beta-Amyloid peptide (Aβ) is a principal component of parenchymal amyloid deposits in Alzheimer's disease. Aβ is derived from amyloid precursor protein (APP) by proteolytic cleavage. APP is subject to N- and O-glycosylation and potential tyrosine sulfation, following protein synthesis, and is then thought to be cleaved in an intracellular secretory pathway after or during these post-translational modifications. Studies utilizing agents that affect a series of steps in the protein secretory pathway have identified the possible intracellular sites of APP cleavage and Aβ generation within the protein secretory pathway. In the present study, using cells with normal protein metabolism, but expressing mutant APP with defective O-glycosylation, we demonstrated that the majority of APP cleavage by α, β, and γ-secretases occurs after O-glycosylation. Cells expressing the mutant APP noticeably decreased the generation of the intracellular APP carboxy-terminal fragment (αAPP-COOH), a product of α-secretase, and both Aβ40 and Aβ42 in medium, a product of β- and γ-secretases. Furthermore, we found that the cells accumulate the mutant APP in intracellular reticular compartments such as the endoplasmic reticulum. Agents that could ambiguously affect the function of specific intracellular organelles and that may be toxic were not used. The present results indicate that APP is cleaved by α-, β-, and γ-secretases in step(s) during the transport of APP through Golgi complex, where O-glycosylation occurs, or in compartments subsequent to trans-Golgi of the APP secretory pathway.

Alzheimer's disease (AD) is characterized by the presence of parenchymal and cerebrovascular β-amyloid (Aβ) deposits (1). Aβ is a 39–43-amino acid peptide that is derived from Alzheimer's amyloid precursor protein (APP). The generation of Aβ is thought to be one of the major events of AD pathogenesis (reviewed in Refs. 3 and 4). APP is an integral membrane protein with a receptor-like structure, existing in several isoforms which, in many tissues, arise by alternative splicing of a single gene (5–12). APP is subject to post-translational modifications such as glycosylation, sulfation, and phosphorylation during transit through the intracellular secretory pathway (13–22). APP isoforms exist as immature (imAPP, N-glycosylated) and mature (mAPP, N- and O-glycosylated, tyrosyl-sulfated) species. The imAPP localizes in the ER and cis-Golgi, and the mAPP localizes in compartments following trans-Golgi and on the plasma membrane. The molecular mechanism(s) and cellular compartment(s) involved in APP cleavage and Aβ production have yet to be fully resolved. Studies using agents (i.e. brefeldin A and monensin) or studies with treatments (i.e. cell culture at low temperature) that interfere with secretory metabolic steps (23–28) suggest that APP cleavage by α-secretase occurs in a secretory step in late Golgi. Although recent reports indicate that the ER is the site for generation of Aβ42 but not Aβ40 in the neuron (29, 30), Aβ in studies using agents that interfere with pH gradients (i.e. chloroquine and ammonium chloride) is believed to be generated in acidic compartments such as endosomes and/or late Golgi (31–33). However, these procedures are toxic, and it is possible that these agents interfere with intracellular protein metabolism through nonspecific and unpredictable mechanisms. To identify potential intracellular compartments involved in the cleavage of APP by secretases without utilizing toxic metabolic inhibitors, we prepared cells expressing mutant APP (APPmut) which is not subject to O-glycosylation. In such cells, all other intracellular protein metabolism is thought to be normal. Taking advantage of the property of the cells expressing APPmut, we examined the processing of APP in healthy cells. Cells expressing the APPmut noticeably decreased the generation of the carboxy-terminal fragment of APP (αAPP-COOH), a product of cleavage by α-secretase, and also failed to generate Aβ40 and Aβ42, products of cleavage by both β- and γ-secretases. The present study shows that, without utilizing metabolic agents which nonspecifically interfere with protein degradation and secretion, APP is cleaved after, or possibly during, maturation (O-glycosylation). These results indicate that APP cleavage occurs in compartment(s) subsequent to trans-Golgi of the protein secretory pathway or possibly during the transport of APP through Golgi complex, where O-glycosylation occurs (34). Generation of Aβ42 in the ER (29, 30) may be a neuron-specific and/or a minor event.
EXPERIMENTAL PROCEDURES

