Expression of β-Amyloid Precursor Protein-CD3γ Chimeras to Demonstrate the Selective Generation of Amyloid β1–40 and Amyloid β1–42 Peptides within Secretory and Endocytic Compartments

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Amyloid β-protein (Aβ) is the main constituent of amyloid fibrils found in senile plaques and cerebral vessels in Alzheimer’s disease (AD) and is derived by proteolysis from the β-amyloid precursor protein (APP). We have analyzed the amyloidogenic processing of APP using chimeric proteins stably transfected in Chinese hamster ovary cells. The extracellular and transmembrane domains of APP were fused to the cytoplasmic region derived from the CD3γ chain of the T cell antigen receptor (CD3γ). CD3γ contains an endoplasmic reticulum (ER) retention motif (RKK), in the absence of which the protein is targeted to lysosomes without going through the cell surface (Letourneur, F., and Klausner, R.D. (1992) Cell 69, 1143–1157). We used the wild-type sequence of CD3γ to create an APP chimera predicted to remain in the ER (γAPPγ). Deletion of the RKK motif at the C terminus directed the protein directly to the lysosomes (γAPPγ). A third chimera was created by removing both lysosomal targeting signals in addition to RKK (γAPPΔγ). This last construct does not contain known targeting signals and consequently accumulates at the cell surface. We show by immunofluorescence and by biochemical methods that all three APP chimeras localize to the predicted compartments within the cell, thus providing a useful model to study the processing of APP. We found that Aβ1–40 is generated in the early secretory and endocytic pathways, whereas Aβ1–42 is made mainly in the secretory pathway. More importantly, we provide evidence that, unlike in neuronal models, both ER/intermediate compartment- and endocytic-derived Aβ forms can enter the secretable pool. Finally, we directly demonstrate that lysosomal processing is not involved in the generation or secretion of either Aβ1–40 or Aβ1–42.

One of the major features of Alzheimer’s disease (AD) is neuropathology, which is the deposition of amyloid β-peptide (Aβ) in brain parenchyma and cerebral vessels. Aβ can be produced as a 40-amino acid peptide (Aβ1–40) or, occasionally, as a more amyloidogenic form of 42–43 amino acids (Aβ1–42). Both forms are generated by the activity of two unknown proteases termed β- and γ-secretase from a larger amyloid precursor protein (APP), a ubiquitously expressed type I membrane glycoprotein (1). Aβ peptide sequence begins at the extracellular domain of APP and ends within its transmembrane domain. In an alternative pathway, APP may be cleaved within the Aβ sequence by another protease termed α-secretase to generate a soluble ~100-kDa N-terminal fragment (αAPPs) and a membrane-retained ~10-kDa C-terminal fragment (2, 3). Because α-secretase activity takes place within the Aβ sequence, generation of intact Aβ and αAPPs are mutually exclusive events.

The identities of α, β, and γ secretases are not known, and the subcellular location of their activities is currently unclear. It is generally thought that α-secretase cleavage occurs at the trans-Golgi network (TGN) or at a late compartment in the constitutive secretory pathway, as well as from the cell surface (4–7). Less clear however is the mechanism and intracellular compartments involved in the production of Aβ1–40 and Aβ1–42. Full-length APP at the plasma membrane may be internalized to generate Aβ in an unidentified intracellular compartment. APP can also be sorted to the endosomal/lysosomal compartments where several Aβ containing C-terminal APP fragments accumulate (8–11). It is not known whether these potentially amyloidogenic fragments are indeed Aβ intermediates or simply undergo lysosomal degradation. Recently it was shown that in transfected COS cells both Aβ1–40 and Aβ1–42 are produced at the cell surface, although the actual sites of production were not identified (12). In contrast, in transfected neurons, Aβ1–40 appears to be produced at the TGN (6) and Aβ1–42 at the endoplasmic reticulum (12–14).

