Identification of a New Presenilin-dependent γ-Cleavage Site within the Transmembrane Domain of Amyloid Precursor Protein* ♦

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γ-Secretase cleavage of β-amyloid precursor protein (APP) is crucial in the pathogenesis of Alzheimer disease, because it is the decisive step in the formation of the C terminus of β-amyloid protein (Aβ). To better understand the molecular events involved in γ-secretase cleavage of APP, in this study we report the identification of a new intracellular long Aβ species containing residues 1–46 (Aβ46), which led to the identification of a novel γ-cleavage site between the known γ- and ε-cleavage sites within the transmembrane domain of APP. Our data clearly demonstrate that the new γ-cleavage is a presenilin-dependent event. It is also noted that the new γ-cleavage site at Aβ46 is the APP717 mutation site. Furthermore, we show that the new γ-cleavage is inhibited by γ-secretase inhibitors known as transition state analogs but less affected by inhibitors known as non-transition state γ-secretase inhibitors. Thus, the identification of Aβ46 establishes a system to determine the specificity or the preference of the known γ-secretase inhibitors by examining their effects on the formation or turnover of Aβ46.

The amyloid deposits in the brain of Alzheimer disease (AD) patients are principally composed of the 39–43-amino acid residue amyloid β-peptide (Aβ), which is derived from a large β-amyloid precursor protein (APP). In the amyloidogenic pathway, APP is first cleaved at the N terminus of Aβ sequence by β-secretase, to produce a soluble ectodomain, sAPPβ, and a membrane-anchored C-terminal fragment, CTFβ. CTFβ is then subsequently cleaved within the transmembrane domain by γ-secretase to produce the full-length Aβ and the intracellular domain (AICD) (1). β-Secretase has been identified as a type I membrane aspartyl protease (2, 3). The findings that knockout of presenilin 1 (PS1) and PS2 results in the abolishment of the γ-secretase cleavage of APP and that two aspartate residues in two transmembrane domains of presenilin have been identified as critical for the γ-secretase activity suggest that presenilin may be the γ-secretase (4–7). Recently, several other molecules, namely nicastrin, Aph-1, and Pen-2, have been identified as essential components of the γ-secretase complex of which presenilin may function as the catalytic subunit (8).

Most of the Aβ species contain 40 or 42 amino acids. Recently, sequence analysis revealed that the N terminus of AICD starts at residue 50 of the Aβ sequence, which is 7–9 amino acids away from the C termini of Aβ40 and Aβ42. This led to the finding of the ε-cleavage site between Aβ49 and Aβ50 (9–12). Now the cleavage at Aβ40/42 has been specifically referred to as γ-cleavage site (12). However, neither the intermediate Aβ peptide, which ends at the ε-cleavage site, nor the C-terminal fragment, which starts with an N terminus generated by γ-cleavage, has ever been detected. One possibility is that γ- and ε-cleavages occur simultaneously. The other possibility is that there may be additional cleavages(s) between γ- and ε-cleavages. Here we report that, in our effort to determine these possibilities, we identified a new cleavage site at Aβ46, which we designated as γ-cleavage site.

MATERIALS AND METHODS

γ-Secretase inhibitors, DAPT, compound E, γ-secretase inhibitor XIX (XIX), L-685,458, and WPE-III-31C (31C), were from Calbiochem and dissolved in dimethyl sulfoxide (Me2SO). Aβ40 was from Biopeptide. Aβ40 and Aβ42 were from American Peptide.

Cell Lines—Human neuroblastoma M17 cells, glioma HS83 cells, and embryonic kidney 293 cells were purchased from American Type Cell Collection. All stably transfected N2a cell lines were established as described previously (13). All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Immunoprecipitation and Western Blotting—Eight hours after treatment with inhibitors, cells were harvested and lysed in Western blot lysis buffer (50 mM Tris-HCl, pH 7.4, 8 mM urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors). Secreted Aβ was immunoprecipitated from conditioned medium (CM) using a Aβ-specific antibody 6E10 (Senetek). Both cell lysates and the immunoprecipitates were analyzed by 10% BisCure/urea SDS-PAGE or 10–18% regular SDS-PAGE. After being transferred to a polyvinylidene fluoride membrane (Millipore) and probed with specific antibodies, the immunoreactivity bands were visualized using ECL-Plus (Amersham Biosciences).

