Secretion of long Aβ-related peptides processed at ε-cleavage site is dependent on the α-secretase pre-cutting

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Received 3 June 2004; revised 14 June 2004; accepted 14 June 2004

Available online 22 June 2004

Edited by Barry Halliwell

Abstract Aβ is the major component of amyloid in the brain in Alzheimer's disease and is derived from Alzheimer amyloid precursor protein (APP) by sequential proteolytic cleavage involving α-, β- and γ-secretase. Recently, γ-secretase was shown to cleave near the cytoplasmic membrane boundary of APP (called the ε-cleavage), as well as in the middle of the membrane domain (γ-cleavage). However, the precise relationship between γ- and ε-cleavage is still unknown. In this paper, I analyzed Aβ-related peptides using immunoprecipitation and liquid chromatography ion trap mass spectrometer and found some long Aβ-related peptides, starting at Aβ residues 16Lys–23Asp and ending at 43Thr–52Leu, in the culture media of COS-1 cells and in human brain extract. These results indicated that longer Aβ-related peptides cleaved at ε-cleavage site were secreted under normal conditions and were dependent on the α-secretase cleavage products.

Keywords: Amyloid precursor protein; Amyloid; Aβ; γ-Secretase; ε-Cleavage; LC/MS/MS

1. Introduction

A significant accumulation of Aβ amyloid is a major pathological feature in the brain in patients with Alzheimer's disease (AD). Amyloid precursor protein (APP) is cleaved by either α- or β-secretase, resulting in secretion of the N-terminal extracellular domain of this protein. Then, the remaining membrane-anchored C-terminal fragment (C83, α-secretase product or C99, β-secretase product) releases P3 or Aβ by γ-secretase cleavage in the middle of the transmembrane domain. This cleavage generates the APP intracellular domain (AICD). Therefore, Aβ is believed to be derived from APP by sequential proteolytic processing involving β- and γ-secretase (Fig. 1, reviewed in [1]).

However, recent reports indicate that AICD is generated by a proteolytic cleavage between amino acids 49 and 50 of the Aβ domain near the cytoplasmic membrane boundary of C-terminal fragment (named ε-cleavage, Fig. 1), rather than by a proteolytic cleavage at the γ-cleavage site in the middle of the membrane domain [2–4]. In addition, AICD binds to a DNA binding protein, Fe65, and translocates to the nucleus [5]. This suggests that like the Notch intracellular domain, AICD plays a role in signal transduction [6]. It has also been reported that γ- and ε-cleavage are clearly dependent on presenilin-1 [3,4,7]. However, the precise relationship between γ- and ε-cleavage is unknown. For many studies related to pathogenesis of AD, it is necessary to determine the relationship between γ- and ε-cleavage. To clarify this relationship, I have been studying APP metabolic pathway. In this paper, I analyzed Aβ-related peptide in the culture media of COS-1 cells and in human brain extract, using immunoprecipitation and liquid chromatography ion trap mass spectrometer (LC/MS/MS). Long Aβ-related peptides processed at ε-cleavage site were secreted under normal conditions.

2. Materials and methods

2.1. Cell culture and transfection

COS-1 cells were cultured as described previously [8]. The C83 and C99 constructs have been reported [9]. Transient expressions of these constructs in COS-1 cells were achieved using LipofectAMINE 2000 (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. After transfection, COS-1 cells were cultured for 72 h and then the culture medium was used for LC/MS/MS analysis.

2.2. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Metabolic labeling with [^{35}S]methionine and immunoprecipitation with antibodies to APP C-terminus, R37 [10], and to Aβ 17–31, E50 [11], were carried out as described previously [8]. The associated Aβ-related peptides were separated in a 12.5% polyacrylamide gel using Tris–tricine system [12]. After electrophoresis, the gel was dried and processed for autoradiography using BAS 3000 (Fuji Photo Film Co., Ltd., Tokyo).