To better understand the role of different subcellular compartments in the production of Aβ1–40 and Aβ1–42 we constructed several APP chimeric proteins. We fused the extracellular and transmembrane domains of APP to the cytoplasmic region derived from the CD3γ chain of the T cell antigen receptor (CD3γ). CD3γ contains an ER retention motif (RKK), in the absence of which the protein is targeted directly from the TGN to the lysosomes without going through the cell surface by virtue of two different lysosomal signals, LL and YQ (15). We used the wild-type sequence of CD3γ to create an APP chimera predicted to remain in the ER (γAPPγ). A second APP chimera was constructed by removing the RKK motif, thus directing the protein directly to the lysosomes. Finally, a third chimera was created by removing both targeting signals in addition to RKK (γAPPΔγ). This ΔLYQ double mutation renders the cytoplas-
mic tail to be devoid of known sorting signals in its sequence, and the resulting mutant protein accumulates at the cell surface at high levels due to impaired internalization (15). This approach therefore provides the opportunity to target APP to multiple intracellular compartments.

Our results suggest that, in transfected Chinese hamster ovary (CHO) cells, APP1-40 is generated in the ER/IC, as well as in the endocytic pathway, whereas, as previously reported for other cell models, APP1-42 is generated in the early secretory pathway, mainly in the ER/IC. More importantly, we provide evidence that, unlike in neuronal models, both ER/IC- and endocytic-derived Aβ forms can enter the secretable pool. Finally, we demonstrate that, in CHO cells, the lysosomes are not involved in the generation or secretion of either APP1-40 or APP1-42.

**MATERIALS AND METHODS**

**Construction of APP Chimeras**—cDNAs encoding the chimeric APP/CD3γ proteins were generated by oligonucleotide-directed mutagenesis with the expression vector pCI-NEO (Promega) from the parental APP1-71 containing the extracellular and transmembrane but lacking the entire cytoplasmic tail (residues 734–770; APP1-770 numbering) fused to the cytoplasmic domain of the γ chain of the CD3 (T cell) receptor (15). Three different APP/CD3γ chimeric constructs were used: full-length tail (Gln-116 to Lys-159) was used to generate γAPPΔK; Gln-116 to Leu-156 (deletion of the C-terminal RKK residues) was used to generate γAPPΔK; and γAPPΔ3δ was obtained by deleting residues LI30L131 and Y137QL140. Both the dileucine- and tyrosine-based targeting motifs were deleted in the last construct. All constructs were verified by DNA sequencing.

**Cell Culture**—CHO cell lines were transfected using Lipofet-AMINE™ (Life Technologies) reagent and selected by G418 resistance. The stably transfected CHO cell lines expressing wild-type APP and various APP chimeras were chosen with comparable levels of expression of the corresponding gene products. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C, with 5% CO2. All experiments using these transfected CHO cells were repeated 3–5 times, and results from either representative experiments or the mean (± S.E.) of all experiments are shown.

**Antibodies**—Monoclonal antibodies 5A3 and 1G7 and polyclonal antibody were expressed in bacterial cultures. The sequence of the cytosolic tail of CD3γ membrane and extracellular domain of APP.

**Immunofluorescence Microscopy**—APP-transfected cells grown on coverslips were fixed and permeabilized in methanol for 5 min at –20 °C. Following extensive washing in PBS, cells were blocked with 3% BSA in PBS (PBS/BSA) and then incubated with 5A3/1G7 (10 μg/ml in PBS/BSA) for 20 min at room temperature. Cells were then extensively washed in PBS and anti-mouse IgG conjugated to fluorescein isothiocyanate (Roche Molecular Biochemicals) for 20 min at room temperature. Control samples were incubated with mouse IgG instead of primary antibody. Immunostained cells were visualized by conventional epifluorescence microscopy.

**Metabolic Labeling and Immunoprecipitation**—Confluent cultures of stable transfected CHO cells were incubated in methionine-free DMEM for 20 min followed by incubation with methionine-free DMEM supplemented with 250 μCi/ml [35S]methionine for 15 min (pulse labeling) or 8 h with 150 μCi/ml (long labeling). In pulse-chase experiments, cells were lysed immediately after brief pulse-labeling or incubated in DMEM with 1% methionine (chase) for the indicated time periods. APP was immunoprecipitated with antibodies 5A3/1G7 or 863 (16) and separated by SDS-polyacrylamide gel electrophoresis. Where indicated, cells were incubated for 4 h with 10 μM proteasome inhibitor MG-132 (Calbiochem) or 8 h with 100 μM leupeptin (Sigma) before immunoprecipitation. Gels were analyzed by phosphorimaging (Bio-Rad).