Mass Spectrometric Analyses—N2a cells cultured in the presence of DAPT were lysed with 1% Nonidet P-40 in IP buffer (50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, protease inhibitor mixture). After centrifugation at 20,000 × g at 4 °C for 15 min, the supernatant was diluted with equal amounts of IP buffer to bring down the concentration of Nonidet P-40 to 0.5%. The intracellular Aβ species were immunoprecipitated using 6E10. The immunoprecipitate was eluted from beads with a buffer of 1% trifluoroacetic acid and 45% acetonitrile and then subjected to matrix-assisted laser desorption/ ionization mass spectrometric (MALDI-MS) analysis performed on a Voyager-DE STR mass spectrometer as described previously (14).
A New Intramembranous Cleavage Site in APP

RESULTS

Identification of a New Intracellular Aβ Species—N2a cells stably expressing wild type PS1 (PS1wt) and the myc-tagged Swedish mutant APP (APPsw), which have been used in previous studies (13, 15), were treated with or without γ-secretase inhibitors: DAPT, DAPM, compound E, and XIX. The cell lysates were analyzed by 10–18% Tris/glycine SDS-PAGE followed by Western blotting, using either 6E10, a monoclonal antibody specific to residues 1–17 of human Aβ, or C15. The blots probed with C15. The second, third, and bottom panels are the blots probed with 6E10. Since the CTFβ and CTFα derived from recombinant APPsw are tagged with a myc epitope, they were detected as bands with slower migration rates than the endogenous CTFα (CTFα/end, top panel) (15). The bottom panel is the Aβ immunoprecipitated from CM. B, cell lysates of human glioma HS683 cells (lanes 1 and 2) and human neuroblastoma M17 cells (lanes 3 and 4) treated with 3 nm of compound E were analyzed for the presence of the new Aβ species. As controls, cell lysates of N2a cells stably expressing APPsw and PS1wt treated with lane 6 or without lane 5) compound E were included. Lane 7 is the mix of synthetic Aβ40 and Aβ42.

FIG. 1. Detection of a new intracellular Aβ species. A, APP derivatives in lysates or media of cells treated with (lanes 2–5) or without (lane 1) various inhibitors (500 nM DAPT, 100 nM DAPM, 3 nM compound E (CPDE), and 5 nM XIX, respectively) for 8 h were analyzed by 10–18% Tris/glycine-SDS-PAGE (top and second panels) or by Bicine/urea SDS-PAGE (third and bottom panels). Lane 6 is the mix of synthetic Aβ40 and Aβ42. Lane 7 is the synthetic Aβ40. The top panel is the top panel is the blot probed with C15. The second, third, and bottom panels are the blots probed with 6E10. Since the CTFβ and CTFα derived from recombinant APPsw are tagged with a myc epitope, they were detected as bands with slower migration rates than the endogenous CTFα (CTFα/end, top panel) (15). The bottom panel is the Aβ immunoprecipitated from CM. B, cell lysates of human glioma HS683 cells (lanes 1 and 2) and human neuroblastoma M17 cells (lanes 3 and 4) treated with 3 nm of compound E were analyzed for the presence of the new Aβ species. As controls, cell lysates of N2a cells stably expressing APPsw and PS1wt treated with lane 6 or without lane 5) compound E were included. Lane 7 is the mix of synthetic Aβ40 and Aβ42.

1, top panel). Surprisingly, when the same samples were probed by 6E10, a band, which is tentatively labeled as Aβ46 (lane 1, second panel) and has a migration rate much faster than that of CTFβ but slightly slower than those of synthetic Aβ40, Aβ42, and Aβ43 (compare lane 1 with lanes 6 and 7, second panel), was detected in untreated cells. More interestingly, this band was markedly increased in cells treated with γ-secretase inhibitors (compare lanes 2–5 with lane 1, second panel). The fact that this band was not detected by C15 but was detected by 6E10 suggests that it is more likely a novel intracellular Aβ-containing peptide with a higher molecular mass than Aβ43. The same samples were also analyzed by 10% Bicine/urea SDS-PAGE as described previously (16). As shown in the third panel, this new Aβ species (lanes 1–5) migrated much faster than Aβ40/42, and Aβ43 (lanes 6 and 7), indicating that this new Aβ species is more hydrophobic than Aβ43. Next, we examined the effects of these inhibitors on the formation of secreted Aβ. As shown in the bottom panel of Fig. 1A, secreted Aβ40 and Aβ42 were detected in CM of untreated cells (lane 1) but not detected in CM of cells treated with inhibitors (lanes 2–5), a result consistent with many previous studies.

