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Deletion of an Endosomal/Lysosomal Targeting Signal Promotes the Secretion of Alzheimer's Disease Amyloid Precursor Protein (APP)[1]

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Alzheimer's disease amyloid precursor protein (APP) generates a β-amyloid protein (Aβ) that is a main component of the senile plaques found in the brains of Alzheimer's disease patients. APP is thought to undergo proteolysis *via* two different pathways, the amyloidogenic pathway which produces Aβ, and the non-amyloidogenic pathway which releases a large N-terminal fragment into the medium. The proteases that mediate these processes remain unidentified. The physiological function of APP is not clear yet. Therefore, the cytoplasmic region of APP has attracted much interest, because this region is highly conserved among species, and members of the amyloid precursor-like protein (APLP) family. Several potentially functional sequences exist in the region, including signal sequences for protein sorting and a G_o-protein binding sequence. We constructed two mutants, 695ΔNPTY and 695ΔGYEN. They lack potential endosome/lysosome targeting signals, NPTY and GY, in the cytoplasmic domain of APP695, respectively. The mutant APPs had longer half-lives and were secreted more easily into the medium than the wild type, suggesting that these sequences are important for the secretion and metabolism of APP.

Key words: Alzheimer's disease, amyloid precursor protein, endosomal/lysosomal pathway, targeting sequence.

Alzheimer's disease amyloid precursor protein (APP) has attracted attention due to its involvement in the deposition of a β-amyloid protein (Aβ) found in the brains of both sporadic and genetic Alzheimer's disease patients (*1, 2*). The APP gene produces several isoforms through alternative splicing, some of which include a Kunitz-type protease inhibitor domain (KPI). Cultured cells produce two main forms, APP770 (KPI+) and APP695 (KPI−) (*3-5*).

It is now accepted that APP undergoes two different types of proteolysis (*6*) catalyzed by three hypothesized proteases, α-, β-, and γ-secretases. One type mediated by α-secretase involves the non-amyloidogenic pathway, which precludes the generation of Aβ (*7*). The N-terminal large fragment of APP is secreted into the serum and is called sAPP. The other type involves the amyloidogenic pathway catalyzed by β- and γ-secretases, and generates Aβ (*8, 9*).

Several reagents that might enhance or suppress the activity of each secretase (*10-13*) have been reported. However, the secretases have not yet been detected as a protein or membrane machinery, and the mechanism by which they process APP remains unknown.

It is thought that the secretases are not a single component but reside in specific compartments of the plasma membrane, because no protease inhibitors specifically inhibit any of the secretases. From the results of kinetical analysis of the processing catalyzed by α-secretase, it is widely accepted that α-secretase functions in the *trans*-Golgi network (TGN) (*14, 15*). It has also been reported that lysosomotropic reagents inhibit both amyloidogenic and non-amyloidogenic processing (*16*). Thus, the possibility that lysosomes or a lysosome-like acid compartment is the processing site cannot be excluded (*17*).

APP is considered to be a type-I membrane protein (*18*), and several functional sequences have been found in its cytoplasmic domain. They are G-protein binding sequences (*19*) and endosome/lysosome targeting signal sequences (*20*). In particular, signal sequences are conserved even among its homologues [APLP1 and APLP2; which lack the β-amyloid sequence (*21*)], and the idea that they locate APP in the region where secretases exist is becoming well established (*22*).

The signal sequences found in the cytoplasmic domain of APP are NPTY and GY. NPTY resembles NPXY (*23*), which exists in the cytoplasmic domain of the LDL receptor. NPXY mediates the receptor internalization from the plasma membrane to clathrin-coated vesicles (CCVs), and its targeting to endosomes/lysosomes. GY is found in the cytoplasmic domain of lysosomal enzymes or membrane proteins (*24, 25*), and is thought to be involved in the sorting from the plasma membrane or TGN to endosomes/lysosomes.

APP has been shown to be endocytosed *via* clathrin-coated vesicles (*26*), while another group has shown that

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APP is present in the lysosomal fraction (*27*). C-Terminal proteolytic fragments have also been detected in lysosomal fractions, indicating that secretases may exist in these compartments.

