ELISA analysis of β-secretase cleavage of the Swedish amyloid precursor protein in the secretory and endocytic pathways

Michelle L. Steinhilb,* R. Scott Turner† and James R. Gaut‡

Institute of Gerontology and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, USA

†*Department of Neurology, University of Michigan Medical Center, Ann Arbor, Michigan, USA*

‡*Veterans Affairs Medical Center Geriatric Research, Education, and Clinical Center, Ann Arbor, Michigan, USA*

Abstract

Limiting beta amyloid (Aβ) production could become an important therapeutic target in Alzheimer's disease (AD). Aβ is derived by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases. A double missense mutation in APP found in a Swedish pedigree (APPsw) elevates Aβ40 and Aβ42 production. Aβ production and, therefore, β-secretase cleavage of APPsw reportedly occur in the endoplasmic reticulum (ER), Golgi and endocytic compartments. However, the relative contribution of β-secretase cleavage occurring in each compartment has not been determined. Experiments described here use a novel ELISA to measure the β-cleaved product, APPswβ. Using this ELISA, we provide new information regarding the relative amount of β-secretase cleavage of APPsw that occurs in secretory and endocytic pathways. Using a dilysine retrieval motif to retain APPsw in the ER, we discovered that less than 15% of the β-secretase cleavage was still detected. Experiments utilizing endocytosis-impaired mutants of APPsw revealed that little or no β-secretase cleavage of APPsw appears to take place through endocytosis. Surprisingly, deletion of the entire cytoplasmic tail increased both APPswβ and Aβ secretion, suggesting that protein interactions with this region normally impede β-secretase cleavage. These results suggest that APPsw is cleaved by β-secretase late in the secretory pathway.

Keywords: Alzheimer's disease, BACE, β-secretase, endoplasmic reticulum.

J. Neurochem. (2002) **80**, 1019–1028.

Amyloid precursor protein (APP) is processed into Aβ40 and Aβ42, by β- and γ-secretases, which, respectively, cleave within the extracellular and the transmembrane regions of this type I integral membrane protein (for review see Selkoe 1998). Alzheimer's disease (AD) is hypothesized to result from the aggregation and deposition of these peptides into amyloid plaques in brain. A double missense mutation in APP identified in a Swedish pedigree with early onset familial Alzheimer's disease (FAD) (APPsw; K651N/M652L) enhances cleavage by β-secretase. This, in turn, increases both Aβ40 and Aβ42 secretion (Citron *et al*. 1994; Haass *et al*. 1995).

To develop strategies for therapeutic treatment of AD, a thorough understanding of all the molecular mechanisms capable of generating amyloid peptides is required. APP bearing the Swedish mutation is an important biochemical tool since it has been established that expression of APPsw in non-neuronal cells more closely mimics processing of APPwt by neurons (Forman *et al*. 1997). A complete understanding of amyloid generation will ensure that a

treatment is also capable of blocking alternate secondary pathways that may become major sites of Aβ generation in neurons if only the primary site is inhibited. Such mechanisms include determining the cellular location(s) of β- and

1019

γ-secretase cleavage of APPsw. However, determining the cellular site(s) of Aβ peptide derivation is more complex than originally thought. Production of Aβ in Chinese hamster ovary (CHO) cells involves internalization of APP from the cell surface (Koo and Squazzo 1994). Furthermore, Aβ secretion is severely decreased in non-neuronal cells expressing APP constructs possessing cytoplasmic tail mutations that impair endocytosis (Perez *et al.* 1999). APPsw processing is different from APP in that blocking endocytosis of the Swedish mutant by deleting the cytoplasmic internalization signal still permits production of Aβ peptide (Citron *et al.* 1995). This suggests that amyloid peptides could be generated from APPsw within the secretory pathway. However, other experiments revealed that Aβ peptide could also be derived through processing of APPsw endocytosed from the cell surface in addition to the secretory pathway (Perez *et al.* 1996). Prior to the studies presented here, it was unclear how much the secretory and endocytic pathways contribute to Aβ peptide production from APPsw.

The specific locations of intracellular amyloid peptide generation within the secretory pathway may be distinctly different for Aβ40 and Aβ42. Treating HEK 293 cells with brefeldin A (BFA) to accumulate APP within the endoplasmic reticulum (ER) results in increased generation of intracellular Aβ peptides that terminated at amino acid 42, but not at 40 (Wild-Bode *et al.* 1997). Similarly, treating human NT2 neurons with BFA eliminated production of intracellular Aβ40 but not Aβ42 (Cook *et al.* 1997). Indeed, evidence of β-secretase cleavage in the ER has been described in NT2 neurons (Chyung *et al.* 1997; Skovronsky *et al.* 1998), hippocampal neurons (Hartmann *et al.* 1997; Annaert *et al.* 1999) and fibroblasts isolated from PS-1 knockout mice (Xia *et al.* 1998). Recently, an enzyme with all the characteristics of β-secretase has been identified and appears to localize to the late Golgi and endosomes (Hussain *et al.* 1999; Vassar *et al.* 1999; Yan *et al.* 1999; Haniu *et al.* 2000). Thus, evidence of β-secretase activity has been reported within the endocytic pathway as well as within the ER, the Golgi and *trans* Golgi network of the secretory pathway in neuronal and non-neuronal cells. Determining the relative amount of β-secretase cleavage of APPsw occurring within each of these subcellular compartments would enhance our understanding of the mechanisms of Aβ peptide generation.

We developed an ELISA capable of measuring APPswβ, a direct product of β-secretase cleavage of APPsw (Steinhilb *et al.* 2001). In experiments described here utilizing the APPswβ ELISA in conjunction with endocytosis-impaired APPsw mutants, we measured the relative contribution of β-secretase cleavage occurring in both the secretory and endocytic pathways. We also determined the relative amount of β-secretase activity occurring within the early secretory compartments by restricting movement of APPsw out of the ER and *cis* Golgi. Finally, we report the surprising finding

that deletion of the cytoplasmic tail of APPsw significantly increased cleavage by β-secretase. This suggests that cytoplasmic proteins that interact with the carboxyl terminal tail of APPsw affect its vulnerability to cleavage by β-secretase.