2.3. Immunoprecipitation of Aβ-related peptides from the culture medium

The capture antibody, E50 (20 μl), was added to 1 ml of the culture medium. After incubation at 4 °C for 16 h, 30 μl of UltraLink Immobilized protein A/G (Pierce, Rockford, IL) was added. The mixture was shaken for 2 h and centrifuged at 5000 × *g* for 5 min. The resulting precipitate was washed with 50 mM Tris–HCl/150 mM NaCl, pH 7.6 (TBS). Immunoreactive Aβ-related peptides were extracted from the resin with 30% formic acid/20% acetonitrile solution. The extract was filtered through a Microcon YM-50 membrane filter (Millipore, Billerica, MA), diluted, and dried in a Speedvac. The resulting extract was dissolved in 50 μl of 2% formic acid/2% acetonitrile solution.

2.4. Immunoprecipitation of Aβ-related peptides from human brain extract

Human brain tissue (0.45 g cortex from a 75-year-old normal male) was homogenized with a Teflon-glass homogenizer in 9 vol of 0.25 M sucrose/10 mM Tris–HCl, pH 7.6, containing protease inhibitor cocktail. After centrifugation at 105 000 × *g* for 60 min, the supernatant fraction (1 ml) was used for the immunoprecipitation with E50 (20 μl) as described above.

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doi:10.1016/j.febslet.2004.06.034

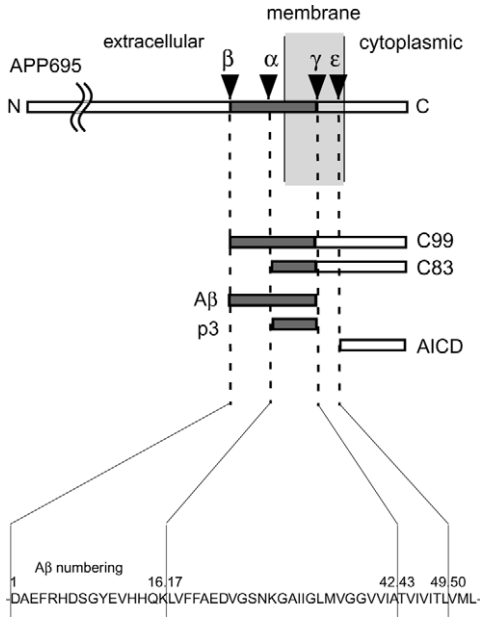


Fig. 1. Schematic diagram of APP and its derivatives. Arrowheads indicate major cleavage sites of α-, β-, γ-, and ε-cleavage.

2.5. LC/MS/MS analysis

Ten μl of the above extract was applied to a Paradigm MS4 (Microm BioResources, Inc., Auburn, CA) HPLC system fitted with HTC-PAL automatic sampler (CHROMSYS LLC, Alexandria, VA). A reverse phase capillary column (ODS-HG5, 0.15 mm i.d. × 50 mm, Nomura Chemical Co., Ltd., Seto, Japan) was used at a flow rate of 2 μl/min with a 4–72% linear gradient of acetonitrile in 0.1% formic acid. Eluted Aβ-related peptides were directly detected with ion trap mass spectrometer, LCQ Advantage (Themo Electron Corporation, Waltham, MA). The obtained data were analyzed with SEQUEST (Themo Electron Corporation, Waltham, MA).

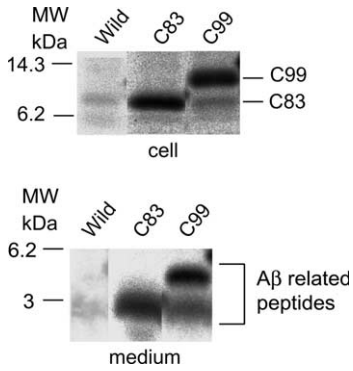


Fig. 2. The generation of APP C-terminal fragments, C83 and C99, and Aβ-related peptides in wild-type COS-1 cells, COS-1 cells transfected with C83 and COS-1 cells transfected with C99. Immunoprecipitates with R37 (upper panel) and with E50 (lower panel) were separated on Tris–tricine system SDS–PAGE. C83, C99 and Aβ-related peptides were shown.

