Increased production of amyloid β peptides ending at position 42 (Aβ42) is one of the pathogenic phenotypes caused by mutant forms of presenilins (PS) linked to familial Alzheimer's disease. To identify the subcellular compartment(s) in which familial Alzheimer's disease mutant PS2 (mt PS2) affects the γ-cleavage of βAPP to increase Aβ42, we co-expressed the C-terminal 99-amino acid fragment of βAPP (C100) tagged with sorting signals to the endoplasmic reticulum (C100/ER) or to the trans-Golgi network (C100/TGN) together with mt PS2 in N2a cells. C100/TGN co-transfected with mt PS2 increased levels or ratios of intracellular as well as secreted Aβ42 at similar levels to those with C100 without signals (C100/WT), whereas C100/ER yielded a negligible level of Aβ, which was not affected by co-transfection of mt PS2. To identify the molecular subdomain of βAPP required for the effects of mt PS2, we next co-expressed C100 variously truncated at the C-terminal cytoplasmic domain together with mt PS2. All types of C-terminally truncated C100 variants including that lacking the entire cytoplasmic domain yielded the secreted form of Aβ at levels comparable with those from C100/WT, and co-transfection of mt PS2 increased the secretion of Aβ42. These results suggest that (i) late intracellular compartments including TGN are the major sites in which Aβ42 is produced and up-regulated by mt PS2 and that (ii) the anterior half of C100 lacking the entire cytoplasmic domain is sufficient for the overproduction of Aβ42 caused by mt PS2.

Alzheimer's disease (AD) is a progressive dementing disorder characterized pathologically by a massive loss of cortical neurons and an accumulation of two types of fibrillar lesions: i.e., amyloid deposits composed of amyloid β peptides (Aβ) and tau-rich paired helical filaments (1). Aβ is produced from β-amyloid precursor proteins (βAPP) through sequential cleavages by proteases originally termed β- and γ-secretases (1, 2); β-secretase has recently been identified as a novel aspartyl protease, BACE (3–5). Deposition of β-amyloid is considered to be closely related to the pathogenesis of AD because (i) deposition of Aβ is a neuropathological change relatively specific to AD; (ii) the diffuse type of senile plaque composed of highly aggregable Aβ42 species (6, 7), as opposed to Aβ40 that comprises the major portion of the secreted form of Aβ (8, 9), is the initial lesion of AD pathology; and (iii) mutations in genes coding for βAPP (10–14) or presenilin 1 (PS1) (15) or 2 (PS2) (16) are linked to some pedigrees of autosomal dominantly inherited familial AD (FAD), and these mutations increase the production of Aβ42 species (12–14, 17–20). Mutations in PS genes that code for multipass integral membrane proteins account for the majority of early onset FAD. Studies in knockout mice or invertebrates demonstrated that PS is involved in γ-cleavage of βAPP (2, 21, 22) as well as in site 3 cleavage of the Notch receptor (2, 23–25), both of which occur within the membrane or at the junction with cytoplasm, although it has not been clear if PS is a co-factor for γ-cleavage or if PS is identical to γ-secretase. However, recent data showing that transition state analogues γ-secretase inhibitors directly and exclusively bound fragment forms of PS strongly support the hypothesis that PS represents the catalytic subunits of γ-secretase (26–28).