Materials and methods

Cell lines, expression systems, and antibodies
Human endothelial kidney 293 (HEK293), Chinese hamster ovary K1 (CHOK1), and mouse neuroblastoma (N2a) cells (American Type Culture Collection, Rockville, MD, USA) were used for transient transfections. All cell lines were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine, non-essential amino acids (Life Technologies, Inc., Rockville, D, USA) and penicillin/streptomycin/fungizone (BioWhittaker, Inc., Walkersville, MD, USA) as described elsewhere (Yang *et al.* 1998). For transfection experiments, cells were seeded 1 day prior to use at 1×10^6 cells/6-cm dish and transfected with LipofectAMINE (Life Technologies, Inc.) reagent as described by the manufacturer. APPsw containing a carboxyl-terminal double lysine motif (KK) was engineered with two single-base changes using PCR site-directed mutagenesis resulting in a QM to KK modification at amino acids 747 and 748, respectively (Q747K/M748K; APP-751 isoform numbering) similar to Chyung *et al.* (1997). The Swedish mutation (K651M/N652L) was introduced into the APP YENP mutants (kindly provided by Dr R.G. Perez, University of Pittsburgh) and all mutants were cloned into the pCDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA, USA). All resulting cDNAs were sequenced to verify that only the correct mutational changes were made. The monoclonal antibody 22C11 was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA; Anti-Alzheimer Precursor Protein A4) and recognizes amino acids 60–100 of the amino terminus. 8E5 is a mouse monoclonal antibody raised to amino acids 444–592 of human APP that does not react with mouse APP and was a generous gift from Dr D. Schenk (Elan Pharmaceuticals).

Pulse-chase analysis, immunoprecipitation, electrophoresis, and western blotting
Forty-four hours after transient transfection, cells were washed in Dulbecco's phosphate-buffered saline (PBS; Life Technologies, Inc.) and incubated in cysteine-free/methionine-free DMEM (Life Technologies, Inc.) for 30 min. One 6-cm plate containing $\sim 2 \times 10^6$ cells was used per sample. Cells were labeled with [^{35}S]methionine/[^{35}S]cysteine (50 μCi/mL; ICN Pharmaceuticals, East Hill, NY, USA) for 60 min. Labeling was terminated by washing the cells with PBS and replacing the medium with DMEM containing unlabeled methionine and cysteine for the appropriate chase time (0–12 h), followed by lysis of cells. Cell lysates were prepared as previously described (Yang *et al.* 1998). Total protein in lysates was quantified (BCA Protein Assay, Pierce Chemicals, Rockford, IL, USA). Specific radiolabeled proteins were immunoprecipitated, separated on an 8% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) and detected by fluorography. Western blot analysis was conducted with the monoclonal antibody, 22C11, as previously described (Yang *et al.* 1998).

ELISAs

Unless otherwise noted, conditioned medium was collected from transiently transfected cells in the 16 h between 24- and 40-h post-transfection. The ELISA is characterized elsewhere (Steinhilb *et al.* 2001) but, briefly, this assay employs a capture antibody (α931) that recognizes the neo-epitope of the N-terminal fragment (NTF) of APPsw that is generated upon β-secretase cleavage. The α931 antibody does not cross-react with the wild-type sequence, nor does it recognize APPsw in the context of the full-length protein (Steinhilb *et al.* 2001). Following capture, 8E5, a monoclonal antibody specific for primate APP, is used to detect β-cleaved APPsw. A horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody is used to develop the ELISA as described (Steinhilb *et al.* 2001). A similar ELISA was adapted to measure full-length APP (and APPsw) by replacing the α931 antibody with another anticarboxyl terminal, α945 (Steinhilb *et al.* 2001). Samples for both the #931 and #945 ELISA were collected within the linear range of each assay (25 μL conditioned media, 10 μg total lysate). ELISA samples were measured in triplicate wells and each experiment was conducted at least three times except where noted. The Aβ40 ELISA was conducted as described (Yang *et al.* 1998).

Results

Confining APP to early secretory compartments extends the half-life of the immature incompletely glycosylated form. We sought to determine more accurately the relative amounts of β-secretase cleavage of APPsw occurring in the early and late secretory pathway as well as in the endocytic pathway. We sought to measure β-secretase cleavage in the ER by introducing an ER retrieval motif directly into APP. The introduction of a dilysine sequence to the cytoplasmic region of a type I transmembrane protein causes its net retention in the ER (Jackson *et al.* 1993; Pond *et al.* 1995). Consequently, the dilysine motif was introduced at the same site in APP as described by Chyung *et al.* (1997) using PCR site-directed mutagenesis.

If introduction of an ER retrieval signal to APP effectively retains APP in the ER, then this should specifically prolong the half-life of the immature, N-linked glycosylated form of APP. To test this prediction, transiently transfected (Tf) HEK293 cells were pulse-labeled with [^{35}S]-methionine/cysteine for 1 h and chased over a period of 12 h. A 1-h pulse was found to be optimal for labeling and monitoring the metabolism of both immature and mature APP. Radiolabeled APP that had been immunoprecipitated (IP) from lysates was resolved by SDS–PAGE. After chasing for just 1 h, the amount of both mature (M) and immature (I) APP was greatly decreased for cells transfected with APP alone (Fig. 1a). This result demonstrates that transiently expressed APP has a half-life of less than 1 h that is similar to previous studies (Weidemann *et al.* 1989).

Experiments were also performed to determine if retention in the ER by addition of a dilysine retrieval motif to APP (APPKK) (see Fig. 6) was sufficient to extend the half-life of immature, N-linked glycosylated APP. As with APP alone,
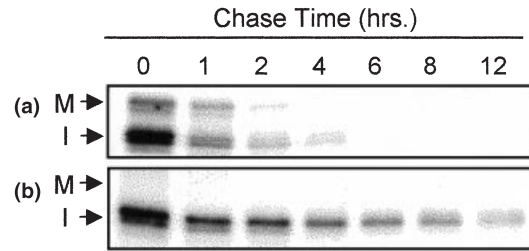
Fig. 1 Pulse-chase analysis indicates that ER retention is sufficient to extend the half-life of immature APP. HEK293 cells were pulse-labeled with [^{35}S]-methionine/cysteine for 1 h and chased in excess unlabeled methionine and cysteine. Radiolabeled lysates were immunoprecipitated (IP) with either Karen (A, B and D) or αBiP antibody (C) and resolved by SDS–PAGE. (a) Transient transfection with APP: immature (I) and mature (M) APP are indicated. (b) Expression of the dilysine retrieval motif (APPKK) results in production of immature APP but not the mature form, consistent with retention of APPKK in the ER.

