Mutagenesis Identifies New Signals for β-Amyloid Precursor Protein Endocytosis, Turnover, and the Generation of Secreted Fragments, Including Aβ42*

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It has long been assumed that the C-terminal motif, NPXY, is the internalization signal for β-amyloid precursor protein (APP) and that the NPXY tyrosine (Tyr742) by APP751 numbering, Tyr482 in APP695) is required for APP endocytosis. To evaluate this tenet and to identify the specific amino acids subserving APP endocytosis, we mutated all tyrosines in the APP cytoplasmic domain and amino acids within the sequence GYENPTY (amino acids 737–743). Stable cell lines expressing these mutations were assessed for APP endocytosis, secretion, and turnover. Normal APP endocytosis was observed for cells expressing Y709A, G737A, and Y743A mutations. However, Y738A, N740A, and P741A or the double mutation of Y738A/P741A significantly impaired APP internalization to a level similar to that observed for cells lacking nearly the entire APP cytoplasmic domain (ΔC), arguing that the dominant signal for APP endocytosis is the tetrapeptide YENP. Although not an APP internalization signal, Tyr746 regulates rapid APP turnover because half-life increased by 50% with the Y743A mutation alone. Secretion of the APP-derived proteolytic fragment, Aβ, was tightly correlated with APP internalization, such that Aβ secretion was unchanged for cells having normal APP endocytosis but significantly decreased for endocytosis-deficient cell lines. Remarkably, secretion of the Aβ42 isofrom was also reduced in parallel with endocytosis from internalization-deficient cell lines, suggesting an important role for APP endocytosis in the secretion of this highly pathogenic Aβ species.

APP1 is a transmembrane protein with homology to glycosylated cell surface receptors (1), can reside at the cell surface (2–4) and is reinternalized via clathrin-coated pits (5, 6) to the endosomal-lysosomal pathway (7, 8). Some internalized APP remains intact to be recycled to the cell surface plasma membrane (9, 10). However, internalized APP can also be proteolytically processed into several distinct secreted fragments, which include the large secreted N-terminal APP ectodomain (APPs), and Aβ, the major protein component of senile plaques in Alzheimer’s disease (AD; reviewed in Ref. 11).

Because Aβ deposition may be central to AD pathogenesis, the mechanism by which Aβ is generated from the precursor is an important focus of AD research. At least two species of Aβ, differing by two amino acids at the C terminus (Aβ40 and Aβ42), are released from cells during normal cellular metabolism (12–14). Aβ42, which readily aggregates in vitro (reviewed in Ref. 15) appears to be more pathogenic and may serve as a seed for plaque formation in individuals with AD (16), hereditary cerebral hemorrhage with amyloidosis Dutch type (17), and Down’s syndrome (18). The source of Aβ deposited in brain tissues is still uncertain. However, cell lines expressing wild type APP can produce and release Aβ primarily after internalization of APP from the cell surface (19, 20). Although familial mutations in APP can enhance Aβ secretion (e.g. the Swedish KM → NL mutation; Refs. 20–23), almost all humans express wild type APP. Therefore, the major pathway for Aβ production appears to involve endocytic recycling of APP from the cell surface. To date, the specific contribution of APP endocytic processing to Aβ42 production in particular has not been established.

APP endocytosis relies on signals in the cytoplasmic C-terminal domain. An NPXY sequence similar to that found in the C terminus of the low density lipoprotein receptor (LDLR, 24) and the LDLR-related protein (25) is also found in APP. Because the tyrosine in NPXY is crucial for LDLR endocytosis (24, 26), it has long been assumed that the homologous tyrosine in the APP NPXY motif is the principal signal for APP endocytosis. In the LDLR, an additional amino acid upstream of the NPXY motif was identified as a contributor to LDLR endocytosis, making FXNXY the accepted LDLR internalization motif (reviewed in Ref. 27). In the only detailed study to date, the APP endocytic signal from an APP-transferrin receptor chimera was localized to the GYENPTY motif (amino acids 737–743), with a potential additional contribution by tyrosine 709 in the YTSI motif adjacent to the membrane (28). However, because the APP-transferrin receptor chimera changed both the orientation and the distance of the potential APP endocytic signal with regard to the plasma membrane, it is uncertain whether this reorientation may have contributed to some of the unexpected findings in that study. In addition, the effect of the endocytic signal on Aβ42 release, in particular, has not been investigated. We therefore undertook extensive mutagenesis

