Retention of the Alzheimer's Amyloid Precursor Fragment C99 in the Endoplasmic Reticulum Prevents Formation of Amyloid β-Peptide*

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γ-Secretase is a membrane-associated endoprotease that catalyzes the final step in the processing of Alzheimer's β-amyloid precursor protein (APP), resulting in the release of amyloid β-peptide (Aβ). The molecular identity of γ-secretase remains in question, although recent studies have implicated the presenilins, which are membrane-spanning proteins localized predominantly in the endoplasmic reticulum (ER). Based on these observations, we have tested the hypothesis that γ-secretase cleavage of the membrane-anchored C-terminal stump of APP (i.e. C99) occurs in the ER compartment. When recombinant C99 was expressed in 293 cells, it was localized mainly in the Golgi apparatus and gave rise to abundant amounts of Aβ. Co-expression of C99 with mutant forms of presenilin-1 (PS1) found in familial Alzheimer's disease resulted in a characteristic elevation of the Aβ42/Aβ40 ratio, indicating that the N-terminal exodomain of APP is not required for mutant PS1 to influence the site of γ-secretase cleavage. Biogenesis of both Aβ40 and Aβ42 was almost completely eliminated when C99 was prevented from leaving the ER by addition of a di-lysine retention motif (KKQN) or by co-expression with a dominant-negative mutant of the Rab1B GTPase. These findings indicate that the ER is not a major intracellular site for γ-secretase cleavage of C99. Thus, by inference, PS1 localized in this compartment does not appear to be active as γ-secretase. The results suggest that presenilins may acquire the characteristics of γ-secretase after leaving the ER, possibly by assembling with other proteins in peripheral membranes.

Amyloid β-peptide (Aβ) is the major molecular component of the cerebral amyloid plaques associated with Alzheimer's disease. The cellular pathways involved in the biogenesis of Aβ have been the subject of intense investigation since the discovery that Aβ originates from intracellular endoproteolytic processing of a type I membrane-spanning glycoprotein termed amyloid precursor protein (APP) (1-4). Extensive studies have established that APP can be processed via two alternative routes, one of which yields the 4-kDa Aβ, whereas the other yields a truncated non-amyloidogenic peptide (p3) (5, 6). In most cells the non-amyloidogenic pathway predominates. The first step involves the cleavage of APP within the Aβ domain by a protease termed α-secretase (7-9). After release of the N-terminal exodomain, the residual 83-amino acid membrane-spanning C-terminal fragment is further processed by another protease termed γ-secretase to remove the cytoplasmic tail and generate p3 (6, 10). Because the latter cleavage occurs within the predicted membrane spanning region of APP (11-13), γ-secretase is generally thought to be an intramembrane protease. In the alternative amyloidogenic pathway, APP is initially cleaved proximal to the Aβ sequence by β-secretase, leaving a 99-amino acid C-terminal fragment (C99) that contains the intact Aβ sequence and the cytoplasmic tail (1, 14, 15). Thus, when γ-secretase cuts the latter substrate, Aβ is released. Cells can generate distinct species of Aβ that differ in chain length (e.g. Aβ40 and Aβ42). It remains unclear whether the different forms of Aβ arise through the action of separate γ-secretases (11, 16, 17) or instead reflect the ability of a single enzyme to cleave C99 at more than one site (18). Although Aβ40 is produced in greater abundance than Aβ42, the longer peptide has particular significance for Alzheimer's disease pathology, since it readily forms insoluble aggregates and accumulates in neuritic plaques (19-21).

A number of reports have provided information about the subcellular compartments where APP is cleaved by the a- and β-secretases. Metalloproteases that function as a-secretases (22, 23) appear to operate on APP at or near the cell surface (9, 24, 25). On the other hand, β-secretase cleavage occurs predominantly in intracellular membrane compartments such as the Golgi apparatus (26-28) and endosomes (29-31). The latter findings have been verified in studies of newly identified aspartic proteases that function as β-secretase (32-35). The identity of γ-secretase remained elusive until a recent series of studies implicated the serpine membrane-spanning proteins, presenilin-1 (PS1) and presenilin-2 (PS2), as catalytic components of this enzyme. Mutations in the presenilins have been linked to familial forms of Alzheimer's disease that are characterized by elevations of the Aβ42/Aβ40 ratio (36-39). It now appears that presenilins may be unique aspartyl proteases that can function as γ-secretase (40-43) or play an essential role in the generation of Aβ.

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‡ The abbreviations used are: Aβ, amyloid β-peptide; APP, amyloid precursor protein; ER, endoplasmic reticulum; PS, presenilin; FAD, familial Alzheimer's disease; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; SREBP, sterol regulatory element-binding protein; FITC, fluorescein isothiocyanate; HSV, herpes simplex virus; wt, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline.

