Study of the Synthesis and Secretion of Normal and Artificial Mutants of Murine Amyloid Precursor Protein (APP): Cleavage of APP Occurs in a Late Compartment of the Default Secretion Pathway

Bart De Strooper, Lieve Umans, Fred Van Leuven, and Herman Van Den Berghe

Center for Human Genetics, University of Leuven, Leuven, Belgium

Abstract. Amyloid precursor protein (APP) secretase plays a pivotal role in the processing of APP since its activity precludes the formation of amyloid peptide in Alzheimer's Disease. The identity and the subcellular localization of this enzyme are at this moment unknown. It is also unclear how APP escapes the activity of this enzyme when amyloid is formed. We have previously shown that APP-secretase activity is not inhibited by exogenously added proteinase inhibitors of different specificity (De Strooper, B., F. Van Leuven, and H. Van Den Berghe. 1992. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 308:50–53). We show here that the primary amine methylamine inhibits the secretion of APP into the medium. Furthermore, we show that a truncated form of APP, devoid of the cytoplasmic domain, is more efficiently cleaved and secreted than wild-type APP, which together with the methylamine block, shows that APP-secretase is located in a late compartment of the default constitutional secretion pathway. The sorting signals in the cytoplasmic domain of APP are therefore important in the deviation of APP from the secretase pathway. Finally we show that mutation of Arg609 to Asp in combination with Lys612 to Glu makes APP a less efficiently cleaved substrate for APP-secretase.

The results are discussed in the context of recent findings on the targeting of APP and a parallel is drawn with some lysosomal glycoproteins that follow similar pathways.

THE amyloid precursor proteins (APP's)[1] are a family of very similar, alternatively spliced proteins, coded by a single gene on chromosome 21, and expressed in brain, and in lesser amounts in other tissues (Kang et al., 1987; Kitaguchi et al., 1988; Tanzi et al., 1988; Ponte et al., 1988; De Sauvage and Octave, 1989; Golde et al., 1992; König et al., 1992). Most APP isoforms contain the β-amyloid region, a short peptide of 42–43 amino acids, which is a constituent of the amyloid plaques in the brains of Alzheimer patients (Glenner and Wong, 1984; Masters et al., 1985). Because several single amino acid substitutions in the APP gene are linked to familial early onset Alzheimer's disease, a direct link between the formation of amyloid plaques and the pathogenesis of this devastating disorder is very likely (Goate et al., 1991; Chartier-Harlin et al., 1991; Murrell et al., 1991; Yoshioka et al., 1991; Fernandez-Madrid et al., 1991; Naruse et al., 1991; Levy et al., 1990; Van Broekhoven et al., 1990; Hendriks et al., 1992). Insight in the cellular metabolism of APP is therefore of uttermost importance for the understanding of the pathogenesis of Alzheimer's disease.

Address correspondence to: Dr. F. Van Leuven, Center for Human Genetics, Campus Gasthuisberg, O & N6, Herestraat 49, B-3000 Leuven, Belgium.

1. *Abbreviation used in this paper*: APP, amyloid precursor protein.

The β-amyloid sequence is located partially in the extracellular and partially in the transmembrane domain of the APP protein. The processing of APP, as studied in different cell types cultured in vitro, includes a proteolytic cleavage step in the amyloid sequence (Weidemann et al., 1989; Sisodia et al., 1990; Esch et al., 1990; Anderson et al., 1991; Wang et al., 1991). This step, mediated by an elusive APP secretase (Selkoe, 1989, 1990; Sisodia, 1992), yields a soluble (secreted) form of APP and a small integral membrane protein (Oltersdorf et al., 1990). Because cleavage of APP occurs in the β-amyloid sequence itself, cleavage and amyloid formation are mutually exclusive processes.

The available information shows that APP is transported via the Golgi-complex to the cell surface and released in a soluble form into the medium (Weidemann et al., 1989). The cytoplasmic carboxyl-terminal domain remains membrane anchored (Oltersdorf et al., 1990) and disappears slowly, probably by endocytosis via coated pits, mediated by the NPXY consensus signal in the cytoplasmic part of the protein (Chen et al., 1990). In some cells, APP appears as an intact protein at the cell surface (Weidemann et al., 1989; Haass et al., 1992*a*). This intact APP is endocytosed (probably mediated by the same signal) and degraded in the lysosomes. This pathway is potentially amyloidogenic (Haass et al., 1992*a*,*b*; Estus et al., 1992; Golde et al., 1992; Shoji et al., 1992). A third pathway is possible in which APP enters directly an endosomal-lysosomal compartment, from

which it either (re)circulates to the cell surface or is transported to the lysosomes. This has been demonstrated for lysosomal membrane glycoprotein lgp120 (Harter and Mellman, 1992).

Two important questions with respect to the intracellular routing of APP remain unanswered at this moment: first, the exact localization of APP-secretase, and second, how intact APP can escape from secretase activity. Either processing by secretase is not very efficient or part of the APP is deviated towards a pathway which does not contain secretase. Finally, although there is evidence (Benowitz et al., 1989; Haass et al., 1992*a*; Estus et al., 1992; Golde et al., 1992) that intact APP can appear in the lysosomes (where it could be potentially amyloidogenic), it remains unclear whether APP enters this compartment directly from the Golgi-apparatus, or passes obligatory via the cell membrane.

The present work was intended to examine these questions. Indirect evidence is provided for an intracellular localization of APP-secretase in a compartment of the constitutional secretion pathway where it cleaves overexpressed APP very efficiently. We find that secretion of normal APP and of a truncated form of APP is inhibited by methylamine. Combined Arg(609) to Asp and Lys(612) to Glu mutations make APP a less good substrate for APP-secretase. We demonstrate that deletion of part of the cytoplasmic domain of APP results in the very efficient secretion of APP, which supports the conclusion that APP-secretase is localized on the (default) constitutional secretion pathway. Our findings are discussed in the light of recently published data on the metabolism and cellular targeting of APP and on the targeting of some lysosomal proteins.