HEK293 cells were transiently transfected with APPKK and subjected to pulse-chase analysis (Fig. 1d). After a 1-h label, immature APP was the predominant form observed, which is consistent with the retention of APPKK in the ER of these cells. This illustrates the efficiency of the dilysine motif in APPKK to retain it in the ER. Compared to APP (Fig. 1a), the half-life of immature APPKK (Fig. 1d) was greatly extended. The immature form of APPKK contains N-linked carbohydrates and is localized to the endoplasmic reticulum as indicated by glycosidase treatment and differential centrifugation (M. L. Steinhilb and J. R. Gaut, unpublished data). Thus, these results demonstrate that introducing a dilysine retrieval motif into the cytoplasmic region is sufficient to retain immature APPKK in the ER.

The Swedish form of APP (APPsw) enhances cleavage by β-secretase, causing an increase in both Aβ40 and Aβ42 relative to APP (Citron *et al.* 1994). To further characterize the effect of ER retrieval on maturation and processing of APP, the dilysine motif was also added to Swedish APP (APPswKK) and compared to wild-type APP and APPKK. HEK293 (Figs 2a,b) and N2a (Figs 2c,d) cells were transiently transfected with APP, APPsw, APPKK, or APPswKK and labeled with [^{35}S]-methionine/cysteine for 1 h. Full-length molecules were immunoprecipitated from the resulting cell lysates and β- or α-secretase cleaved fragments were immunoprecipitated from conditioned media with the amino terminal-specific Karen antibody. In cells transfected with APPKK or APPswKK there is only a trace of the mature form (Fig. 2a and c, lanes 2 and 4) compared to APP and APPsw (Fig. 2a and c, lanes 1 and 3, respectively). This indicates that like APPKK, APPswKK is efficiently retained in the ER/IC of both HEK293 and the mouse neuroblastoma cell line, N2a. Because Karen antibody recognizes human APP, the small amount of mature fully glycosylated protein recovered in HEK293 transfections (Fig. 2a) with APPKK
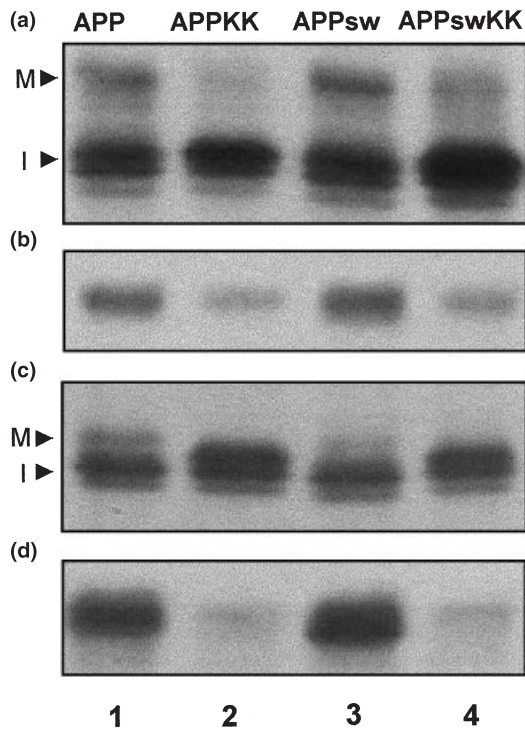
Fig. 2 Addition of a dilysine retrieval sequence to both the wild-type and the Swedish form of APP blocks maturation and secretion of cleaved fragments. HEK293 (a,b) and N2a (c,d) cells were transiently transfected with pCDAPP, pCDAPPKK, pCDAPPsw, or pCDAPPswKK. Cells were labeled with [^{35}S]-methionine/cysteine for 1 h and cell lysates (a,c) were immunoprecipitated with Karen antibody. Both the α- and β-secretase cleaved soluble fragments were immunoprecipitated from conditioned media (b,d) by Karen antibody and resolved as a single diffuse band. The mature (M) and immature (I) full-length forms immuno-isolated from HEK293 and N2a cell lysates are indicated in (a) and (c), respectively.

and APPswKK may be endogenous APP. Restriction to the ER/IC using the dilysine retrieval signal also impeded the secretion of the soluble APP and APPsw fragments into conditioned media in HEK293 and N2a cells (Fig. 2b and d, respectively). Similar results were also obtained in CHOK1 cells (data not shown). We conclude that maturation is impaired when a dilysine retrieval motif is added to either APP or APPsw. In turn, this block in maturation decreased, but did not eliminate the subsequent secretion of cleaved amino terminal fragments (NTFs) due to retention of full length APPswKK in the ER/IC. Furthermore, these data establish that in both neuronal and non-neuronal cell lines, Swedish APP and wild-type APP undergo similar processing and metabolic fates.

Restriction of APPsw to the ER/IC strongly inhibits β-secretase cleavage

The studies thus far have shown that addition of a dilysine retrieval signal can effectively confine APPsw to the ER/IC.

Furthermore, we found that retention in the ER impeded, but did not eliminate, secretion of NTFs. Therefore, experiments were next conducted to determine the proportion of β-secretase cleavage occurring when APPsw is retained in the ER. Our laboratory recently developed and optimized an ELISA to measure β-secretase cleavage of APPsw (Steinhilb *et al.* 2001 and Materials and methods). This previously characterized ELISA is highly sensitive and specific to β-secretase cleaved APPsw and not β-secretase cleaved endogenous APP (Steinhilb *et al.* 2001). In addition, it does not recognize α-secretase cleaved APPsw or endogenous APP. Thus, we used this assay to first determine how much β-secretase cleavage occurred when APPsw was retained in the ER.

If a small amount of β-secretase cleavage occurs in the ER/IC, then it should be detectable when APPsw is retained in this compartment by introducing a dilysine retrieval motif. HEK293 and CHOK1 cells were transiently transfected with empty vector, APPwt, APPsw, or APPswKK. Secreted APPswβ was measured in conditioned media using the #931 ELISA. In both CHOK1 and HEK293 cells transfected with APPwt a slight elevation of signal above empty vector was measurable (Fig. 3a). Thus, this constituted the background for the ELISA in subsequent analysis. Compared to APPsw, β-secretase cleavage of APPswKK was significantly decreased ($p < 0.001$) in both HEK293 and CHOK1 cells. However, a small amount of β-secretase cleaved APPswKK was reproducibly detected in the conditioned media. The amount of APPswβ detected in the condition medium of APPswKK-transfected HEK293 and CHOK1 cells was about 9% and 14%, respectively, of the amount measured when the same cells were expressing similar levels of APPsw. It was possible that more APPswβ was derived in the early secretory pathway, but this pool was not secreted from the cell. Therefore, intracellular APPswβ (iAPPswβ) was immunoprecipitated from CHOK1 cell lysates with #931 and immunoblotted with 22C11 (Fig. 3b). Densitometric analysis revealed that cells expressing APPswKK contained about 15% of the amount of iAPPswβ present in APPsw expressing cells. Thus, the decrease in secreted APPswβ in APPswKK expressing cells is not due to intracellular accumulation of the cleaved product. Furthermore, APPswβ production by cells expressing APPswKK was comparatively the same whether measured intracellularly or after secretion in the media.