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20267
role in regulating γ-secretase activity (44, 45). The subcellular location of the γ-secretase cleavage step has not been established definitively, but several studies (46–48) have suggested that it occurs in the ER, where most of the presenilin is localized (49–51). If this is true, it would imply that the C83 and C99 fragments generated by α-secretase and β-secretase in the plasma membrane and Golgi/endosomal compartments, respectively, must be transported back to the ER for the final cleavage by γ-secretase. However, the evidence supporting γ-secretase processing of APP in the ER is not definitive. For example, in neurons Aβ42 is found in the ER, whereas Aβ40 is localized in the trans-Golgi network (52), but it is difficult to determine if the steady-state distribution of the Aβ peptides reflects the primary site where they are generated. Other evidence pointing to the ER as a site of γ-secretase activity centers around the observation that intracellular production of total Aβ (46) or Aβ42 (47) can continue unchecked when ER → Golgi transport is blocked by brefeldin A. However, the interpretation of these observations is complicated by the fact that brefeldin A causes mixing of various membrane compartments, i.e., the Golgi apparatus merges with the ER and the trans-Golgi network fuses with endosomes (53, 54). A final line of evidence supporting the existence of γ-secretase activity in the ER comes from a report by Soriano et al. (48), who found that secretion of Aβ40 and Aβ42 was not markedly reduced when APP was retained in the ER by fusing the extracellular and transmembrane domains with the cytoplasmic region of the T cell antigen receptor CD3γ chain. However, from this study it is not entirely clear whether the susceptibility of the chimeric APP/CD3γ substrate to the ER protease accurately represents the behavior of the native APP or C99. Indeed, there is considerable evidence that the cytoplasmic domain of APP, which was removed in the aforementioned study, contains important sorting determinants (55) and sequence elements required for interaction with proteins like X11 (56) and nicastrin (57), which may influence APP processing and Aβ secretion.

In the present study we examined directly the contribution of the ER compartment to γ-secretase processing of the membrane-anchored C99 stump of APP in transfected cells, using two different strategies. The first strategy involved the addition of minimal tetrapeptide extensions to the C terminus of C99 to either retain the protein in the ER (KKQN) or allow its retention in the aforementioned study, contains important sorting determinants (55) and sequence elements required for interaction with proteins like X11 (56) and nicastrin (57), which may influence APP processing and Aβ secretion.

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sample buffer containing 8 mM urea, 4% SDS w/v, 10% 2-mercaptoethanol v/v, 20% glycerol v/v, and 125 mM Tris-HCl, pH 6.8. Proteins were subjected to SDS-PAGE, using 10% polyacrylamide gradient gels (NOVEX, San Diego, CA). Proteins were transferred to Immobilon-P (Millipore Corp, Bedford, MA), and the membranes were preincubated for 1 h in blotting solution (PBS containing 5% w/v powdered milk and 0.05% v/v Tween 20). Immunoblotting was carried out as described previously (27, 58). Intracellular APP and total secreted forms of APP were detected with the monoclonal antibody 8E5 as described previously (27, 58). Intracellular APP and total secreted APP were detected with antibodies 2H3 (27) and SW192 (31), respectively. PS1 sequence (Senetek, Napa, CA), or 13G8, which recognizes the last 20 residues at the C terminus of APP (gift from P. Seubert and D. Schenk, Elan Pharmaceuticals, South San Francisco, CA). Rab1B was detected with an affinity-purified polyclonal antibody from Zymed Laboratories Inc. (South San Francisco, CA). SwAPP (58), the 108-kDa form of SwAPP with the KKQN motif di-lysine ER retention motif. As determined previously for Rab1B, the 112-kDa form of Rab1B is used as the reporter in the assays of s-APPβo or s-APPαo (Fig. 3). Monoclonal antibody 266 (specific for residues 13–28 of Aβ) was used as the reporter in the assays of cells transfected with full-length APP (Fig. 3). Monoclonal antibody 266 (specific for residues 13–28 of Aβ) was used as the reporter in the studies where C99 was co-expressed with PS1 (Fig. 11). The 6H9 monoclonal antibody (see below) was used as the reporter in the assays of mediolucinal-norleucinal to the medium 3.5 h prior to harvest. Cells from four dishes were divided and fractionated into membrane components and nuclear extracts as described by Hua et al. (75). Aliquots of protein from the membrane fraction (100 µg) and nuclear extract (185 µg) were subjected to SDS-PAGE and immunoblot analysis using a monoclonal antibody to APP or polyclonal antibodies to the protein C99, which recognizes the full-length protein and the N-terminal fragment (71). The secondary antibody was Texas Red-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Where indicated, cells expressing C99 were incubated with a rabbit polyclonal anti-Myc antibody (Upstate Biotechnology, Inc., Lake Placid, NY) combined with a mouse monoclonal antibody H4B4 against the lysoosomal membrane protein LAMP-2 (73), obtained from the Developmental Studies Hybridoma Bank, University of Iowa. To visualize PS1, cells were incubated with an affinity-purified rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA). SwAPP with the normal C-terminal ending (QMQN) or a modified version of SwAPP under the control of the thymidine kinase promoter (74). Parallel 10-cm dishes of 293 cells were co-transfected with 2 µg of pT7-HSV-BP2 combined with either 2 µg of pCMV5Rab1B( wt) or pCMV5Rab1BN121I. Immediately after transfection, cultures were fed with DMEM containing 10% delipidated FBS and 25 µM lovastatin to promote sterol depletion and SREBP cleavage. Cells were collected 20 h after transfection, with addition of 25 µg/mL N-acetyl-leucinal-leucinal-norleucinal to the medium 3.5 h prior to harvest. Cells from four dishes were divided and fractionated into membrane components and nuclear extracts as described by Hua et al. (75). Aliquots of protein from the membrane fraction (100 µg) and nuclear extract (185 µg) were subjected to SDS-PAGE and immunoblot analysis using a monoclonal antibody to APP or polyclonal antibodies to the protein C99, which recognizes the full-length protein and the N-terminal fragment. Immunoblots were scanned and quantified with a Kodak 440CF Image Station.