Although there is ample evidence that mutations in PS genes increase the production of Aβ42 (29–32), the intracellular compartment(s) in which mutant forms of PS interact with βAPP and promote γ-cleavage at the Aβ42 position has not been clearly identified. Generation of intracellular Aβ42 has been shown to occur in endoplasmic reticulum (ER) of cultured neurons (33, 34) or in human embryonic kidney 293 cells (35), whereas the trans-Golgi network (TGN) (36) or endocytic pathway (37, 38) are also implicated in the generation of secretable Aβ42. Although the ER localization of PS dovetails with the former data, others have suggested that Golgi may be related to the abnormal effect of mt PS1 to increase Aβ42 (39, 40). Furthermore, subdomains in βAPP proteins that are required for this interaction with mt PS to increase production of Aβ42 have not been definitively identified. In this study, we studied the intracellular compartment and intramolecular subdomain of βAPP that are relevant to the abnormal effects of mutant PS2 to affect γ-cleavage and increase production of Aβ42. For these purposes, we expressed modified forms of a C-terminal fragment of βAPP tagged with targeting signals to specific com-
portments or harboring deletion of defined cytoplasmic subdomains. We show here that TGN and other late intracellular compartments are the major sites where mt PS2 up-regulates Aβ42 production and that the cytoplasmic domain of bAPP is dispensable for the overproduction of Aβ42 caused by mt PS2.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids—**A cDNA encoding the C-terminal 99 amino acids of bAPP fused to a signal peptide for rat preproenkephalin cDNA (C100) was previously described (32, 41). C100 peptides tagged with sorting signals to ER or TGN were constructed by using a C9 cDNA constructed in a murineelin vector pBS1023 as a template. Briefly, oligonucleotides encoding the rat preproenkephalin signal peptide were used as a sense polymerase chain reaction primer: 5′-TTTAAAGTCTTCCACATGGCGGAGTCTCCTG-3′ for C100/ER, 5′-GGGCGCTATGGCGGAGTCTCCTG-3′ for C100/TGN, and 5′-AAAGGATCTCAGTTGTTCTGCAATTATGCTGATCAATCAATCAATTC-3′ for C100 (without signal motif). Amplification of cDNAs was performed using PhiTuru DNA polymerase (Stratagene). Amplified DNA fragments were digested with Hana I and Bgl II and ligated into pcDNA3.1-Hygro vector (Invitrogen). bAPP695 tagged with KNLN or SDQYRQL (for TGN) were used as antisense polymerase chain reaction primers: 5′-CCCCGGATCCCTAATTCAGATTATTGTTCTGCAATTATGCTGATCAATTC-3′ for C100/ER, 5′-CCCCGGATCCCTAATTCAGATTATTGTTCTGCAATTATGCTGATCAATTC-3′ for C100/TGN, and 5′-AAAGGATCTCAGTTGTTCTGCAATTATGCTGATCAATTC-3′ for C100 (without signal motif). Amplification of cDNAs was performed using PhiTuru DNA polymerase (Stratagene). Amplified DNA fragments were digested with Hana I and Bgl II and ligated into pcDNA3.1-Hygro vector (Invitrogen). bAPP695 tagged with KNLN or SDQYRQL (for TGN) were used as antisense polymerase chain reaction primers:

**Cell Culture, Transfection, and Caspase Inhibitor Treatment—**Mouse neuro2a (N2a) neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. N2a cells were destined to be secreted, we transiently expressed cDNAs coding for C100, C100/ER, or C100/TGN tagged at the C terminus in mouse N2a cells using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Expression of transfected proteins was enhanced by treatment with 10 μg/ml tunicamycin for 24 h prior to harvesting cells or culture supernatants. For the inhibition of caspase activities, cells were treated with a 100 μM concentration of a pancaspase inhibitor, zVAD-fmk, for 24 h prior to analysis by Western blotting.

**Immunoblot Analysis of bAPP or PS2 Derivatives and Cell-associated Aβ—**Cells were lysed in 2% SDS sample buffer and briefly sonicated. Samples were separated by SDS-polyacrylamide gel electrophoresis using a Tris-Tricine gel system, transferred to polyvinylidene difluoride membrane (Millipore Corp.), and probed with monoclonal antibodies BAN50 (specific for human Aβ1–16) for the detection of C100 derivatives. A rabbit polyclonal antibody anti-G2N4 raised against a recombinant protein corresponding to the N-terminal residues 2–59 of human PS2 was used to probe PS2 and its derivatives. For the detection of Aβ in RIPA-soluble fractions of cell lysates, samples were initially lysed in RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate), and the supernatants after centrifugation at 15,000 × g for 5 min were immunoprecipitated by BAN50 using protein G-agarose and then analyzed by immunoblotting with BA27, BC05, or BAN50, using previously described procedures (44, 45, 47). Extraction of cell-associated Aβ by formic acid was performed as described (48). Briefly, cell pellets were lysed in 1% SDS and then solubilized by ultrasonication followed by incubation in 100 μl of 70% formic acid at room temperature for 30 min. Supernatants after centrifugation at 100,000 × g for 20 min were desiccated and then solubilized in 100 μl of SDS sample buffer. Samples containing Aβ were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with antibodies after boiling (44, 46, 47). The immunoblots were developed using an ECL system (Amersham Pharmacia Biotech) or Immunostar (Walco Pure Chemicals).