Introduction of Random Mutation on APP cDNA and Construction of Plasmid—cDNA encoding human APP770 was cloned from a zAP HeLa cell cDNA library by immunoscreening with anti-APP antibody, G-369 (35). The cDNA was subcloned into pcDNA3 (Invitrogen) at HindIII/XhoI sites (Fig. 1a). A sequence of APP770 extracellular domain, 379–666 (the numbering for APP770 and also 304–591 for APP695 forms) which includes potential N-glycosylation sites (13), 1 and 20), was deleted by exclusion of XhoI/BglII fragment. The 3′ recessed termini were filled with dNTP and ligated in frame (pAPP770mut) (Fig. 1a (i)). To produce EcoO65I site in the cytoplasmic domain of pAPP770mut, site-directed mutagenesis was included with PCR as follows: primer 1, 5′-GCCGCCGTCACCCAGAGAGCGACACTCTGTC-3′ and primer 2, 5′-ATTAGTACAGCTATGATAGT-3′ (SP6 promoter primer), were used in PCR with PWO DNA polymerase (Boehringer Mannheim) in the presence of plasmid pAPP770mut. Primer 3, 5′-TCTGGGTGACCGCGGTCACTCTCAC-3′ (the nucleotide underlined was changed to produce EcoO65I site (A to C)), and primer 4, 5′-TAATAGATCCTACTATGAGG-3′ (TT promoter primer), were used in PCR with PWO DNA polymerase in the presence of plasmid pAPP770mut. Both PCR products were digested with EcoO65I, ligated, and then inserted into pcDNA3 at HindIII/XhoI sites. Production of the EcoO65I site does not change the amino acid sequence in the APP protein, and this PCR procedure with PWO DNA polymerase did not induce nucleotide mutations. The position and direction of primers is indicated in Fig. 1b.

The pAPP770mut that introduced EcoO65I site was further amplified between primers 3 and 4 with Taq DNA polymerase (Takara Co., Kyoto, Japan). The Taq DNA polymerase introduces nucleotide mutations on newly synthesized DNA strands with a frequency of one base per approximately 400 bases (36). The resulting PCR products were digested with pAPP770mut, in which the 3′ downstream sequence from EcoO65I site of APP770 has been inserted into pcDNA3, at HindIII and EcoO65I sites (Fig. 1a (ii)). The constructs for mutant pAPP770, pAPP770mut, were subcloned and transfected into 293 cells (human transfected primary embryonal kidney) with Lipofectin, and cell lines that expressed stably-transfected pAPP770mut were isolated. Among the cell lines isolated, cells displaying aberrant APP metabolism were further characterized. The site of mutation was detected by sequencing the DNA inserted in pAPP770mut, and the resulting amino acid substitution was listed in Table 1. The mutation was also introduced into APP695cDNA to construct pAPP695mut by exchanging the HindIII/XcmI fragment from APP695cDNA with that from pAPP770mut which carries the mutation (Fig. 1b (ii)). Cell lines that express stably-transfected pAPP695mut were also isolated and analyzed for APP metabolism. APP695mut contains all the N-glycosylation sites and the complete amino acid sequence of APP695 except for the mutated site(s).

Detection of APP—Intracellular APP and the truncated cytoplasmic domain, αAPPmut, derived from APP cleaved by α-secretase were detected by a combination of immunoprecipitation and immunoblot with anti-APP cytoplasmic domain antibody, UT-421, which is raised against a peptide (CysoAPP770-675) (the numbering for APP695 isomorph). UT-421 is specific to APP, and does not react with any myeloid precursor-like proteins, APLP1 and APLP2. 293 cells (2–3×10⁵ cells) were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal bovine serum. APP and αAPPmut were recovered through immunoprecipitation as described (15, 21). Immuno precipitates were analyzed by SDS-PAGE (7.5% (w/v) polyacrylamide for ΔAPP770 and APP695 and 15% (w/v) polyacrylamide for αAPPmut) and transferred electrophoretically to a nitrocellulose membrane. The membrane was probed with UT-421 antibody by 125I-protein A (Amersham Corp., Laclay, France). Specific identity and identification of the immunoprecipitants were examined by a competition study with a peptide (amino acids 675–765 as described previously) (18, 22). The radioactivity of the immunoblot was quantitated using a Fuji BAS 2000 Imaging Analyzer (Tokyo, Japan) or by autoradiography.

Enzymatic Deglycosylation—Deglycosylation of APP was performed with a procedure described previously (19). Antibody (UT-421) complex was recovered from cell lysates following addition of protein A-Sepharose (Pharmacia Biotech Inc.). The beads were washed twice with wash buffer, 10 mM Tris-Cl (pH 8.0), 2.25 mM MgCl₂, and then incubated with 1 milliunit of O-glycanase and/or 10 milliunits of neuraminidase (Seikagaku Co., Tokyo, Japan) in the same reaction buffer containing protease inhibitors as follows: 200 μg/ml (w/v) pepstatin A, 200 μg/ml (w/v) chymostatin, and 200 μg/ml (w/v) leupeptin. In a separate study, the beads were washed twice with reaction buffer, 50 mM citrate buffer (pH 5.5), and then incubated with 4 milliunits of endoglycosidase H (Seikagaku Co.) in the same reaction buffer containing protease inhibitors as follows: 200 μg/ml (w/v) pepstatin A, 200 μg/ml (w/v) chymostatin, and 200 μg/ml (w/v) leupeptin. After overnight digestion at 37 °C, the samples were subject to SDS-PAGE (7.5% (w/v) polyacrylamide) and analyzed by immunoblot using UT-421.