We constructed deletion mutants to investigate the functions of the two intracellular targeting signal sequences of APP. We deleted NPTY and GYEN, respectively, to adjust the number of deleted residues. We used site directed mutagenesis to delete each of these domain sequences, introduced the mutant cDNAs into COS7-cells, and investigated the sorting of the protein products. The results indicate that the C-terminal domain of APP is important for the secretion of APP.

MATERIALS AND METHODS

1. Cell Culture and Transformation of COS-7 Cells—COS-7 cells were cultured in DMEM and transfected by electroporation (*28, 29*) using plasmid pSRD (4.0 μg) (*30, 31*) containing the APP constructs described below for 5.0×10^6 cells.

2. Metabolic Labeling—Forty-eight hours after transfection, cells were pulse-labeled for 30 min in 0.7 ml of methionine- and cysteine-free DMEM containing 0.17 mCi/ml Pro-mix™ L-[^{35}S] *in vitro* cell labeling mix (Amersham) and 10% (v/v) dialyzed fetal calf serum, and then chased for the indicated periods.

The labeled cells were solubilized with RIPA (1% NP40/0.1% sodium deoxycholate/150 mM NaCl/50 mM Tris-HCl, pH 7.5). The medium and solubilized cells were centrifuged, and the supernatant was subjected to immunoprecipitation as described below.

3. Plasmids and Construction—Human APP695 was subcloned in the pSRD-plasmid under the control of the early SV40 promotor.

$N_{684}PTY_{687}$ and $G_{681}YEN_{684}$ of APP695 were deleted by the method of Kunkel (*32*) using the following oligonucleotides: AC-GGC-TAC-GAA-TTC-TTT-GAG-CA (⊿NPTY) and CAG-CAG-AAC-CCT-ACG-TAC-AAG-TTC-TTT-G (⊿GYEN). The mutants were screened and their sequences were confirmed.

4. Antiserum and Immunoprecipitation—A monoclonal antibody, 22C11, raised against residues 66-81 (*33*) was purchased from Boehringer Mannheim. Rabbit antiserum (anti-PN) was raised against the secreted N-terminal fragment of APP695. Rabbit antiserum (anti-β1-16) was raised against the N-terminal 16 amino acids of β-amyloid. Rabbit polyclonal antibody anti-AC against residues 675-695 was a gift from Dr. E. Kominami (Juntendo University, Tokyo).

The medium and cells, prepared as described above, were preincubated with protein A-Sepharose for 1 h and then centrifuged at $20,000\times g$ for 15 min. The supernatant was incubated with anti-PN at 1:70-1:100 dilution for 1 h. Immunocomplexes were precipitated with protein A-Sepharose at $20,000\times g$ for 15 min, boiled in 15 μl sample buffer, and then analyzed by 7.5% SDS-PAGE. Fluorography and quantitative densitometry were performed with an Image-analyzer BAS1000 (Fuji-Film).

5. Subcellular Fractionation by Centrifugation—Experiments were carried out at 4°C. Confluent transfected cells were homogenized in buffer A [0.25 M sucrose/10 mM Tris-HCl, pH 7.5/1 mM dithiothreitol (DTT)] with 10 strokes of an overhead stirrer (Wheaton) set at level 3. The lysate was centrifuged at $650\times g$ for 5 min to remove nuclei. The supernatant was centrifuged at $3,000\times g$ for 30 min, at $7,500\times g$ for 30 min, and then at $100,000\times g$ for 60 min. The precipitates were denoted as the heavy lysosomal and mitochondrial fraction, the light lysosomal and mitochondrial fraction, and the microsomal fraction, respectively. The supernatant was denoted as the cytosol.

6. Percoll Density Cell Fractionation—Cell fractionation was performed as described previously (*34*) except that the samples were centrifuged at $30,000\times g$ for 45 min in 20% Percoll (starting density, 1.057 g/ml), and marker enzymes were assayed (*35, 36*). The samples were boiled with an equal volume of sample buffer and then analyzed by SDS-PAGE (*37*).

7. Western Blot Analysis and Measurement of APP—Western blot analysis was performed essentially as described previously (*38*). Proteins separated on SDS-polyacrylamide gels were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Quantitative densitometry was performed with an Imagemaster densitometer (Pharmacia).