**Immunofluorescence Microscopy—**Transiently transfected N2a cells were cultured on glass coverslips for 48 h. Cells were fixed by incubation with phosphate-buffered saline (10 mM phosphate buffer, pH 7.4) containing 4% paraformaldehyde for 20 min, and then permeabilized and blocked with PBS-TB (phosphate-buffered saline containing 150 mM NaCl, 0.1% Triton X-100 and 3% bovine serum albumin) for 30 min at room temperature. Coverslips were then incubated with primary antibodies (a rabbit polyclonal antibody, C4, against the C terminus of bAPP (48) and monoclonal antibodies specific for Aβ (BAN50, using previously described procedures (44, 45, 47)). BA27 and BC05 that specifically recognize the C terminus of bAPP (48), i.e. C100 (without signal motif) and 15-kDa amyloidogenic C-terminal truncated C100 (C100/stop56, and C100/stop52) were generated by polymerase chain reaction and ligated into pcDNA3.1-Hygro vector similarly as C100/ER, and C100/stop52 was used to probe PS2 and its derivatives. A rabbit polyclonal antibody anti-G2N4 raised against a recombinant protein corresponding to the N-terminal residues 2–59 of human PS2 was used to probe PS2 and its derivatives. For the detection of Aβ in RIPA-soluble fractions of cell lysates, samples were initially lysed in RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate), and the supernatants after centrifugation at 15,000 × g for 5 min were immunoprecipitated by BAN50 using protein G-agarose and then analyzed by immunoblotting with BA27, BC05, or BAN50, using previously described procedures (44, 45, 47). Extraction of cell-associated Aβ by formic acid was performed as described (48). Briefly, cell pellets were lysed in 1% SDS and then solubilized by ultrasonication followed by incubation in 100 μl of 70% formic acid at room temperature for 30 min. Supernatants after centrifugation at 100,000 × g for 20 min were desiccated and then solubilized in 100 μl of SDS sample buffer. Samples containing Aβ were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with antibodies after boiling (44, 46, 47). The

**RESULTS**

**Effects of FAD Mutant PS2 on bAPP Production from bAPP C100 Targeted to ER or TGN—**To identify the intracellular compartments where Aβ, especially Aβ42, is generated and destined to be secreted, we transiently expressed cDNAs coding for the C-terminal 99 amino acids of human bAPP harboring a signal peptide at the N terminus (C100) or C100 tagged with sorting signals for retention to ER (C100/ER) or for recycling to TGN (C100/TGN) tagged at the C terminus in mouse N2a cells (Fig. 1, A–C). C100, C100/ER, or C100/TGN was expressed as a monoclonal antibody, 13-kDa (monoclonal antibody, 8-kDa polypeptide for immunoblots, the latter corresponding to fragments cleaved by caspases (see below), and the banding patterns were similar between C100 with and without sorting signals (Fig. 1B). Immunocytochemistry by C4 (against the cytoplasmic tail of bAPP (48)) combined with anti-BiP antibody that specifically reacts with a KDEL sequence (ER marker) or anti-adaptin-γ

**Immunofluorescence Microscopy—**
FIG. 1. Expression of βAPP C100 harboring sorting signals to ER or TGN and αβ secretion in N2a cells. A, schematic depiction of C100 tagged with sorting signals to ER (C100/ER) or trans-Golgi network (C100/TGN). C100/WT is C100 without a sorting signal. The shaded area represents Aβ flanked by cleavage sites for β- and γ-secretases (arrows). B, Western blot analysis of C100 harboring sorting signals expressed in N2a cells with BAN50. An arrow indicates the holoprotein of C100, mock, cells expressing an empty vector alone. Molecular markers are shown in kilodaltons. C, double fluorescence immunocytochemistry of N2a cells expressing C100/ER, C100/TGN, or C100/WT labeled by C4 (probe for C100; green) and anti-BIP (ER marker, left lane; red) or anti-adaptin-γ (Ady, TGN marker, right lane; red) and viewed with a confocal microscope. Areas visualized in yellow represent co-localization of C100 and ER or TGN markers. Transfected cDNAs and primary antibodies are shown to the left of and above the panels, respectively. Scale bar, 10 μm. D, levels of secreted Aβ- (1–40) (open column) and Aβ- (1–42) (closed column) quantitated by two-site ELISAs. Mean values ± S.E. in four independent experiments are shown. Transfected C100 cDNAs are shown below the columns.

