A Basic Amino Acid in the Cytoplasmic Domain of Alzheimer's β-Amyloid Precursor Protein (APP) Is Essential for Cleavage of APP at the α-Site*

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In Alzheimer's disease (AD), the β-amyloid peptide (Aβ) is thought to be produced as a result of the aberrant metabolism of β-amyloid precursor protein (APP). We report that the APP cytoplasmic domain contains a novel and important signal for APP metabolism. A single amino acid mutation that changed arginine at amino acid 747 of APP770 (corresponding to position 672 of APP695) to a non-basic amino acid greatly increased the production of intracellular APP carboxy-terminal fragment(s) cleaved at β-site(s) (CTFβ), but did not result in increased secretion of Aβ40 and Aβ42. This was not due to a simple intracellular accumulation of CTFβ resulting from a lack of γ-secretase. CTFβ derived from this mutant APP was generated and degraded as efficiently as CTFβ derived from wild-type APP. This result indicates that the increase in the quantity of CTFβ does not always give rise to more Aβ production, as was previously suggested by studies of a familial AD mutation of APP. These findings suggest that APP carrying the substitution mutation at this basic amino acid may be metabolized by another protein secretory pathway. Although these results have not completely elucidated why CTFβ derived from the mutant APP escapes from subsequent cleavage by γ-secretase, analysis of the processing pathway of this mutant APP should provide insights into the pathogenesis of the sporadic type of AD.

A principal component of amyloid plaques in Alzheimer's disease (AD)1 is a 38–43-amino acid β-amyloid peptide (Aβ) (1–3), which is derived from the β-amyloid precursor protein (APP) (4–11). Production and accumulation of Aβ is a major event believed to be involved in the etiology of AD (reviewed in Refs. 12 and 13). APP is an integral membrane protein with a short intracellular carboxyl terminus. Normal proteolytic processing of APP generates two fragments, a large extracellular amino-terminal domain (sAPPα) and a truncated carboxy-terminal fragment (CTFα), both of which are products of cleavage within the Aβ domain. Another form of proteolytic processing occurring at β-site(s) gives rise to limited production of carboxy-terminal fragments (CTFβ) and Aβ in unidentified intracellular compartments of the protein secretory pathway (14). Previous studies suggest that the α-secretase responsible for cleavage of APP at the α-site is active in the trans-Golgi network or other end-stage compartments of the protein secretory pathway (15, 16). Studies utilizing the APP of familial Alzheimer's disease (FAD), such as APP with the “Swedish” double mutation (K59N/M591L, numbering for the APP695 isoform), suggest that APP cleavage at the β-site by β-secretase occurs in the medial-Golgi network and in unidentified compartments proximal to the plasma membrane (17, 18), although a recent report suggests that the endoplasmic reticulum is the site of generation of Aβ42 (but not of Aβ40) in neurons (19). Furthermore, it is generally held that the increased production of CTFβ always gives rise to more Aβ, although the molecular mechanisms of CTFβ production by β-secretase from wild-type APP and of subsequent Aβ production following the cleavage of CTFβ by γ-secretase are still unknown in sporadic AD patients, who constitute the majority of AD cases.

The APP cytoplasmic domain is thought to be responsible for the intracellular transport of APP. Two internalization signals, NPTY (APP770 amino acids 759–762 and APP695 amino acids 684–687) and YTSI (APP770 amino acids 728–731 and APP695 amino acids 653–656), have been identified (20, 21). The NPXY motif in the cytoplasmic domain of membrane proteins is believed to mediate interactions between internalized proteins and the clathrin cage of the clathrin-coated vesicle (22). The YXXI motif conforms to a potential 4-residue tyrosine-based internalization signal consensus sequence (21). Furthermore, the sorting signal(s) in the cytoplasmic domain of APP are thought to play an important role in the distribution of APP within the protein secretory pathway (15), although the sorting signal has not been well analyzed. Therefore, using site-directed mutagenesis, we performed further analysis of the sorting signal(s) in the cytoplasmic domain of APP, which may be responsible for the regulation of APP metabolism such as the production of CTFβ and/or Aβ. Identification and characterization of the signal(s) in the APP cytoplasmic domain are important for our understanding of the molecular mechanism that directs APP into a protein secretory pathway where CTFβ and/or subsequent Aβ production occurs without an FAD mutation.