To determine the physiological relevance of this new Aβ species, we examined its presence in other types of cells. As shown in the top panel of Fig. 1B, in the presence of 3 nm compound E, this new Aβ species was also detected in human glioma HS683 cells (lane 2) and human neuroblastoma M17 cell (lane 4) without overexpressing APP. This Aβ species was also detected in human embryonic kidney 293 cells (data not shown). When analyzed by a Bicine/urea SDS-PAGE (bottom panel), as expected, the new Aβ species migrated much faster than Aβ40/42 (compare lanes 2, 4, and 6 with lane 7). The detection of this new Aβ species in different types of cells, and specifically, in cells without overexpressing APP indicates that this new Aβ species is a normal metabolic product of APP.

Identification of a New γ-Cleavage Site at Aβ46—To determine the identity of the novel Aβ-containing peptide, mass spectrometric analysis was carried out. The new Aβ species was immunoprecipitated with 6E10 from the lysates of cells treated with DAPT and analyzed by MALDI-MS. As shown in Fig. 2, a major spectral peak with a mass of 4927.53 Da, which is in agreement with the expected mass of 4926 for Aβ46, was observed. Thus, we determined the new Aβ species as Aβ46 and identified a new cleavage site at Aβ46 within the transmembrane domain of APP between the known γ-cleavage site at Aβ40/42 and the ε-cleavage site at Aβ40/42. We named this new cleavage site as “ε-cleavage” site, following the tradition by which other APP processing sites were named. It is noted that in addition to Aβ46, a weak spectral peak corresponding to Aβ43 (measured mass = 4614.86 Da; expected mass = 4615 Da) was detected in the immunoprecipitate.

γ-Cleavage Is PS1-dependent—The novel finding that the new γ-cleavage product Aβ46 was not inhibited but rather increased by the known γ-secretase inhibitors DAPT etc. prompted us to determine whether γ-cleavage is dependent on presenilin. To this end, we examined the effect of the dominant negative aspartate mutant PS1 on the formation of Aβ46. In addition to the cells that stably express both APPsw and PS1wt, another line of cells, APPsw/PS1(D385A), which stably expresses both APPsw and the dominant negative mutant PS1(D385A) and has been used in a previous study (13), was employed. Cells were treated with or without 0.5 μM DAPT for 8 h. The cell lysates and the secreted Aβ immunoprecipitated from CM were analyzed by 10–18% SDS-PAGE followed by Western blotting using specific antibodies. As shown in the top panel of Fig. 3, full-length PS1 was detected in all cells. The PS1 processing product, the N-terminal fragment of PS1, was
detected in cells expressing wild type PS1 (lanes 1 and 2, top panel) but not in cells expressing the dominant negative PS1(D385A) mutant (lanes 3 and 4, top panel), which has been shown not to undergo endoproteolytic processing (7). As shown in the middle panel, in the presence of DAPT, Aβ46 was detected in cells expressing wild type PS1 (lane 2) with concomitant decrease in secreted Aβ (bottom panel, lane 2). Interestingly, Aβ46, as well as secreted Aβ, was not detected in cells expressing dominant negative PS1 mutant, regardless of the presence or absence of DAPT (lanes 3 and 4), indicating that Aβ46 formation is PS1-dependent.

**Transition State Analog Inhibitors Inhibit ω-Cleavage**—The data presented in Fig. 1 show that the known γ-secretase inhibitors DAPT etc. at the concentrations used, completely inhibited the formation of secreted Aβ40 and Aβ42 produced by γ-cleavage but had less effect on the formation of Aβ46 produced by ω-cleavage. This observation raises a possibility that these inhibitors may have differential effects on γ- and ω-cleavages. In this regard, it was noted that the inhibitors tested, namely DAPT, DAPM, compound E, and XIX, are known as non-transition state inhibitors. This prompted us to determine the effect of the inhibitors known as transition state analogs on the new ω-cleavage. As shown in Fig. 4, in cells treated with DAPT, DAPM, compound E, and inhibitor XIX (lanes 10–17 and lanes 20–27), a dose-dependent decrease in secreted Aβ40 and Aβ42 in CM (compare with samples from untreated control cells in lanes 9 and 19, lower panel) and, specifically at the lower range of concentrations of these inhibitors, a concomitant increase in CTFβ and Aβ46 (upper panel) were observed for all these inhibitors. Surprisingly, it was found that Aβ46 was not detected in the cells treated with transition state analogs, L-685,458 and 31C (lanes 2–8, upper panel), although these inhibitors caused a marked and dose-dependent decrease in secreted Aβ40 and Aβ42 in CM (compare lanes 2–8 with lane 1, lower panel). This result reveals an important notion that ω-cleavage, which is responsible for the formation of Aβ46, is specifically inhibited by inhibitors known as transition state analogs and is less affected by inhibitors known as non-transition state inhibitors. All inhibitors examined at the ranges of concentrations used were found to have no effect on the cell growth and the expression and processing of PS1 (data not shown).