**RESULTS**

**Addition of a Di-lysine Motif to SwAPP Prevents Golgi-dependent Maturation of the Protein**—Numerous studies have established that type I transmembrane proteins containing C-terminal KXXK motifs are retrieved from early Golgi and intermediate compartments and retained in the ER (76–78). To determine whether the addition of such a motif to the cytoplasmic tail of APP would prevent the protein from being transported beyond the cis-Golgi compartment, plasmids encoding SwAPP with the normal C-terminal ending (QMKN) or a modified sequence, KQKN, were transfected into 293 cells, and the transiently expressed proteins were monitored by pulse-chase analysis of the [35S]methionine-labeled protein. Radiolabeled APP was immunoprecipitated from parallel transfected 293 cell cultures, either immediately after a 15-min pulse with [35S]methionine or after a 45-min chase to allow time for nascent APP to undergo ER → Golgi transport and oligosaccharide maturation. Consistent with previous observations, SwAPP migrates as two major bands with the slower form (~130 kDa) representing the mature protein that has undergone O-glycosylation in the medial-late Golgi compartment and the faster form (~108 kDa) representing the immature form of the protein that is localized predominantly in the ER (76–78). As shown in Fig. 1A, the nascent 108-kDa form of SwAPP was not detected beyond the ER. This experiment indicates that the protein detected in the cultures immediately after the pulse. Conversion of immature SwAPP to the mature form was readily detected after the 45-min chase in the cells overexpressing SwAPP with the normal C-terminal sequence but not in the cells expressing the protein with the di-lysine ER retention motif. As determined previously for SwAPP (58), the 108-kDa form of SwAPP with the KQKN motif...
was sensitive to digestion with endoglycosidase H (not shown), indicating that it contains N-linked high mannose oligosaccharides typical of immature glycoproteins that have not yet been trimmed by α1,2-mannosidase II in the Golgi compartment (81). The ability of the KKQN motif to retain SwAPP in the ER was confirmed by SDS-PAGE and immunoblot analysis to monitor steady-state levels of immature versus mature intracellular APP, using the 8E5 monoclonal antibody, which recognizes all forms of APP.

**Addition of a Di-lysine ER retention motif prevents Golgi-dependent maturation of SwAPP in 293 cells.** A, cells were transiently transfected with vectors encoding SwAPP or the same protein in which the last amino acids were altered to form a KKQN motif. Parallel cultures that were not transfected (lanes marked None) were used to determine the background levels of endogenous APP expressed in the 293 cells. Pulse-chase analysis of APP processing was performed 24 h later. Cells were harvested immediately after pulse-labeling with [35S]methionine (0 min) or after the chase with unlabeled methionine (45 min) as indicated. APP was immunoprecipitated and subjected to SDS-PAGE and fluorography. The positions of the mature O-glycosylated form of APP (m) and the immature protein (i) are indicated by the arrows. B, immunoblot analyses were performed on aliquots of the cell lysates from the cultures harvested at the end of the chase period to determine the steady-state levels of immature and mature intracellular APP, using the 8E5 monoclonal antibody, which recognizes all forms of APP.

**Addition of a Di-lysine ER retention motif inhibits the secretion of soluble exodomain fragments (s-APP) released by α-secretase or β-secretase.** HEK293 cells were transfected with vectors encoding SwAPP with the normal C-terminal ending (SwAPP) or the di-lysine ER retention motif (SwAPP-KKQN). Parallel cultures that were not transfected (None) were used to assess background levels of s-APP in the medium. The medium was removed from all cultures 24 h after transfection. Immunoblot assays were performed on equal aliquots of medium using the following: A, monoclonal antibody 8E5, which detects both s-APPα and s-APPβ (s-APPtotal); B, polyclonal antibody SW192, which detects only s-APPβ derived from the Swedish variant of APP; or C, monoclonal antibody 2H3, which detects s-APPα. The results (mean ± S.E.) from separate determinations on three cultures are shown in the bar graphs next to the representative blots. Intracellular levels of expressed SwAPP and SwAPP(KKQN) were very similar, so that essentially identical results were obtained when the extracellular s-APP values were normalized to the intracellular levels of SwAPP (not shown).