3. Results and discussion

As shown in Fig. 2, a faint Aβ-related band was observed in wild-type COS-1 cells, while clear Aβ-related bands were observed in COS-1 cells transfected with C83 or C99. To analyze these Aβ-related peptides, I carried out immunoprecipitation with E50, followed by LC/MS/MS analysis. Immunoprecipitation followed by MS is reliable, easy method for the identification and semi-quantification of Aβ [13–15]. Using this technique, however, some Aβ molecules are indistinguishable, e.g., Aβ 2–41(4326.213, monoisotopic molecular mass) and 3–42 (4326.213), or Aβ 6–37 (3454.721) and 5–35 (3454.732). Therefore, the information of amino acid sequence is necessary for identifying these peptides. Consequently, I combined the

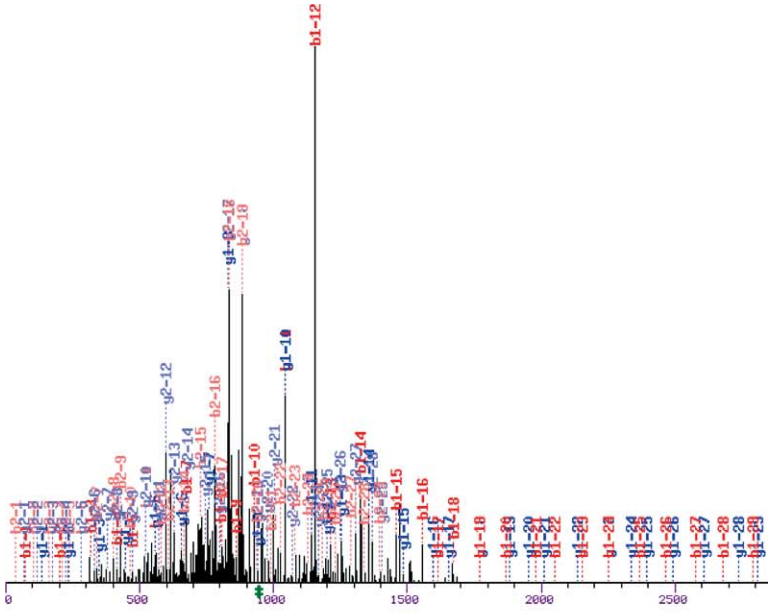
Table 1
Aβ-related peptides identified by LC/MS/MS

TIC	MW	MW observed	Numbering	Amino acid sequence
Wild				
1.4e6	1886.0	1887.8	21–40	AEDVGSNKGAIIGLMVGGVV
2.1e6	3463.6	3463.3	19–52	FFAEDVGSNKGAIIGLMVGGVVIATVIVITLVM*L
C83				
3.5e7	1886.0	1885.7	21–40	AEDVGSNKGAIIGLMVGGVV
3.5e7	2825.6	2828.1	21–49	AEDVGSNKGAIIGLM*VGGVVIATVIVITL
C99				
9.5e5	1511.9	1512.6	27–42	NKGIIGLMVGGVVIA
3.5e6	1776.2	1778.6	23–43	DVGSNKGAIIGLM*VGGVVIAT
5.7e7	2724.6	2722.2	23–50	DVGSNKGAIIGLM*VGGVVIATVIVITLV
5.7e7	1814.9	1815.1	22–40	EDVGSNKGAIIGLMVGGVV
8.9e6	1687.8	1689.1	21–38	AEDVGSNKGAIIGLMVGG
3.5e7	1886.0	1883.9	21–40	AEDVGSNKGAIIGLMVGGVV
5.9e6	1902.0	1902.4	21–40	AEDVGSNKGAIIGLM*VGGVV
9.7e5	2498.4	2497.1	21–46	AEDVGSNKGAIIGLM*VGGVVIATVIV
3.5e7	2825.6	2825.3	21–49	AEDVGSNKGAIIGLM*VGGVVIATVIVITL
3.5e6	1778.0	1778.6	20–37	FAEDVGSNKGAIIGLMVG
5.0e6	1834.9	1834.1	20–38	FAEDVGSNKGAIIGLMVGG
8.8e5	3818.2	3816.8	16–52	KLVFFAEDVGSNKGAIIGLM*VGGVVIATVIVITLVM*L
1.1e6	3672.8	3673.7	1–33	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG
2.6e6	4130.0	4130.7	1–38	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
Human brain				
2.1e6	2856.6	2855.1	22–50	EDVGSNKGAIIGLM*VGGVVIATVIVITLV
8.5e5	2791.3	2791.9	17–44	LVFFAEDVGSNKGAIIGLM*VGGVVIATV