antibody (TGN marker) showed retention of C100/ER in a meshwork-like pattern overlapping with an immunolabeling with the ER marker (Fig. 1C, top left), whereas immunoreactive pattern for C100/TGN completely overlapped with that for adaptin-γ (Fig. 1C, middle right), suggesting proper localization of C100 variants at the intended sites. C100/WT showed a combined ER and TGN localization (Fig. 1C, lower panels). We then quantitated Aβ- (1–40) and Aβ- (1–42) secreted from N2a cells expressing C100 variants by two-site ELISAs using BAN50 as a capture antibody, which specifically detects human Aβ but not endogenous murine Aβ (Fig. 1D). Cells transfected with C100/TGN secreted ~2000 pg Aβ- (1–40) and ~200 pg Aβ- (1–42), which were at comparable levels with those secreted from cells expressing C100/WT. In contrast, C100/ER did not secrete detectable levels of Aβ- (1–40) or Aβ- (1–42) as in N2a cells transiently transfected with an empty vector. Those results suggested that C100 would be the major intracellular site in which γ-cleavage to yield Aβ- (1–40) as well as Aβ- (1–42) that are destined for secretion takes place.

We then expressed C100/ER, C100/TGN, or C100/WT in N2a cells that stably express WT or N141I or M239V FAD mt PS2 and examined the production of secreted or intracellular Aβ. N2a cells expressing WT PS2 transiently transfected with C100/WT or C100/TGN secreted similar levels of Aβ- (1–40) and Aβ- (1–42), whereas those with C100/ER did not secrete detectable levels of Aβ, in almost identical patterns to those observed in cells without exogenous PS2. In contrast, cells expressing mt PS2 transiently transfected with C100/WT or C100/TGN secreted larger amounts of Aβ- (1–42) compared with Aβ- (1–40), whereas those with C100/ER did not secrete detectable Aβ (Fig. 2A). These data suggest that TGN or later intracellular compartments, but not ER, are the intracellular site where mt PS2 affects γ-cleavage of βAPP to promote secretion of Aβ42 in N2a cells.

To verify the intracellular production of Aβ- (1–40) and Aβ- (1–42) from C100 with or without sorting signal motifs, we analyzed RIPA-extracted lysates of N2a cells expressing C100/WT, C100/ER, or C100/TGN together with WT or N141I mt PS2 by Western blotting with antibodies to βAPP (i.e. BAN50 against the Aβ N terminus) or with those against C termini of Aβ after immunoprecipitation with BAN50 (Fig. 2B). C100/WT, C100/ER, or C100/TGN were expressed as ~13-kDa as well as ~8-kDa polypeptides as observed without co-expression of PS2. In N2a cells co-expressing WT PS2, C100/TGN yielded a ~4-kDa polypeptide positive for Aβ40 as well as an equally intense Aβ42-positive ~4-kDa polypeptide, in a similar pattern to those observed with C100/WT. In N2a cells expressing N141I or M239V mt PS2, however, C100/WT and C100/TGN yielded comparable levels of Aβ42-positive 4-kDa bands, whereas only trace amounts of Aβ40-positive bands were detected in cell lysates, despite robust expression of C100 and its derivatives. In contrast, detectable levels of Aβ40 or Aβ42-positive polypeptides were not observed in Western blots of cell lysates expressing C100/ER together with WT or N141I or M239V mt PS2. To confirm the lack of detectable cell-associated Aβ in cells expressing C100/ER, we solubilized N2a cells expressing C100/ER or C100/WT in 70% formic acid and analyzed the extracted proteins by Western blotting with BA27 and BC05. Comparable levels of Aβ40-positive and Aβ42-positive ~4-kDa proteins were detected in cells expressing C100/WT, whereas no Aβ-positive bands were detectable in cells expressing C100/ER despite robust expression of C100/ER holoprotein (Fig. 2C), suggesting that the levels of formic acid-extractable ER-associated Aβ are very low, if any, in our N2a cells overexpressing C100 derivatives.