**DISCUSSION**

In this study we detected a new intracellular Aβ-containing peptide, Aβ46. The presence of Aβ46 in intact cells was confirmed in different types of cells. The finding of this new intracellular Aβ46 is also supported by recent observations that Aβ species ending at residue 46 can be detected in human tissue of subjects with and without AD (17, 18). It is noted that Aβ46 exists at very low concentration in the absence of inhibitors. This may accounts for the difficulty in detecting it in living cells.

The identification of this new Aβ46 offers several novel insights into the mechanism of the normal and pathogenic intramembranous processing of APP and the formation of Aβ.

First, the identification of Aβ46 reveals a novel cleavage site, ω-cleavage site between the two known γ- and ε-cleavage sites. Our data also clearly reveal that this new ω-cleavage occurs as a presenilin-dependent event, indicating that it is a new intramembranous cleavage of APP by γ-secretase or related protease activity. Moreover, the facts that Aβ46 can be detected in the absence of inhibitors and it is the predominant intracellular Aβ species as determined by both Western blot and mass spectrometry analyses strongly indicate that the cleavage site at Aβ46 is another major cleavage site in APP, besides the known γ-cleavage site at Aβ40/42 and the ε-cleavage site at

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**Fig. 2.** MALDI-MS spectra for Aβ species immunoprecipitated from lysate of cells treated with DAPT. Mass spectrometric analysis was performed as described previously (14) except that after applying the sample matrix solution to the sample plate, formic acid and isopropanol were added to a final concentration of 30% each.

**Fig. 3.** Requirement of presenilin for Aβ46 formation. Cell lines are labeled at the top. Lysates of cells cultured in the presence (+) or absence (−) of 0.5 μM DAPT were analyzed by Western blot for the expression of PS1 (top panel) using anti-PSIN, which is specific for the N-terminal domain of PS1 and was raised against a peptide of 24 amino acids corresponding to residues 27–50 of human PS1. Full-length APP (fAPP), CTFβ, and Aβ46 in the cell lysate were detected by 6E10 (middle panel). Secreted Aβ and soluble APP (sAPP) immunoprecipitated from CM were also detected by 6E10 (bottom panel).
Aβ49. It is also noted that the new ζ-cleavage site at Aβ46 is the site at which the AD-linked APP mutation, APP717 mutation also known as London mutation, occurs (19, 20). Thus, our finding reveals an interesting fact that the well characterized AD-linked APP717 mutation actually occurs at a major Swedish processing site, the ζ-site near the C terminus of Aβ. Also the other well characterized AD-linked APP mutation, the Swedish mutation occurs at another major cleavage site, the β-site at the N terminus of Aβ.

Second, our dose-curve experiments clearly demonstrate that, at the lower range of concentrations, DAPT, DAPM, compound E, and XIX cause a dose-dependent decrease in secreted Aβ40/42 and a concomitant increase in Aβ46, suggesting a possible precursor-product relationship between Aβ46 and Aβ40/42. However, the observation that ζ-cleavage can be differentially inhibited by γ-secretase inhibitors known as transition state analogs but less affected by the inhibitors known as non-transition state inhibitors also raises a possibility that ζ-cleavage may be catalyzed by a γ-secretase-like activity. According to this model, the dose-dependent decrease in secreted Aβ40/42 and the concomitant increase in Aβ46 caused by non-transition state inhibitors at the lower range of concentrations may be related to the substrate availability, i.e., when the true γ-secretase, which cleaves APP at Aβ40/42, is inhibited, it makes more CTFβ available for the putative γ-secretase-like activity, which produces Aβ46. At higher ranges of concentrations, these inhibitors also caused a dose-dependent increase of CTFβ, suggesting an allosteric mechanism of the inhibitory effects of these inhibitors on the turnover of Aβ46 and CTFβ.