In this study, we introduced an alanine-scanning mutation into the cytoplasmic domain of APP and established many cell lines that expressed stably transfected APP carrying a single amino acid mutation in its cytoplasmic domain. Among the cell

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1 The abbreviations used are: AD, Alzheimer's disease; Aβ, β-amyloid peptide; APP, β-amyloid precursor protein; sAPP, large extracellular amino-terminal domain truncated at the a-site (sAPPα) and/or the β-site (sAPPβ); CTFα, carboxy-terminal fragment of APP cleaved at the a-site; CTFβ, carboxy-terminal fragment of APP cleaved at the β-site; FAD, familial Alzheimer's disease; PCR, polymerase chain reaction; wt, wild-type; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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lines, cells that expressed APP carrying a mutation at amino acid 747 of APP770 (position 672 of APP695) generated a greater amount of CTFβ, but this did not result in an increase of Aβ secretion, indicating that the intracellular increase in CTFβ is not necessarily related to an increase in Aβ production. This effect was not due to a simple intracellular accumulation of CTFβ resulting from a lack of γ-secretase. These results suggest that the basic amino acid at position 747 of APP770 (position 672 of APP695) plays an important role in the direction of APP into the normal secretory pathway, in which APP is cleaved preferentially at the α-site. Thus, APP carrying this mutation may be metabolized in a different protein secretion pathway, which suggests that the basic amino acid may act by altering the conformation of the protein, thereby affecting the recognition by γ-secretase.

**Experimental Procedures**

Introduction of Substitution Mutation and Plasmid Construction—cDNA encoding human APP770 (10) was cloned from a λZAP HeLa cDNA library by immunoscreening with anti-APP antibody, G-369 (23). A new cDNA library (λZAP HeLa) was performed with primers APP695 cDNA (681–700) (forward, 5’-GTTCCGGAGGG-GTAGATTGTTG-3’) and APP695 cDNA (1922–1940) (reverse, 5’-GCATCCTCAGCTTCTAGC-3’). A 1.2-kilobase PCR product was digested with XcmI and the resulting fragment (APP695 cDNA (990–1916)) was recombined into the larger fragment from XcmI/XniI digestion of APP770 to delete Kunitz-type protease inhibitor (exon 7) and APP770-specific (OX-2), exon 8) domains of APP770 and to construct a complete APP695 cDNA (see Fig. 1a) (5). The cDNAs were subcloned into pCDNA (Invitrogen) at HindIII/Asai sites. A sequence of the APP770 extracellular domain between amino acids 379 and 666 was deleted by exclusion of the XhoI/BglII fragment (p2APP770wt) (see Fig. 1a) (24).

Site-directed mutations were introduced using PCR (25) with the following primers: primer 1, 5’-GCCGGGTGCTACCCGAGGAGGCCACCTGTTCC-3’ (the underlined nucleotides were changed to produce an EcoO65I site (T to G) and the Arg-to-Ala mutation (CG to GC)) and primer 2, 5’-ATTTAGTGACACTATAATGAC-3’ (SP6 promoter primer), in the presence of plasmid p2APP770wt and Pwo DNA polymerase (Boehringer Mannheim). Primer 3, 5’-TCTGGGGTTCGGCCG-GGTCACCCCTCCACCAGGGGACACCC-CACCTGTTCC-3’ (the underlined nucleotides were changed to produce an EcoO65I site (T to G) and the Arg-to-Ala mutation (CG to GC)) and primer 2, 5’-ATTTAGTGACACTATAATGAC-3’ (SP6 promoter primer), in the presence of plasmid p2APP770wt and EcoO65I ligated, and then inserted into pCDNA3 at the HindIII/XniI sites. The resulting plasmid, pΔAPP770wt and p2APP770wt were digested with EcoO65I and ligated, and then inserted into pCDNA3 at the HindIII/XniI sites. The resulting plasmid, pΔAPP770R747A, encodes an amino acid sequence identical to that of APP770wt except for the substitution of Ala for Arg 747. The introduction of this single amino acid sequence mutation into the APP cytoplasmic domain allows one to introduce another single amino acid substitution into the APP cytoplasmic domain. The sites of restriction enzymes and the positions of primers used in this study are shown in Fig. 1a.