The #945 antibody, used to capture full-length APP, did not recognize APPKK, which precluded using the ELISA to measure cellular APP levels. To verify equivalent expression of the APP mutants, lysates from transiently transfected cells were immunoprecipitated with 8E5 antibody, which recognizes an epitope in the lumenal domain of APP. The immunoprecipitated full-length protein was identified by western blot using 22C11 (Fig. 3c). Although Fig. 3(c) would suggest a more significant increase, densitometric analysis of western blots indicated that protein expression levels of APPswKK are about 5% greater than APPsw.

(a)

(b)

(c)

pCDNA3   APPwt   APPsw   APPswKK
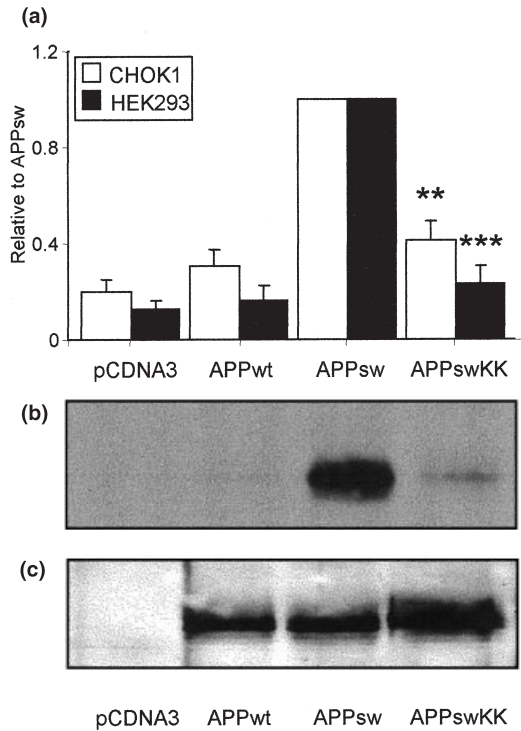
Fig. 3 Addition of a dilysine retrieval motif to the carboxyl-terminus of APPsw significantly decreases APPswβ levels in conditioned media. HEK293 and CHOK1 cells were transiently transfected with pCDNA3, pCDAPPwt, pCDAPPsw, or pCDAPPswKK as indicated, and conditioned media was collected for APPswβ ELISA. (a) Compared to APPsw, β-secretase cleavage of dilysine-modified APPsw (APPswKK) decreased secreted APPswβ levels in conditioned media by 91% and 86% in HEK293 (■) and CHOK1 cells (□), respectively. ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t-test). (b) Lysates from CHOK1 cells used for the #931 ELISA in (a) above were immunoprecipitated with #931 antisera and immunoblotted with 22C11 to visualize intracellular APPswβ (iAPPswβ). Compared to cells expressing APPsw (lane 3), lysates from APPswKK-expressing cells had only ~15% iAPPswβ (lane 4). (c) The same lysates used in (b) above were immunoprecipitated with 8E5 (recognizes epitopes within the aminoterminal ectodomain of APP) and immunoblotted with 22C11 to visualize full-length APP. Densitometric analysis of this Western blot confirmed equivalent protein expression levels (data not shown).

Determining the amount of β-secretase cleavage of APPsw occurring in the secretory and endocytic pathways. β-secretase cleavage of APPsw occurs in both the secretory and endocytic pathways (Perez *et al.* 1996). However, the relative contributions by these two pathways to the total amount of APPswβ secreted into the medium is uncertain. Using our specific sandwich ELISA, we sought to measure the amount of β-secretase cleavage taking place only within the secretory pathway. When APP lacks a cytoplasmic tail (APPΔC), its endocytosis is inhibited (Koo and Squazzo 1994; Perez *et al.* 1999), and thus, it is restricted to the secretory pathway. β-secretase cleavage was still detectable

(a)

(b)

pCDNA3   APPsw   APPswΔC
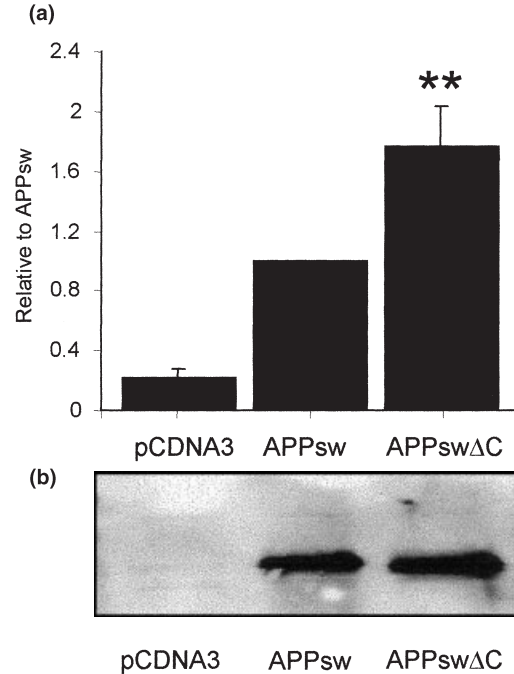
Fig. 4 Deletion of the cytoplasmic tail of APPsw causes a significant increase in β-secretase cleavage. Following transient transfection with pCDNA3, pCDAPPsw (APPsw), or pCDAPPswΔC (APPswΔC), conditioned media from CHOK1 cells were collected for APPswβ ELISA. Cellular lysates were immunoprecipitated with 8E5 antibody and visualized by western blot using 22C11. (a) Compared to APPsw, expression of the truncated form of APPsw (swΔC) causes a 99% increase in the level of APPswβ detected in conditioned media ($n = 3$, $^{**}p < 0.01$, Student's t-test). (b) Immunoprecipitation of lysates with an antibody raised to the ectodomain of APP (8E5) followed by immunoblotting with 22C11 demonstrated that APPswΔC expression is similar to that for APPsw in CHOK1 cells (confirmed by densitometry, data not shown).