TIC, total ion current, M*, oxidized Met.

immunoprecipitation of Aβ-related peptides with LC/MS/MS analysis. As shown in Table 1, 2, 2, and 14 Aβ-related peptides were identified in the culture medium of wild-type COS-1 cells, COS-1 cells transfected with C83, and COS-1 cells transfected with C99, respectively. The MS/MS data of Aβ 21–49 peptide, which obtained from the culture medium of COS-1 cells transfected with C83, are shown in Fig. 3. Other Aβ-related peptides were also identified in the same manner (date not shown). The N-terminal and C-terminal regions were heterogeneous, starting at Aβ residues, 1Asp, 16Lys, 19Phe, 20Phe, 21Ala, 22Glu, 23Asp and 27Asn, and ending at Aβ residues, 33Gly, 37Gly, 38Gly, 40Gly, 42Ala, 43Thr, 46Val, 49Leu, 50Val and 52Leu (Table 1). Some Aβ-related peptides were oxidized at 35Met or 51Met residue. This oxidation might have occurred in vivo or might have resulted from the extraction process using formic acid [16]. No other post-translational modifications were found. Next, Aβ-related peptides in human brain extract were analyzed using the same method. As shown in Table 1, Aβ 22–50 and Aβ 17–44 peptides were identified in human brain extract.

One peptide in wild-type and C83 COS-1 cells, five peptides in C99 COS-1 cells and two peptides in human brain extract



Seq	#	b	y	(+1)
A	1	72.0	2809.6	29
E	2	201.1	2738.6	28
D	3	316.1	2609.5	27
V	4	415.2	2494.5	26
G	5	472.2	2395.4	25
S	6	559.2	2338.4	24
N	7	673.3	2251.4	23
K	8	801.4	2137.3	22
G	9	858.4	2009.2	21
A	10	929.4	1952.2	20
I	11	1042.5	1881.2	19
I	12	1155.6	1768.1	18
G	13	1212.6	1655.0	17
L	14	1325.7	1598.0	16
M	15	1456.7	1484.9	15
V	16	1555.8	1353.9	14
G	17	1612.8	1254.8	13
G	18	1669.9	1197.8	12
V	19	1768.9	1140.8	11
V	20	1868.0	1041.7	10
I	21	1981.1	942.6	9
A	22	2052.1	829.5	8
T	23	2153.2	758.5	7
V	24	2252.2	657.5	6
I	25	2365.3	558.4	5
V	26	2464.4	445.3	4
I	27	2577.5	346.2	3
T	28	2678.5	233.1	2
L	29	2791.6	132.1	1

Seq	#	b	y	(+2)
A	1	36.5	1405.3	29
E	2	101.0	1369.8	28
D	3	158.6	1305.3	27
V	4	208.1	1247.8	26
G	5	236.6	1198.2	25
S	6	280.1	1169.7	24
N	7	337.1	1126.2	23
K	8	401.2	1069.2	22
G	9	429.7	1005.1	21
A	10	465.2	976.6	20
I	11	521.8	941.1	19
I	12	578.3	884.6	18
G	13	606.8	828.0	17
L	14	663.4	799.5	16
M	15	728.9	743.0	15
V	16	778.4	677.4	14
G	17	806.9	627.9	13
G	18	835.4	599.4	12
V	19	885.0	570.9	11
V	20	934.5	521.3	10
I	21	991.0	471.8	9
A	22	1026.6	415.3	8
T	23	1077.1	379.8	7
V	24	1126.6	329.2	6
I	25	1183.2	279.7	5
V	26	1232.7	223.2	4
I	27	1289.2	173.6	3
T	28	1339.8	117.1	2
L	29	1396.3	66.6	1