Detection of Intracellular Carboxyl-terminal Fragments of APP—293 cells were transfected with plasmid DNA, and several independent clones stably expressing APP were isolated for each construct. Identical cells were transfected with plasmid DNA, and several independent clones were subjected to SDS-PAGE (15% (w/v) polyacrylamide) using a mod-

**Results**

**Production of Intracellular Carboxyl-terminal Fragments (CTFα and CTFβ) in Cells That Express APP Carrying a Single Amino Acid Mutation in Its Cytoplasmic Domain—** It is well known that APP is cleaved preferentially at the α-site to produce CTFα (Fig. 1a) (29). Intracellular products of APP cleaved at the β-site are extremely rare in healthy cells if APP does not carry an FAD mutation such as the Swedish double amino acid substitution (30). However, the production of intracellular CTFβ (Fig. 1a) and the secretion of Aβ increase in the sporadic type of AD even if the resident APP gene carries an entire sequence (31, 32). In the case of sporadic AD, one of the most reasonable explanations for the increased production of CTFβ and the secretion of Aβ is abnormality in the protein secretory
pathway. To explore the signals that direct APP into the protein secretory pathway, we introduced a series of single amino acid substitutions into the 735–755-amino acid region of the cytoplasmic domain of ΔAPP770, which lacks the 287-amino acid region between amino acids 379 and 666 of APP770, but undergoes identical intracellular metabolism as endogenous APP in transfected cells (24). The mutants were then examined for their level of production of APP carboxy-terminal fragments, CTFα and CTFβ (Fig. 1). The two known functional internalization signal sequences, NPTY and YTSI (underlined in Fig. 1b), were excluded from the analysis because they have already been well characterized (21).

293 cells (human transfected primary embryonal kidney) were transfected with wild-type (pΔAPP770wt) and mutant (pΔAPP770mt) plasmids (see “Experimental Procedures” for plasmid construction), and then several independent clones of cells expressing stably transfected ΔAPP770 were isolated. CTFβ was recovered from 293 cells expressing wild-type (ΔAPP770wt) or mutant (ΔAPP770mt) APP by immunoprecipitation with polyclonal anti-APP cytoplasmic domain antibody UT-421 (epitope(s) exists within APP770 (759–770)) and was detected by immunoblot analysis using the same antibody (Fig. 2). CTFα, which is a product of cleavage by α-secretase, was detected in 293 cells expressing ΔAPP770wt, (Fig. 1a (part ii) and arrows in Fig. 2 (a and b)). The CTFα from endogenous APP was below the level of detection (not shown). CTFβ with a higher molecular mass than expected was detected (Fig. 1a (part iii) and arrowheads in Fig. 2 (a and b)) in 293 cells expressing ΔAPP770 R747A (substitution of Ala for Arg747) mutant protein. A weak CTFβ band (20–30% of the amount of CTFα in terms of radioactivity) was also detectable in cells expressing ΔAPP770wt after longer exposure of the autoradiogram (Fig. 2, b and c) or after metabolic labeling of protein with [35S]methionine (see Fig. 5a). A moderate amount of CTFβ (~50% of the amount of CTFα in terms of radioactivity) was also detectable in cells expressing ΔAPP770 L749A (substitution of Ala for Leu749) and ΔAPP770 R751A (substitution of Ala for Lys751) mutants (arrowheads in Fig. 2 (b and c)). Although the differences in the production level of CTFα among the substitution mutants reflected the expression level of ΔAPP770mt in the cell, none of the alanine-scanning mutants, except for ΔAPP770 R747A, containing a substitution within the 735–755-amino acid region of ΔAPP770, led to greater CTFβ production (~130% of the amount of CTFα) when compared with ΔAPP770wt (see the ratio CTFβ/CTFα in Fig. 2c and data not shown for the site between Val730 and Thr732). This result suggests that the argininyl residue at amino acid 747 of APP770, or possibly the domain consisting of 5 amino acids from Arg747 to Lys751 of APP770, plays an important role in APP cleavage at the α-site.