for APPswΔC, but the relative amount of secreted APPswβ compared to full-length APPsw was not determined (Citron *et al.* 1995). We hypothesized that APPswΔC would undergo less β-secretase cleavage than APPsw, which has access to β-secretase cleavage in both the secretory and endocytic pathways. CHOK1 cells were transiently transfected with empty vector, APPsw or APPswΔC. Conditioned media was collected as previously described and analyzed by ELISA for APPswβ secretion. Surprisingly, rather than a decrease in β-secretase cleavage, cells expressing the APPswΔC mutant secreted APPswβ at significantly higher levels than APPsw (Fig. 4a). Western blot and densitometric analysis of proteins immunoprecipitated from cell lysates with 8E5 revealed that APPsw and APPswΔC expression levels were equivalent (Fig. 4b) and could not account for the increase in β-secretase cleavage of APPswΔC. This suggests that the cytoplasmic tail of APPsw influences its trafficking and β-secretase cleavage. Deletion of the carboxyl terminus
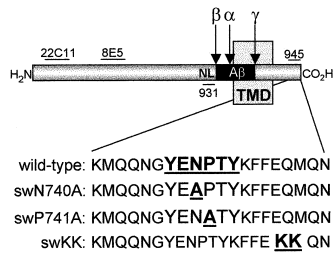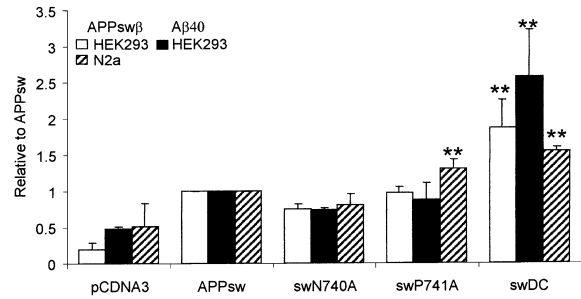
Fig. 5 Location of antibody recognition sites within APPsw and sites of mutagenic modifications. Antibody recognition sites are indicated above and below the schematic of APPsw (APP751 numbering). The K651M/N652L mutation of APP to generate APPsw is shown in bold at the β-secretase cleavage site. The last 20 amino acids of APPsw are enlarged to illustrate the position of the three point mutations within the YENP internalization signal, as well as the location of the dilysine (KK) mutation. The APPswΔC mutant lacks all but four amino acid residues of the entire cytoplasmic tail (truncated from Y709 to N^{751}). TMD, transmembrane domain; Aβ, amyloid-β peptide; α β γ, arrows indicate α-, β-, γ-secretase cleavage sites.

Fig. 6 Point mutations in the endocytic signal sequence within the cytoplasmic tail of APPsw do not significantly affect β-secretase cleavage or Aβ40 peptide generation. HEK293 cells were transiently transfected with the following: pCDNA, pCDAPPsw (APPsw), pCDAPPswN740A (swN740A), pCDAPPswP741A (swP741A), and pCDAPPswΔC (swΔC). Conditioned media were collected for both APPswβ- and Aβ-ELISA analysis. Data are plotted relative to APPsw (APPsw = 1.0). The percent changes were calculated after subtracting the signal obtained with empty vector. Compared to APPsw, expression of the truncated APPsw mutant (swΔC) resulted in a 107% increase in APPswβ levels (white bars) ($n = 3$, $p < 0.08$, Student's t-test). Conversely, the swN740A and the swP741A mutants have levels of APPswβ, respectively, corresponding to a 30% decrease and a negligible 4% decrease, compared to APPsw. Aβ ELISA analysis ($n = 3$) of conditioned media closely corresponded with that for APPswβ. Compared to APPsw, swΔC has an increase in Aβ peptide production of 305% (**$p < 0.01$, Student's t-test). The swN740A mutant produces only 48% of the Aβ peptide production obtained with APPsw. The swP741A mutant had an insignificant decrease in Aβ compared to APPsw. Full-length protein expression (data not shown) of the three point mutants and the C-terminal truncation were equivalent to APPsw. N2a (black bars, $n = 3$) cells were transfected as described for HEK cells. The conditioned media were analyzed by APPswβ ELISA. Data are plotted relative to APPsw (APPsw = 1.0). For most samples, N2a cells produced the same levels of APPswβ relative to APPsw as in HEK293 cells. However, a significant increase in APPswβ (65%) in N2a cells, compared to APPsw. (**$p < 0.01$, Student's t-test).

makes APPswΔC more vulnerable to β-secretase cleavage within the secretory pathway.

Several proteins have been reported to bind to different regions of the cytoplasmic tail of APP (Nishimoto *et al.* 1993; Borg *et al.* 1996; Chow *et al.* 1996; Guenette *et al.* 1996; Homayouni *et al.* 1999). The exact functions of these interactions remain to be determined. Deletion of the cytoplasmic tail not only disrupts endocytosis, but also prevents the interactions of these APP binding proteins. On the other hand, the increase in secreted APPswβ may be because a β-secretase is present at or near the cell surface to cleave APPswΔC molecules that accumulate there due to a block in endocytosis. If this latter explanation were true, then specific point mutations known to disrupt endocytosis of APP should also elevate APPswβ secretion as observed for APPswΔC. Therefore, additional experiments were conducted to measure the effect these specific point mutants that impair APP endocytosis have on APPswβ secretion.

Cytoplasmic tail point mutations that block endocytosis have little or no effect on β-secretase cleavage of APPsw. It has been demonstrated that a block in endocytosis was produced when specific mutations (N740A or P741A; Fig. 5) were made in the carboxyl terminus of APP (Perez *et al.* 1999). Therefore, we sought to measure β-secretase cleavage of APPsw in the secretory versus the endocytic pathways by expressing these endocytic point mutants in different cell types and measuring secreted APPswβ. We reasoned that such specific point mutations would block endocytosis without completely disrupting binding of all cytoplasmic proteins to other regions of the carboxyl terminus as occurred with the APPswΔC deletion mutant. Thus, experiments were conducted in which HEK293 and N2a (Fig. 6) cells were transiently transfected with pCDNA3, APPsw, APPswΔC, APPswN740A or APPswP741A. Conditioned media and cell

lysates were collected for ELISA analysis of APPswβ and full-length APPsw, respectively. Mutations within the YENP internalization signal of APPsw that block endocytosis (Perez *et al.* 1999) did not greatly affect β-secretase cleavage (Fig. 6). Unlike APPswΔC, cells expressing the APPswN740A or APPswP741A point mutants did not elevate APPswβ levels in the conditioned medium. Instead, the amount of APPswβ secreted by HEK cells expressing APPswP741A was not significantly different from that secreted by cells expressing APPsw. Surprisingly, a reproducible increase in APPswβ secretion was detected when APPswP741A was expressed in the neuroblastoma cell line, N2a.