**Decreased Formation of Amyloid β-Peptide**

Because the preceding findings indicated that SwAPP-(KKQN) was not efficiently cleaved by β-secretase, we postulated that the amount of Aβ produced by cells expressing this construct would be significantly diminished. This hypothesis was tested by measuring the concentrations of Aβ released into the culture medium using ELISAs specific for long and short forms of the peptide. As shown in Fig. 3, addition of the KKQN motif to SwAPP caused a 75–80% decrease in the amount of Aβ42 released into the medium. The amount of Aβ40 was similarly reduced, so that percentage of Aβ42 relative to Aβ40 (−5%) was not significantly altered.
Retention of the APP C99 Domain in the ER Decreases the Production of Aβ—The foregoing studies indicate that the biogenesis of Aβ from full-length SwAPP is greatly diminished when the latter is retained in the ER. The decline in Aβ seen in these studies is probably due to the reduced contact of the SwAPP substrate with active β-secretase, based on the parallel reduction in the output of s-APPβ (Fig. 2B). The small amount of residual Aβ produced in the cells overexpressing SwAPP-(KKQN) could be related to incomplete retention of the protein in the ER, possibly due to saturation of cotransfer binding sites (82). Alternatively, it could reflect the presence of γ-secretase activity in the ER, possibly allowing a small pool of SwAPP to be transported to the β-secretase compartment after removal of the C-terminal tail containing the KKQN motif. In light of these uncertainties, we felt it was important to obtain a more direct assessment of the activity of γ-secretase in the ER. Toward this end, we generated a C99(KKQN) construct that corresponds to the C-terminal portion of APP that remains after β-secretase cleavage. To facilitate detection, we added a short Myc epitope tag to the C terminus, followed by the ER retention motif, KKQN (Fig. 4A). To verify that the C-terminal additions did not in themselves affect γ-secretase cleavage, an identical construct was generated with QLQN instead of KKQN following the Myc tag, i.e. C99(QLQN). Immunoblot analyses using different antibodies to detect epitopes on the N-terminal or C-terminal ends of the molecule indicated that both C99(QLQN) and C99(KKQN) were transiently expressed in an intact form at similar levels in 293 cells (Fig. 4B). However, the cells expressing the construct with the di-lysine ER retention motif showed a striking decrease in the amount of Aβ deposited into the culture medium (Fig. 4C). To verify that the decrease in extracellular Aβ reflects a decrease in the biogenesis of the peptide rather than a block in its secretion, we also measured intracellular Aβ in cells expressing the two C99 constructs (Fig. 4C). Consistent with the absence of Aβ in the medium, intracellular Aβ was nearly undetectable in cells expressing C99(KKQN).
To confirm that these observations were pertinent to human neurons as well as 293 cells, the C99(QLQN) and C99(KKQN) constructs were subcloned into Sindbis virus vectors and expressed in stable differentiated NT2N neurons. As shown in Fig. 4, D and E, radiolabeled Aβ was clearly detected above background levels in medium from NT2N cultures expressing C99(QLQN) but not in cultures expressing similar amounts of C99(KKQN).

**Subcellular Localization of C99** —When 293 cell lysates were subjected to differential centrifugation, the expressed C99(QLQN) and C99(KKQN) polypeptides were found predominantly in membrane-enriched particulate fractions (not shown). However, because C99 lacks the glycosylation sites of the APP exodomain, it was not possible to verify retention of C99(KKQN) in the ER by monitoring Golgi-dependent post-translational modifications of the protein. As an alternative, we compared the subcellular distribution of C99(QLQN) and C99(KKQN) with the distribution of an ER marker protein, calreticulin (83), by immunofluorescence analysis. As shown in Fig. 5A, C99(QLQN) was concentrated in a discrete region adjacent to the nucleus, with almost no overlap with calreticulin. In contrast, C99(KKQN) was localized in a perinuclear ring and a diffuse reticular network throughout the cytoplasm. The staining pattern of C99(KKQN) was nearly identical to that of the calreticulin marker (Fig. 5A). In the absence of the ER retention signal, most of the overexpressed C99(QLQN) was co-localized with Rab6 GTPase, which is known to function within the medial and trans-Golgi compartments (84, 85) (Fig. 5B). Consistent with its retention in the ER, C99(KKQN) showed little or no co-localization with Rab6 (Fig. 5B). It should be noted that in the merged image of C99(QLQN) with Rab6 (Fig. 5B), a small portion of the C99 did not overlap with the Golgi marker. This non-Golgi pool of C99 may be localized in the endocytic compartment, based on partial overlap of C99(QLQN) with LAMP-2, a marker for late endosomes and lysosomes (73) (Fig. 5C).

**Subcellular Localization of C99 in Relation to PS1** —Because PS1 has been proposed as a possible γ-secretase or a critical interacting protein controlling γ-secretase activity, we decided to compare the subcellular distribution of C99(QLQN) and C99(KKQN) with the distribution of PS1. Previous studies have established that endogenous PS1 is localized predominantly in the perinuclear region and the ER, with very little protein localized in post-Golgi compartments (50, 86). The staining around the nuclear envelope has been attributed to the full-length protein, whereas the protein in the ER appears to arise from the N- and C-terminal fragments (50). In Fig. 6A we examined the distribution of overexpressed PS1, using a previously characterized antibody that recognizes the full-length protein and the N-terminal fragment. We observed both a perinuclear ring and a predominant ER staining pattern. The latter was verified in separate studies where there was good coincidence between PS1 and the ER markers, protein disulfide isomerase, and calnexin (not shown). Identical results were obtained when the localization studies were repeated with an antibody against the C-terminal loop domain of PS1 (not shown), consistent with the notion that the N-terminal and C-terminal polypeptides remain together after endoproteolytic cleavage of PS1 (51, 87). As expected, based on its demonstrated retention in the ER (Fig. 5), C99(KKQN) was localized almost entirely in compartments that contained PS1 (Fig. 6A). In contrast, the staining pattern for C99(QLQN), which was localized mainly in the Golgi and endosomal compartments, showed much less overlap with PS1 (Fig. 6B). Thus, the sharp decline in Aβ production that we observed when C99 was retained in the ER (Fig. 4) occurred despite an increased co-localization of C99(KKQN) with PS1.

**Retention of C99 in the ER Affects the Biogenesis of Both Aβ40 and Aβ42** —Because the immunoblot methods used in the preceding studies did not distinguish between long and short forms of Aβ, it was conceivable that retention of C99 in the ER selectively prevented the production of the more abundant Aβ40, while having little or no effect on the formation of smaller amounts of Aβ42. To examine this possibility, the comparative studies of C99(QLQN) and C99(KKQN) were repeated in 293 cells, using specific and highly sensitive ELISAs to quantify Aβ40 and Aβ42 (Fig. 7). The concentration of Aβ42 fell by ~98% in the cultures expressing C99 with the ER retention motif, in accord with the major loss of Aβ signal in the earlier immunoblot studies. In addition, these studies clearly revealed that the concentration of the less abundant Aβ42 underwent a parallel 96% decline in the same cultures. It should be noted that the

![Fig. 5. Immunofluorescence localization of C99 proteins with or without an ER retention motif.](image)
The reporter antibody used in these assays (6H9) is specific for the region of Aβ distal to the α-secretase cleavage site. Thus, the results indicate that retention of C99 in the ER caused a decline in all potential γ-secretase products, including any N-terminal “ragged” forms of Aβ that could arise from β-secretase cleavage at alternative sites (88), as well as any p3 peptides formed from γ-secretase cleavage of C83 fragments remaining after α-secretase cleavage of endogenous APP in the transfected cells.