The CTFβ derived from ΔAPP770 R747A was identified by monoclonal anti-αβ antibody 2D1, which recognizes a human-specific amino acid sequence, FRH (APP770 amino acids 676–678 and APP695 amino acids 600–602), between the β- and α-secretase sites (Fig. 3). In Fig. 3, we show that CTFβ was recovered by immunoprecipitation with antibodies UT-421 and 2D1. When the immunoprecipitates were subjected to electrophoresis, both CTFα (arrow) and CTFβ (arrowheads) were detected with antibody UT-421. CTFβ was clearly competed out by the addition of antigen peptide, APP770-(751–770). Antibody 2D1 recognized four CTFβ species (arrowheads 1–4), but not CTFα (arrow), and these four CTFβ species were competed out by the addition of the Aβ (1–15) (APP770-(672–687) peptide. These results confirm that CTFβ derived from ΔAPP770 R747A is a product formed by the action of β-secretase and that it contains the β-amyloid domain.

To further analyze the function of the amino acid at position 747 of APP770, we substituted another basic amino acid, lysine (ΔAPP770 R747K), and an acidic amino acid, glutamic acid (ΔAPP770 R747E) for Arg747 (Fig. 4a). Identical substitution mutations were also introduced into the entire length of APP695, a neuron-specific isoform (Fig. 4b), as described under “Experimental Procedures.” The 293 cells stably expressing these APP proteins with a mutation at amino acid 747 of ΔAPP770 (position 672 of APP695) were also isolated and analyzed for intracellular CTFβ production. The production of the heterogeneous CTFβ species was also clearly observed when Arg747 of ΔAPP770 (Arg672 of APP695) was replaced by Glu (ΔAPP770 R747E and APP695 R672E), but not when replaced by Lys (ΔAPP770 R747K and APP695 R672K) (arrowheads in Fig. 4). Because only small quantities of CTFβα and CTFβγ were usually detected (Figs. 2 and 3), all CTFβα species cannot be observed in Fig. 4. The substitution of the acidic amino acid
at position 747 of ΔAPP770 (position 672 of APP695) with a basic amino acid seemed to have more drastic effects on the production of the CTFβ species compared with that seen after substitution with alanine. The slight upward mobility shift of the substitution mutants of CTFα (arrows) and CTFβ (arrowheads) may be due an alteration of the charge of CTF (Figs. 2 and 4) because this shift was not observed in ΔAPP770 R747K or APP695 R672K, where the basic amino acid was replaced by other basic amino acids (Fig. 4). There was no difference between the ΔAPP770 and APP695 isoforms with respect to these mutations. This result indicates that the basic amino acid at position 747 of ΔAPP770 (position 672 of APP695) is essential for the cleavage of APP at the α-site and that the substitution of a non-basic amino acid with a basic amino acid alters APP metabolism in the protein secretory pathway.