**Cell Surface Biotinylation**—Confluent cultures of stably transfected CHO cells were surface-biotinylated on ice using sulfo-NHS-biotin (sulfo-NHS-biotin, Pierce) as described previously (16). Cells were then extensively washed and the resulting mutant protein accumulates at the cell surface and is endocytosis-deficient. The sequence of the cytosolic tail of CD3γ (amino acids 116 to 159) is represented at the bottom with the motifs LL and YQ in bold.

**RESULTS**

**Expression and Localization of APP and APP/CD3γ Chimeras**—Stably transfected wild-type APP and APP/CD3γ (Fig. 1) CHO lines with comparable levels of expression were analyzed by immunoprecipitations after 8 h of [35S]-methionine labeling. The results are shown in Fig. 2, a and b. Essentially, no differences in the levels of accumulated “mature” and “immature” forms of APP were found between APP, γAPPΔK, and γAPPΔ3δ, indicating that at this level of analysis, all three forms are appropriately post-translationally processed through the secretory pathway. In contrast, very low levels of γAPPΔK mature forms were detected, suggesting that the majority of the newly synthesized protein is, as expected, retained in the endoplasmic reticulum and does not undergo normal maturation.

To further investigate the subcellular localization of the APP chimeras, we first performed immunofluorescence on methanol fixed/permeabilized cells. As expected, APP localized mainly to diffuse vesicular structures with a juxtanuclear distribution (Fig. 3), consistent with Golgi localization (20). In contrast, γAPPΔK staining pattern showed a strong perinuclear ring ex-

**FIG. 1. Schematic diagram of APP and CD3γ/APP chimeras containing the cytoplasmic tail of the CD3γ chain and the transmembrane and extracellular domain of APP.** Panel a, schematic diagram of wild-type APP; panel b, schematic diagram CD3γ/APP chimeras. γAPPΔK contains the two lysosomal sorting motifs, “LL” and “YQ”, as well as the “RKK” ER retention motif and is predicted to stay in the ER. γAPPΔK lacks the motif RKK and is directed to the lysosomes from the TGN without sorting to the cell surface. γAPPΔ3δ lacks both the lysosomal signals LL and YQ and the ER retention motif RKK. This chimera accumulates at the cell surface and is endocytosis-deficient. The sequence of the cytosolic tail of CD3γ (amino acids 116 to 159) is represented at the bottom with the motifs LL and YQ in bold.
tending into fine reticular structures, a distribution characteristic of ER resident proteins. γAPP<sub>LYS</sub>, distribution partially overlapped with that of APP at a juxtanuclear region due to the presence of both proteins in the biosynthetic pathway, but also

![Diagram](image1.png)

**FIG. 2. Expression of APP and CD3γAPP chimeras.** Panel a, total APP was immunoprecipitated with antibodies 5A3/1G7 from cell lysates after 8 h ([<sup>35</sup>S]methionine labeling. Note the absence of high molecular weight mature forms (m) from the γAPP<sub>LYS</sub> chimera (∗, immature forms). Results from a representative experiment are shown. Panel b, quantitation of the ratio of mature to immature forms of APP shows a significant decrease for γAPP<sub>LYS</sub>, again representative of its lack of maturation. The results are mean ± S.E. from three experiments.