RESULTS

1. Secretion of APP695⊿NPTY and APP695⊿GYEN—Wild-type APP695 and mutant-APPs, APP695⊿NPTY and APP695⊿GYEN (Fig. 1), were expressed in COS7-cells and APP species secreted into the culture medium were examined. The molecular weights of the full-length and secreted mutant APPs were indistinguishable from that of wild-type APP695 (Fig. 2). Western-blot analysis with anti-β1-16 suggested that the deletion of the two 4 residue sequences did not alter the site of cleavage catalyzed by α-secretase or the secretion of the N-terminal fragment (sAPP).

2. Deletion of the NPTY and GYEN Sequences Increases

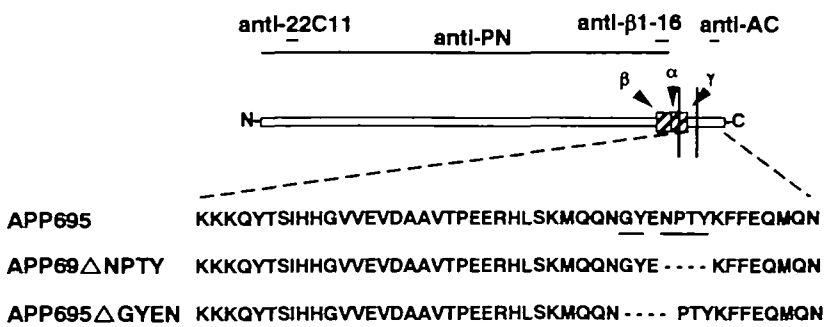
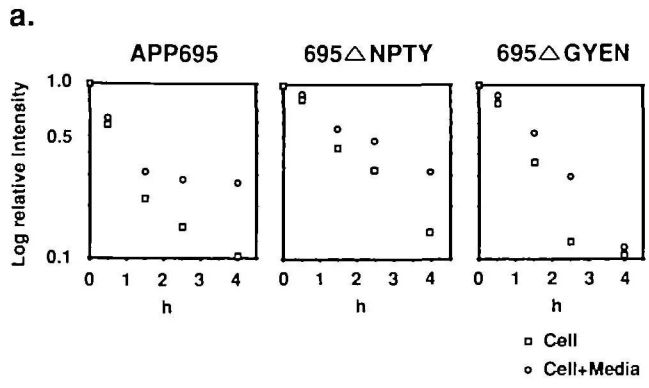


Fig. 1. **Schematic representation of APP processing and the deletion mutants.** The hatched box indicates the Aβ domain and the vertical lines indicate the cell membrane. Arrows indicates the α-, β-, and γ-secretase cleavage sites. Bars indicate epitopes of the antibodies and antisera used in this study. The amino acid sequence of the cytoplasmic domain of APP is enlarged. The potential signal sequences, NPTY and GY, are underlined. The abbreviations for each construct are given at the left.

the Secretion of APP—The NPTY sequence in the cytoplasmic domain of APP has been proposed to mediate the endocytosis of APP. Therefore, we examined the effect of the deletion of this sequence on secretion. The medium and cells from each APP-transfected cell culture were collected 24, 48, and 72 h after transfection. The samples were quantitatively analyzed by Western blotting with 22C11 and anti-AC staining, respectively. The ratio of sAPP to intracellular full-length APP was calculated for each mutant, and normalized as to the value of APP695 at 24 h.

As shown in Fig. 2, the rates of APP secretion from both 695⊿NPTY and 695⊿GYEN were higher than the control values at 24 and 48 h. This does not contradict the previously reported data on the disruption of the NPTY sequence or other C-terminal truncation (*14*). This implies the possibility that these sequences interfere with the processing by α-secretase or the degradation by the cellular proteolytic machinery. Since the cell number began to decrease after 72 h transfection, we used cells within 60 h after transfection in the following experiments.

3. The Metabolism of APP Is Affected by the Deletion of NPTY and GYEN—To study the possibility that these deleted sequences play a role in the cellular metabolism of APP, we performed pulse-chase experiments on transfected COS7-cells. The total radioactivity represents the sum of the radioactivity of immunocomplexes from the medium and cells. The total radioactivity collected at each time point is given as the ratio against that at 0 h. $t_{1/2}$ was calculated from the total radioactivity and cellular radioactivity (Fig. 3).



b.