Compared to APPsw, the APPswN740A mutation decreased APPswβ secretion in both cell types. In fact, in HEK293 cells, the amount of β-secretase cleavage detected with the APPswN740A mutant was about 65% of APPsw.

Thus, our results indicate that β-secretase cleavage of APPsw through the endocytic pathway accounts for no more than 35% of the cleavage that takes place in these cells. Lysates of N2a cells were tested for full-length protein expression using the #945 ELISA and western blot analysis. The differences in APPswβ levels measured in our #931 ELISA of conditioned media were not due to differences in expression levels of the different point mutants of APPsw. Cross-reactivity with endogenous human APP prevented measuring full-length protein levels in HEK293 cells.

The effect of endocytosis mutations on secreted Aβ40 produced by transiently transfected cells

In Fig. 6, we demonstrated that point mutations in the cytoplasmic tail that blocked endocytosis did not strongly influence β-secretase cleavage of APPsw. On the other hand, deletion of the entire cytoplasmic tail significantly increased β-secretase cleavage of APPsw. Experiments were next conducted to determine if these same point and deletion mutations had similar affects on Aβ40 secretion, as would be predicted. HEK293 cells were transiently transfected with empty vector, APPsw, or the APPsw C-terminal mutants and the resulting conditioned media were analyzed by ELISA for Aβ40. The Aβ42 levels were below the threshold for detection. The levels of Aβ40 (Fig. 6, black bars) released into the media from the endocytosis-impaired APPsw mutants were found to closely parallel the ELISA results described for secreted APPswβ (Fig. 6, white bars). Cells expressing APPswΔC produced a significantly increased amount of Aβ40 compared to APPsw-expressing cells. The amount of Aβ40 generated by the endocytosis mutant, APPswP741A, did not significantly differ from that generated by APPsw. Finally, as observed for APPswβ secretion, cells expressing APPswN740A produces a slight, but statistically insignificant decrease in Aβ40. Thus, mutations that increase or decrease secretion of APPswβ as measured by our ELISA resulted in a corresponding and proportional change in Aβ40 production.

Discussion

Aβ peptides are produced from APP at several different locations within the cell. The experiments presented here determined the relative importance these distinct cellular locations have in contributing to the amount of β-secretase-cleaved soluble product, APPswβ, which is secreted into the media. Aβ peptides are reportedly derived as early as the endoplasmic reticulum (Cook *et al*. 1997; Wild-Bode *et al*. 1997; Skovronsky *et al*. 1998; Annaert *et al*. 1999). This would suggest that both β- and γ-secretase cleave APP early in the secretory pathway. Presenilins, which are closely associated with γ-secretase activity, are predominantly located in the ER and Golgi compartments (Xia *et al*. 1998). Thus, they could be responsible for the carboxyl

terminal cleavage necessary to generate Aβ peptide in the ER or intermediate compartment. However, the recently cloned β-secretase, BACE1, primarily localizes to the Golgi apparatus, *trans* Golgi network and possibly endosomes (Vassar *et al*. 1999). Two explanations for the β-secretase activity in the ER have been proposed: either APP is cleaved at the amino terminus by a novel β-secretase in the ER that is distinct from BACE1 (Chyung *et al*. 1997) or there is a low level of BACE activity in this compartment (Annaert *et al*. 1999). Regardless of which is correct, the relative amount of β-secretase cleavage occurring in the ER has not been previously measured.

To measure ER β-secretase activity, we introduced an ER retrieval signal to the cytoplasmic tail of APP and APPsw. Others have introduced this dilysine motif to measure the amount of Aβ peptide generated in the ER/IC (Cook *et al*. 1997; Skovronsky *et al*. 1998; Annaert *et al*. 1999). However, the effectiveness of this motif to retain APP has not been thoroughly characterized. We found that introduction of a dilysine retrieval motif extended the half-life of immature APP by approximately fourfold. The amount of APPswβ secreted by cells expressing APPswKK was significantly decreased to between 9 and 15% of that secreted by cells expressing APPsw. Yet, it was not completely eliminated. We previously reported that binding of a mutant of the ER resident molecular chaperone, BiP, to either APP or APPsw significantly inhibited secretion of Aβ40 and Aβ42 (Yang *et al*. 1998). Retention of APPsw in the ER through binding mutant BiP may explain the decrease in Aβ peptide secretion that we previously observed if this effect was a result of blocking movement to the site of β- or γ-secretase cleavage (Yang *et al*. 1998). When HEK cells were transfected with mutant BiP and APPsw, a significant decrease in APPswβ secretion similar to that obtained with APPswKK transfection (data not shown). Thus, we show for the first time that the maximal amount of β-secretase cleavage that takes place in the ER is less than 14% of the total cleavage detected. Analysis of cell lysates revealed a similar amount (∼15%) of intracellular APPswβ present in cells expressing APPswKK compared to APPsw. This latter observation indicates that β-secretase cleavage of APPswKK does not create a separate intracellular pool of APPswβ. This result also confirms the measurements obtained for secreted APPswβ by ELISA for cleavage of APPswKK in the ER.

There is another explanation for these results besides β-cleavage occurring in the early secretory pathway. Although no fully glycosylated APPsw protein was detected (see Fig. 2), some APPswKK molecules may escape the cellular mechanisms of ER retention to be cleaved in the Golgi by BACE. Therefore, the 15% of β-secretase cleavage measured here must be considered as the maximum amount of cleavage that could occur in the ER/IC. Nevertheless, this amount of β-secretase cleavage may be significant, if the resulting carboxyl terminal fragment were preferentially

converted to the more amyloidogenic, Aβ42 peptide. Results of others indicate that this might be the case. Treating cells with brefeldin A (BFA) blocks secretion and redistributes the Golgi into the ER (Doms *et al*. 1989; Lippincott-Schwartz *et al*. 1989). Incubation of HEK293 cells stably expressing APP with BFA increased intracellular levels of Aβ1–42 and Aβx−42 (Wild-Bode *et al*. 1997). Similarly, BFA treatment of human NT2 neurons blocked production of intracellular Aβ40, but not Aβ1–42 (Cook *et al*. 1997). Immunostaining and electron microscopic examination of primary hippocampal neurons also identified the ER as a site of Aβ1–42 generation (Hartmann *et al*. 1997). Still other results using NT2 neurons expressing APPKK demonstrate that a large portion of the intracellular Aβ1–42 generated in the ER/IC is an insoluble pool (Skovronsky *et al*. 1998). Thus, while the amount of β-secretase cleavage of APPsw we detect in the ER/IC may be low, its role in the pathogenesis of Alzheimer's disease requires further investigation.