Pulse-Chase Study—Pulse-chase labeling of cells was carried out with [35S]methionine (1 μCi/ml, NEN Life Science Products, NEN-072). 2D1, raised against stably-transfected ΔAPP770 and ΔAPP770mut were labeled metabolically for 30 min, followed by a chase period as indicated. The chase was initiated by replacing the labeling medium with medium containing excess unlabeled methionine. ΔAPP770 was immunoprecipitated using UT-421 and analyzed with Fuji BAS 2000 Imaging Analyzer or autoradiography following SDS-PAGE (7.5% (w/v) polyacrylamide).

Immunocytochemistry—Cultured cells were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS (pH 7.4) containing 0.12 mM sucrose, permeabilized with 0.3% (w/v) Triton X-100 for 5 min, and blocked in 10% (w/v) solution of bovine serum albumin. The cells were incubated with the affinity purified primary antibody, UT-421, and then with fluorescein isothiocyanate-conjugated secondary antibody (Zymed, San Francisco, CA). The same procedure was double-stained with rhodamine-conjugated ConA (Vector Laboratories, Burlingame, CA) which binds with high affinity to glycoproteins in the ER plus cis-Golgi and with rhodamine-conjugated WGA (Vector Laboratories) which binds with high affinity to glycoproteins in medial- plus trans-Golgi (37, 38). The coverslips were mounted in Immersion oil type B (R. P. Cargille Laboratory Inc., Cedar Grove, NJ), and cells were viewed using a confocal laser scanning microscope, Bio-Rad MRC 600.

ELISA Analysis—Three monoclonal antibodies that recognize distinct portions of Aβ were used for quantification of Aβ species in medium. 2D1, raised against Aβ1–27, recognizes a human-specific epitope FRHeco–63 between the β-and α-secretase sites. 4D1, raised against Cys + Aβ32–40, recognizes Aβi peptides truncated at Aβ40 but not Aβ42, 4D8, raised against Gly-Gly + Aβ37–42, recognizes Aβ derivatives truncated at Aβ42 but not Aβ40.

All monoclonal antibodies were purified with protein G-Sepharose (Pharmacia) from the ascites. Purified 2D1 was biotinylated with ECL protein biotinylation module (Amersham, RPN 2202). Conditioned medium from cells (2 × 10⁵ cells) were collected 18–20 h after medium change. Cells were counted with the monoclonal Aβ end-specific antibody, 4D1 or 4D8 (0.3 μg of antibody in a phosphate-buffered saline (PBS, 140 mM NaCl, 10 mM sodium phosphate (pH 7.2)), washed with PBS containing 0.05% (v/v) Tween 20 (washing buffer, WB), blocked with bovine serum albumin (3% (w/v) in PBS), washed with WB, and then a sample (100 μl) diluted suitably with WB containing 1% (w/v) bovine serum albumin (dilution buffer, DB) was incubated together with 2D1 and Aβ1–40 or Aβ1–42 peptides (synthesized at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University). After washing, wells were treated with biotinized 2D1 (12.5 ng in DB), washed, and incubated with 100 μl of a streptavidin-horseradish peroxidase conjugate (1:2000 dilution: Amersham RPN1051). The plate was further washed, and 100 μl of 2,2'-azino-bis(3-ethylbenothiazoline-6-sulphonic acid) peroxidase substrate solution (Bio-Rad, Mike 602-01, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was added to wells and then incubated at room temperature. Reaction was stopped by addition of 100 μl of 1% (w/v) SDS, and the absorbance at 405 nm was determined. This procedure can quantify <0.4 ng of Aβ40 and Aβ42 in 100 μl of medium.

To estimate the level of APP695 expression, APP from cells that expressed stably-transfected plasmids was immunoprecipitated from the same amount of protein lysate, detected by immunoblot with UT-421 following SDS-PAGE, and quantified using a Fuji BAS 2000 Imaging Analyzer. The level of APP695mut expression was normalized to the level of APP695mut expression, which was assigned a reference value of 1.0 and was indicated as a relative ratio. Quantity of Aβ40 and Aβ42 (fmol/100 μl of medium) was divided by the relative APP695 ratio and was indicated as an Aβ/APP ratio.