	APP695	△NPTY	△GYEN
[Cell + Media]	0.9	2.2	1.5
[Cell]	0.7	1.5	1.0

(h)

Fig. 3. **Pulse chase experiments with COS7-cells expressing wild-type and mutant-APPs.** Cells were metabolically labeled for 30 min with [^{35}S]methionine and cysteine, and then chased in the presence of excess amounts of unlabeled methionine and cysteine for the indicated times. (a) Total radioactivity (○) and cellular radioactivity (□) at each time point. Data are the ratios to the 0 h value. (b) The half-lives of the wild-type and mutant-APPs were calculated from (a).

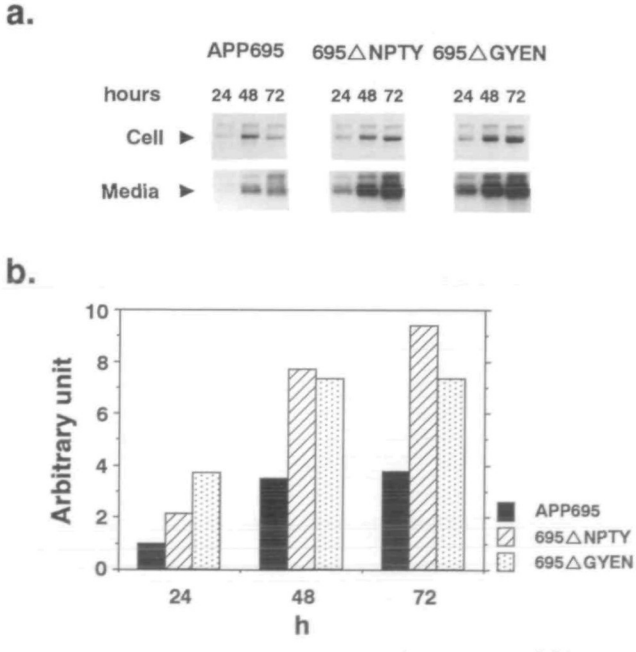


Fig. 2. **APP mutants expressed in COS7-cells.** (a) COS7-cells were transfected with wild-type and mutant APP695 cDNAs. At the indicated times after transfection, cells and media were collected, and the cells were solubilized in RIPA buffer and analyzed by SDS-gel electrophoresis (a). The upper bands represent the endogenous APP770 expressed in COS7-cells. APPs in cell lysates and media were detected with anti-AC and anti-22C11, respectively, and quantified (b). The ratios of sAPP to intracellular full-length APP were calculated. Data were normalized as to the value of wild-type APP695 after 24 h.

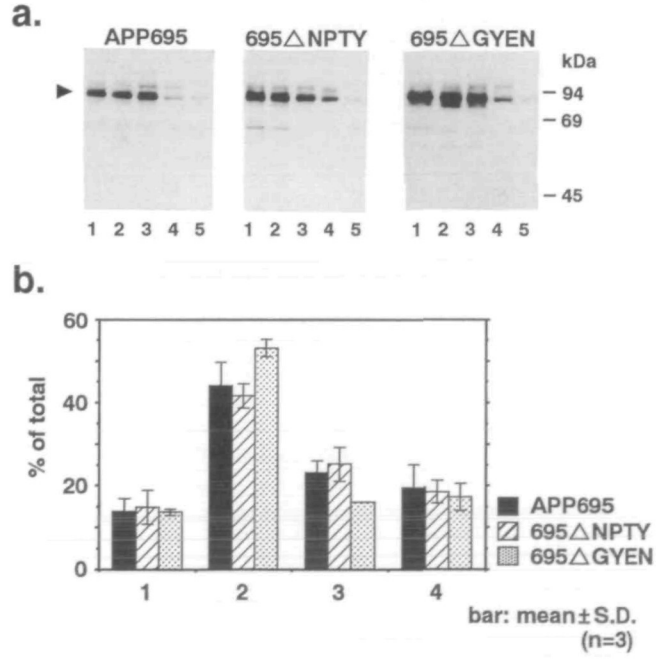


Fig. 4. **Subcellular fractionation of transfected COS7-cells.** Fractionation was performed and each sample was as follows: (1) nuclei, (2) mitochondria and lysosomes (heavy), (3) mitochondria and lysosomes (light), (4) microsomes, and (5) cytosol. (a) Samples were subjected to SDS-PAGE (7.5%), followed by Western-blotting with anti-AC staining. (b) Quantitation of the data shown in (a). Data are expressed as the % of total intracellular full-length APP. The cytosol fractions were omitted. The majority of full-length APPs was in the lysosome/mitochondria fraction.