Fig. 3. MS/MS analysis data of Aβ 21–49 obtained from culture medium of COS-1 cells transfected with C83. The correlation of experimental mass spectrum with predicted fragment ion peaks of a candidate peptide is shown.

were longer Aβ-related peptides with C-termini between residues Aβ 43Thr and 52Leu. These results are supported by a recent report by Roher et al. [16] in which longer Aβ-related peptides, ending at Aβ residues 43–54, existed in amyloid deposits from the brains of Val717Phe familial AD individuals. Of these longer peptides, peptides ending at Aβ residues 49Leu, 50Val, and 52Leu were ε-cleavage products. As shown in Table 1, their total ion current (TIC) values indicated that these peptides were major products. These suggest that ε-cleavage products, as well as γ-cleavage products (Aβ and p3), were secreted under normal conditions. Although the precise relationship between γ- and ε-cleavage is still unknown, the C-terminal heterogeneity of longer Aβ-related peptides as shown in Table 1 suggests that carboxypeptidases could have degraded the longer Aβ to sequentially generate shorter Aβ peptides as described previously [16].

Most of these ε-cleavage products have N-termini starting at Aβ residues 17leu–21Ala, suggesting that these were derived from α-secretase cleavage product, C83. This result is in good agreement with our previous finding that AICD production was preferentially derived from C83 by ε-cleavage [9]. Additionally, I could not detect ε-cleavage products, which had N-termini starting at Aβ residues 1Ala–15Gln, in the medium of COS-1 cells transfected with C99, suggesting that C99 was not directly processed at ε-cleavage site under normal conditions. Therefore, in COS-1 cells transfected with C83, expressed C83 is directly processed at the ε-cleavage site, while in wild-type COS-1 cells or COS-1 cells transfected with C99, C83 produced by α-secretase cleavage is processed at the ε-cleavage site.

The relationship between these longer Aβ-related peptides and amyloid fibril formation is interesting. In general, Aβ with a longer C-terminus can more readily polymerize into amyloid filaments [1]. However, identified longer peptides in the culture media and in brain extract lack the N-terminal region of Aβ (residues 1–16), which is the essential region for amyloid fibril formation [17]. Therefore, these longer Aβ peptides, which are secreted under normal conditions, may not be involved in amyloid fibril formation. Recent report that no peptides longer than 40 and 42 amino acids have been recovered in the amyloid deposits obtained from sporadic AD supports this hypothesis [16].

In this experiment, I showed that ε-cleavage related to the production of long Aβ-related peptides was dependent on C83. Additionally, I previously showed that ε-cleavage relevant to the production of AICD was also dependent on C83 [9] and that γ-cleavage related to the production of Aβ and p3 was not dependent on the size of APP C-terminal fragments [8]. These suggest that γ- and ε-cleavage in APP are differently regulated. Recent report that Aβ and AICD production are not temporally linked events but rather two separately regulated, proteolytic events supports my results [18]. It has been reported that AICD interacts with Fe65 [5] and c-Jun N-terminal kinase interacting protein-1 [19] to induce gene activation, indicating that AICD plays a role in signal transduction. Interestingly, some groups report that some PS1 mutations causing familial AD inhibit the generation of AICD [20–22]. Therefore, the failure of ε-cleavage on C83 relevant to the production of long Aβ-related peptide and AICD may be involved in the pathogenesis of AD [9]. Further studies are necessary to clarify the producing mechanism of Aβ-related peptides and the potential pathological activity.

Finally, the information of amino acid sequences and post-translational modifications can be obtained using LC/MS/MS. I have shown that the combination of immunoprecipitation of Aβ and LC/MS/MS is a sensitive, reliable, and powerful tool for Aβ analysis.

Acknowledgements: I thank Dr. Hideaki Kume (Tohoku University Graduate School) for support and discussion. This work was supported in part by a grant from Health, Labor and Welfare of Japan.

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