**Co-expression of C99 with a Dominant-negative Rab1B GTPase Inhibits Aβ Production**—Although the preceding studies strongly suggested that reduced Aβ formation in cells expressing C99(KKQN) was due to retention of the γ-secretase substrate in the ER, we could not rule out the alternative possibility that the introduction of the two lysine residues at the C terminus of the polypeptide, and its consequent association with the COP-I coatomer complex, might somehow interfere with the recognition of C99 by γ-secretase. To address this issue, we used a different approach to retain C99 in the ER. This entailed co-expressing C99 with either wild-type Rab1B or a dominant-negative Rab1B mutant, i.e. Rab1B(N121I). Previous studies have established that the Rab1 GTPase functions as a molecular switch in the ER → Golgi transport pathway (89, 90). Introduction of the amino acid substitution, N121I, into Rab1B drastically reduces its affinity for GTP and renders the protein a dominant suppressor of protein trafficking between the ER and Golgi compartments (58, 91).

Immunofluorescence analysis confirmed that Rab1B(N121I) had the predicted effect on C99 localization (Fig. 8). That is when C99(QLQN) was co-expressed with Myc-tagged Rab1B(N121I), it assumed a diffuse reticular staining pattern similar to that previously observed for C99(KKQN) (see Fig. 5). On the other hand, when C99(QLQN) was co-expressed with wild-type Rab1B, it accumulated mainly in the juxtanuclear Golgi region (Fig. 8).

We next examined the effect of the dominant-negative Rab1B(N121I) on Aβ production in 293 cells. Because Rab mutants that fail to bind guanine nucleotides are unstable, they do not accumulate to the same extent as their wild-type counterparts when transiently expressed in cultured cells. This is evident in Fig. 9A, where the upper band, representing the Myc-tagged Rab1B(N121I), it assumed a diffuse reticular staining pattern similar to that previously observed for C99(KKQN) (see Fig. 5). On the other hand, when C99(QLQN) was co-expressed with wild-type Rab1B, it accumulated mainly in the juxtanuclear Golgi region (Fig. 8).

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expression completely blocks ER \to Golgi trafficking of the low density lipoprotein receptor (63) and APP (58). As shown in Fig. 9C, co-expression of C99(QLQN) with the Rab1B(N121I) caused a 90% reduction in extracellular Aβ, compared with cells expressing the same C99 construct with Rab1B(wt). As determined previously for cells expressing C99 with the di-lysine retention motif, the decline in extracellular Aβ caused by co-expression of C99(QLQN) with Rab1B(N121I) was matched by a similar reduction of Aβ in the cell lysates (not shown), indicating that it was not due to intracellular sequestration of the peptide. Furthermore, the reduction in Aβ seen in the cells expressing the dominant-negative Rab1B mutant could not be explained by an inhibitory effect of Rab1B(N121I) on overall expression of the C99(QLQN) substrate, which showed similar steady-state levels in cells expressing either the wt or N121I Rab1B constructs (Fig. 9B). In this regard, it might at first seem puzzling that intracellular levels of the C99 substrate did not increase noticeably in conjunction with the block in Aβ production caused by the Rab1B mutant. However, this was not entirely unexpected since, under conditions of continuous C99 overexpression, the fractional conversion of C99 to Aβ is actually much lower than is suggested by direct comparison of the blots shown in Fig. 9, B and C. The Aβ blots shown in Fig. 9C were subjected to a special high sensitivity detection method involving biotinylated IgG and streptavidin-horseradish peroxidase. Under the standard ECL conditions and exposure times used for C99 (Fig. 9B), Aβ was almost undetectable. It is also important to mention that other proteases besides γ-secretase probably contribute to the turnover of C99. For example, recent studies (92, 93) have documented the existence of calpain-like proteases that can degrade C99 in the ER. It is possible that these enzymes may assume a greater role in C99 degradation when the protein is prevented from exiting the ER in cells expressing Rab1B(N121I).

To verify that the reduced γ-secretase cleavage of C99 in the preceding studies was related specifically to the inability of cells expressing Rab1B(N121I) to transport the substrate protein from the ER to the Golgi compartment, we carried out several follow-up studies to rule out a global perturbation of membrane-associated proteases or general disruption of cell function.

First, we asked whether or not the dominant-negative Rab1B mutant would similarly impair the endoproteolytic cleavage of presenilin-1 (PS1). In contrast to the striking reduction of Aβ production (Fig. 9C), we observed no difference in the production of PS1 N-terminal fragments when PS1 was co-expressed with either Rab1B(wt) or Rab1B(N121I) in 293 cells (Fig. 9D).