![Diagram](image2.png)

**FIG. 3. Localization of APP and CD3γAPP chimeras in CHO cell lines by immunofluorescence.** Cells were fixed/permeabilized in methanol for 5 min at −20 °C and stained with monoclonal antibodies 5A3/1G7. APP shows a mainly juxtanuclear Golgi distribution, whereas γAPP<sub>ER</sub> staining pattern reveals a strong perinuclear ring extending into fine reticular structures, a distribution characteristic of ER resident proteins. γAPP<sub>LYS</sub> shows the accumulation of APP in vesicular structures throughout the cytoplasm. γAPP<sub>Δ3</sub> was found at the cell surface (arrowheads, γAPP<sub>Δ3</sub> panel) in addition to the juxtanuclear staining also present in both APP and γAPP<sub>LYS</sub>. Double staining experiments also showed that γAPP<sub>ER</sub> co-localized with calnexin, whereas γAPP<sub>LYS</sub> co-localized with the lysosomal markers LAMP-1 and LAMP-2 (not shown).

**Biochemical Characterization of APP/CD3γ Chimeras—**Because accurate targeting of APP chimeras to different subcellular locations is essential in this study, additional biochemical approaches were used to further define the subcellular localization of the three APP/CD3γ chimeras. Recently, it became clear that selective degradation of ER membrane proteins occurs mainly through the ubiquitin-proteasome pathway (21, 22). Accordingly, if γAPP<sub>ER</sub> is an ER resident protein, it should accumulate after treatment with MG-132, a specific proteasome inhibitor that has no apparent effect on lysosomal proteases (23). As shown in Fig. 4, there was no effect on APP (Fig. 4b), γAPP<sub>LYS</sub> or γAPP<sub>Δ3</sub> (not shown), but γAPP<sub>ER</sub> increased substantially after MG-132 treatment, indicating that the latter chimera is retained in the ER.

To further confirm that the vesicular staining seen for γAPP<sub>LYS</sub> corresponds to the lysosomes, the transfected cells were treated with leupeptin to inhibit lysosomal proteases. As presented in Fig. 4a, leupeptin treatment results in accumulation of γAPP<sub>LYS</sub> but not APP (Fig. 4a) or the other γAPP chimeras (not shown). A control immunoblot for β-tubulin (Fig. 4a) shows that the modest increase in γAPP<sub>LYS</sub> after leupeptin treatment is not due to differences in the amount of protein loading.

In the absence of targeting signals, γAPP<sub>Δ3</sub> is predicted to be mainly cell surface distributed and deficient in endocytosis (15). Consequently, cell surface accumulation of γAPP<sub>Δ3</sub> was further examined by surface biotinylation, followed by lysis and immunoprecipitation of total cellular APP. Surface and total amounts of APP were estimated by dividing the amount of reactivity of the biotinylated versus total pools of APP (see “Materials and Methods”). The results showed that cell surface levels of γAPP<sub>Δ3</sub> were ~2.5-fold higher than those seen in APP.

![Diagram](image3.png)

**FIG. 4. Effect of protease inhibitors on the accumulation of APP and APP chimeras.** Panel a, cells were incubated in the absence or presence of leupeptin (100 μM) for 8 h. At the end of the incubation period, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, and the levels of APP were analyzed by Western blotting. A modest accumulation of γAPP<sub>LYS</sub>, but not APP, is apparent after leupeptin treatment. Parallel samples were blotted for β-tubulin to confirm protein loading. Panel b, proteasome inhibitor MG-132 (10 μM) was added to cells for 4 h, and levels of APP were analyzed as above. The strong accumulation of γAPP<sub>ER</sub> after MG-132 indicates that its predominant degradation route is, as expected, in the ER. Results from a representative experiment are shown.
levels of biotinylated APP were analyzed by phosphorimaging after enhanced chemiluminescence using an antibody against biotin. The relative amounts of APP at the cell surface are shown as the ratio of biotinylated versus total APP. The results are the mean ± S.E. of three experiments.