We next analyzed the relationship between the subcellular localization of PS2 and the intracellular production site of Aβ42. To this end, we expressed C100/WT together with WT or N141I mt PS2, separated the cells by iodixanol density fractionation, and analyzed the fractions by Western blotting (Fig. 2D). N-terminal fragments of PS2 were chiefly distributed in fractions 4–10, which overlapped with those positive for a TGN marker (adaptin-γ, fractions 3–10). In contrast, the distribution of full-length PS2 (fractions 11 and 12) was limited to those positive for an ER marker (i.e. BiP; fractions 10–12). The distribution patterns of PS2 and its derivatives were similar between WT and N141I mt PS2. ELISA quantitation of cell fraction-associated Aβ42 showed that cells expressing N141I mt PS2 harbored elevated levels of Aβ- (1–42) in fractions 8 and 9, which corresponded to those positive for PS2 N-terminal fragment as well as for a TGN marker, supporting the notion that TGN is the site in which mt PS2 affects γ-cleavage of C100 to promote Aβ42 production (Fig. 2E).

To examine if the conclusions drawn from experiments using tagged C100 above are applicable to full-length βAPP, we transiently co-expressed human βAPP tagged with KKLNM (βAPP/ER) or SDYQRL (βAPP/TGN) or without the tags (βAPP/WT) in N2a cells that stably express WT or N141I or M239V FAD mt PS2 and examined the production of secreted Aβ. N2a cells expressing WT PS2 transiently transfected with βAPP/WT or
transfected with C100/WT. Each fraction was analyzed by Western blotting with anti-G2N4 (for full-length and N-terminal fragments of PS2), density gradient fractionation of N2a cells stably expressing WT (upper two panels). The numbers of the fractions are indicated in four independent experiments are shown. Transfected C100 cDNAs are shown with detectable Aβ, and the results were similar to those in cells expressing C100 and its derivatives. In contrast, cells expressing N141I or M239V mt PS2 transiently transfected with C100 were detectable in cells expressing WT, or mock-transfected cells (left lane; mock). Expression of C100 in cell lysates probed by BAN50 is shown in upper panel. D, iodixanol density gradient fractionation of N2a cells stably expressing WT (upper two panels) or N141I FAD mt (middle two panels) PS2 transiently transfected with C100/WT. Each fraction was analyzed by Western blotting with anti-G2N4 (for full-length and N-terminal fragments of PS2), BAN50 (for C100), anti-adaptin-γ (marker for TGN), or anti-BiP (marker for ER). The numbers of the fractions are shown above. Molecular mass markers are shown in kilodaltons. E, levels of cell-associated Aβ from residue Asp 1 of Aβ to promote secretion of Aβ-(1–42), whereas those with BAN50/Western blot analysis of RIPA-extractable cell-associated Aβ (Aβ-(1–40), middle panel; Aβ-(1–42), lower panel) and C100 (upper panel) in cells stably expressing WT (left), N141I (middle), or M239V (right) mt PS2 and transiently transfected with C100 with targeting signals (ER, C100/ER; TGN, C100/TGN; wt, C100/WT) quantitated by two-site ELISAs. Mean values ± S.E. of four independent experiments are shown. In four independent experiments are shown. Transfected C100 cDNAs are shown with detectable Aβ, and the results were similar to those in cells expressing C100 and its derivatives. In contrast, cells expressing N141I or M239V mt PS2 transiently transfected with C100 were detectable in cells expressing WT, or mock-transfected cells (left lane; mock). Expression of C100 in cell lysates probed by BAN50 is shown in upper panel. D, iodixanol density gradient fractionation of N2a cells stably expressing WT (upper two panels) or N141I FAD mt (middle two panels) PS2 transiently