**Intracellular Generation and Degradation of CTFs**—To examine for eventual differences between the intracellular generation and degradation of CTFα and CTFβ deriving from APP695 R672A and those derived from APP695 wt, a pulse-chase study was performed (Fig. 5). Cells expressing APP695 R672A and APP695 wt were labeled with [35S]methionine and chased for the indicated times (Fig. 5a). The radioactivities of CTFα and CTFβ were quantified, and the relative ratios with respect to the maximum level were calculated (Fig. 5b for CTFα and Fig. 5c for CTFβ). CTFα (squares) and CTFβ (circles) derived from APP695 wt (open symbols) were produced and degraded at almost the same rate (Fig. 5, b and c). These results clearly indicate that both CTFα and CTFβ deriving from APP695 R672A are generated and degraded in the same way as those derived from APP695 wt, except for APP695 R672A, which was more efficiently cleaved by β-secretase. Thus, the increase in the level of CTFβ resulting from the APP695 R672A mutation, which was detected by immunoblot analysis (Figs. 2–4), is not due to a simple intracellular accumulation of CTFβ. The CTFs from APP695 wt, as well as those from APP695 R672A, are subject to further degradation.

**Secretion of sAPP from Cells Expressing APP695 R672A**—Because the large extracellular amino-terminal domain truncated at α- or β-sites (sAPPα or sAPPβ) is believed to be always secreted into the medium, we examined the secretion of sAPPα/β derived from APP695 R672A and compared it with that derived from APP695 wt. The culture medium of the pulse-chase studies (Fig. 5) was collected; sAPPα/β was recovered by immunoprecipitation with anti-APP extracellular domain antibody 22C11; and the immunoprecipitates were subjected to SDS-PAGE (7.5% (w/v) polyacrylamide), followed by autoradiography (Fig. 5a). It was not possible to distinguish sAPPα from sAPPα on SDS-PAGE as described previously (33). The arrow in Fig. 6a indicates a band containing both sAPPα and sAPPβ. The secretion of sAPPα/β from APP695 wt (open circles) appeared slightly earlier than that from APP695 wt (open circles) in the medium (Fig. 6b). The secretion of sAPPα/β reached the maximum level after 3 h (APP695 R672A) or after 5 h (APP695 wt). The slight difference may be due to the fact that sAPPβ from APP695 R672A probably contains more sAPPβ than sAPP from APP695 wt. This result suggests the possibility that sAPPβ may be secreted earlier than sAPPα, although we did not detect any remarkable differences between the metabolic rates of intracellular APP695 (Fig. 6c) and CTFs (Fig. 5). The secretion level of sAPPα/β from APP695 R672A seemed to be low when compared with the level of APP expression (Fig. 6a). Therefore, the relative ratio of the level of sAPPα/β to that of intracellular APP was determined at different time points during the pulse-chase experiment (Fig. 6d). The secretion level of sAPPα/β from APP695 R672A was lower (~50% at 3 h and ~30% at 5 h of APP695 wt) compared with that from APP695 wt when the values were standardized with the level of APP expression (Fig. 6d). This may be a reason why the secretion of sAPP from APP695 R672A reached a plateau at 3 h. This result suggests that sAPPα/β from APP695 R672A is not always secreted into the medium and that a certain amount of sAPPα/β from
APP695 R672A is subject to intracellular degradation prior to secretion.

**Secrecion of Aβ from Cells Expressing APP695mt**—If CTFβ is always cleaved by γ-secretase, cells expressing APP695 R672A should generate a greater amount of Aβ, as the pulse-chase study of Fig. 5 indicates that CTFβ from APP695 R672A did not accumulate intracellularly, instead but was subject to further metabolism. To examine this possibility, the amount of Aβ in the medium was analyzed with sandwich ELISA using three types of monoclonal anti-Aβ antibodies: 2D1, which recognizes the human-specific amino acid sequence FRH (amino acids 600–602) of APP695 in the Aβ sequence; 4D1 (epitope is the terminal of Aβ40); and 4D8 (epitope is the terminal of Aβ42), which recognizes APP derivatives truncated at Aβ42 (24). Combinations of antibodies 2D1 and 4D1 or 4D8 in sandwich ELISA allows the specific measurement of the amount of Aβ40 or Aβ42 secreted into the medium. We established additional 293 cell lines that stably expressed APP695 carrying a FAD mutation and APP695 carrying both the FAD mutation and an R672A substitution. We did this because it is well known that APP carrying an FAD mutation is subject to active β-site cleavage and subsequent secretion of Aβ species following the cleavage of CTFβ by γ-secretase (30, 34, 35). When 293 cells (2 × 10^6 cells) expressing APP695wt were cultured in 3 ml of medium for 24 h and 100 μl of the medium was quantified for Aβ, usually 40–50 fmol of Aβ40 and 20–30 fmol of Aβ42 were detected. Because the amount of Aβ secretion is affected by the level of APP expression in the established cell lines, we have indicated the secretion level of Aβ as a ratio of the level of APP expression that was determined from the intracellular APP content (Fig. 7). The APP695 R672A mutation suppressed the production of Aβ40 more efficiently than that of Aβ42. The data suggest that the APP695 R672A mutation does not contribute to the generation the Aβ40 and Aβ42, at least in 293 cells, despite the fact that a greater amount of CTFβ was generated intracellularly. This result also indicates that the increase in CTFβ does not always result in increased secretion of Aβ and that CTFβ derived from the APP695 R672A mutation is subject to degradation prior to cleavage by γ-secretase.