While conducting ELISA analysis, we found that APPswβ secretion exactly mirrored Aβ secretion. Since Aβ peptide generation requires β- followed by γ-secretase cleavage, β-secretase cleavage indirectly regulates γ-secretase cleavage of APP and Aβ generation. This makes our findings regarding β-secretase cleavage in the secretory and endocytic pathway especially pertinent to therapeutic intervention strategies. In fact, our observation is consistent with β-secretase cleavage of APP being the rate-limiting step of *in vivo* Aβ production (Sinha and Lieberburg 1999). In addition to the utility of the #931 ELISA to measure APPswβ, it is possible that this ELISA may serve as a proxy for measuring Aβ.

APP and APPsw processing into Aβ peptides in nonneuronal cells appears to utilize different cellular mechanisms. Radiolabeling of cell surface APP in CHO cells revealed that amyloid peptide could be derived through endocytosis (Koo and Squazzo 1994). Furthermore, deletion of the carboxyl terminal tail from APP to block endocytosis strongly inhibits Aβ peptide secretion. On the other hand, when similarly truncated APPsw is expressed in HEK293 cells, Aβ peptide is still detected in conditioned media (Citron *et al*. 1995). This implies that APPsw could be cleaved within the secretory pathway. Yet, APPsw processing into Aβ peptide occurs through the endocytic pathway in addition to the secretory pathway (Perez *et al*. 1996). Moreover, cell surface-radioiodination of APPsw molecules resulted in the release of twofold more iodinated Aβ peptide compared to APP (Perez *et al*. 1996). The proportion of Aβ derived from APPsw processing in the secretory pathway compared to the endocytic pathway has not been previously determined. Therefore, we measured the amount of APPswβ and Aβ40 secreted by cells expressing endocytosis-impaired mutants of APPsw and compared them to normal APPsw.

Endocytosis of APP is dependent on a highly conserved signal sequence within its cytoplasmic tail (Koo and Squazzo

1994). Two residues, N740 and P741, are part of the YXNP motif important for endocytosis of APP (Perez *et al*. 1999). Internalization of full-length APP is significantly impaired when either of these residues in the cytoplasmic tail is mutated to alanine (Perez *et al*. 1999). In fact, the N740A mutant is as effective as deleting the entire cytoplasmic domain at impeding endocytosis. The block in endocytosis by these point mutants, in turn, caused a substantial reduction in the amount of secreted Aβ peptide compared to wild-type APP. Consequently, we introduced these same point mutants into APPsw to block its endocytosis. When expressed in HEK293 cells, APPswP741A had little or no effect on APPswβ secretion. Curiously, a slight increase in APPswβ secretion was reproducibly measured in N2a cells expressing APPswP741A, suggesting that this mutation has an effect on neuronal-specific cytoplasmic proteins. As mentioned, the N740A mutation more severely impaired endocytosis of APP than did the P741A mutation (Perez *et al*. 1996). This may explain why, compared to APPsw-expressing cells, APPswβ secretion was lower in cells expressing APPswN740A but not in cells expressing APPswP741A. In HEK293 cells, this decrease was 30%. This decrease in β-secretase cleavage of APPswN740A resulted in a corresponding decrease in secreted Aβ40. These results indicate that the amount of β-secretase cleavage of APPsw taking place within the endocytic pathway is not more than one-third of the total activity within these cells. However, it should be noted that our ELISA specifically recognizes APPswβ cleaved between L671 and D672. β-secretase cleavage that occurs at alternate sites would not be captured by the #931 antibody. Therefore, if endocytosed APPsw is cleaved at such alternate sites, then our ELISA would not detect this activity. We conclude from these studies that β-secretase cleavage of APPsw does occur within the endocytic pathway as reported (Perez *et al*. 1996). However, our results indicate that the majority of β-secretase cleavage of APPsw occurs in compartments of the late secretory pathway.

Like the point mutations in the YENP protein binding motif, deletion of the cytoplasmic domain of APP (APPΔC) strongly impairs endocytosis (Perez *et al*. 1999). Surprisingly, we discovered that the same deletion in APPsw (APPswΔC) significantly increased Aβ40 secretion compared to full-length APPsw. We also observed a corresponding increase in the amount of APPswβ secreted by cells expressing APPswΔC compared to those expressing APPsw. Others, who have previously observed that APPswΔC-expressing cells secreted Aβ peptide, did not report such an increase (Citron *et al*. 1995). Western blot analysis does not indicate any difference in the amount of intact APPswΔC compared to full-length APPsw. It is possible that deletion of the cytoplasmic tail interferes with insertion of APPswΔC into the membrane, making it more vulnerable to β-cleavage. Alternatively, deletion of the cytoplasmic tail from APPsw could make APPswΔC more susceptible to β-secretase

cleavage. This, in turn, could lead to increased Aβ40 generation. Such an increased susceptibility to β-secretase can not involve endocytosis since this effect was not observed with the N740A or P741A point mutants. A number of cytoplasmic proteins have been reported to bind to the exposed carboxyl terminal tail of APP (Nishimoto *et al.* 1993; Borg *et al.* 1996; Chow *et al.* 1996; Guenette *et al.* 1996; Homayouni *et al.* 1999). Mutations in the YENP sequence within the APP tail are reported to alter binding of two such cytoplasmic proteins: FE65 and X11 (Borg *et al.* 1996; Guenette *et al.* 1996). The role of these interacting proteins in APP processing is unclear. We hypothesize that such an endogenous protein binds to the cytoplasmic tail of APPsw making it less vulnerable to β-secretase cleavage. We speculate that C-terminal–protein interactions may be especially important for manifesting this shielding effect as APPsw is processed through the secretory pathway. Experiments are currently underway in our laboratory to explore this hypothesis.

Acknowledgements

References

Annaert W. G., Levesque L., Craessaerts K., Dierinck I., Snellings G., Westaway D., George-Hyslop P. S., Cordell B., Fraser P. and De Strooper B. (1999) Presenilin 1 controls gamma-secretase processing of amyloid precursor protein in pre-Golgi compartments of hippocampal neurons. *J. Cell Biol.* **147**, 277–294.

Borg J. P., Ooi J., Levy E. and Margolis B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol. Cell. Biol.* **16**, 6229–6241.

Chow N., Korenberg J. R., Chen X. N. and Neve R. L. (1996) APP-BP1, a novel protein that binds to the carboxyl-terminal region of the amyloid precursor protein. *J. Biol. Chem.* **271**, 11339–11346.