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The abbreviations used are: APP, β-amyloid precursor protein; AD, Alzheimer’s disease; LDLR, low density lipoprotein receptor; CHO, Chinese hamster ovary; WT, wild type; Triton, N,N-dihydroxy-1,1-binhydromethylglycine; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance. APPs, secreted Nt-terminal ectodomain of APP.
studies to identify the specific amino acids required for APP endocytosis and to measure their potential effects on APP processing.

Because tyrosines are associated with endocytosis of multiple proteins (29, 30), we generated point mutations of all the APP cytoplasmic domain tyrosines as well as of the amino acids G, N, and P in the GYENPTY motif. We studied the effects of these mutations in stably transfected cell lines and now report that single amino acids contribute substantially to both APP endocytosis and the release of APP secreted fragments. Of particular note, we observed that although Tyr138, the first tyrosine in GYENPTY, contributed to APP endocytosis, neither the second tyrosine in this motif (Tyr143) nor Tyr709 near the membrane were functional endocytic signals. Nonetheless, Y743A mutant cell lines exhibited slower APP turnover. Moreover, we documented a very tight correlation between the degree of APP endocytosis and Aβ secretion. Interestingly, changes in Aβ42 and total Aβ secretion generally paralleled each other, suggesting that endocytic processing contributed to the secretion of both the pathogenic Aβ42 peptide and the more abundantly produced Aβ40 peptide by our cell lines.

EXPERIMENTAL PROCEDURES

Cell Lines—Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (Mediatech/CellGro, Herndon, VA) containing 10% fetal calf serum (HyClone, Logan, UT). Stably transfected CHO cells, selected by G418 (Life Technologies, Inc.) resistance, were maintained in 200 μg/ml G418. The cell lines expressing wild type APP751 (WT) or APP751 with almost the entire cytoplasmic domain deleted (ΔC) have been described (19, 20, 31, 32). CMVAPP751 cDNAs with Y653A and Y682A mutations, kindly provided by Dr. Christian Haass (33), were subcloned into APP751 to produce the Y709A and Y738A mutations. Alanine substitutions at Tyr709, Gly737, Tyr738, Asn46, Pro44, and Tyr43 were performed by polymerase chain reaction, confirmed by sequencing, and expressed in CHO cells using pClneo (Promega, Madison, WI). Stably transfected CHO cell lines were selected for comparable biosynthetic rates of APP proteins as determined by metabolic labeling and immunoprecipitation using APP ectodomain antibodies (19, 20) to immunoprecipitate full-length APP as described below. A schematic representation of the APP C terminus and the mutated amino acids is illustrated in Fig. 1.