Second, we examined the effect of the dominant-negative Rab1B(N121I) on the proteolytic cleavage of a membrane protein unrelated to APP, i.e. SREBP2. The latter is localized in the ER, where sterol depletion triggers the release of the N-terminal transcriptional regulatory domain through the action of two sequential site-specific endoproteases, the second of which cleaves within a transmembrane region (94). As shown in Fig. 9E, co-expression of SREBP2 with Rab1B(N121I) did not have a major effect on the release of the N-terminal domain, which is typically found in the nuclear compartment (75). Since the cleavage of SREBP2 depends on the sequential action of two endoproteases, as well as an interaction with a sterol-sensing activating protein (95), these results strongly suggest that the block of ER \to Golgi transport by the Rab1B mutant does not cause a global disruption of protein function in the ER.

Finally, to explore further the possibility of general cell damage, we performed an experiment to determine if the suppression of Aβ production by the dominant-negative Rab1B(N121I) was reversible. The cDNAs encoding Rab1B wt and N121I were subcloned into the pTRE vector, where gene expression is controlled by the Tet operator. Each of these constructs was then transfected together with pCMV-C99(QLQN) into a 293 cell line (Tet-Off®) where the stably expressed tetracycline-controlled transactivator complex (tTA) strongly represses transcription in the presence of doxycycline. When the transfected cells were maintained in the absence of doxycycline for 24 h, Rab1B(wt) and N121I were transiently expressed at levels comparable to those previously seen with unregulated cytomegalovirus vectors (Fig. 10, A and B). Also, as expected, the dominant-negative mutant completely blocked Aβ production during this period (Fig. 10C). When expression of the Rab1B constructs was subsequently suppressed by addition of doxycycline to the medium, Myc-Rab1B(N121I) declined to an undetectable level within 24 h (Fig. 10B), whereas the more stable Myc-Rab1B(wt) declined more slowly (Fig. 10A). Of particular importance, Fig. 10C shows that, in parallel with the disappearance of Rab1B(N121I), the production of Aβ was restored to levels comparable to those detected in cultures expressing Myc-Rab1B(wt) or no exogenous Rab (control with doxycycline always present).

Effects of PS1 Mutations on Aβ Production from C99—Sev-
eral studies have shown that co-expression of APP with mutant forms of PS1 can cause an increase in cellular production of $A\beta_{42}$ versus $A\beta_{40}$ (38, 39, 64, 96). This implies that interactions between APP and presenilins play a critical role in determining the exact site where $\gamma$-secretase cuts the polypeptide chain at the C-terminal end of the $A\beta$ sequence. Although it is generally assumed that APP is cleaved by $\beta$-secretase after the N-terminal exodomain is removed by either $\alpha$-secretase or $\beta$-secretase, it remains unclear whether or not the exodomain of APP is involved in the initial protein interactions with $\gamma$-secretase/

![Image](80x465 to 267x730)

**FIG. 9.** Retention of $C99$($QLQN$) in the ER by co-expression with a dominant-negative Rab1B mutant inhibits $A\beta$ production but does not block the cleavage of $PS1$ or SREBP2. HEK293 cells were co-transfected with vectors encoding $C99$($QLQN$) and either Rab1B(wt) or Rab1B(N121I). A and B, expression of the recombinant proteins was verified by SDS-PAGE and immunoblot analysis of equal aliquots of cell lysate using polyclonal antibody against Rab1B (A) or 6E10 antibody to detect C99 (B). A, the recombinant Rab1B is distinguished from the endogenous protein by its slightly slower mobility, which is due to the presence of an N-terminal Myc epitope tag (arrows). C, equal aliquots of medium from the same cultures were subjected to electrophoresis and immunoblot analysis to detect $A\beta$ as described under "Experimental Procedures." The blots shown in the illustration are representative of determinations performed on three parallel cultures. The $A\beta$ values in the culture expressing Rab1B(N121I) were reduced to 8.5 ± 1.9 (S.E.) percent of the values in the cultures expressing Rab1B(wt). D shows an immunoblot performed with an antibody against the N terminus of PS1 in 293 cells where PS1 was co-expressed with either Rab1B(wt) or Rab1B(N121I), as indicated. The full-length PS1 (FL) and N-terminal fragments (NTF) are indicated to the right of the panel. Material above 50 kDa represents PS1 aggregates typically seen on SDS gels. The lane on the right contains lysate from 293 cells transfected only with the Rab1B(N121I) construct. Expression levels of the wild-type and N121I Rab1B constructs (not shown) were similar to those in A. E shows immunoblots of membrane and nuclear fractions from sterol-deprived 293 cells co-expressing HSV-tagged SREBP2 with either Rab1B(wt) or Rab1B(N121I). Relative expression levels of the wild-type and N121I Rab1B constructs (not shown) were similar to those in A. Full-length SREBP2 and the N-terminal fragment (NTF) detected by the anti-HSV antibody are indicated by the arrows. Other bands detected by the anti-HSV antibody are nonspecific, as indicated by their presence in non-transfected 293 cells (control). Scanning of the blots indicated that ratio of nuclear NTF to full-length SREBP in the cells expressing Rab1B(N121I) was ~62% of the ratio determined in the cells expressing Rab1B(wt). Similar results were obtained in two separate experiments.

