precursor protein (APP). In this study, C-terminal fragments of APP in a Tris/Triton soluble fraction were partially purified from DS brain by heparin-affinity and reverse phase chromatography, and analyzed by N-terminal amino acid sequencing after SDS polyacrylamide gel electrophoresis and Western blotting. We found at least six different C-terminal fragments including those with the entire Aβ region. These results suggest that secretory processing of APP is heterogeneous and generates amyloidogenic C-terminal fragments.

Key words: Alzheimer's disease; Down's syndrome; Amyloid β protein precursor; Aβ

1. Introduction

Aβ (β/A4) is the major constituent of senile plaque and cerebrovascular amyloid in Alzheimer's disease (AD), Down's syndrome (DS) and normal aged persons [1–3]. This protein is thought to be derived by proteolysis from a transmembrane glycoprotein precursor, termed APP [2,4,5]. The C-terminal one-third of the Aβ sequence spans part of the transmembrane domain and N-terminal two-thirds extends into the adjacent extracellular domain of APP [4]. Secretory forms of APP have been detected in brain and cerebrospinal fluid (CSF) [6–8]. In previous studies, APP was found to be cleaved in the interior of the Aβ sequence, which would preclude amyloid formation [9–11]. Recent studies show that an intact Aβ is produced in soluble form during normal cellular metabolism [12–14] and is released into the CSF even in normal subjects [12,13]. One possible source of Aβ is cell surface APP that has been reinternalized and metabolized in the lysosome [15,16]. Alternatively, intact Aβ is also left behind on the amyloidogenic C-terminal fragment of APP produced by alternative processing at or near the cell surface [7,8,17]. In order to shed light on the origin and mechanism of amyloid formation, we are attempting to fully characterize the various derivatives of APP in the brain itself. We have previously reported that the secretory form of APP-695 in human brain and CSF lacks β/A4 immunoreactivity, suggesting alternative processing of APP to leave the entire Aβ sequence behind on the membrane [7,8]. To confirm this result, we have analyzed C-terminal fragments of APP in the membrane fraction of DS brain.

2. Materials and methods

2.1. Isolation of C-terminal fragments

DS brain cortex (55-year-old female) was homogenized with a teflon-glass homogenizer in 9 vol. of 10 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 0.5 mM PMSF and 1 mM EDTA and centrifuged at 47,400 × *g* for 60 min. The pellet was rehomogenized and centrifuged as before. The pellet was homogenized in 10 mM Tris-HCl, 0.2 M NaCl, 0.5% Triton X-100, 0.1 mM PMSF, 1 mM EDTA, pH 7.5, and centrifuged again. The supernatant fraction was applied on an Affigel-Heparin (Bio-Rad) column. The unbound fraction was applied on an Aquapore Prep-10 C-4 (Perkin Elmer) HPLC column and eluted with 60% acetonitrile. For further purification, the eluted fraction was adjusted to 20% acetonitrile/0.05% TFA. This fraction was applied on a column of Bakerbond Wide-Pore C4 (Baker Bond) and eluted with acetonitrile (20–75%) in 0.05% TFA. The anti-C-terminal R37 immunoreactive fractions were pooled.

2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Purified APP was analyzed with SDS-PAGE (15–25% gradient Laemmli gel and 10–16.5% gradient gel of Tris-Tricine system) and immunoblotting [18–20]. For N-terminal amino acid sequencing, the Laemmli system was used since the difference of the resolution of C-terminal fragments between Laemmli system and Tris-tricine system was not seen. Antibodies to subsequences of APP, 757 (Aβ 1–10) and C-terminal R37 (residues 681–695 of APP-695), were produced as described previously [21]. Antiserum to Z31 (residues 577–596 of APP-695) was a gift from Dr. H. Yamaguchi.

2.3. N-Terminal amino acid sequence analysis of C-terminal fragments

Partially purified C-terminal fragments, derived from APP, were separated by SDS-PAGE and then electroblotted onto a PVDF membrane [22]. The C-terminal fragment bands were cut out and applied on an automatic protein sequencer (Perkin Elmer 477A/120A).

3. Results and discussion

C-terminal fragments obtained from TB/T fraction had molecular weights of 12–16 kDa (Laemmli system, as shown in Fig. 1) and 8–12 kDa (Tris-Tricine system, data not shown). These bands were labelled with anti-C-terminal R37 antibody and none of them were labelled with antiserum to Z31. The anti-757 antibody to Aβ (1–10) appeared to react with at least two bands in the molecular weight range 15–16 kDa. A band at 16 kDa was the same molecular size as APPC-100 [23], as shown in Fig. 1. C-terminal fragment fractions were further separated by reverse phase HPLC [23] as shown in Fig. 1. The N-terminal amino acid sequences of each fraction are listed in Table 1 and summarized in Fig. 2. From this data it is clear that none of the sequenced bands are derived from APP-like molecules [24,25]. We were not able to determine N-terminal sequences of other anti-R37 positive fractions because of contamination with other proteins and/or blocking of their N-termini.