Metabolic Labeling and Antibodies Used—Nearly confluent cultures of APP transfected CHO cells were incubated in methionine-free Dulbecco's modified Eagle's medium supplemented with 50 μCi/ml [35S]methionine (NEN Life Science Products) for 16 h. Media were collected, cells were lysed using 1% Nonidet P-40 buffer supplemented with protease inhibitors, and APP proteins were immunoprecipitated with APP-specific antibodies (R1282 for the APP ectodomain, antibodies 5A3 and 1G7 to measure full-length APP and all species of secreted APP, as described previously) (19, 20). In three independent experiments, cultures were pulse labeled for 15 min with 150 μCi/ml [35S]methionine and either not chased (for time 0) or chased for 1, 2, or 4 h for measuring the half-life of full-length APP. For these studies, APP turnover was immunoprecipitated with the APP ectodomain antiserum, 861 (34). Half-life was calculated by taking the ratio of acid-resistant to acid-labile counts provided a measure of the cellular expression for each cell line analyzed. Proteins were separated by SDS-polyacrylamide gel electrophoresis (6–10% Tris-glycine gels for higher molecular weight proteins and 16.5% Tris-Tricine gels for lower molecular weight proteins). Dried gels were exposed using Transcreen LE with BioMax AR film (Kodak, Rochester, NY) and/or a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for visualizing phosphorimaging signal loads 1, 2, and 4 h divided by the signal at time 0, thus normalizing the data for cellular expression for each cell line analyzed. Uptake Assay—Triplicate cultures of CHO cells were grown in 12-well tissue culture plates. To measure internalization of cell surface APP, the APP ectodomain antibody (to the APP ectodomain was iodinated as described previously to a specific activity of ~3–6 μCi/g (10), added to binding medium (RPMI 1640 supplemented with 20 mM Hepes + 0.2% bovine serum albumin) and applied to confluent layers of CHO cells and incubated at 37 °C for 30 min. After incubation, cells were rapidly chilled on ice, and the reaction was quenched by the addition of ice-cold binding medium. After 10 min, chilled cells were washed multiple times with ice-cold Dulbecco's phosphate-buffered saline (Life Technologies, Inc.) to eliminate unlabeled antibody. Residual antibody bound to surface APP (designated the acid-labile fraction) was then detached from cell surfaces by two 5-min washes with ice-cold phosphate-buffered saline, pH 2.5, and collected into sample tubes. Cells lysed in 0.2 M NaOH (yielding the acid-resistant fraction) were collected into sample tubes. Acid-labile and acid-resistant APP antibody counts were measured by γ counting. In control experiments, the acid wash consistently detached 90–95% of cell surface antibody radioactivity from cells that had not been allowed to undergo endocytosis. The ratio of acid-resistant to acid-labile counts provided a measure of the internalized versus cell surface pools of APP. Specific binding was calculated after subtraction of the radioactive counts obtained from parallel cultures of untransfected CHO cells that have minimal amounts of APP on the cell surface.

ELISA—Aβ sandwich ELISAs were performed as described previously (35, 36). Briefly, capture antibody 266 (to Aβ13–28) was used for total Aβ, and antibody 21F12 (to Aβ residues 33–42) was used for Aβ42. The reporter antibody for both assays was 3D6 (to Aβ residues 1–5). All experiments were repeated 2–3 times on triplicate samples for each cell line.

RESULTS

Specific Amino Acids in the APP C Terminus Contribute to Endocytosis of Full-length APP.—To assess APP endocytosis in control and mutant stable cell lines we used a well established protocol (10) in which iodinated 1G7 antibody to the APP ectodomain was applied to cultured cells, followed by incubation at 37 °C to monitor the internalization of APP. The APP WT control cell line has been extensively characterized, and it has been confirmed to undergo rapid internalization, as well as secretion, in this line (10). As expected, WT cells exhibited robust uptake of the specifically surface-bound APP antibody at 37 °C (Fig. 2). APP internalization, indistinguishable from that of WT cells, was observed for cells expressing Y709A, Y737A, and Y743A mutations (Fig. 2), indicating that endocytosis of APP was unchanged for these mutant cell lines. In contrast, cells expressing Y738A, N740A, and P741A mutations or the double mutation Y738A/P741A showed dramatically reduced APP internalization (Fig. 2; ANOVA, p < 0.001). The degree of reduction in APP internalization for these four mutant cell lines was comparable with that observed for the

FIG. 1. Schematic diagram of the APP751 C terminus and mutations. The inner aspect of the plasma membrane is represented by the shaded vertical rectangle on the left. APP cytoplasmic domains are represented by horizontal rectangles projecting from the plasma membrane with the APP amino acid sequence indicated using the single-letter code. The top rectangle shows the truncated C terminus of the APP ΔC mutant with its 4-amino acid C-terminal tail, KKKQ. The bottom rectangle represents the native 47-amino acid APP C terminus with the mutated amino acids indicated by arrows and labeled using the number and letter code. The inner aspect of the plasma membrane is represented by the shaded vertical rectangle.
cytoplasmic deletion line ΔC (Fig. 2), which has been shown previously to be internalization-deficient (31). These results suggest that the amino acids, Y, N, and P in the YXY motif (amino acids 738–741) confer the principal ability for APP to undergo rapid endocytosis.