Next examined the levels of Aβ1–40 and Aβ1–42 in CD3γAPP Chimeras—We next examined the levels of Aβ1–40 and Aβ1–42 in cell lysates and conditioned media from cells stably transfected with APP or γAPP chimeras using sandwich ELISA (see “Materials and Methods”). In comparison with APP, all γAPP chimeras showed differences in the levels of Aβ1–40 and Aβ1–42, although in different ways. First, with respect to APP, there was a dramatic decrease in the secreted and intracellular pools of Aβ1–40 in γAPP<sub>LYS</sub> and γAPP<sub>ΔΔ</sub> expressing cells (Fig. 7a). Because all three proteins presumably share similar processing steps through the secretory pathway up to the TGN, the differences in Aβ1–40 production are likely to originate late in the secretory pathway (i.e. a post-TGN compartment) and/or the endocytic pathway. Neither γAPP<sub>LYS</sub> nor γAPP<sub>ΔΔ</sub> undergo efficient endocytosis, because in the former construct, the molecules were sorted away from the cell surface levels (Fig. 5) and in the latter construct, internalization was substantially impaired. Taken together, these results suggest that the endocytic pathway is a major site of production and subsequent release of Aβ1–40 (~60–70% of the total detected), the remaining presumably originated in the early secretory pathway (i.e. ER, Golgi, and/or TGN, the three compartments shared by both chimeras).

In contrast, however, levels of Aβ1–40 from γAPP<sub>ER</sub>-expressing cells showed different changes with respect to wild-type APP. Specifically, although intracellular levels of Aβ1–40 were almost 75% higher, secreted levels were approximately one-half those of APP cells (Fig. 7a). This suggested that only a portion of intracellularly generated Aβ1–40 molecules are released. Moreover, because γAPP<sub>ER</sub> is retained in the ER and endocytic processing is largely absent, the high levels of intracellular Aβ1–40 are unexpected. We hypothesized that the accumulation of Aβ1–40 in γAPP<sub>ER</sub> transfected cells derives from its retention in the ER, where there is an enhancement of substrate available for γ-secretase activity. To test this hypothesis, we measured the half-life of APP and the three γAPP chimeras by pulse-chase labeling. Indeed, both γAPP<sub>ΔΔ</sub> and γAPP<sub>ER</sub> showed markedly longer half-lives than wild-type APP (135.1 ± 3.38 min and 86.6 ± 4.3 min, respectively, versus 53.3 ± 6.9 min, n = 3, p < 0.001, analysis of variance) (Table I).

In contrast to Aβ1–40 levels, Aβ1–42 levels (Fig. 7b) were surprisingly unchanged in both γAPP<sub>LYS</sub> and γAPP<sub>ΔΔ</sub> expressing cells as compared with wild-type APP. This was seen in both intracellular and secreted pools of Aβ1–42, a finding that suggests that even the secreted pool of Aβ1–42 was generated predominantly in the ER/IC.

γAPP<sub>ER</sub> behaved very differently from the other three constructs. As with Aβ1–40, the intracellular levels of Aβ1–42 were substantially higher in γAPP<sub>ER</sub> cells as compared with wild-type APP and the other two APP/CD3γ chimeras (Fig. 7b). In contrast to Aβ1–40, even the secreted levels of Aβ1–42 were also higher. In summary, with respect to wild-type APP, levels of Aβ1–42 from γAPP<sub>ER</sub>-expressing cells were increased ~10- and ~4-fold in the intracellular and secreted pools, respectively.

Are Lysosomes Involved in the Generation of Aβ?—Lysosomes have been hypothesized as a possible site of Aβ production. Several studies have indirectly addressed this question, but, to date, the role of the lysosomes in Aβ production is still unclear (24–26). Efficient targeting of APP to lysosomes en-
been dedicated to the study of Aβ pathogenesis of the disease (1). Consequently, much effort has focused on understanding the role of such subcellular locations in the generation and degradation of Aβ proteins predicted to localize to particular organelles, to study the mechanisms underlying the proteolytic processing and is turned over more slowly. In the case of APP, it has been shown that the targeting motifs from the lysosomes from the TGN, indicating that lysosomes are not involved in Aβ generation (Fig. 7a). The same can be concluded for Aβ1–42. Because no differences are found between APP, γAPPlys, and γAPPΔγ (Fig. 7b), the lysosomal compartment is unlikely to be a major source of either Aβ1–40 and Aβ1–42 generation.