Previously, we reported that some secreted APP-695 in

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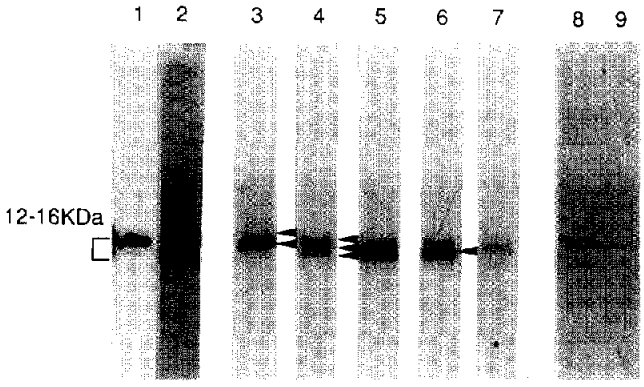

Fig. 1. Immunoblots of partially purified C-terminal fragments of APP from DS brain. Lanes 1–7: anti-C-terminal R37 antibody; lanes 8 and 9: anti-757 antibody. Lane 1, APP-C100 [23]; lane 2, crude C-terminal fragment of APP; lane 3 and 8, reverse-phase HPLC fr. 35, lane 4, fr. 36; lane 5 and 9, fr. 37; lane 6, fr. 38; lane 7, fr. 39. Bands corresponding to identified N-terminal amino acid sequence are indicated by arrowheads (35a and 35b from fraction 35, lane 3; 36a, b, and c from fraction 36, lane 4; and 38 from fraction 38, lane 6, with 'a' being in each lane the uppermost arrowhead).

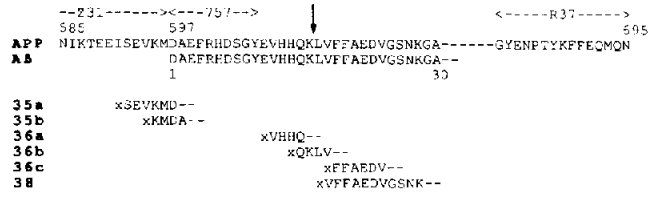

Fig. 2. Summary of N-terminal amino acid sequences of C-terminal fragments derived from APP. Arrow indicates the cleavage site of 'alpha-secretase' [9–11]. Aβ consists of 39–43 amino acid residues with ragged C-termini [2].

human CSF and brain is produced by an alternative cleavage on the N-terminal side of Aβ, and that this alternative cleavage would leave behind a membrane-bound fragment containing the whole of the Aβ sequence, which could be further processed to release the amyloid peptide [7,8]. Seubert et al. have also reported that a substantial portion of the APP secreted by human mixed brain cell cultures, as well as that present in human CSF is of a novel form cleaved precisely at the amino terminus of Aβ [17]. To confirm these results, we have analyzed the C-terminal fragments of APP in the membrane fraction of DS brain.

We detected at least six different lengths of C-terminal fragment as shown Fig. 2. These results suggest that the secretory processing of APP may be heterogenous in DS brain, although some effects of post-mortem proteolysis cannot be completely ruled out. However, similar results to ours have been reported recently by Zhong et al. and Cheung et al. [26,27] in culture cells, who noted cleavage of APP near the membrane surface at several different sites. Additionally, a C-terminal fragment starting at residue −30 (Aβ numbering) has been reported in lymphoblastoid cells obtained from patients with early-onset and late-onset familial AD, and in platelets of normal subjects [28,29].

Table 1
Amino acid sequences of the C-terminal fragments derived from APP

	Yield of PTH-amino acid (pmol)					
Cycle	35a	35b	36a	36b	36c	38c
1	X	X	X	X	X	X
2	S 1.48	K 1.33	V 1.28	Q 1.85	F 2.81	V 4.23
3	E 2.56	M 0.62	H 1.61	K 1.39	F 2.99	F 1.70
4	V 1.02	D 0.92	H 1.87	L 0.67	A 4.76	F 2.48
5	K 0.88	A 0.75	Q 1.27	V 1.08	E 1.28	A 4.93
6	M 0.72				D 4.41	E 1.83
7	D 1.64				V 1.60	D 3.90
8						V 1.33
9						G 4.11
10						S 1.48
11						N 2.46
12						K 1.32

Unidentifiable residues are shown X.

Processing at -Glu^{-7}-Ile^{-6} and -Glu^{-4}-Val^{-3} sites would generate C-terminal fragments including the complete Aβ sequence, with 3 or 6 additional amino acids (see Fig. 2). This would be compatible with our previous reports of secretory forms of APP in CSF [8] and brain [7] that appear to lack the Z31 sequence immediately prior to the amyloid region. Recently, it has been reported that N-terminal sequences of soluble Aβ and the 3 kDa product generated by subsequent processing of the non-amyloidogenic C-terminal fragment are heterogeneous [12,13,30–34]. The various N-terminal sequences described in these reports are comparable to the secretory processing sites of APP described here. Our results suggests that soluble Aβ and the 3 kDa product are generated from C-terminal fragments after secretory processing. However, we cannot rule out the possibility that Aβ is generated directly from intact APP via the lysosomal passway. Further extensive studies will be required to clarify this point.

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