Third and more importantly, the identification of Aβ46 made it possible to determine the specificity of the known γ-secretase inhibitors. Our experiment examining the effects of the known inhibitors on the formation and turnover of the newly identified Aβ46 led to a novel finding that the newly identified ζ-cleavage is specifically inhibited by inhibitors known as transition state analogs and is less affected by inhibitors known as non-transition state inhibitors. According to the amyloid hypothesis (1), the longer and more hydrophobic and more amyloidogenic Aβ is more toxic and pathogenic. In this regard, it is notable that DAPT, DAPM, compound E, and XIX, which were previously known to inhibit the formation of secreted Aβ, cause an intracellular accumulation of an even longer Aβ species, Aβ46. This finding provides information important for the strategy of prevention and treatment of AD aimed at the design of γ-secretase inhibitors. Therefore, the observation that ζ-cleavage is differentially inhibited by so-called transition state inhibitors and is less affected by non-transition state inhibitors is significant because it establishes, for the first time, a system to determine the specificity or the preference of the known γ-secretase inhibitors by examining their effects on the formation or turnover of Aβ46. In general, the identification of the intracellular Aβ46 and the new ζ-cleavage site may provide a potential new therapeutic target for the prevention and treatment of AD.

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REFERENCES

1. Selkoe, D. J. (2001) Physiol. Rev. 81, 741–766
2. Tisserat, B., Bennett, B. D., Koo, E. H., Kasaian, S., Kohn, S., Mendieta, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeﬄof, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarriën, S. M. A., Bierie, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
3. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brasheer, J. R., Stratum, N. C., Mathews, R. W., Buhl, A. E., Carter, D. B., Tomaselli, A. G., Purdom, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Nature 402, 533–537
4. De Strooper, B., Saﬁtig, P., Cressersett, K., Vandeneul, H., Guilde, G. Annaert, W., Van Figura, K., and Van Leuven, F. (1998) Nature 391, 287–290
5. Herreman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L., and De Strooper, B. (2000) Nat. Cell. Biol. 2, 461–462
6. Zhang, L., Nadeau, P., Song, W., Denovell, D., Yuan, M., Bernstein, A., and Yankner, B. (2000) Nat. Cell. Biol. 2, 463–467
7. Wolfe, M. S., Xia, W., Otsuzawski, B. L., Dieli, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 398, 513–517
8. Kimberly, W. T., and Wolfe, M. S. (2003) J. Neurosci. Res. 74, 353–360
9. Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takik, K., and Ibara, Y. (2001) J. Biol. Chem. 276, 35235–35238
10. Sastre, M., Steiner, H., Fu¨h, K., Capell, A., Mullhaup, G., Condon, M. M., Teplow, D. B., and Haas, C. (2001) EMBO Rep. 2, 835–841
11. Yu, C., Kim, S. H., Ikeuchi, T., Xu, H., Gasparin, L., Wang, R., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43756–43760
12. Weidemann, A., Egert, S. E., Rennecke, F. B., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beyreuther, K., and Evin, G. (2002) Biochemistry 41, 2825–2835
13. Kim, S. H., Lee, J. Y., Lah, J. J., Shunt, H. H., Levey, A. I., Thinakaran, G., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43343–43350
14. Chen, F., Gu, Y., Hasegawa, H., Ruan, X., Arawaka, S., Fraser, P., Westaway, D., Mount, H., and St George-Hyslop, P. (2002) J. Biol. Chem. 277, 36521–36526
15. Ikeuchi, T., Dolios, G., Kim, S. H., Wang, R., and Sisodia, S. S. (2003) J. Biol. Chem. 278, 7010–7018
16. Wiltfang, J., Smirnov, A., Schneizer, B., Kelken, G., Matthies, U., Klafki, H. W., Staufenbibel, M., Huther, G., Ruther, E., and Kornhuber, J. (1997) Electrophoresis 18, 527–532
17. Kuo, Y. M., Kojohun, T. A., Watson, M. D., Wood, A. S., Cotter, R. J., Sue, L. I., Kalback, W. M., Emmerling, M. H., Beach, T. G., and Rober, E. A. (2000) Am. J. Pathol. 156, 797–805
18. Roher, A. E., Kojohun, T. A., Esh, C., Weiss, N., Childress, J., Kalback, W., Luerss, D. C., Lopez, J., Brune, D., Kuo, Y. M., Farlow, M., Murrell, J., Vidal, R., and Gheti, B. (2004) J. Biol. Chem. 279, 5829–5836
19. Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Hoyner, A., Irving, N., and James, L. (1991) Nature 349, 704–706
20. Hardy, J. J. (1992) Nat. Genet. 1, 233–234