RESULTS

Analysis of APPmut—To differentiate exogenous transfected APP from endogenous APP in 293 cells, a cDNA (pΔAPP770mut) was constructed, encoding APP770 lacking 287 amino acids
(APP770379–666; numbering for APP770 isoform) of the extracellular domain (Fig. 1a) as described under “Experimental Procedures.” An immunoblot with UT-421 showed that 293 cells, expressing pΔAPP770mut, presented two isoforms (Fig. 2a). The deleted region contains two potential N-glycosylation sites (Fig. 1 and Refs. 13, 15, and 20), and it is well-characterized that endoglycosidase H removes the N-glycan portion of glycoproteins (reviewed in Refs. 39 and 40). Treatment of ΔAPP770mut with endoglycosidase H, isolated from cells which expressed it stably, did not alter the mobility of ΔAPP770 on SDS-PAGE (Fig. 2a). This confirms that N-glycosylation sites are deleted from the pΔAPP770 cDNA, and the resulting ΔAPP770mut is not subject to N-glycosylation in 293 cells. On the other hand, we found that the ΔAPP770mut is modified by O-glycosylation with a terminal neuraminic acid of O-glycan because the treatment of ΔAPP770mut, isolated from the cell, with neuraminidase and a combination of neuraminidase and O-glycanase increased the mobility of ΔAPP770 on SDS-PAGE (Fig. 2a). The treatment of ΔAPP770mut with O-glycanase alone had no effect because the sialic acid first needs to be removed to release O-glycan from the protein (data not shown). We tentatively assigned different ΔAPP770 species as follows; a high molecular weight O-glycosylation form is ogΔAPP770, and a low molecular weight non-glycosylated form is nonΔAPP770. The ΔAPP770mut treated with a combination of neuraminidase and O-glycanase does not show identical mobility with nonΔAPP770 on SDS-PAGE (compare Neu. + O-gly, with Control in Fig. 2a). The ΔAPP770 may be subject to further unidentified modification. We also define, in a broad sense, this ΔAPP770, which may be carrying only the unidentified modification, as nonΔAPP770.

When O-glycosylation and degradation of ΔAPP770mut were compared with those of endogenous APP in a pulse-chase study (Fig. 3a), we found that the respective metabolic rate of nonΔAPP770 and endogenous mAPP and that of ogΔAPP770 and endogenous mAPP were identical (Fig. 4, a and b). These results indicate that the intracellular metabolism of ΔAPP770mut is normal.

To introduce a mutation into its extracellular domain, pΔAPP770 was amplified with primers 3 and 4 using Taq DNA polymerase as shown in Fig. 1a (i). The PCR fragments were substituted for a fragment from HindIII/EcoO65I digestion of pΔAPP770mut and subcloned into pcDNA3 vector as described under “Experimental Procedures.” The plasmid carrying a potential mutation (denoted as × in Fig. 1a (ii)), pΔAPP770mut, was transfected into 293 cells, and approximately 100 independent clones of cells expressing ΔAPP770mut stably were tested for intracellular APP metabolism with immunoblot using UT-421 antibody. A cloned cell line that expresses ΔAPP770mut1 presented with abnormal APP metabolism (Fig. 2b). The cells contained large amounts of nonΔAPP770 and relatively little ogΔAPP770. Treatment with glycosidases of APP recovered from the cells using UT-421 does not affect its mobility on SDS-PAGE when detected by immunoblot using UT-421 (Fig. 2b). The mobility is identical to that of ΔAPP770mut treated with a combination of neuraminidase and O-glycanase (compare Neu. + O-gly in Fig. 2a with Control in Fig. 2b). These results indicate that ΔAPP770mut1 is not subject to O-glycosylation. DNA sequence analysis of pΔAPP770mut1 revealed that Ser-124 (all numbering for

FIG. 1. Schematic model for construction of ΔAPP770 and APP695 mutants. a, construction of pΔAPP770mut. (i) To construct pΔAPP770mut, the N-glycosylation site was excluded by deletion of 0.9 kilobase pairs of XhoI/BglII fragment (APP770379–666; numbering for APP770 isoform). Primers 1 and 2 were used to produce EcoO65I site. The pΔAPP770mut was amplified with Taq DNA polymerase with EcoO65I (#3) and HindIII (#4) primers. (ii) The PCR product was ligated with pAPP770 lacking HindIII/EcoO65I fragment. The resulting pΔAPP770mut contains 2–4 substitution mutations. ΔAPP770mut1 contains mutations at the sites of Ser-124 and Leu-172 (denoted as × and see Table I). b, construction of pAPP695mut1. (i) pAPP695 cDNA. (ii) HindIII/XcmI fragment containing mutation at the sites of Ser-124 and Leu-172 was dissected from pAPP770mut1 and exchanged to a HindIII/XcmI fragment from pAPP695 to construct pAPP695mut1.