Materials and Methods

Cell Culture, Metabolic Labeling and Double-immune Precipitation

Neuro 2a neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DME/F12 (GIBCO BRL, Gaithersburg, MD) with 10% FCS. For metabolic labeling experiments, 400,000 cells were seeded into a T25 flask (Falcon Labware, Becton, Dickinson and Company, Oxnard, CA) and after 24 h medium was replaced by DME/F12 without serum. In these conditions cells develop overnight multiple and long neuritic extensions (De Strooper et al., 1992).

Pulse labeling of the cells was done with 150 μCi per ml [^{35}S]methionine (NEN) in methionine-free DME for 20 min. After a chase-period performed in DME/F12, APP was isolated by double-immune precipitation as described previously (De Strooper et al., 1991*a*). The polyclonal antibody B2/3 against murine APP was raised in rabbits by immunization with a fusion protein between the T7 gene 10 protein (pGEMEX, Promega Corp., Madison, WI) and the carboxy terminal part of the mouse APP695 starting from amino acid 321 (De Strooper et al., 1991*b*). The antibody was used at a dilution 1/16. In the conditions specified below, immune precipitation of APP was quantitative both from cell extracts and from medium (result not shown). Carboxy-terminal specific antibodies (R2292) were generously provided by Dr. C. Abraham (Boston University School of Medicine, Boston, MA) and used at 1/50 dilution. Precipitation of APP was quantitative as illustrated in Fig. 9 *b*. mAb 22C11 against APP was obtained from Boehringer Mannheim Corp. (Germany).

After incubation for 1 h with the first antibody, the immune complexes were precipitated overnight with protein A Sepharose (Pharmacia, Uppsala, Sweden) saturated with polyclonal swine anti-rabbit antibodies (Dakopatts, Dako S. A., Denmark).

Stock solutions of methylammoniumchloride, chloroquine, and ammoniumacetate were sterilized and stored in small aliquots at −20°C until used. The solutions were added directly to the culture medium of the cells 30 min before the start of the experiment.

COS-1 cells were cultured in DME/F12 medium containing 10% FCS. For transfection, cells were plated at 700,000 cells in a T25 flask and after 24 h, processed as described below. All experiments were performed on COS-1 cells before the tenth passage.

Secretion of APP695 and mutated forms into the medium was followed by SDS-PAGE (7% gels, unless otherwise indicated) by applying normalized amounts of complete medium directly or after double-immune precipitation of APP. Normalization was done towards β-galactosidase activity, which was cotransfected with the APP-constructs (see below). Fluorography was done as described (De Strooper et al., 1991*a*). Quantitative densitometry was performed with a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA). The density of the bands was calculated by volume integration as described by the manufacturer. Individual experiments were analyzed by comparing absolute values. For calculations concerning the mean of the secretion rate of APP over several independent experiments at once, absolute densitometric volumes were converted towards relative volumes by normalization towards the volume obtained for APP695 after 2 h in the separate experiments. Linearity of the obtained signals was evaluated by measuring the signals from serial dilution curves of metabolic labeled APP and by exposing autoradiograms for variable time intervals (4–60 h in the experiments shown in this publication).

The sensitivity of the double-immune precipitation assay is illustrated in Fig. 1, which shows the result of double-immune precipitations on equal amounts of progressively diluted medium of COS-1 cells transfected with APP695. It was found that up to 1% of the amount of APP present in the samples used is readily detectable after an exposure of 60 h.

Transformation of COS-1 Cells

COS-1 cells were transfected with the DEAE-dextran method (Luthman and Magnusson, 1983). Briefly, cells were washed twice with PBS and incubated with a mixture of DME/F12, 20 μg/ml DEAE-dextran, 10 μg/ml plasmid PSG5 containing the APP-constructs as described below and 5 μg/ml pSVL β-galactosidase plasmid (Promega Corp., Madison, WI). After 2 h, the cells were washed twice with PBS, and then treated with DME/F12 containing 0.15 mM chloroquine. After 2 h, cells were washed and further cultured in complete medium for 48 h. In all steps, antibiotics (ampicillin-streptomycin, GIBCO BRL) were included in the culture medium.

The pSG5 plasmid contains the early SV40 promotor, a β-globin intron, the cloned APP or its mutants and a polyadenylation signal and directs the synthesis of APP-RNA. The pSVL plasmid directs the RNA synthesis for β-galactosidase from a SV40 late promotor. The β-galactosidase activity was assessed with the β-galactosidase enzyme assay system (Promega Corp.) and used as a measure for the transfection efficiency.

Transfection of COS-1 cells with pSG5 containing the cDNA for murine α2-macroglobulin was done as described (Van Leuven et al., 1993).

Plasmids and Constructions

Mouse APP695 was isolated by screening a mouse brain cDNA library (Uni-Zap™ XR, Stratagene Inc., La Jolla, CA) as a full insert clone in