transfected with C100/WT. Each fraction was analyzed by Western blotting with anti-G2N4 (for full-length and N-terminal fragments of PS2), BAN50 (for C100), anti-adaptin-γ (marker for TGN), or anti-BiP (marker for ER). The numbers of the fractions are shown above. Molecular mass markers are shown in kilodaltons. E, levels of cell-associated Aβ from residue Asp 1 of Aβ to promote secretion of Aβ-(1–42), whereas those with BAN50/Western blot analysis of RIPA-extractable cell-associated Aβ (Aβ-(1–40), middle panel; Aβ-(1–42), lower panel) and C100 (upper panel) in cells stably expressing WT (left), N141I (middle), or M239V (right) mt PS2 and transiently transfected with C100 with targeting signals (ER, C100/ER; TGN, C100/TGN; wt, C100/WT) quantitated by two-site ELISAs. Mean values ± S.E. of four independent experiments are shown. Transfected C100 cDNAs are shown with detectable Aβ, and the results were similar to those in cells expressing C100 and its derivatives. In contrast, cells expressing N141I or M239V mt PS2 transiently transfected with C100 were detectable in cells expressing WT, or mock-transfected cells (left lane; mock). Expression of C100 in cell lysates probed by BAN50 is shown in upper panel. D, iodixanol density gradient fractionation of N2a cells stably expressing WT (upper two panels) or N141I FAD mt (middle two panels) PS2 transiently transfected with C100/WT. Each fraction was analyzed by Western blotting with anti-G2N4 (for full-length and N-terminal fragments of PS2), BAN50 (for C100), anti-adaptin-γ (marker for TGN), or anti-BiP (marker for ER). The numbers of the fractions are shown above. Molecular mass markers are shown in kilodaltons. E, levels of cell-associated Aβ from residue Asp 1 of Aβ to promote secretion of Aβ-(1–42), whereas those with BAN50/Western blot analysis of RIPA-extractable cell-associated Aβ (Aβ-(1–40), middle panel; Aβ-(1–42), lower panel) and C100 (upper panel) in cells stably expressing WT (left), N141I (middle), or M239V (right) mt PS2 and transiently transfected with C100 with targeting signals (ER, C100/ER; TGN, C100/TGN; wt, C100/WT) quantitated by two-site ELISAs. Mean values ± S.E. of four independent experiments are shown. Transfected C100 cDNAs are shown with detectable Aβ, and the results were similar to those in cells expressing C100 and its derivatives. In contrast, cells expressing N141I or M239V mt PS2 transiently transfected with C100 were detectable in cells expressing WT, or mock-transfected cells (left lane; mock). Expression of C100 in cell lysates probed by BAN50 is shown in upper panel. D, iodixanol density gradient fractionation of N2a cells stably expressing WT (upper two panels) or N141I FAD mt (middle two panels) PS2 transiently transfected with C100/WT. Each fraction was analyzed by Western blotting with anti-G2N4 (for full-length and N-terminal fragments of PS2), BAN50 (for C100), anti-adaptin-γ (marker for TGN), or anti-BiP (marker for ER). The numbers of the fractions are shown above. Molecular mass markers are shown in kilodaltons. E, levels of cell-associated Aβ from residue Asp 1 of Aβ to promote secretion of Aβ-(1–42), whereas those with BAN50/Western blot analysis of RIPA-extractable cell-associated Aβ (Aβ-(1–40), middle panel; Aβ-(1–42), lower panel) and C100 (upper panel) in cells stably expressing WT (left), N141I (middle), or M239V (right) mt PS2 and transiently transfected with C100 with targeting signals (ER, C100/ER; TGN, C100/TGN; wt, C100/WT) quantitated by two-site ELISAs. Mean values ± S.E. of four independent experiments are shown. There is a 1:1 correspondence between the fractions indicated below the columns and the order shown in D.
Aβ42 Effect of PS2 on βAPP C-terminal Fragment