FIG. 2. Characterization of ΔAPP770mut and ΔAPP770mut1. ΔAPP770mut (a) and ΔAPP770mut1 (b) were recovered by immunoprecipitation from 293 cells that express corresponding cDNA and were tested for intracellular APP metabolism with immunoblot using UT-421 antibody. A cloned cell line that expresses ΔAPP770mut1 presented with abnormal APP metabolism (Fig. 2b). The cells contained large amounts of nonΔAPP770 and relatively little ogΔAPP770. Treatment with glycosidases of APP recovered from the cells using UT-421 does not affect its mobility on SDS-PAGE when detected by immunoblot using UT-421 (Fig. 2b). The mobility is identical to that of ΔAPP770mut treated with a combination of neuraminidase and O-glycanase (compare Neu. + O-gly in Fig. 2a with Control in Fig. 2b). These results indicate that ΔAPP770mut1 is not subject to O-glycosylation. DNA sequence analysis of pΔAPP770mut1 revealed that Ser-124 (all numbering for
a and D glycosylated) endogenous APP; as indicated. mAPP, mature (N- and O-glycosylated) endogenous APP; imAPP, immature (N-glycosylated) endogenous APP; ogΔAPP770, O-glycosylated ΔAPP770wt and ΔAPP770mut1; nonΔAPP770, naked ΔAPP770wt and ΔAPP770mut1 without glycosylation.

FIG. 3. Autoradiogram of pulse-chase study of ΔAPP770wt and ΔAPP770mut1. 293 cells expressing ΔAPP770wt (a) and ΔAPP770mut1 (b) were pulse-labeled with [35S]methionine for 30 min and chased for periods (0–3 h) as indicated. mAPP, mature (N- and O-glycosylated) endogenous APP; imAPP, immature (N-glycosylated) endogenous APP; ogΔAPP770, O-glycosylated ΔAPP770wt and ΔAPP770mut1; nonΔAPP770, naked ΔAPP770wt and ΔAPP770mut1 without glycosylation.

FIG. 4. Intracellular metabolism of ΔAPP770wt and ΔAPP770mut1. The relative ratios of mature endogenous APP (mAPP), immature endogenous APP (imAPP), O-glycosylated ΔAPP (ogΔAPP770), and naked ΔAPP770 (nonΔAPP770) are indicated relative to maximum levels, which were assigned a reference value of 1.0. a, metabolism of mAPP and nonΔAPP770wt. b, metabolism of mAPP and ogΔAPP770wt. c, metabolism of mAPP and nonΔAPP770mut1. d, metabolism of mAPP and ogΔAPP770mut1. Results are averages of duplicate pulse-chase studies, and the error bars are indicated.

TABLE I

| APP       | Amino acid position (APP695) |
|-----------|-----------------------------|
| wt        | Ser | Leu | Leu | Pro |
| mut 1     | Cys |     |     |     |
| mut 1a    | Cys |     |     |     |
| mut 1b    |     |     | Pro |     |
| mut 2     |     |     |     | Pro |
| mut 3     |     | Pro |     |     |
| mut 4     |     |     |     | Pro |
| mut 5     |     |     |     |     |
| mut 6     |     |     |     |     |

Amino acid positions is for the APP695 isoform) was substituted for cysteine (Ser-124 → Cys), and Leu-172 was substituted for proline (Leu-172 → Pro) (Table I). It is reasonable to assume that either or perhaps both mutations interfere with the O-glycosylation of APP. Pulse-chase studies also confirmed aberrant metabolism of ΔAPP770mut1 (Fig. 3b). Very small amounts of ΔAPP770mut1 were O-glycosylated, and the majority of nonΔAPP770mut1 accumulated intracellularly without O-
glycosylation (Figs. 3b and 4c). However, once ΔAPP770mut1 is O-glycosylated, ogΔAPP770mut1 is degraded in a process similar to that for endogenous mAPP (Fig. 4d). The results indicate that ΔAPP770mut 1 is metabolized normally if it is modified with O-glycan, although the cellular content of ogΔAPP770mut1 is extremely low (Figs. 2b and 3b).

Identical results were obtained when the mutation was carried on the APP695 isoform. To construct pAPP695mut1, a fragment containing the mutations, Ser-124 → Cys and Leu-172 → Pro, which was derived from HindIII/XcmI digestion of pAPP770mut1, was substituted for a fragment from HindIII/ XcmI of pAPP695 wild type (pAPP695wt) and subcloned (Fig. 1b). pAPP695mut1 encodes the entire amino acid sequence including the N-glycosylation sites, except for the two amino acid mutations (Fig. 1b (ii)). When 293 cells stably expressing pAPP695mut1 were selected and analyzed for APP metabolism with immunoblot, a result identical to that for ΔAPP770mut1 was observed (Fig. 5). Because 293 cells do not endogenously express APP695, a neuron-specific APP isoform, it is easy to identify exogenous APP695mut1. In the cells expressing APP695mut1, imAPP695 accumulated in large quantities, whereas only very small amounts of mAPP695 were detected (Fig. 5). The results confirm that the mutation, mut1, inhibits O-glycosylation of APP.