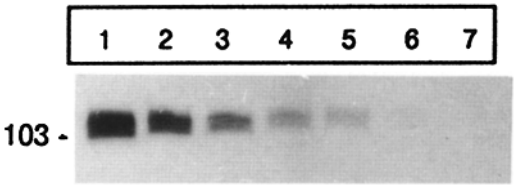
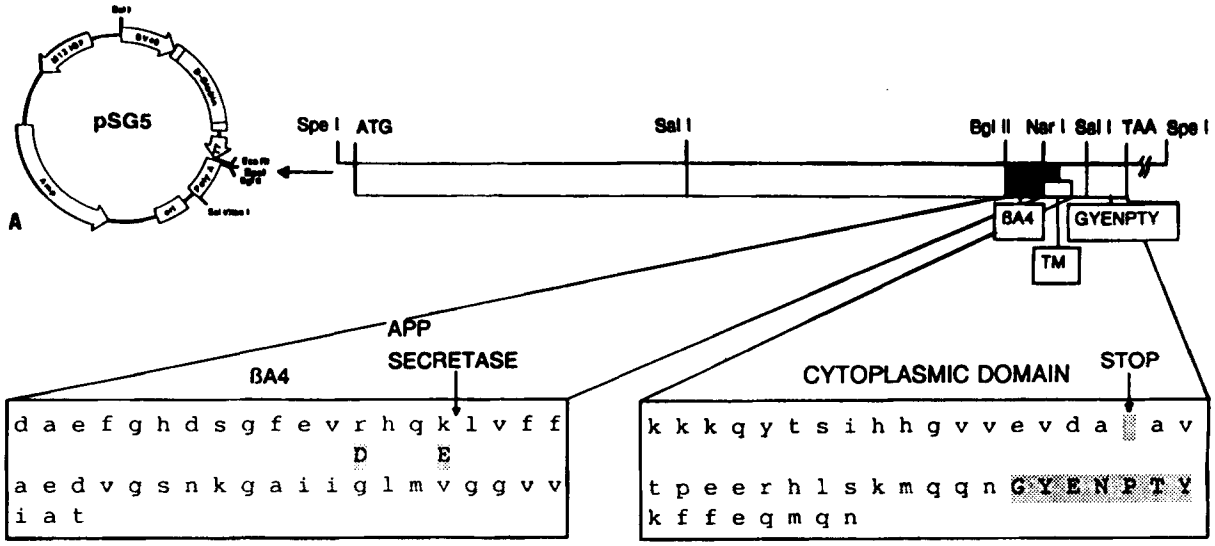


Figure 1. Sensitivity of the immune precipitation assay. Double-immune precipitations with B2/3 (1/16) were performed on a series of diluted medium of COS1-cells transfected with APP695 (see legend to Fig. 9 for further experimental details). Exposure was 30 h. (Lane *1*) Standard amount of medium of transfected COS-1 cells used in the experiments is taken as 1/1. (Lane *2*) 1/2 dilution; (lane *3*) 1/4 dilution; (lane *4*) 1/10 dilution; (lane *5*) 1/20 dilution; (lane *6*) 1/100 dilution; (lane *7*) no label added. The weak signal in lane *6* is clearly observed after 60 h of exposure of the autoradiogram, which shows that the immune precipitation assay is able to detect levels of APP 100 times lower than the level in the control experiments.



T C G A C G C C T A G T A C T G T T A A C G
G C G G A T C A T G A C A A T T G C A G C T

C

Figure 2. Eukaryotic expression vectors for APP695, APP695R/KMUT and APP695TRUNC. *A* shows the plasmid pSG5 with its essential features. Mouse APP695 (De Strooper et al., 1991*b*) was cloned into the multiple cloning site as a SpeI fragment. The β-amyloid region (*hatched*) and the transmembrane domain (*white box*) are indicated, together with putative sorting signals in the cytoplasmic domain. *B* shows the βA4-amyloid sequence and the exact cleavage site of APP-secretase. The mutated amino acids in APP695R/KMUT are boxed. The right displays the cytoplasmic domain of APP. The position of the stop-codon in APP695TRUNC is indicated, together with the deleted putative sorting signals. *C* shows the synthetic oligoduplex used to make APPTRUNC.

pBluescript. The deduced amino acid sequence is 97.6% identical to the human sequence (De Strooper et al., 1991*b*). APP695 was cloned as a SpeI fragment in the adapted multiple cloning site of a pSG5-plasmid (Stratagene Inc.) under the control of the early SV40 promotor (Fig. 2 *A*).

APP695R/KMUT was prepared by deletion of a BglII and NarI fragment (see Fig. 2 *A*) and replacing it with a synthetic oligonucleotide cassette containing the mutations (*boxed*, Fig. 2 *B*). The presence of the mutations was confirmed by sequencing. The mutated APP695R/KMUT was further cloned as a SpeI fragment in pSG5.

APP695TRUNC was prepared by partial digesting pBluescript/APP695 with SalI (Fig. 2 *A*), and introduction of a synthetic oligonucleotide cassette containing a stop codon, a ScaI and an unique HpaI site (Fig. 2 *C*). The correct insertion in the second SalI site was selected for and confirmed by sequencing. The APP695trunc construct was cloned in pSG5 as described above.

Synthetic oligonucleotides were synthesized on a 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). For the APP695R/KMUT a sense oligonucleotide of 105 nucleotides (spanning nucleotides 1770 to 1874 of APP695, numbering as in De Strooper et al., 1991*b*) and an antisense of 103 nucleotides (nucleotides 1774 to 1876) were made. The codon at nucleotide 1825 (CGC, Arg609) was mutated to GAT (Asp). The codon at nucleotide 1834 (AAA, Lys612) was mutated to GAG (Glu). The cassette used for APP695TRUNC is shown in Fig. 2 *C*.

Results

Methylamine Inhibits APP Secretion in Neuroblastoma Cells

Mouse neuro 2a neuroblastoma cells, differentiated as described in Materials and Methods, secreted APP in their culture medium as shown in Fig. 3 *a*. We found previously that the addition of proteinase inhibitors of all classes of proteinases, including the broad spectrum proteinase inhibitor α2-macroglobulin (Van Leuven, 1982) to the culture medium had no effect on APP-secretion (De Strooper et al., 1992), which suggested that APP-secretase is located intracellularly. We tested therefore, inhibitors of lysosomal and endosomal proteinases. In the presence of relatively high concentrations of primary amines, the secretion of APP decreased to ~50% of the control with chloroquine and with ammoniumacetate but to 15% of the control with methylamine (Fig. 3 *b*). However, the effect of chloroquine seemed small because total incorporation of label into cellular protein, assessed by TCA precipitation, was also decreased to ~50%. Methylamine, on the other hand, was clearly less toxic because in all experiments its effect on APP secretion was much more pronounced than its effect on protein synthesis. Moreover, the inhibition of secretion was dose dependent and was always paralleled by an increase of cell associated immune precipitable APP (Fig. 3 *c*). This demonstrated that the effect of methylamine was caused not by an inhibition of APP synthesis, but by an inhibition of APP secretion into the medium. To observe the effect, it was necessary to pretreat the cells for 30 min, indicating an intracellular site of action.