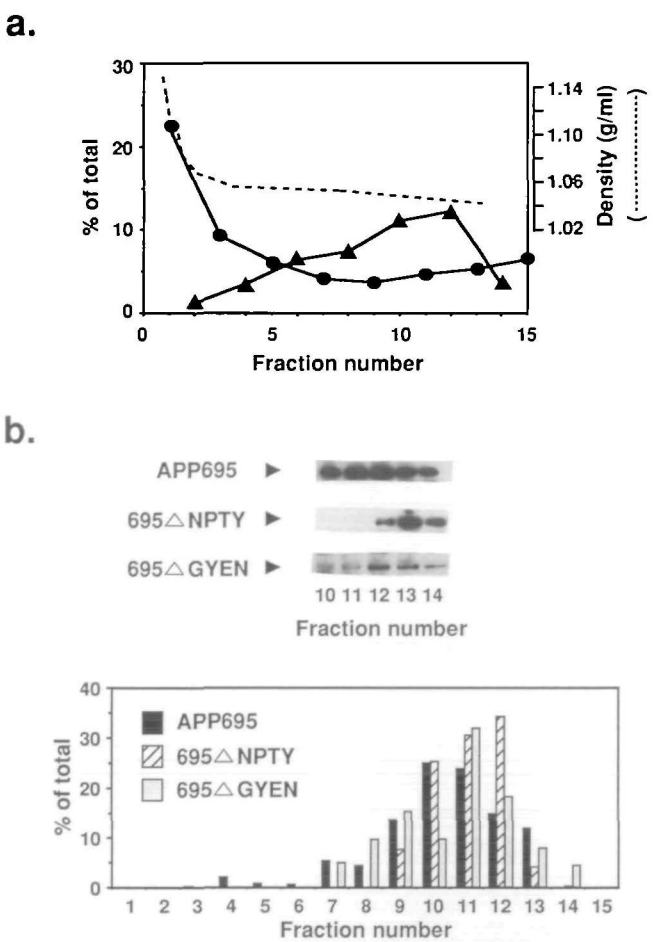


Fig. 5. **Distribution of full-length APP in mitochondrial and lysosomal fractions.** (a) Mitochondrial-lysosomal fractions were prepared from COS-7 cells expressing the wild-type and mutant-APPs, and then subjected to Percoll density gradient centrifugation (1.057 g/ml), 15 fractions of 0.5 ml each being collected. The activities of cathepsin B (●) and succinate-2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium-reductase (▲) were measured. Data are expressed as percentage of the total activities. The graph is a representative one. (b) Each sample was subjected to SDS-PAGE (7.5%), followed by detection by Western-blotting, and then the distribution of full-length APP was determined.

It is obvious that APP695 with the deletion has a longer life ($t_{1/2}=2.2$ and 1.5 h, respectively) than the wild type ($t_{1/2}=0.9$ h), suggesting that some portion of APP695 escapes from rapid degradation. The radioactivity of sAPP released from cells increased more rapidly for both deletion mutants. The secretion pathway and the degradative pathway possibly compete with each other.

4. Subcellular Fractionation of Transfected COS7-Cells—Mutant transfected COS7-cells were fractionated to determine whether or not the deletion of these putative signal sequences affects the cellular localization of APP. Full-length APP in each fraction was measured using anti-AC and the ratios to the total cellular full-length APP are shown in Fig. 4. The distribution of full-length APP did not change remarkably in the mutants. The majority of full-length APP was observed to be colocalized with lysosomes and mitochondria. We therefore tried to separate these fractions more precisely.