**Fig. 3.** Aβ production in N2a cells expressing full-length βAPP tagged with sorting signals. A, levels of Aβ-(1–40) (open column) and Aβ-(1–42) (closed column) secreted from N2a cells stably expressing WT PS2 (left), N141I (middle), or M239V (right) FAD mt PS2 and transiently transfected with full-length βAPP with targeting signals (ER, βAPP/ER; TGN, βAPP/TGN; wt, βAPP/wt) quantitated by two-site ELISAs. Mean values ± S.E. in four independent experiments are shown. Transfected C100 cDNAs are shown by *lines below the columns.* B, Western blot analysis of formic acid-extracted Aβ40 (middle panel) by BA27 and Aβ42 (lower panel) by BC05 in cells overexpressing βAPP/ER (middle lane), ER (βAPP/WT, right lane; wt), or mock-transfected cells (left lane; mock). Expression of full-length βAPP in cell lysates probed by BAN50 is shown in the upper panel. Molecular mass markers are shown in kilodaltons.

-52 showed similar meshwork-like staining patterns accentuated in perinuclear areas, suggesting the ER/Golgi localization of these C100 derivatives (Fig. 4E). We then quantitated Aβ secreted from N2a cells transiently transfected with these C100 derivatives (Fig. 4F). All C-terminally deleted C100 derivatives showed robust secretion of Aβ-(1–40) at similar levels ranging from 1500 to 1800 pM. C100/stop68 and C100/stop56 (1–42), which comprised only 

**Fig. 4.** Expression of βAPP C100 truncated at the cytoplasmic domain and Aβ secretion in N2a cells. A, schematic depiction of C100 truncated at the cytoplasmic domain. C100/stop58 is truncated at Asp⁶⁸ (starting from Asp¹ of the Aβ sequence), which is inferred as the caspase-3 cleavage site; C100/stop56 retains the membrane-flanking four amino acid residues KKKQ; and C100/stop52 lacks the entire cytoplasmic domain. B, Western blot analysis of C-terminally truncated C100 transiently expressed in N2a cells with BAN50. An asterisk shows the co-migration of an ~8-kDa polypeptide derived from C100/WT with C100/stop68. Molecular mass markers are shown in kilodaltons, and the names of transfected cDNAs are indicated above each lane. C, inhibition of the generation of ~8-kDa band (asterisk) from C100/WT by a caspase inhibitor zVAD-fmk. N2a cells transfected with C100/WT without zVAD-fmk treatment. zVAD, N2a cells transfected with C100/WT treated with 100 μM zVAD-fmk for 24 h. D, differential extraction of C-terminally truncated C100 by Na₂CO₃ or Triton X-100. Microsomal fractions of N2a cells transiently expressed C100/WT (wt), C100/stop58 (stop58), C100/stop56 (stop56), C100/stop52 (stop52) were extracted by 0.5 M Na₂CO₃ (pH 11.0) or 1% Triton X-100, and solubilized proteins (S) and insoluble pellets (P) were analyzed by Western blotting with BAN50. E, immunofluorescence localization of C-terminally truncated C100 in N2a cells revealed by BAN50. Scale bar, 10 μm. F, levels of Aβ-(1–40) (open column) and Aβ-(1–42) (closed column) secreted from transiently transfected N2a cells quantitated by two-site ELISAs. Note that C100/stop52 yielded significantly reduced levels of Aβ-(1–42) (*, p < 0.01 by analysis of variance). Mean values ± S.E. in four independent experiments are shown. Transfected C100 cDNAs are shown below each column.

the secretion of Aβ42 in cells stably expressing N141I or M239V mt PS2. These data suggest that the anterior half of the C100 (i.e. Aβ sequence plus the following intramembranous portion of βAPP) is sufficient for the full effect of mt PS2 to increase secretion of Aβ-(1–42).

**DISCUSSION**

In this study, we showed that (i) TGN is one of the major intracellular sites in which a secretable pool of Aβ42 is produced and up-regulated by the abnormal function of FAD mt...
PS2, (ii) lack of the entire cytoplasmic domain of C100 selectively decreases the production of Aβ42, and (iii) the anterior half of C100 lacking the entire cytoplasmic domain is sufficient for the abnormal function of mt PS2 to increase production of Aβ42.

The intracellular site of Aβ42 generation in relation to PS function has been a matter of controversy (38). Here we showed that C100 targeted to TGN (C100/TGN) yielded similar levels of intracellular as well as secreted forms of Aβ-(1–40) and Aβ-(1–42), compared with those derived from C100 without sorting signals, whereas C100 targeted to ER (C100/ER) did not produce detectable levels of intracellular as well as secreted Aβ. Moreover, FAD-linked N141I and M239V mt PS2 fully increased the production of Aβ-(1–42) from C100/TGN at an extent comparable with that for C100 without signals, whereas production of intracellular as well as secreted Aβ-(1–42) was not up-regulated by co-expression of C100/ER. Use of C100 that does not require β-cleavage, which is presumed to occur in the post-Golgi compartments (52), to trigger γ-cleavage enabled us to directly address the intracellular site where γ-cleavage takes place and is affected by the abnormal effects of mt PS2. It has been reported that cultured neurons (33, 34) as well as human embryonic kidney 293 cells (35) produce Aβ-(1–42) in ER upon overexpression of βAPP. These findings were in good agreement with the predominant ER localization of PS, which has been implicated in γ-cleavage. However, it was subsequently shown that the ER-associated Aβ42 was not directly secreted and was considered to comprise a distinct pool from the secreted Aβ (53). The reason for our failure to detect ER-associated Aβ in our cell system is not clear at present; however, the following possibilities could be considered to explain these discrepancies: (i) full-length Aβ may be detected in ER only by extremely high level expression of APP (e.g. by Semliki Forest virus infection) (33), and modest levels of overexpression of βAPP or C100 fail to produce detectable levels of Aβ; (ii) in N2a cells, small amounts of Aβ42 truncated at the N terminus, but not full-length species, have been detected in ER (36, 47), which escaped our detection system specific for human full-length Aβ; and (iii) ER-derived Aβ is present at a relatively small amount compared with those in late compartments, the former being lower than the detection limit of our highly sensitive immunoblot assay.