Methylamine Inhibits APP Secretion in COS-Cells

To avoid the heterogeneity of the APP-proteins in the neuro-

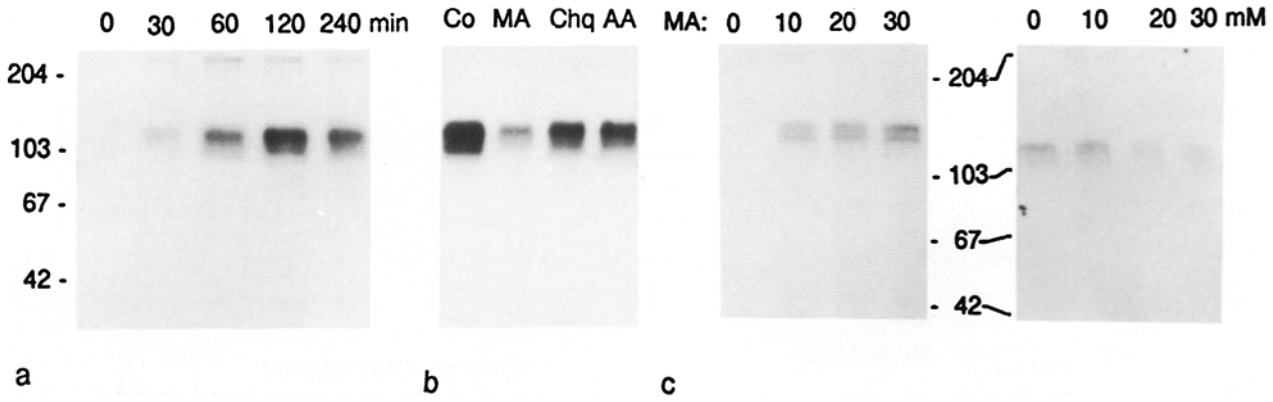


Figure 3. APP-secretion in differentiated Neuro 2a neuroblastoma cells. Cells were pulse-labeled for 20 min and chased either as indicated or for 2 h. Double-immune precipitation was done with polyclonal antibodies B2/3 against a fusion protein of mouse APP with the T7 gene 10 protein. It recognizes epitopes on both sides of the secretase cleavage site and was used at a 1/16 dilution. Precipitation is quantitative as evaluated by consecutive immune precipitations (not shown). A 7% homogeneous gel was used. (*a*) Appearance of APP in the medium. After 2 h, the secretion of APP is maximal. The reason for the slight decrease of APP at 4 h is unknown, but could be caused by proteolytic degradation, or uptake by the cells (Knauer and Cunningham, 1984). (*b*) Effect of primary amines and chloroquine on APP-secretion after 2 h of chase. (*Co*) Control; (*MA*) 30 mM methylamine; (*Chq*) 0.3 mM chloroquine; (*AA*) 50 mM ammoniumacetate. At these high concentrations only methylamine has a significant effect on APP-secretion. (*c*) Dose-response curve with methylamine. (*Left*) Double-immune precipitations of the cell extracts show a concentration dependent accumulation of APP reactive material in the cells. Total incorporated label varied ~20% in the four lanes. (*Right*) Shows a concentration dependent inhibition of secretion of APP into the medium.

blastoma cells (Fig. 3, De Strooper et al., 1992) and to be able to test mutated forms of APP, we decided to use transient transfection of COS-1 cells which express only very low levels of endogenous APP (Fig. 4 *a*, lane *1*). These cells were transfected with constructs containing mouse APP695 under the control of the early SV40 promotor, allowing synthesis, maturation and secretion of APP695 by these cells (Fig. 4 *a*, lane *2*). In this system also, methylamine (30 mM) inhibited efficiently the secretion of mouse APP695. Moreover, the electrophoretic mobility of the cellular APP695 in the methylamine treated cells was identical to that of the untreated controls (Fig. 4 *b*).

Truncation of the Cytoplasmic Domain Increases the Secretion of APP

The cytoplasmic domain of APP contains the NPXY consensus sequence for coated pit mediated endocytosis (Chen et al., 1990). A stop codon was introduced upstream of this signal to delete most of the cytoplasmic domain (Fig. 2 *B*). This truncated APP695 was secreted more efficiently by transfected COS-cells than the wild-type APP695 (Figs. 5 and 7). Significantly, methylamine was again observed to be an efficient inhibitor of this process (Fig. 6 *a*). The secretion (but not the biosynthesis) of transfected murine α_2-macroglobulin, was also inhibited by methylamine, indicating that the methylamine effect is not limited to APP (Fig. 6 *b*).

An Arg609 to Asp and Lys612 to Glu Mutation in the Secretase Recognition Site of APP695 Decreases the Rate of APP695 Cleavage and APP695 Secretion into the Medium

The secretase recognition site in APP695 is located COOH terminal of a lysine residue, preceded two amino acids upstream by another basic residue (arginine in mouse and histidine in humans), (Sisodia et al., 1990; Esch et al., 1990; Anderson et al., 1991; Wang et al., 1991; Yamada et al., 1987; De Strooper et al., 1991*b*). Most other amino acids in this region are either acidic or not charged (Fig. 2 *B*). We opted to mutate both basic residues and replaced the arginine609