![Image](335x372 to 528x730)

**FIG. 10.** Effects of the dominant-negative Rab1B(N121I) on $A\beta$ production are reversible. Parallel cultures HEK293 Tet-off cells were co-transfected with pCMV3-C99($QLQN$) and either pTRE-Rab1B(wt) (A) or pTRE-Rab1B(N121I) (B). All cultures were grown without doxycycline (~dox) for the initial 24 h after transfection to allow expression of the Rab1B proteins. At this point one set of cultures was harvested for immunoblot analysis to determine the expression levels of Myc-Rab1B relative to endogenous Rab1B. The remaining cultures were changed to medium containing 1 μg/ml doxycycline (+dox) and harvested for immunoblot analysis at the indicated times (times are total hours after transfection). C, equal aliquots of medium (conditioned for 24 h) were removed from the cultures analyzed in A and B and subjected to immunoblot analysis. $A\beta$ was detected as described under "Experimental Procedures," and the results were expressed as percent of the $A\beta$ values determined for matched control cultures where doxycycline was added prior to transfection to suppress the expression of the Rab1B constructs throughout the entire 72-h post-transfection period.

PS1. By using our $C99$ construct, we have been able to explore this issue by co-expressing $C99$ with wild-type or mutant forms of PS1 in 293 cells and then determining the concentrations of $A\beta_{42}$ and $A\beta_{40}$ in the conditioned culture medium. Immunoblot analysis of PS1 in the transfected cells indicated that the wild-type and mutant forms of the protein were very similar with respect to overall expression and the extent of endoproteolytic cleavage (Fig. 11A). Quantification of long and short forms of $A\beta$ in these cells revealed a striking 90% increase in the ratio of $A\beta_{42}$ to $A\beta_{40}$ when $C99$ was co-expressed with either PS1(M146L) or PS1(L286V), compared with cells co-expressing $C99$ with $PS1$wt (Fig. 11B). This increase was comparable to that previously observed when the same PS1 mutants were co-expressed with full-length SwAPP (64). Based on
FIG. 11. Mutant forms of PS1 increase the production of $\beta_42$ versus $\beta_40$ from C99. HEK 293 cells were co-transfected with the vector encoding C99(QLQN) combined with vectors encoding either PS1(wt), PS1(M146L), or PS1(L286V) as indicated. Cells and medium were collected 24 h after transfection. A, immunoblot assays were performed on aliquots of the cell lysates to confirm that comparable levels of C99 and PS1 expression were obtained in all of the cultures. The major PS1 bands at $\sim 23$ and 45 kDa represent the N-terminal fragment and the full-length protein, respectively. Endogenous PS1 was not detectable at the exposure times used for these blots (not shown). The blots shown in the illustration are representative of determinations performed on three parallel cultures. B, equal aliquots of medium from the same cultures were subjected to ELISA to quantify $\beta_42$ and $\beta_40$, as described under “Experimental Procedures.” Each bar shows the mean ± S.E. of separate determinations from three parallel cultures.

this finding, it appears that the exodomain of APP is not required for the elevation of the $\beta_42/\beta_40$ ratio by the PS1 mutants.

DISCUSSION

$\gamma$-Secretase catalyzes the terminal step in the proteolytic processing of APP, leading to the release of $\beta$. The subcellular localization of $\gamma$-secretase remains uncertain, although a few reports have implied that this activity may exist in early compartments of the secretory pathway (46–48). The first part of this study was designed to test the hypothesis that one or more $\gamma$-secretase activities reside in the ER. We began by adding a di-lysine ER retention motif to SwAPP. Retention of this protein in the ER was clearly demonstrated by 1) the impairment of Golgi-dependent post-translational modifications and 2) reduction of exodomain products normally released after SwAPP is cut by $\alpha$-secretase or $\beta$-secretase in medial or late compartments of the secretory pathway. These results confirm previous reports (26, 28, 50, 97) indicating that most of the $\beta$-secretase activity resides in subcellular compartments distal to the ER. Moreover, they suggest that the recently described maturation and cleavage of the $\beta$-secretase pro-peptide after it leaves the ER (98) may be required for activation of the enzyme.

In the cells expressing SwAPP(KKQN), the amounts of both $\beta_42$ and $\beta_40$ released into the medium were markedly reduced. Because the full-length SwAPP(KKQN) construct was unable to progress beyond the first step in the amyloidogenic pathway (i.e. the translocation of APP to sites containing active $\beta$-secretase), its utility for assessing $\gamma$-secretase localization was limited. To circumvent this problem and obtain a direct measure of $\gamma$-secretase activity, we generated a C99 construct, C99(QLQN) which represents the C-terminal stump of APP that remains after $\beta$-secretase cleavage, with a C-terminal Myc epitope and a tetrapeptide extension similar to the normal APP C-terminal sequence, QMQN. Our initial studies showed that this construct was a good physiological substrate for $\gamma$-secretase, giving rise to abundant amounts of $\beta_42$ and $\beta_40$ that could be easily detected with several different antibodies directed against epitopes at the N terminus and C terminus of the peptide. When the di-lysine motif, KKQN, was added to C99, retention of the protein in the ER was indicated by a marked change in its immunofluorescence localization from a predominant Golgi-like pattern to one that closely matched the ER marker, calcereulin.