Aβ1–42/Aβ1–40 Ratios Increase in All γAPP Chimeras—Combining all the Aβ results of our study (Fig. 7), we have analyzed the relative ratios of Aβ1–42/Aβ1–40 expressed in CHO cells. Extracellularly, Aβ1–42 is consistently a minor fraction of total Aβ, and wild-type APP showed the expected ~1:1 ratio of Aβ1–42 to Aβ1–40. The intracellular ratio is ~6:10, a value similar to that reported in neurons (13). However, there was an increase in the 42:40 ratios in both intracellular (between 2–3:1) and secreted (~4:10) pools for all γAPP chimeras. And despite the differences in absolute levels of each Aβ species, the relative levels are surprisingly similar to each other. Intracellularly, this was achieved by either a large increase in Aβ1–42 (γAPPter; although attenuated by a slight increase in Aβ1–40) or by a decrease in Aβ1–40 (γAPPlys and γAPPΔγ). A similar, although more modest trend, is seen extracellularly. The secreted 42:40 ratio increase was achieved by either a decrease of Aβ1–40 and an increase in Aβ1–42 (γAPPter) or by a decrease in Aβ1–40 secretion (γAPPlys and γAPPΔγ).

**DISCUSSION**

Aβ is the major component of senile plaques in the Alzheimer’s brain, and it is thought to play an important role in the pathogenesis of the disease (1). Consequently, much effort has been dedicated to the study of Aβ generation in a variety of models. Here, we have chosen to generate several chimeric APP proteins predicted to localize to particular organelles, to study the role of such subcellular locations in the generation and subsequent secretion of Aβ1–40 and Aβ1–42. Although several recent reports have successfully used chimeric APP proteins to study the proteolytic processing of APP (27–29), our study is the first to use a common approach to target APP to multiple organelles and directly analyze the intracellular formation and secretion of Aβ1–40 and Aβ1–42. The strategy that we followed for APP subcellular targeting is based on a well characterized model, that of the y chain of the CD3 receptor (Fig. 1) (15). Because the targeting of the γAPP chimeras to specific organelles is central to our approach, we ascertained by morphological and biochemical studies the predicted localization of the chimeric proteins. Accordingly, several lines of evidence demonstrated that the targeting motifs from γCD3 are fully functional when fused to the APP transmembrane and extracellular domains, thereby providing a valuable tool for the analysis of Aβ formation in different subcellular organelles. Thus, we showed that γAPPter is localized to the ER with impaired post-translational processing and is turned over more slowly. In the case of γAPPlys, we showed that this chimeric protein is predominantly sorted to lysosomes, bypassing the cell surface, after maturation. Finally, we demonstrated that in the absence of the two targeting motifs, γAPPΔγ accumulates at the cell surface and, not surprisingly, showed impaired internalization.

As expected, a consequence of the latter abnormality is an increase in APPs in the medium (16).

Recently, it has become clear that the sites of generation and degradation of Aβ forms are complex and may be cell type dependent. Specifically, in neuronal cells, Aβ1–42 is produced in the ER/Golgi compartment (12, 13, 30), whereas Aβ1–40 is apparently derived from the TGN or beyond (12). In contrast, APP695 transfected COS-7 cells generate both Aβ forms at the plasma membrane (12) and are undetectable intracellularly. A nonneuronal cell type, kidney 293 cells, when stably transfected with APP, shows detectable levels of intracellular Aβ1–42 but not Aβ1–40 (31). Therefore, our analysis is important from the standpoint that the different intracellular processing pathways can be simultaneously analyzed with respect to both Aβ1–40 and Aβ1–42. Our results indicate that in CHO cells (1) both secretory and endocytic processing contribute to the production of Aβ1–40 whereas Aβ1–42 derives mainly from the secretory pathway (2) both endocytic and, to a lesser extent, ER/IC-derived Aβ forms can enter the secretable pool, and (3) lysosomes are not a major site of Aβ generation.