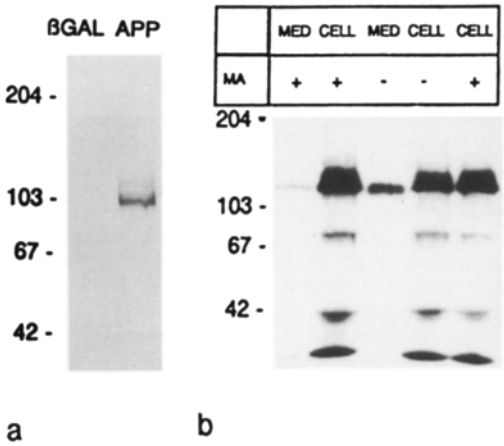


Figure 4. Mouse APP695 in COS-1 cells. (*a*) COS-1 cells were transfected with APP695 or pSVLβGal as detailed in Material and Methods. Total cell extracts were applied on SDS-PAGE and immunoblotted with mAb 22C11 (Weidemann et al., 1989). Negligible to very weak immunoreaction is observed in the control cells transfected with pSVLβgal (*βGal*), whereas in the APP695 transfected cells (*APP*), a strong reaction of the precursor form and weak reaction of the maturated form is seen. (*b*) Cells, transfected as in *a*, were metabolically labeled for 2 h in the presence (+) or absence (−) of 30 mM methylamine (*MA*). Double-immune precipitations were performed with antibody B2/3 (1/16) against the mouse APP fusion protein, either on the medium (*MED*) or on the cell extracts (*CELL*). Methylamine inhibits clearly secretion of APP. The mobility of cell associated APP in both methylamine treated and untreated cells is identical in 7% SDS-PAGE. The accumulation of APP in the methylamine treated cells is obvious in less exposed autoradiograms.

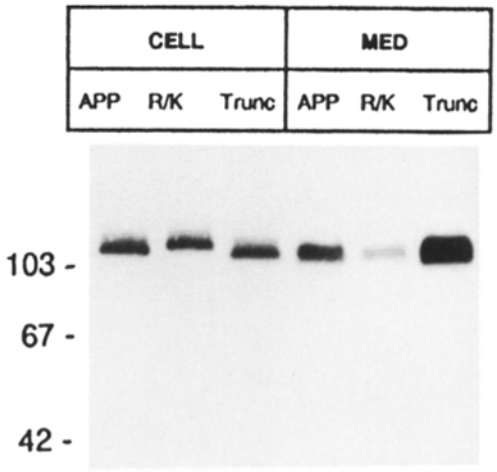


Figure 5. Secretion of mutated forms of APP. COS-1 cells transfected with either APP695 (*APP*), APP695R/KMUT (*R/K*) and APP695TRUNC (*Trunc*) were metabolically labeled for 60 min and then processed by double-immune precipitations on the cells (*CELL*) or on the medium (*MED*) with antibody B2/3 (1/16). Notice the decreased secretion of the APPR/KMUT and the enhanced secretion of APPTRUNC, while the synthesis of precursor APP is in the three instances almost equal. Analysis was done on 7% SDS-PAGE.

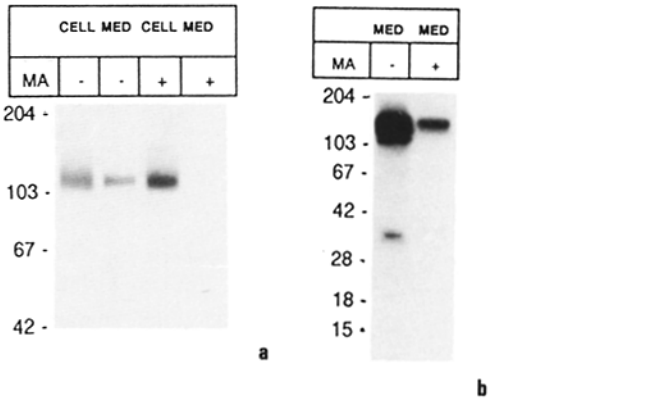


Figure 6. Inhibition of secretion of APP695TRUNC in the presence of 30 mM methylamine. (*a*) COS-1 cells, transfected with APP695TRUNC, were metabolically labeled either without or with addition of 30 mM methylamine. Double-immune precipitation on comparable amounts of either the cell extracts (*CELL*) or the media (*MED*) revealed that secretion is strongly decreased in the presence of methylamine. There is also an increase in cell associated material in the presence of methylamine. Analysis was done on a 7% SDS-PAGE. (*b*) COS-1 cells, transfected with pSG5 vector containing the cDNA for mouse α_2-macroglobulin, were metabolically labeled for 1 h in the absence or presence of 30 mM methylamine. Double-immune precipitation was performed with rabbit anti-mouse α_2-macroglobulin antiserum (Nordic), diluted 1/16. Analysis was done on 6–20% gradient SDS-PAGE. Note the 185-kD, the 165-kD, and the 35-kD subunits of mouse α_2-macroglobulin as described in detail elsewhere (Van Leuven et al., 1992). In the presence of methylamine secretion is strongly inhibited.

by an aspartic acid and lysine612 by a glutamic acid (see Materials and Methods).

When this construct (APP695R/KMUT) was transfected into COS cells, APP695 secretion was decreased although not completely abolished (Fig. 5).

Quantitative measurements of secreted APP were performed to compare the effect of the mutations to that of methylamine (Fig. 7). After 2 h, the secretion of APP695TRUNC and APP695R/KMUT was, respectively, 181 and 56% of control APP695 (Fig. 7). The secretion of APP695 in the presence of 30 mM methylamine was 11% of the control (Fig. 7). All measurements were normalized towards cotransfected β-galactosidase activity in the cell extracts.

The Bulk of Cell-associated APP is Uncleaved

To investigate whether cleaved APP could accumulate in any of the conditions tested, we performed immune precipitations with an antiserum against cleaved and intact APP (rabbit antiserum B2/3) and with an antiserum against the carboxyl terminus of APP (rabbit antiserum R2292) on continuous metabolically labeled COS cells. As shown in Fig. 8, no major differences were observed in immune precipitated material with either antiserum, from cells transfected with APP695 and treated with methylamine or from cells transfected with APP695R/KMUT, when compared with controls transfected with APP695. As anticipated, the carboxy terminal directed antibodies R2292 did not immune precipitate APP695TRUNC (result not shown).

These results indicated qualitatively that most of the cell associated APP contained the 20 carboxy-terminal amino acids, and is thus uncleaved.

The accumulation of APP695 in the presence of methylamine was investigated in further quantitative detail by pulse chase analysis. As shown in Fig. 9 *a*, an important increase (250%) of cell associated APP695 is observed after a chase of 2 h in the presence of methylamine when compared to the control. No major differences were observed in the material precipitated from cell extracts with antiserum B2/3 against complete APP or R2292 against the carboxyl terminus of APP. In contrast, the carboxyl terminus specific antibodies did not precipitate APP from the culture medium (Fig. 9 *a*).