The Swedish mutation (APP695sw), which is thought to enhance the production of Aβ by increasing its sensitivity to β-secretase (30, 34, 36, 37), caused a 25-fold increase in Aβ40 and a 6-fold increase in Aβ42 production. Another FAD mutation, the “London (Hardy)” mutation (APP695 V642F) (38, 39), also caused a remarkable increase in Aβ40 and Aβ42 secretion. The results of this study are consistent with these previous reports describing the effect of the FAD mutation on Aβ production (35). Interestingly, the level of Aβ42 secretion from cells carrying a double mutation of R672A and FAD, Swedish R672A/sw and London R672AV642F, was less than that from cells carrying the original FAD mutation alone (Fig. 7a). This result indicates that the R672A mutation induces a decrease in the Aβ40 secretion from cells carrying the FAD mutation, as in the case for wild-type APP. An identical double mutation of R672A and FAD caused no remarkable decrease in Aβ42 secretion (Fig. 7b), which suggests that the secretion of Aβ42 may

![Figure 6](image)  
**Fig. 6.** Secretion rate of sAPP, intracellular metabolic rate of APP, and secretion level of sAPP. sAPPα/β was recovered from the medium, and APP was recovered from the cell lysate of the pulse-chase experiment (see Fig. 5) by immunoprecipitation. a, shown are autoradiograms of sAPPα/β from APP695wt (left) and APP695 R672A (right). b and c, the incorporation of radioactivity into sAPPα/β and APP was determined as described under “Experimental Procedures.” and the relative ratios of the levels of sAPPα/β (b) and APP (c) are indicated relative to the maximum levels, which were assigned a reference value of 1.0. d, the level of sAPPα/β secretion was normalized to the level of APP expression and assigned an sAPP/APP ratio. Results are the average of duplicate assays, and error bars are indicated.

![Figure 7](image)  
**Fig. 7.** Secretion of Aβ from cells expressing APP695wt and APP695sw. Aβ40 (a) and Aβ42 (b) in the medium were quantified by sandwich ELISA with Aβ40 (4D1) and Aβ42 (4D8) end-specific antibodies. To estimate the level of APP695 expression, APP from cells stably transfected with plasmids was immunoprecipitated, detected by immunoblotting with antibody UT-421 following SDS-PAGE (7.5% w/v) polyacrylamide gel), and quantified using a Fuji BAS 2000 imaging analyzer (data not shown). The level of APP695 expression (a relative ratio) was normalized to the amount of APP from a clone expressing APPwt. The amount of Aβ (fmol/100 μl of medium) was divided by the relative APP695 ratio and is expressed as the Aβ/APP695 ratio. wt, cells expressing APP695wt; R672A, cells expressing APP695 R672A mutation; V642F, cells expressing APP695 V642F (London mutation); R672A/V642F, cells expressing a double mutation of APP695 R672A and APP695 V642F; sw, cells expressing the Swedish mutation (a double mutation of APP695 K595N and APP695 M596L); R672A/sw, cells expressing a double mutation of APP695 R672A and the Swedish mutation. Results are the average of several independent studies with independent clones (n = 4–7), and error bars indicate S.D. *, p < 0.01; ***, p < 0.001.
be different from that of Aβ40. Furthermore, these observations support the notion that APP carrying the R672A mutation is cleaved in a secretory pathway that differs from the normal secretory pathway, where APP carrying the FAD mutation is cleaved.