Our studies showed clearly that both Aβ1–40 and Aβ1–42 species are readily detectable in APP-transfected CHO cells and that the secretory pathway plays a role in the generation of both forms (Fig. 7, a and b). This is apparent from the fact that both Aβ1–40 and Aβ1–42 are increased when APP is artificially retained in the ER (γAPPter) via the presence of a retention signal engineered into the cytoplasmic domain. However, we reasoned that the majority of Aβ1–40 (~70–75%) originates from endocytic processing because of the loss of Aβ1–40 in the γAPPlys and γAPPΔγ cells as compared with wild-type APP cells (Fig. 7a). Furthermore, the fact that retention of APP in the ER results in accumulation of intracellular Aβ1–40 (Fig. 7a, compare γAPPter with APP) indicates that the ER itself contains an Aβ1–40-specific γ-secretase activity. This is a surprising finding, because Aβ1–40-specific γ-secretase activity has only been reported previously at the TGN or at the plasma membrane. One explanation of our finding is that this population of ER-derived Aβ1–40 is generated from secondary cleavage of Aβ1–42, a postulate that is consistent with evidence arguing against distinct Aβ1–40 and Aβ1–42 γ-secretase activities (32).

In contrast, consistent with published reports, Aβ1–42 may be derived primarily from processing in the early secretory pathway (12, 13). As shown in Fig. 7b, APP and both γAPPlys and γAPPΔγ produce and secrete comparable amounts of Aβ1–42, indicating that a main site of production is in a compartment common to all three APP forms, i.e., ER, Golgi, or the TGN. Again, the fact that retention of APP in the ER causes intracellular accumulation of Aβ1–42 points to that organelle as a major site for Aβ1–42 production. However, our results cannot rule out the late secretory and/or the endocytic pathways as additional sites of production for Aβ1–42. Specifically, because the γAPP chimeras have very different cytosolic tails, direct comparison to APP may be misleading. This is evident from the fact that all three γAPP chimeras show substantially higher Aβ1–42/Aβ1–40 ratios. Therefore, the possibility remains that the decrease seen in Aβ1–42 production from γAPPlys and γAPPΔγ, with respect to γAPPter, rather than to wild-type APP, may be due to the deficient endocytosis from the former chimeras and not only to the accumulation of γAPPter in the ER. This concept would be consistent with the observation that secretion of both Aβ1–40 and Aβ1–42 is diminished when the endocytic signal is removed from the cytoplasmic domain of APP (33). We should emphasize that retaining APP in the ER is equally artificial, and we cannot ascertain the degree to which Aβ1–42 generation has been abnormally increased. Furthermore, the normal interactions with proteins that associate with the APP...
cytoplasmic domain are absent in the chimera. Thus, the Fe65/X11 family of proteins that bind to APP at or near the YENPTY domain and alter APP trafficking, translocation to the plasma membrane, sAPP secretion, and Aβ production (34, 35) are ineffectual in the APP chimeric molecules. Interestingly, overexpression of X11 as well as the Y743A mutation (in the NPTY motif) both decreased the turnover of APP but with different effects on Aβ generation (33, 35). Although γAPPER also increased the APP half-life, direct comparison between these three conditions is not possible. First, as mentioned above, γAPPER contains a completely different cytoplasmic domain. Second, the other studies did not examine the levels of intracellular Aβ. Third, the mechanisms underlying the reduced turnover of APP are different in all three cases: in γAPPER, the molecule is postulated to remain in the ER; in APP Y743A mutant, a lysosomal targeting signal may be lost (33); and in X11, the effect is presumably due to delayed protein maturation (35).

Our results also showed that not all intracellular Aβ1-40 and Aβ1-42 is released. It has been argued that in neurons, the secreted and the intracellular pools of Aβ are produced independently and that the secreted pool is endocytosis-dependent (12, 36). Subsequent studies (13) showed that the intracellular pool of Aβ1-42 derives from the ER and does not enter the secreted pool. This was shown by insertion of a KK ER-retention motif to the cytosolic tail of APP (13), an approach similar to the γAPPER construct, and by BFA treatment of wild-type APP-expressing cells, although protein traffic distal to the ER was indirect evidence suggesting that an acidic compartment, perhaps a lysosome, is the site of production of either Aβ1-40 or Aβ1-42. Subsequently, we showed that in CHO cells the lysosomes are not likely to rep-