**Cell Lines That Do Not Efficiently Internalize APP from the Cell Surface Secret More APP.**—To determine whether diminished APP uptake also may have affected APP proteolytic processing, we next measured the secretion of the total pool of APPs, the major N-terminal ectodomain fragments released from both control and mutant cell lines after cleavage by α- or β-secretase. Equivalent normal levels of total APPs were released into the medium of metabolically labeled WT cells and cells expressing APP with point mutations of Y709A, G737A, and Y743A (Fig. 3) entirely consistent with the endocytosis results (Fig. 2). As expected, significantly more total APPs was secreted by cell lines with diminished APP uptake, i.e. ΔC cells and the cells expressing point mutations Y738A, N740A, P741A, and Y738A/P741A (Fig. 3, ANOVA, \( p < 0.001 \)).

Taken together the data in Figs. 2 and 3 indicate that when APP endocytosis is normal, APPs secretion is unchanged. Interestingly, both uptake and APPs secretion were normal for cells with tyrosine mutations in the YTSI and NPXY motifs. Although neither Tyr709 in YTSI nor Tyr743 in NPXY contributed significantly to APP endocytosis and APPs secretion, we further analyzed these cell lines to determine whether other aspects of APP processing may have been altered by these mutations.

**Mutation of the NPXY Tyrosine but Not the YTSI Tyrosine Affects Full-length APP Turnover.**—Full-length APP turnover was measured by pulse chase analysis for WT cells and cells expressing point mutations in the three C-terminal tyrosines, Y709A, Y738A, and Y743A, as well as for the ΔC mutant. No difference in APP turnover was observed for Y738A (Fig. 4), and Y709A or ΔC cell lines (not shown) as compared with WT cells. In contrast, Y743A cells appeared to metabolize APP differently. By pulse chase analysis, Y743A cells showed a significant −50% increase in half-life as compared with WT and Y738A cells (Fig. 4A; WT = 64 min ± 7.2, Y738A = 56 min ± 7.8, Y743A = 86 min ± 5.0; ANOVA, \( p < 0.02 \), data are the averages (± S.E.) from three independent experiments). The ratio of mature to immature APP reveals a significant increase for Y743A cells compared with both WT and Y738A cells. Most full-length APP has been metabolized by 4 h for all cell lines (A). The ratio of mature to immature APP was calculated based on the time 0 signal for each cell line, all data are normalized for APP expression and thus unaffected by variations observed in individual experiments.

Moreover, when we compared the levels of mature and imm-

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**Fig. 2. Endocytosis of full-length APP is markedly diminished in cells expressing Y738A, N740A, P741A, Y738A/P741A, or the ΔC mutation.**—Uptake of iodinated 1G7 antibody bound to cell surface APP was nearly identical for WT cells and for cells expressing Y709A, G737A, and Y743A mutations. In contrast, cells expressing Y738A, N740A, P741A, Y738A/P741A, or the large APP cytoplasmic deletion (ΔC) had a significantly reduced ratio of internal to surface APP. All data are the means (± S.E.) of four to nine independent experiments, each performed in triplicate and adjusted to the level of full-length APP expression for each cell line. *, significance at \( p < 0.001 \).

**Fig. 3. Release of APPs, is increased by cells expressing Y738A, N740A, P741A, Y738A/P741A, or the ΔC APP C-terminal deletion.** Total APPs, was immunoprecipitated from the conditioned media of nearly confluent cells labeled 16 h with \(^{35}\)S)methionine, using the APP ectodomain antibodies 1G7 and 5A3 (19, 20), which recognize all APPs species. APPs was separated using SDS-polyacrylamide gel electrophoresis and quantified by phosphorimage analysis. The data are the means (± S.E.) from three independent experiments and adjusted to the level of full-length APP expression for each cell line. *, significance at \( p < 0.01 \) or better.