Our data indicating that TGN harbored elevated levels of “secretable” Aβ42 upon co-expression of mt PS2 strongly support the view that the active form of PS (i.e. endoproteolytic fragments that are stabilized (44) and form a high molecular weight complex (54)) resides in Golgi/TGN as well as in additional late intracellular compartments and that mt PS1 up-regulates production and secretion of Aβ42 in these compartments (39, 40). In this case, a relatively small amount of “active” presenilin complex may be sufficient for the generation of Aβ in the late compartments. Notwithstanding the present data, the problem of the “spatial paradox” (38) between the localization of PS and γ-secretase activities has not been completely clarified. Further careful studies on the intracellular distribution of presenilin complex and γ-cleavage activities for the processing of βAPP as well as Notch in different types of cells will be needed.

We and others have shown that co-expression of C100 that lacks the majority of the extracellular domain of βAPP with FAD-associated mt PS1 (40) or PS2 (32) is sufficient to induce overproduction of Aβ42. To examine whether the cytoplasmic domain of βAPP is required for the abnormal effect of mt PS, we co-expressed C-terminally truncated forms of C100 and evaluated the secretion of Aβ. Unexpectedly, we found that the expression of C100 lacking the entire cytoplasmic domain (C100/stop52), with or without co-expression of WT PS2, dramatically reduced the secretion of Aβ42, although the total levels of Aβ secretion were not significantly altered. Similar results were obtained also in COS cells (data not shown). The mechanism whereby γ-secretase differentially cleaves Aβ40 and Aβ42 within the transmembrane segment of βAPP is not well understood. However, accumulating data suggest that γ-cleavage occurs in a position-dependent manner within the membranous portion, irrespective of the amino acid sequences (54, 55). The lack of the cytoplasmic domain including the KKKQ motif at the membrane-flanking portion, which is presumed to work as a membrane anchor (50), may destabilize the positioning of the transmembrane domain of βAPP, thereby leading to predominant cleavage at the Aβ40 position. Moreover, C100 ending at the putative caspase-3 cleavage site (C100/stop68) did not change the level or proportion of secreted Aβ. It has been shown that βAPP truncated at the caspase-3 cleavage site increased the secretion of Aβ40 (49). The reason for this discrepancy is unknown, but it is possible that the caspase-cleaved βAPP may promote β-cleavage (49), thereby

![Fig. 5. Secretion of Aβ-(1–40) and Aβ-(1–42) from N2a cells stably expressing WT or FAD mt PS2 and transiently transfected with C-terminally truncated C100 or βAPP. Levels of secreted Aβ-(1–40) (open columns) and Aβ-(1–42) (closed columns) from N2a cells stably expressing WT (left columns), N141I (middle columns), or M239V (right columns) FAD mt PS2 and transiently transfected with C-terminally truncated 100(A) or βAPP(B) quantitated by two-site ELISAs. Mean ± S.E. in four independent experiments are shown in A and B.](http://www.jbc.org/content/21683/1/2860/images)
increasing Aβ40 secretion.

Finally, we have shown that the cyttoplasmic domain of C101 is dispensable for the abnormal effects of mt PS2 to increase production of Aβ42. This domain is implicated in a number of βAPP functions including interaction with a number of binding proteins (i.e. FE65 (57) or X11 (58, 59)) as well as endocytosis (51), all of which are known to alter Aβ production (60). However, co-transfection of mt PS2 fully increased the secretion of Aβ42 from all types of C101 truncated at the cyttoplasmic domain. It has recently been suggested that PS serves as a chaperone for APP (56, 57) and for cleavage by β-secretase (58). We have shown that the normal function of PS2 and PS1 may be dispensable for the abnormal effects of mt PS2 to increase the production of Aβ. This may be due to a mechanism whereby PS leads to increased production of AD as well as of the unusual but important proteolytic mechanism more recently referred to as regulated intramembrane proteolysis (62).

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Subcellular Compartment and Molecular Subdomain of \( \beta \)-Amyloid Precursor Protein Relevant to the \( \beta \)-42-promoting Effects of Alzheimer Mutant Presenilin 2
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