Because it remained possible that a small amount of cleaved APP in the extracts was obscured by the large amount of uncleaved APP in the cells, cell extracts of methylamine treated cells were first cleared by three consecutive immune precipitations with the carboxyl-terminus directed antibody. This effectively removed all uncleaved APP from the cell extract as shown in Fig. 9 *b*. Thereafter, an immune precipitation was performed with the antibody against complete APP. A small, but significant amount of APP was precipitated (1–5% when compared to the amount of uncleaved APP initially precipitated with R2292). As a positive control, the same experiment was done on cell extract combined with an equivalent quantity of medium from untreated cells, containing cleaved APP. As shown in Fig. 9 *b*, cleaved APP was, as anticipated, quantitatively recovered at the end of the assay. This experiment excluded proteolysis or other artifacts during the assay.

That the double-immune precipitation assay was sensitive and linear in a range up to 1% of the material used, was already demonstrated in Fig. 1.

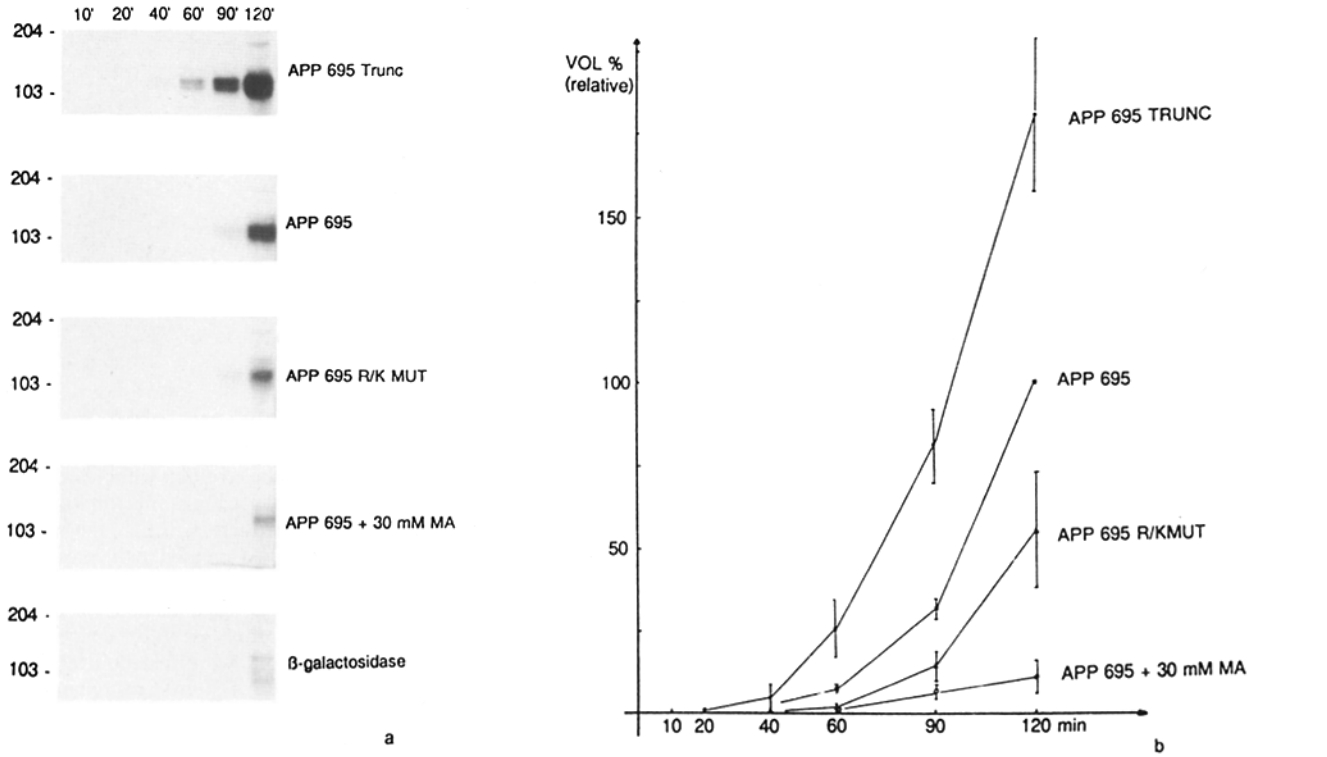


Figure 7. Secretion of APP695TRUNC, APP695 and APP695R/KMUT in the medium of transfected COS-1 cells. (*a*) COS-1 cells, transfected with the indicated expression vectors for the APP constructs and β-galactosidase were metabolically labeled for 2 h. At the indicated time intervals, 100 μl of medium was taken for analysis. At the end of the experiment, cell layers were solubilized and β-galactosidase activity was measured by a colorimetric assay (see Material and Methods). This was taken as a measurement for transfection efficiency and a normalized amount of medium was directly applied on 7% SDS-PAGE for every time point. The autoradiogram labeled β-galactosidase is a negative control. *b* shows the appearance of APP in the medium as determined by densitometry of autoradiograms displayed in *a*. To allow comparison between separate experiments, obtained densitometric volumes (see Material and Methods) were normalized to the volume obtained at 2 h for APP695. For the APP constructs the mean ± SEM for four independent transfection experiments are indicated. For APP695+30 mM methylamine only three independent experiments were performed.