Determination of Mutation Site Inhibiting O-Glycosylation—ΔAPP770mut1 and APP695mut1 contain two amino acid substitutions, Ser-124 → Cys and Leu-172 → Pro. To determine which mutation inhibits O-glycosylation, we constructed plasmids (pAPP695mut) carrying several mutations including a single amino acid substitution for Ser-124 → Cys and Leu-172 → Pro as follows: pAPP695mut1a carries Ser-124 → Cys, pAPP695mut1b carries Leu-172 → Pro, pAPP695mut2 carries a mutation of leucine at position 172 changed to alanine (Leu-172 → Ala), pAPP695mut3 carries a mutation of leucine at position 171 changed to proline (Leu-171 → Pro), pAPP695mut4 carries a double mutation of Leu-172 → Pro and a mutation of proline at position 173 changed to leucine (Pro-173 → Leu), and pAPP695mut5 carries a mutation of leucine...
127 changed to proline (Leu-127 → Pro) (Table I). These mutant APP plasmids were stably expressed in 293 cells, and APP metabolism was examined (Fig. 6a). Among the mutants, APP695mut1b, APP695mut3, and APP695mut4 presented with abnormal imAPP695 accumulation. The ratio of mAPP695 to total APP695 was estimated (Fig. 6b). Generally the ratio of mAPP695 to total APP695 in pAPP695wt was 0.2–0.3 (mAPP695/total APP695). The ratio of mAPP695/total APP695 in cells expressing APP695mut1b, APP695mut3, and APP695mut4 was approximately 0.05, which is identical to that for APP695mut1a. The ratio of mAPP695/total APP695 for APPmut1a, APPmut2, and APPmut5 is identical to, or slightly lower, that of APPwt but is significantly higher than that of APPmut1 (the ratio of mAPP695/total APP695 is >0.15). These results indicate that it is the Leu-172 → Pro substitution that affects O-glycosylation (or maturation) of APP, although a Ser-124 → Cys substitution in APPmut1 may contribute to the aberrant metabolism of APP. However, a Leu-172 → Ala substitution (mut2) does not appear to inhibit O-glycosylation.

When an unrelated leucyl residue at position 127 was changed to proline, Leu-127 → Pro (mut5), O-glycosylation was observed in the same manner as APP695wt.

Intracellular Distribution of APPmut1—To study the intracellular localization of APPmut1, 293 cells expressing APP695mut1 and APP695mut4 were double-stained with UT-421 and ConA (ER plus cis-Golg marker) or WGA (medial- plus trans-Golg marker) and then observed under a confocal laser scanning microscope (Fig. 7). We confirmed that the APP695wt was distributed in ER and Golgi apparatus (Fig. 7, a and b) as described previously (19, 41–44). The APP695wt co-localized with the staining of the ER plus cis-Golg with ConA (Fig. 7a) and of medial- plus trans-Golg with WGA (Fig. 7b). However, APP695mut1 seemed to be distributed in cytoplasm, including ER, but not in late Golgi because the distribution of APP695mut1 was identical with the staining using ConA (Fig. 7c) but not using WGA (Fig. 7d). When non-transfected 293 cells were stained, only a background level of fluorescence was observed (Fig. 7e) because the level of expression of endogenous APP is very low in 293 cells (Fig. 5). Therefore, the immuno-staining observed in this study is thought to be due to the result of transfected exogenous APP. The stainings of ER plus cis-Golg with ConA and of medial- plus trans-Golg with WGA in the transfected cells (Fig. 7, a–d) showed an identical pattern to that in non-transfected 293 cells (Fig. 7e). These results clearly indicate that the intracellular distribution of APP695mut1 is abnormal and that the majority of APP695mut1 is distributed in the ER, in contrast to APP695wt, which is distributed in both the ER and the Golgi equally.