Measurements of $\beta$ in the medium from cells expressing C99(KKQN) revealed a near-complete block in deposition of both $\beta_40$ and $\beta_42$ compared with cells expressing C99(QLQN). If the decline in extracellular $\beta$ due to block in peptide secretion, we would have expected to see an increase in the intracellular $\beta$ pool accompanied the decreased peptide output. Instead, we observed a corresponding decrease in intracellular $\beta$, strongly suggesting that the ER retention signal was preventing biogenesis of the peptide. To confirm that the reduced $\beta$ production was specifically related to retention of C99 in the ER, rather than to the di-lysine motif interfering with $\gamma$-secretase substrate interactions, we retained C99 in the ER without the di-lysine signal by co-expressing it with a dominant-negative Rab1B mutant that blocks ER $\rightarrow$ Golgi trafficking (58, 63). These studies showed that C99(QLQN), which normally gave rise to substantial amounts of $\beta$ when expressed in 293 cells, was unable to generate $\beta$ when its transport out of the ER was blocked. The latter effect was readily reversed when expression of the Rab1B mutant was suppressed, and the functions of other ER endoproteases operating on PS1 and SREBP2 were not disrupted. Taken together, these observations lead us to conclude that the ER is not a major site for $\gamma$-secretase processing of the APP C99 fragment in the well-characterized 293 cell model. Our results contrast with those of Soriano et al. (48), who found no reduction of $\beta_42$ or $\beta_40$ secretion in Chinese hamster ovary cells expressing an APP/CD3$\gamma$ chimera that was retained in the ER. At present we cannot offer a simple explanation for these conflicting results. One possibility is that major cell type-specific differences exist in the distribution of $\gamma$-secretase. However, the marked reduction of $\beta$ that we observed when C99(KKQN) was expressed in NT2N neurons indicates that these cells are similar to 293 cells insofar as most of the $\gamma$-secretase activity appears to exist outside of the ER.

It has been proposed that $\beta_40$ and $\beta_42$ are generated by different $\gamma$-secretases (16, 17), with the enzyme responsible for producing the long form of $\beta$ residing in the ER and the enzyme producing $\beta_42$ localized mainly in the endosomes or other peripheral compartments (52). Although the present study does not directly rule out the existence of multiple $\gamma$-secretases that cut C99 at different sites, the parallel reductions in the amounts of $\beta_40$ and $\beta_42$ produced by 293 cells expressing C99(KKQN) argues against the idea that a different $\gamma$-secretase responsible for generating $\beta_42$ versus $\beta_40$ is selectively compartmentalized in the ER. It remains to be determined precisely where, beyond the ER, the final step in $\beta$ biogenesis actually occurs and how the peptide is released from the cell. Under normal circumstances it is difficult to detect substantial amounts of C99 or C83 in cultured cells, suggesting that these intermediate fragments are rapidly cleaved by $\gamma$-secretase. However, in cells transfected with the C99 plasmids, overexpression of the substrate appears to saturate the endogenous $\gamma$-secretase processing pathway, with consequent
accumulation of a large C99 pool that can be readily detected by immunoblot analysis. The presence of this material in the Golgi apparatus, and to a lesser extent in the endosomes, could indicate that these are normal sites of γ-secretase processing. This would be consistent with numerous studies pointing to the invovlement of acidic compartments in Aβ biogenesis (6, 30, 31, 99).

The apparent absence of substantial γ-secretase activity in the ER has important implications for understanding the relationship between presenilins and APP processing. Mutations in PS1 (100) and PS2 (101) have been linked to early-onset familial forms of Alzheimer’s disease, with the majority of the known mutations occurring in PS1 (102, 103). Several of the amino acid substitutions in the presenilins have been shown to cause alterations in the amyloidogenic processing of APP, such that cells produce an increased amount of Aβ42 relative to Aβ40 (36–39, 104). The molecular mechanism underlying this effect remains controversial. Presenilins are serpentine polyptides with multiple hydrophobic membrane-spanning segments and a large cytoplasmic loop (105, 106), and there is some evidence for a direct physical interaction between PS1 and APP, based on co-immunoprecipitation studies (107). Both endogenous and overexpressed presenilins are localized predominantly in the perinuclear region and the ER (49–51, 108, 109). Nascent presenilins undergo endoproteolytic cleavage in vivo to produce stable N-terminal and C-terminal derivatives (87, 110, 111). This “presenilinase” activity is completely abolished by substitutions of two conserved aspartate residues that lie in the transmembrane domains flanking the cleavage site (40, 41). Interestingly, the same studies show that the aspartate substitutions in presenilin also reduce γ-secretase activity in cell lysates, raising the prospect that presenilins are autoactivated aspartyl proteases that function as γ-secretases. Further support for this hypothesis comes from recent studies in which photoactivated γ-secretase inhibitors have been shown to bind directly to the cleaved forms of PS1 and PS2 (42, 43). If PS1 and γ-secretase are truly the same molecule, then one might expect to observe an increase in Aβ production in cells where the C99 substrate is retained in the ER where most of the PS1 resides, compared with cells where C99 is exported to other compartments. In fact, our studies show the exact opposite, with Aβ42 relative to Aβ40 when expressed with only the C99 domain of APP. Specifically, the increase in Aβ42/Aβ40 seen when C99 was co-expressed with PS1 L286V or M146I was similar in magnitude to that previously reported when the same mutants were expressed with full-lengthSwAPP (64). This demonstrates that the exodomain of APP is not required for the relevant PS1 interactions. When combined with earlier observations indicating that the association of PS1 with APP can occur in the absence of the APP cytoplasmic tail (107), our results suggest that the critical domain for interaction with PS1 may be confined to a narrow region of APP that lies between the β-secretase cleavage site and the end of the transmembrane domain. This conclusion is consistent with the mutagenesis studies of Lichtenthaler et al. (114), which indicate that the cleavage specificity of γ-secretase (resulting in Aβ42 or Aβ40) is determined largely by the eight amino acid residues immediately downstream of the cleavage site within the transmembrane region of C99. Thus, it is likely that modified C99 constructs will be particularly useful in future studies aimed at defining the specific structural domains involved in the physical interactions between PS1 and the transmembrane region of APP.

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Decreased Formation of Amyloid β-Peptide
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