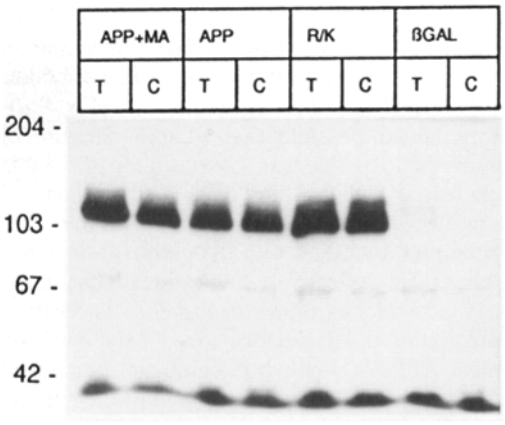


Figure 8. Analysis of cell associated APP in COS-cells. The cell extracts of transfected COS-cells metabolically labeled for 2 h and obtained from the quantitative experiments in Fig. 6 were double-immune precipitated with polyclonal antibody B2/3 at a dilution 1/16. B2/3 recognizes epitopes at both sides of the secretase cleavage site of murine APP and precipitates therefore both cleaved and uncleaved APP (*T*). Polyclonal antibody R2292 (kindly provided by Dr. C. Abraham, Boston University School of Medicine, Boston, MA) against the carboxyl terminus of APP, recognizes only epitopes at the carboxyterminal side of the cleavage site. It was used at a 1/50 dilution. Corresponding lanes are labeled (*C*) in the figure. Above each lane, the construct used to transfect the cells is indicated. Shorter exposures of the autoradiograms did also not reveal any quantitative differences. R2292 did not react with APP695-TRUNC (result not shown).

Discussion

Insight in the cellular processing of APP will eventually lead to the understanding at the molecular level of the formation of the amyloid peptide and of amyloid plaques in Alzheimer's disease and other cerebral disorders. The APP secretase plays a pivotal role in the processing of APP, because only APP that escapes this activity can give rise to amyloid peptide.

We have previously demonstrated that proteinase inhibitors specific for the four major classes of proteinases were not able to inhibit secretase activity at the cell surface of mouse neuroblastoma cells. This led us to the conclusion that secretase is located in an intracellular compartment (De Strooper et al., 1992). Only methylamine inhibited efficiently the secretion of APP into the medium (Fig. 3). Methylamine treatment led also to the accumulation of two APP-forms in Neuro 2a cells. It is unclear whether they rep-

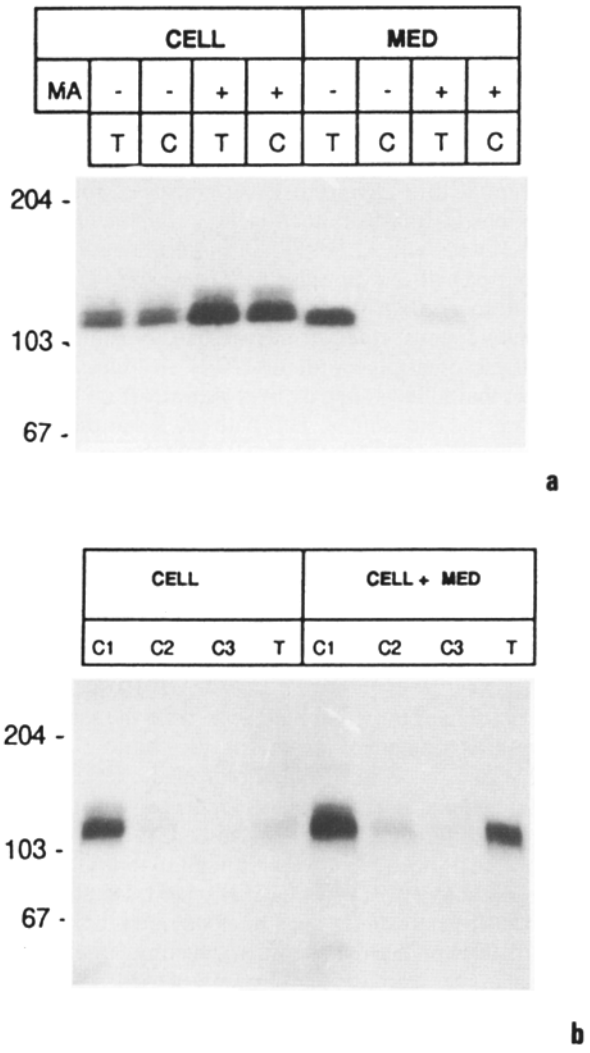


Figure 9. Quantitative analysis of cleaved APP in methylamine treated cells. COS-1 cells transfected with APP695 and β-galactosidase containing vectors, were pulse labeled for 20 min and chased for 2 h in the absence or presence of 30 mM methylamine. Double-immune precipitation was done on comparable amounts of cell extracts or medium in all lanes. Normalization was done towards β-galactosidase activity. (*a*) Normalized amounts of cell extracts (*Cell*) or medium (*Med*) were immune precipitated with antibody B2/3 (1/16 dilution) or R2292 (1/50 dilution) and precipitates were analyzed on 6% SDS-PAGE. Notice the slower metabolism of APP in COS-1 cells compared to neuroblastoma cells (Fig. 3 *c*), the increase of cell associated APP after methylamine treatment (lane *3* compared with lane *1*) concomitant with the decreased secretion of APP into the medium (lane *7* compared with lane *5*). Most of the cell associated APP is recognized by antiserum B2/3 (*CELL*, lanes labeled *T*) as well as by antiserum R2292 (*CELL*, lanes labeled *C*). R2292 does not react with cleaved APP (compare lane *6* with lane *5*). (*b*) Identical amounts of cell extracts from methylamine treated cells (*a*, lanes *3* and *4*) were supplemented either with buffer (*CELL*, lanes *1–4*) or with an equivalent amount of medium of untreated cells (*CELL + MED*, lanes *5–8*). The extracts were immune precipitated three times with antibody R2292 to remove all APP containing the carboxyterminal part and finally with antibody B2/3 to precipitate any remaining cleaved APP. Note that after three sequential immune precipitations all APP reacting with R2292 has been removed. A small quantity (1–5% of the material precipitated in lane *1*) is, however, immune precipitated in the fourth step with B2/3, which indicated that a minor amount of cell associated APP is cleaved. The other half of the gel (lanes *5–8*) illustrates that externally added cleaved APP is quantitatively recovered at the end of this assay. If APP would accumulate as a cleaved form in methylamine treated cells, the magnitude of the signal should be comparable to that obtained in lane *4*.

resent cleaved and uncleaved forms of APP, alternatively spliced forms of the APP-mRNA or different stages of glycosylation of APP. To avoid this problem and to test the effect of mutations in APP on secretion, we decided to use transient transfection in the COS-system and restricted the study to only one form, APP695. Transfected COS cells produced precursor and mature forms of APP, and secreted APP into their medium. Consistent with the observations in the neuroblastoma cells, methylamine inhibited APP secretion also in transfected COS cells.