**Fig. 4. Mutation of the Tyr743 in the NPXY motif significantly alters turnover of full-length APP751.** In this representative experiment, full-length APP was immunoprecipitated from cell lysates of WT, Y738A, and Y743A cells pulse-labeled with \(^{35}\)S)methionine for 15 min and chased for 0, 1, 2, and 4 h from a representative experiment (A). At time 0, all APP consisted of the immature N-glycosylated species. Both N-glycosylated (immature) and N- and O-glycosylated (mature) species are abundant at 1 h for all three cell lines. More full-length APP is present at 1 and 2 h for Y743A cells compared with both WT and Y738A cells. Most full-length APP has been metabolized by 4 h for all cell lines (A). The ratio of mature to immature APP was calculated based on the time 0 signal for each cell line, all data are normalized for APP expression and thus unaffected by variations observed in individual experiments.

Moreover, when we compared the levels of mature and im-
mature APP for these same three cell lines, Y743A cells had a significantly greater ratio of mature to immature full-length APP at both 1 h and 2 h than either WT or Y738A cells (Fig. 4B, p < 0.001). These data indicate that both mature and immature full-length APP were retained at later time points by cells expressing the Y743A mutation compared with WT or Y738A cells. Therefore, although Tyr743 does not appear to be an endocytic signal, this APP tyrosine contributes to normal APP turnover.

Total Aβ Secretion Is Dramatically Reduced by Cells Expressing ΔC, Y738A, N740A, P741A or, Y738A/P741A Mutations—To determine whether other aspects of APP processing were affected by the C-terminal mutations, we next measured the metabolic production and release of Aβ from our WT and mutant stable CHO cell lines. Total Aβ secretion was measured using antibody R1282 to immunoprecipitate all Aβ species from conditioned medium of cells labeled overnight with [35S]methionine. WT cells and cells expressing Y709A, G737A, and Y743A mutations secreted equivalent amounts of total Aβ consistent with their ability to internalize full-length APP (Fig. 5). In contrast, cells expressing Y738A, N740A, P741A, or Y738A/P741A of large APP C-terminal deletion, ΔC, have significantly reduced levels of total Aβ secretion, as seen in a representative autoradiogram (B). The Aβ levels in B, although not normalized for APP expression, were obtained from cells expressing comparable levels of APP. Data in A are the means (± S.E.) from 4–8 independent experiments, normalized for full-length APP expression. *, significance at p < 0.01 or better.

Aβ42 Secretion Is Dramatically Reduced by Mutations That Diminish APP Endocytosis—We measured both total Aβ (principally Aβ40) and Aβ42 levels in media conditioned for 24 h by WT cells and by cells expressing the various C-terminal mutations using a well-characterized and sensitive sandwich ELISA (35, 36). In striking parallel to their abilities to efficiently internalize APP, WT cell lines and the Y709A, G737A, and Y743A mutant cell lines released similar levels of total Aβ and Aβ42, suggesting that Aβ release was normal for these cell lines. Not surprisingly, the ratios of Aβ42 to total Aβ in medium were unchanged in these three mutant cell lines (Fig. 6). In sharp contrast, cells expressing mutations that diminished APP endocytosis showed significantly reduced Aβ42 secretion. The C-terminal deletion, ΔC, and point mutations of Y738A, N740A, P741A, and Y738A/P741A of GYENPTY all resulted in significantly less Aβ42 secretion relative to total Aβ than that observed for the WT, Y709A, G737A, and Y743A cell lines (Fig. 6) revealing for the first time that mutations that diminish APP internalization dramatically reduce Aβ42 secretion. These data suggest that recycling of full-length APP contributes significantly to the pool of both Aβ40 and Aβ42 produced and released from cells during normal metabolic processing.