Cleavage of APP Occurs after O-Glycosylation—It has been well characterized that APP is cleaved preferentially at the α-site compared with the β-site and that the carboxyl-terminal fragment of APP, αAPPCOOH, is generated intracellularly. The generation of αAPPCOOH from APP695mut1 was examined. αAPP695COOH was recovered by immunoprecipitation with UT-421 from the lysates of 293 cells expressing APP695wt and APP695mut1, separated by SDS-PAGE (15% (w/v) polyacrylamide gel), and analyzed by immunoblot using UT-421 (Fig. 8). APP695wt generates a 14–15-kDa αAPPCOOH (αAPPCOOH presents a higher molecular weight on the SDS-PAGE than its actual molecular weight) that has been fully characterized (18, 45, 46). Because expression of endogenous APP in 293 cells is extremely low (Figs. 5 and 7e) and production of endogenous αAPP695COOH was under the detectable level (data not shown), it is clear that the detected αAPP695COOH in Fig. 8a is derived from transfected exogenous APP695wt. Only extremely low levels of αAPP695COOH were detected in cells expressing APP695mut1 (Fig. 8a), although the level of APP695mut1 expression was almost identical to that of APP695wt (Figs. 2, 5, 6, and 7). The lower production of αAPP695COOH was observed in several independently cloned cells (mut1–3) that stably express APP695mut1. The production of αAPP695COOH was quantified and indicated as a ratio of αAPP695COOH to total APP (αAPP695COOH/total APP) in Fig. 8b. The results indicate that APP cleavage by α-secretase occurs after, or possibly during, O-glycosylation of APP.

We also quantified the amount of Aβ40 and Aβ42, the major Aβ isomers, in the medium of 293 cells expressing APP695mut1 by sandwich ELISA using an Aβ-specific monoclonal antibody 2D1 (epitope is FRβ600–602), and 4D1 or 4D8, the Aβ carboxyl-terminal end-specific monoclonal antibodies that recognize Aβ40 and Aβ42, respectively. The level of APP expression was quantified with immunoblot as described under
Experimental Procedures. We indicated the level of Ab production as a ratio of the amount of Ab to APP expression level (Ab/APP) in Fig. 9. Production of both Ab40 (Fig. 9a) and Ab42 (Fig. 9b) from APP695mut1 was found to be very low. Identical results were also obtained from the study with 293 cells expressing DAPP770wt and DAPP770mut1 (data not shown). These results suggest that the majority of APP cleavage at β- and γ-sites also occurs after, or possibly during, O-glycosylation of APP.

DISCUSSION

APP is thought to be cleaved by α-, β-, and γ-secretases in the protein secretory pathway. Previous studies using protein metabolic inhibitors (chloroquine, brefeldin A, bafilomycin A1, etc.) suggest that APP cleavage by α-secretase occurs in the trans-Golgi network or other late compartments of the protein secretory pathway (23, 24, 26–28), and that cleavage by β-secretase occurs in acidic compartments such as endosome and/or late Golgi (31–33). These results are plausible, but one must consider the fundamental problem of drugs, which may affect protein metabolism nonspecifically, that were used in the previous studies. The results obtained from such studies may have been due to indirect or generic effects on APP metabolism. Furthermore, recent reports suggest that the production of Ab42 but not Ab40 occurs in the ER (29, 30). However, the biochemical quantification of Ab42 production in ER without toxic drugs has not been performed. Therefore, we conducted...
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Fig. 8. Detection of intracellular αAPP<sub>COOH</sub>, a product by θ-secretase. A, autoradiogram of αAPP<sub>COOH</sub> derived from APP695. αAPP<sub>COOH</sub> was recovered by immunoprecipitation with UT-421 antibody from two independent clones of 293 cells expressing APP695<sub>wt</sub> and three independent clones of 293 cells expressing APP695<sub>mut1</sub>. Arrow indicates αAPP<sub>COOH</sub>, wt-1 and wt-2, independent clones of 293 cells expressing APP695<sub>wt</sub>; mut1-1, mut1-2, and mut1-3, independent clones of 293 cells expressing APP695<sub>mut1</sub>. 20.1 and 14.4 are standard molecular mass (kDa) of protein. B, quantification of αAPP<sub>COOH</sub>. APP and αAPP<sub>COOH</sub> were quantified using a Fuji BAS 2000 Imaging Analyzer. The level of αAPP<sub>COOH</sub> production was normalized to the amount of APP. Quantity of αAPP<sub>COOH</sub> was divided by the relative APP ratio and indicated as αAPP<sub>COOH</sub>/total APP ratio. Results are the average of five independent studies (n = 5), and the error bar indicates standard deviation. wt, 293 cells expressing APP695<sub>wt</sub>; mut1, 293 cells expressing APP695<sub>mut1</sub>.

Fig. 9. Analysis of Aβ in the medium. Aβ40 (a) and Aβ42 (b) were quantified as described under “Experimental Procedures.” Quantity of Aβ40 (a) and Aβ42 (b) (fmol/100 μl of medium) was divided by the relative level of total APP and indicated as the ratio of Aβ/APP. Results are the average of eight independent clones (n = 8 for APP695<sub>wt</sub>) and that of two independent clones (n = 4 for APP695<sub>mut1</sub>). The error bar indicates standard deviation (***, p < 0.001; **, p < 0.05).

Further biochemical studies to confirm the previous results that had been obtained using metabolic inhibitors.