5. The Cytoplasmic Domain of APP Mediates the Translocation—Percoll density gradient fractionation was performed to distinguish the lysosomal fraction from the mitochondrial fraction. Cathepsin B activity, a lysosomal marker, and succinate-2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium-reductase activity, a mitochondrial marker, were assayed. The distribution of full-length APP was analyzed by Western blotting (Fig. 5). Selective accumulation of full-length APP was not detected in the lysosomal fraction.

The wild-type was detected in fractions 4 to 13 suggesting that a small portion of full-length APP exists in the lysosomal fraction. But no accumulation of full-length APP was detected in the lysosomal fraction for either deletion mutant. The 695⊿NPTY mutant was distributed in lower density fractions than the wild-type. This tendency was also observed for the 695⊿GYEN mutant, but the trend was weaker (Fig. 5).

The fraction between the lysosomal fraction and the mitochondrial one is rich in endosomes. The above results imply that the mutants are inefficiently translocated from endosomes to lysosomes or another related compartment where the degradation occurs.

DISCUSSION

The deletion of the NPTY and GYEN sequences from the cytoplasmic domain of APP 695 did not alter the hydrolysis by α-secretase, but it changed two parameters of APP metabolism.

First, the ratio of sAPP to cellular full-length APP was elevated in both mutants. This indicates that the deletion of each signal sequence makes the APP molecule more susceptible to processing by α-secretase.

Second, the half-life of cellular full-length APP was longer for the deletion mutants. In comparison with the wild-type, $t_{1/2}$ was longer by 1.3 h for 695⊿NPTY and 0.6 h for 695⊿GYEN. The total radioactivity at 0 h was almost identical for wild-type APP and both mutant-APPs. Therefore, the half-life does not depend on the quantity of expressed APP. The questions are what causes this delay in metabolic degradation, and what is responsible for the increase in the secretion.

It is supposed that only a small portion of full-length APP is actually retained on the plasma membrane. The results of an immunofluorescence study on wild-type APP695 indicated that full-length APP is intracellularly distributed in a vesicular form and little is on the plasma membrane (data not shown). Therefore, longer retention of APP at the plasma membrane may suppress the degradation by inhibiting lysosomal proteolysis after endocytosis. Previous studies have also demonstrated that secretases do not exist on the plasma membrane (*39*).

The same tendency was observed for the 695⊿GYEN mutant. This mutant also showed delayed degradation, but it was secreted a little more rapidly than 695⊿NPTY. 695-⊿GYEN should be internalized as efficiently as wild-type APP695 if its NPTY sequence in the C-terminal region functions properly, as described previously. The results indicate that the sequence, GYEN, mediates two independent types of sorting; one is from TGN to the endosomes/lysosomes, and the other from the plasma membrane to the endosomes/lysosomes except for internalization.

Two methods of fractionation were used, but the deletion of NPTY or GYEN did not change the distribution of full-length APP in cells discretely, nor completely abolished the translocation of APP. This is different from the case of the lysosomal membrane glycoproteins, lamp2 and lap. In these proteins, an amino acid substitution, such as tyrosine to alanine, causes the absence of these proteins in the lysosomal fraction (*25*).

On Percoll density gradient fractionation, we obtained one remarkable result. The 695⊿NPTY mutant accumulated in lower density fractions, not in the lysosomal fraction. The 695⊿GYEN mutant also showed this tendency. We believe that our results show that the mutants did not migrate like the wild-type due to the deletion of the signal sequence.

Since proteins that are endocytosed by endosomes migrate from lower density fractions to higher density fractions and fuse with the lysosomal fraction, lysosomal proteases can degrade APP very rapidly, and some degraded products were detected in other studies (*15*). In our case, however, no specific band was detected.

From this point of view, the fact that the deletion of the two sequences affects this step differently is quite interesting. For 695⊿NPTY, a reduction in the proportion of APP existing in the endosomes occurred, but for 695⊿GYEN, the speed of APP migration from the endosomes to the lysosomes might be directly reduced. Our experiments were carried out using COS7-cells. It may be a future problem as to whether the above results can be extended to other cell types including neurons. However, we think COS7-cells are suitable for examining the effect of a mutation introduced into APP molecules, because COS7-cells have been proved to possess 3 kinds of secretase activity (*28*).

Further analysis is required to define more precisely the function of each signal sequence in APP metabolism.

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