**DISCUSSION**

Stably transfected CHO cells have been shown in numerous studies to process APP similarly to other cells (e.g., Refs. 7 and 37–39) and thus provide a good model for studying APP trafficking and metabolism (6, 10, 19, 20, 28, 31). Analyses of our stably transfected CHO cells expressing WT APP or APP with various C-terminal mutations have generated three major and novel findings regarding APP trafficking and processing. First, we have identified the tetrapeptide motif, YXNP (amino acids 738–741 in APP751 or 682–685 in APP695) in the APP C terminus as the dominant APP endocytic signal. Second, we observed that whereas mutation of Tyr743 in the NPXY motif had no effect on APP endocytosis, this mutation significantly altered metabolism (half-life) of full-length APP. Third, we identified dramatic and parallel effects on the secretion of APP, total Aβ, and Aβ42 in association with C-terminal mutations that altered normal APP endocytosis.

Some APP holoprotein, including cell surface APP, is found...
in clathrin-coated vesicles (5, 6), indicating that APP utilizes a clathrin-based endocytic pathway. Many integral membrane proteins, including APP, undergo continued recycling and trafficking throughout their lifetime, a phenomenon common to cell surface receptors (extensively reviewed in Ref. 27). A key receptor molecule used to define endocytic recycling is the LDLR (e.g. 24, 26, 27). Based on its role in LDLR endocytosis, NPXY has been assumed to be the signal for APP endocytosis (24). In addition to the four amino acids of this NPXY motif, an aromatic amino acid residue upstream of NPXY also contributes to LDLR endocytosis, making the recognized endocytic signal for this molecule the hexapeptide motif FXNPyX (24).

Once FXNPyX had been defined as the LDLR endocytic motif, an analogous sequence in the APP C terminus, YENPTY, became the primary focus for APP endocytosis and trafficking studies (19, 28, 31, 33, 37). Although studies suggested that the APP endocytic signal was localized within this YNPyX motif, the specific amino acids underlying APP endocytosis remained undefined. Furthermore, potential contributions to endocytosis had also been suggested for the proximal tyrosine Tyr709 (in the YTSI motif), and for the glycine (Gly737) in GYENPTY of APP (28). Therefore, we systematically analyzed the residues thought to contribute to APP endocytosis using a well characterized method for evaluating APP endocytosis (10).

In our study, we show that mutations of Tyr, Asn, or Pro or a double mutation of Tyr and Pro but not a mutation of the C-terminal Tyr in YENPTY significantly diminish APP endocytosis. Point mutations within the YXNP motif all reduced APP internalization dramatically. Therefore, our data strongly suggest that the four amino acid sequence, YENP, is the major signal for clathrin-mediated APP endocytosis. The single amino acid Asn740 appears to be a major component of the APP endocytic signal because the N740A mutation alone reduced endocytosis to a level similar to that observed for cells (ΔC) lacking nearly the entire APP cytoplasmic domain.

In addition, our data showed that neither Tyr709 nor Gly737 contributed to APP internalization in our studies, suggesting that these amino acids, like Tyr743 in NPXY, are not part of the APP endocytic signal. In native APP, Tyr709 lies 5 amino acids from the plasma membrane, rather than 46 amino acids from the plasma membrane as was the case in the reported APP-transferrin-receptor chimera (28), and this may explain why the YTSI tyrosine promoted internalization in the latter model. Although N-terminal ectodomain residues of APP may play a role in basolateral sorting in polarized epithelial cells (37), support for the YTSI tyrosine subserving basolateral sorting of APP has also been shown (33). It may be that the YTSI tyrosine (Tyr709 in APP751 and Tyr653 in APP695) interacts with the newly identified PAT1 molecule (a protein with kinase homology that interacts with microtubules) to sort APP to the basolateral compartment in polarized epithelial cells (40).

Regarding the role of Tyr743 in APP processing we found that Tyr743 in the NPXY motif is not an APP endocytic signal and that cells overexpressing APP with this mutation produced normal levels of Aβ. Although endocytosis was not measured in their studies, other investigators using cells with mutations of the NPXY tyrosine have also observed normal Aβ secretion from their cell lines (41, 42). Although therefore not an endocytic signal, Tyr743, when mutated, reduced the otherwise rapid turnover of APP. Although the APP half-life was longer in Y743A cells, APP was converted from immature to fully glycosylated APP similarly to WT and Y738A APP, suggesting that trafficking through the Golgi compartment was normal for these cell lines. Thus, we speculate that Tyr743 may function, in part, as a lysosomal targeting signal for APP.