In the present study, we found that APP<sub>mut1</sub> is defective for O-glycosylation and is metabolized aberrantly in normal cells. APP<sub>mut1</sub> contains two sites of substitution mutation, Ser-124 → Cys and Leu-172 → Pro, and ΔAPP770 also has a deleted sequence within the extracellular domain, including N-glycosylation sites. Present results demonstrate that only the Leu-172 → Pro mutation (mut1b) is effective in inhibiting O-glycosylation of APP. Two explanations for altered metabolism of APP by the mutation are possible. One is that the amino acid sequence around Leu-172 is essential for O-glycosylation itself and/or for recognition by the enzyme(s) which is responsible for O-glycosylation. There are several seryl and threonyl residues, Ser-162, Thr-163, Ser-193, Ser-198, and Ser-206, around the position of Leu-172. Generally, seryl and threonyl residues are candidates for modification by O-linked carbohydrates. However, the amino acid sequences around those seryl and threonyl residues do not appear to contain the recognition motif for GalNAC-transferase, although the peptide motifs that allow for O-glycosylation have not been identified (47). Another possibility is that the mutation of Leu-172 → Pro (mut1b) may inhibit O-glycosylation by causing a partial mis-folding of the APP amino-terminal which in turn may cause failure of APP transport within the Golgi complex. Because the Leu-172 → Ala mutation (mut2) is not effective but the Leu-171 → Pro mutation (mut3) and a double mutation, Leu-172 → Pro/Pro-173 → Leu (mut4), showed an effect identical to the Leu-172 → Pro mutation (mut1b), it is thought that the prolinyl residue at position 171 or 172 may induce a conformational change and then mis-folding of the APP amino-terminal. This may be critical for O-glycosylation or the passing of APP into the Golgi complex. APP<sub>mut1</sub> does not exhibit a conformational change in its β-amyloid and carboxyl-terminal domains because APP<sub>mut1</sub> is metabolized normally if it is modified with O-glycosylation, but this modification is very rare in APP<sub>mut1</sub>. This result indicates that the mutation does not alter the substrate specificity to the secretases.

The amino acid sequence of the deleted region, APP770<sup>40</sup> to 779<sup>3666</sup>, does not contain any known functional domains for APP metabolism. ΔAPP770<sub>wt</sub> matures and degrades identically to APP770. Furthermore, the Leu-172 → Pro mutation does not affect N-glycosylation, as imAPP695<sub>mut1</sub> showed identical mobility to imAPP695<sub>wt</sub> on SDS-PAGE. The cells expressing APP<sub>mut1</sub> present a reliable system to analyze whether APP is cleaved after O-glycosylation, without using drugs which inhibit intracellular protein metabolism indiscriminately. The present results clearly demonstrate that the majority of APP<sub>mut1</sub> is not cleaved by θ-secretase and αAPP<sub>COOH</sub> is not generated. The results indicate, without using cytotoxic drugs, that APP cleavage at the θ-site occurs in a metabolic step following trans-Golgi after proteins have completed O-glycosylation, although we cannot rule out the possibility that the cleavage occurs during a metabolic step of O-glycosylation. Our results agree with previous observations using monensin and brefeldin A (25, 42, 48).

Quantification, using sandwich ELISA, of Aβ40 and Aβ42 in the medium of 293 cells expressing APP695<sub>mut1</sub>, indicates that a majority of APP cleavage at the β- and γ-sites also occurs after O-glycosylation, although we cannot rule out the possibility that a small quantity of APP is subject to cleavage by secretases at an earlier step during O-glycosylation modification. Previous reports using ammonium chloride and chloroquine suggest that Aβ may be generated in acidic compartments following medial-Golgi (31–33). However, the present study suggests that both Aβ40 and Aβ42 are generated subsequent to trans-Golgi, as in the case of θ-cleavage in 293 cells. However, it is not known whether the molecular mechanism of APP processing in 293 cells is identical to that in neurons. Furthermore, recent reports suggested that Aβ42 but not Aβ40 is able to accumulate intracellularly (29, 30, 48, 49). Although we do not rule out a possibility of a very minor ratio...
of intracellular Aβ accumulation in 293 cells expressing APP695mut1, the intracellular accumulation of Aβ, which is not secreted, may not be critical for the pathogenesis of AD because it has been well analyzed that the Aβ accumulation is an extracellular event in the brain of AD patient.

In previous studies, to identify the intracellular site of APP cleavage by β-secretase, APP carrying the Swedish double mutation was often utilized (50). We have not used such an FAD mutant APP because we feel that the mechanisms of cleavage at the β-site of APP carrying an FAD mutation differ from those of non-FAD patients. Therefore, our approach may be more useful in understanding the molecular mechanism of the pathogenesis of non-FAD.

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