Chyung A. S. C., Greenberg B. D., Cook D. G., Doms R. W. and Lee V. M. Y. (1997) Novel β-secretase cleavage of β-amyloid precursor protein in the endoplasmic reticulum intermediate compartment of NT2N cells. *J. Cell Biol.* **138**, 671–680.

Citron M., Teplow D. B. and Selkoe D. J. (1995) Generation of amyloid beta protein from its precursor is sequence specific. *Neuron* **14**, 661–670.

Citron M., Vigo-Pelfrey C., Teplow D. B., Miller C., Schenk D., Johnston J., Winblad B., Venizelos N., Lannfelt L. and Selkoe D. J.

(1994) Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc. Natl Acad. Sci. USA* **91**, 11993–11997.

Cook D. G., Forman M. S., Sung J. C., Leight S., Kolson D. L., Iwatsubo T., Lee V. M. Y. and Doms R. W. (1997) Alzheimer's Aβ (1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat. Med.* **3**, 1021–1023.

Doms R. W., Russ G. and Yewdell J. W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61–72.

Forman M. S., Cook D. G., Leight S., Doms R. W. and Lee V. M. (1997) Differential effects of the Swedish mutant amyloid precursor protein on beta-amyloid accumulation and secretion in neurons and non-neuronal cells. *J. Biol. Chem.* **272**, 32247–32253.

Guenette S. Y., Chen J., Jondro P. D. and Tanzi R. E. (1996) Association of a novel human FE65-like protein with the cytoplasmic domain of the beta-amyloid precursor protein. *Proc. Natl Acad. Sci. USA* **93**, 10832–10837.

Haass C., Lemere C. A., Capell A., Citron M., Seubert P., Schenk D., Lannfelt L. and Selkoe D. J. (1995) The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. *Nat. Med.* **1**, 1291–1296.

Haniu M., Denis P., Young Y., Mendiaz E. A., Fuller J., Hui J. O., Bennett B. D., Kahn S., Ross S., Burgess T., Katta V., Rogers G. and Citron M. (2000) Characterization of Alzheimer's beta-secretase protein BACE. A pepsin family member with unusual properties. *J. Biol. Chem.* **275**, 21099–21106.

Hartmann T., Bieger S. C., Brühl B., Tienari P. J., Ida N., Allsop D., Roberts G. W., Masters C. L., Dotti C. G., Unsicker K. and Beyreuther K. (1997) Distinct sites of intracellular production for Alzheimer's disease Aβ40/42 amyloid peptides. *Nat. Med.* **3**, 1016–1020.

Homayouni R., Rice D. S., Sheldon M. and Curran T. (1999) Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. *J. Neurosci.* **19**, 7507–7515.

Hussain I., Powell D., Howlett D. R., Tew D. G., Meek T. D., Chapman C., Gloger I. S., Murphy K. E., Southan C. D., Ryan D. M., Smith T. S., Simmons D. L., Walsh F. S., Dingwall C. and Christie G. (1999) Identification of a novel aspartic protease (Asp 2) as beta-secretase. *Mol. Cell. Neurosci.* **14**, 419–427.

Jackson M. R., Nilsson T. and Peterson P. A. (1993) Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* **121**, 317–333.

Koo E. H. and Squazzo S. L. (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J. Biol. Chem.* **269**, 17386–17389.

Lippincott-Schwartz J., Yuan L. C., Bonifacino J. S. and Klausner R. D. (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801–813.

Nishimoto I., Okamoto T., Matsuura Y., Takahashi S., Okamoto T., Murayama Y. and Ogata E. (1993) Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G (o) [see comments]. *Nature* **362**, 75–79.

Perez R. G., Soriano S., Hayes J. D., Ostaszewski B., Xia W., Selkoe D. J., Chen X., Stokin G. B. and Koo E. H. (1999) Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Aβ42. *J. Biol. Chem.* **274**, 18851–18856.

Perez R. G., Squazzo S. L. and Koo E. H. (1996) Enhanced release of amyloid beta-protein from codon 670/671 'Swedish' mutant beta-amyloid precursor protein occurs in both secretory and endocytic pathways. *J. Biol. Chem.* **271**, 9100–9107.

Pond L., Kuhn L. A., Teyton L., Schutze M. P., Tainer J. A., Jackson M. R. and Peterson P. A. (1995) A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. *J. Biol. Chem.* **270,** 19989–19997.

Selkoe D. J. (1998) The cell biology of β-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol.* **8,** 447–453.

Sinha S. and Lieberburg I. (1999) Cellular mechanisms of beta-amyloid production and secretion. *Proc. Natl Acad. Sci. USA* **96,** 11049–11053.

Skovronsky D. M., Doms R. W. and Lee V. M. (1998) Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture. *J. Cell Biol.* **141,** 1031–1039.

Steinhilb M. L., Turner R. S. and Gaut J. R. (2001) The protease inhibitor, MG132, blocks maturation of the amyloid precursor protein swedish mutant preventing cleavage by beta-secretase. *J. Biol. Chem.* **276,** 4476–4484.

Vassar R., Bennett B. D., Babu-Khan S., Kahn S., Mendiaz E. A., Denis P., Teplow D. B., Ross S., Amarante P., Loeloff R., Luo Y., Fisher S., Fuller J., Edenson S., Lile J., Jarosinski M. A., Biere A. L., Curran E., Burgess T., Louis J. C., Collins F., Treanor J., Rogers G. and Citron M. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE [see comments]. *Science* **286,** 735–741.

Weidemann A., Konig G., Bunke D., Fischer P., Salbaum J. M., Masters C. L. and Beyreuther K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57,** 115–126.

Wild-Bode C., Yamazaki T., Capell A., Leimer U., Steiner H., Ihara Y. and Haass C. (1997) Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42. *J. Biol. Chem.* **272,** 16085–16088.

Xia W. M., Zhang J. M., Ostaszewski B. L., Kimberly W. T., Seubert P., Koo E. H., Shen J. and Selkoe D. J. (1998) Presenilin 1 regulates the processing of β-amyloid precursor protein C-terminal fragments and the generation of amyloid β-protein in endoplasmic reticulum and Golgi. *Biochemistry* **37,** 16465–16471.

Yan R., Bienkowski M. J., Shuck M. E., Miao H., Tory M. C., Pauley A. M., Brashier J. R., Stratman N. C., Mathews W. R., Buhl A. E., Carter D. B., Tomaselli A. G., Parodi L. A., Heinrikson R. L. and Gurney M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity [see comments]. *Nature* **402,** 533–537.

Yang Y., Turner R. S. and Gaut J. R. (1998) The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Aβ40 and Aβ42 secretion. *J. Biol. Chem.* **273,** 25552–25555.