We previously showed that cells expressing C-terminal deletions have reduced levels of total Aβ secretion (19, 31). As anticipated, total Aβ release was again diminished by cells expressing mutations that reduced APP endocytosis. Specifically, Y738A, N740A, P741A, Y738A/P741A, and ΔC cell lines all had dramatically reduced total Aβ secretion as measured by immunoprecipitation. No decrease in total Aβ secretion was observed for Y709A, G737A, or Y743A, cell lines that also had normal APP endocytosis. These data strongly reinforce the importance of the endocytic pathway for the generation of total Aβ released by cells.

When we measured Aβ42 secretion by sandwich ELISA we were surprised to see that in parallel with the diminution of total Aβ secretion, the Y738A, N740A, P741A, Y738A/P741A, and ΔC cell lines also secreted less Aβ42. This unexpected finding suggested that processing of APP in the endocytic pathway contributes substantially to Aβ42 secretion. However, non-neuronal (20, 42–45) and neuronal cells (46–48) are known to produce and, under some conditions, accumulate intracellular Aβ. Because several studies have identified Aβ42 production in the early compartments of the secretory processing pathway (42, 45, 47–50), we were surprised to see that endocytic processing appeared to contribute significantly to the pool of Aβ42 secreted from our mutant cell lines. However, it is not known whether the intracellular Aβ identified in the earlier studies ultimately gets secreted by the cells. Our results suggest that perhaps most Aβ42 does not become secreted, because the level of Aβ42 released into the medium was strongly dependent on the endocytic signal. Because we did not measure intracellular Aβ species in our current studies, measurements for total Aβ, Aβ40, and Aβ42 in cell lysates or in isolated endoplasmic reticulum or Golgi vesicles of our C-terminal mutants will be required to determine whether or not a deficiency in endocytosis can affect the various intracellular Aβ species. Furthermore, it should be noted that although our data indicate that mutations that affected APP internalization reduced Aβ secretion, whether the same mutations may have affected exocytic APP trafficking remains to be elucidated.

Although it is unknown how the amino acids in the YXNP motif mediate endocytosis, it is appealing to speculate that it may occur by interaction with APP-binding proteins such as BP1, X11, or Fe65 (reviewed in Ref. 51). Recent data in which APP processing was assessed in cells overexpressing X11 or Fe65 (52, 53) suggest that the interaction of APP with these molecules may occur in different pathways and that Aβ production is perturbed by altered protein-protein interactions of this sort. X11 may bind to APP primarily in the secretory pathway because overexpression of X11a reduced APPcleaved by α-secretase (52), possibly because of reduced amounts of APP arriving at the cell surface. Conversely, interaction with Fe65 may occur in an early stage of the endocytic pathway because overexpression of human Fe65 (hFe65L) in H4 neuroglioma cells increased APPsecretion (53), an effect possibly related to retention of APP at or near the cell surface, where it could undergo enhanced α-secretase cleavage, as recently reported for MDCK cells overexpressing rat Fe65 (54). This enhancement of α-secretase cleavage may be associated with APP binding to caveolin-1 within plasma membrane microdomains (55). Additionally, an NPXY motif-dependent interaction between APP and the receptor LDLR-related protein by way of the cytosolic adaptor proteins mDab (mammalian Disabled protein) and Fe65 has also been identified that may affect APP trafficking and processing (56). These possibilities require additional experimental validation.

In conclusion, our data indicate that the dominant signal for APP endocytosis resides in the motif YXNP, that other signals in the APP C terminus contribute to different APP processing/
trafficking pathways, and that endocytic trafficking of APP contributes importantly to both APP\(_\beta\) and A\(_\beta\) secretion. Further understanding of the mechanisms by which cells convert APP into secreted fragments may provide new insights for designing or testing the efficacy of inhibitors of A\(_\beta\) generation and secretion as potential treatments for AD.

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