**DISCUSSION**

Previous studies in cells expressing APP with a truncated cytoplasmic domain have not been able to clarify the function of this cytoplasmic domain in detail because the cytoplasmic domain is thought to contain multiple signal domains regulating APP metabolism (15, 40). Therefore, we tried to explore the intracellular sorting signals in the APP cytoplasmic domain with an alanine-scanning mutation, a single amino acid substitution of alanine for the original amino acid. We focused on the production of intracellular CTFs in cells expressing APP carrying the alanine-scanning mutation because an alteration of the protein secretion system should first of all induce CTFβ generation and because the APP cytoplasmic domain may function to direct APP into the normal secretory pathway. In this study, we found that the basic amino acid at position 747 of APP770 (position 672 of APP695) in the cytoplasmic domain plays an important role in the cleavage of APP at the α-site. This basic amino acid seems to be essential for the normal metabolism of APP in the normal secretory pathway, in which APP is cleaved preferentially by α-secretase rather than by β-secretase when APP does not carry the FAD mutation (Fig. 8). The increased production of CTFβ derived from APP695 R672A did not result in increased secretion of Aβ and sAPP. The most likely interpretation of these observations is that CTFsβ do not migrate to the compartment of the normal secretory pathway and are subject to degradation prior to cleavage by γ-secretase (Fig. 8). The alternative possibility, that the arginyl residue at amino acid 672 of APP695 is critical for γ-secretase recognition, does not seem plausible because the production of Aβ42, at least, was not affected by the R672A mutation of APP695 and because a double mutation of R672A and FAD increased Aβ secretion above the level seen with the R672A mutation alone. Therefore, we postulate a default secretory pathway in which APP carrying the R672A mutation is cleaved by β-secretase as well as by α-secretase (Fig. 8).

The present observation also showed that CTFβ does not always generate Aβ and that sAPP is not always secreted, although the increased production of CTFβ is thought to result in increased secretion of Aβ and sAPP in the case of APP carrying the FAD mutation (Fig. 8). The decreased secretion of sAPP derived from APP695 R672A may be due to the intracellular degradation of sAPP in the default secretory pathway. CTFs from the APP695 R672A mutation, as well as CTFs from APP695wt, do not accumulate in the cell and are degraded. This may be the reason why CTFβ does not generate Aβ in the case of the APP695 R672A mutation. These facts may be relevant to Aβ production in the case of the sporadic type of AD. The molecular mechanism that results in APP695 migration into a default secretory pathway that would be enriched in γ-secretase in the R672A mutant has not been elucidated. Further analyses of the intracellular metabolism of APP695 R672A will be important for our understanding of what regulates γ-secretase processing of CTFβ and determines γ-secretase activation in the default secretory pathway.

APP695 R672A may be directed into a default secretory pathway by a putative cytoplasmic factor that can associate with the APP cytoplasmic domain sequence around Arg747 of the amyloidogenic metabolism of APP, the cleavage of APP at the default secretory pathways, the normal (upper) and default (lower) secretory pathways of APP secretion. In healthy (normal) cells, APP is generally directed into the normal secretory pathway, in which APP is cleaved preferentially at the α-site. An FAD mutation such as the "Swedish" mutation (**) enhances the amyloidogenic metabolism of APP, the cleavage of APP at the β-site, in the normal secretory pathway. Then, CTFβ is always cleaved by γ-secretase, and the resulting Aβ and p3 fragment are secreted from the cell. sAPPα/β is also secreted. On the other hand, the APP770 R747AAPP695 R672A mutation is thought to direct APP into the default secretory pathway due to inefficient function of putative cytoplasmic factor(s). APP in the default secretory pathway is cleaved at the α-site as well as the β-site. The resulting sAPPα/β is not always secreted, and CTFβ is not always subject to subsequent cleavage by γ-secretase. Some sAPPα/β and CTFβ is degraded intracellularly prior to secretion. When the regulatory steps of γ-secretase in the default secretory pathway become aberrant for some reason such as aging, the relatively large amount of CTFβ will be further cleaved by γ-secretase prior to degradation, and an increased amount of Aβ will be generated. This may explain how the pathogenic state of sporadic AD continues to develop.

**FIG. 8. Putative secretory pathways of intracellular APP metabolism.** Cells are postulated to have at least two protein secretory pathways, the normal (upper) and default (lower) secretory pathways of APP secretion. In healthy (normal) cells, APP is generally directed into the normal secretory pathway, in which APP is cleaved preferentially at the α-site. An FAD mutation such as the "Swedish" mutation (**) enhances the amyloidogenic metabolism of APP, the cleavage of APP at the β-site, in the normal secretory pathway. Then, CTFβ is always cleaved by γ-secretase, and the resulting Aβ and p3 fragment are secreted from the cell. sAPPα/β is also secreted. On the other hand, the APP770 R747AAPP695 R672A mutation is thought to direct APP into the default secretory pathway due to inefficient function of putative cytoplasmic factor(s). APP in the default secretory pathway is cleaved at the α-site as well as the β-site. The resulting sAPPα/β is not always secreted, and CTFβ is not always subject to subsequent cleavage by γ-secretase. Some sAPPα/β and CTFβ is degraded intracellularly prior to secretion. When the regulatory steps of γ-secretase in the default secretory pathway become aberrant for some reason such as aging, the relatively large amount of CTFβ will be further cleaved by γ-secretase prior to degradation, and an increased amount of Aβ will be generated. This may explain how the pathogenic state of sporadic AD continues to develop.
APP770 and Arg^{672} of APP695. The protein may play a role in the direction of APP into the normal secretory pathway (Fig. 8). The effect of the APP770 R747A (APP695 R672A) mutation on CTFβ production probably results from interference with the binding of this hypothetical factor to the APP cytoplasmic domain. One recent report, which indicates that transgenic mice expressing CTFβ develop extracellular Aβ with age (41), may be in support of this idea. Aging may induce a loss of function of the cytoplasmic factor and activate γ-secretase activity in a default secretory pathway. Another recent report, which indicates that knockout mice for presenilin-1 have increased levels of CTFβ, but decreased secretion of Aβ (42), may also be relevant to our present results. The basic amino acid (Arg^{672}) of APP695 may play an important role in the APP secretory pathway related to presenilin-1.

Furthermore, when compared with the CTFβ production in cells expressing APP695 R672A, secretion of Aβ into the medium was reduced slightly (Aβ40) or did not change (Aβ42). This result is not necessarily cell-type specific, at least in non-neuronal cells, because identical results, i.e. increased CTFβ but unchanged secretion of Aβ, were obtained when APP695 R672A was stably expressed in Chinese hamster ovary cells (data not shown). Double mutants of APP695 R672A and FAD also suppressed the secretion of Aβ40, but not Aβ42, when compared with the FAD-only mutant, indicating that the mechanism of Aβ42 production is different from that of Aβ40 production. It has been shown clearly that the FAD mutation increases the secretion of both Aβ40 and Aβ42, which are thought to be generated in the normal secretory pathway (30, 34, 36, 37). Therefore, these results suggest that the cleavage of APP695 R672A by β-secretase occurs in a compartment different from that used for the cleavage of APPwt and APP carrying the FAD mutation at their β-sites (Fig. 8). Further analysis of the metabolism of APP695 R672A in this default secretory pathway should contribute to our understanding of the molecular mechanism of APP processing in the sporadic type of AD pathology.

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