Primary amines such as methylamine, ammoniumacetate, and chloroquine act by alkalinization of intracellular acidic compartments and are therefore used to inhibit lysosomal degradation. These drugs inhibit however also recirculation of endocytosed cell membrane receptors from the endosomal compartment towards the cell surface (Van Leuven et al., 1980) and cleavage and secretion of proteins along the default secretory pathway in hepatocytes (Oda and Ikehara, 1985; Oda et al., 1986; Lammers and Jamieson, 1989). While all these drugs have similar effects on lysosomal processing of proteins, we find here that methylamine has a more pronounced effect on the cleavage and secretion of APP. As discussed below, this methylamine block is located in the late acidic compartments of the default secretion pathway and inhibits also the constitutive secretion of the proteinase inhibitor α_2-macroglobulin. The exact molecular mechanism awaits further investigation.

Human APP follows the normal constitutive secretion pathway into the Golgi apparatus, where maturation of glycosyl residues is followed by cleavage and secretion of soluble APP (Weidemann et al., 1989). It is not clear whether after maturation of the carbohydrate chains, APP enters the endosomal compartment or follows the default pathway taken by most integral membrane and constitutively secreted proteins. In the first option, secretase would be located in the endosomes, in the second case, secretase could be located either in the late Golgi, in the TGN or in a transport vesicle. Putative sorting signals in the cytoplasmic domain of APP are expected to be important in that respect and their deletion would favor the default secretion pathway. As demonstrated here, a truncated form of APP is cleaved and secreted more efficiently than the wild-type form. Hence, we conclude that APP secretase is located in the secretion pathway. The fact that methylamine inhibited the secretion of the truncated APP695, led us to the conclusion that the methylamine block of APP secretion is also located in the constitutive secretion pathway. This conclusion is corroborated by the observation that the constitutive secretion of mouse α2-macroglobulin transfected in COS-1 cells (Van Leuven et al., 1993) was also inhibited by methylamine treatment (Fig. 6 *b*). This methylamine block of protein secretion is located in the acidic, late Golgi compartment, at least in hepatocytes (Oda et al., 1986; Lammers and Jamieson, 1989). Methylamine did not interfere with glycosyl-residue maturation in these cells, but inhibited very efficiently the proteolytic cleavage of proalbu-

min, of a proform of complement C3, and of a membrane anchored sialyltransferase in the late Golgi (Oda and Ikehara, 1985; Oda et al., 1986; Lammers and Jamieson, 1989). Because methylamine inhibited also the secretion, but not the cleavage of haptoglobin, which is proteolytic processed in the ER, the effect of methylamine on protein secretion is more general than interference with proteolytic maturation alone (Oda et al., 1986). Although our findings in COS cells showed no major differences between immune precipitated APP695 from control or methylamine-treated transfected COS cells after 2 h continuous labeling (Fig. 4 *b*) and showed accumulation of APP with the mobility of mature APP in methylamine treated cells after a 2-h chase (Fig. 9 *a*), it remains to be further investigated whether the methylamine block on the secretion pathway in COS cells observed in our study is similarly restricted to the late Golgi. Our results indicate however that the cell-associated APP accumulating in the presence of methylamine is mainly uncleaved. This led us to the conclusion that methylamine blocks the transport of APP towards the secretase compartments or inhibits directly the activity of secretase in the secretase compartment.

Our previous published data, which showed that proteinase inhibitors acting at the cell surface did not inhibit APP secretion (De Strooper et al., 1992), indicated that secretase is located intracellularly. The fact that small amounts of cleaved APP are associated with cell extracts (Fig. 9 *b*) is also compatible with an intracellular localization of secretase, although endocytosis from the medium remains a possibility as well. Recent evidence suggests however that this soluble APP is indeed produced intracellularly (Sambamurti et al., 1992). Because the methylamine block of protein secretion is located in the late, acidic Golgi compartments of the default secretion pathway, we conclude that secretase is mainly located in this compartment or in the transport vesicles towards the cell surface. The fact that almost no mature APP695 is detected in transfected COS cells (Fig. 4 *a*), suggests also that final glycosyl maturation and cleavage of APP are closely associated processes in time and therefore in space (Weidemann et al., 1989).

Based on the observation that surface labeled APP was secreted almost immediately into the culture medium after warming up cell layers to 37°C, it was recently suggested that secretase is present at the cell surface (Sisodia, 1992). This finding is not necessarily in contradiction with our observations: it is conceivable that secretase is not completely limited to the late compartment of the default secretion pathway. Because it is well established that escape and retrieval processes of proteins in the different compartments of this pathway are the rule, a similar mechanism can explain why minor amounts of APP at the cell surface can be cleaved without being endocytosed. The fact that proteinase inhibitors added at the cell surface have no major effects on the cleavage of APP, indicate however that the bulk of the processing takes place intracellularly (De Strooper et al., 1992, 1993).

Endocytosis, recently demonstrated for APP (Haass et al., 1992*a*), mediated by the consensus NPXY signal in the cytoplasmic domain, could target intact APP at the cell surface towards the lysosomes. Interestingly, the NPXY signal is also present in lysosomal acidic phosphatase, a protein which recirculates 10–15 times between the endosomes and the cell surface before reaching its final destination in the lysosomes (Braun et al., 1989; Eberle et al., 1991). Deletion of this signal in the APP695trunc mutant yielded systematically more soluble APP695 in the medium compared to the wild-type APP (Figs. 5 and 7). Because similar amounts of APP695-vector were used in all experiments, and because cotransfection with a β-galactosidase construct ruled out large variations in transfection efficiency, we conclude that wild-type APP695 (and APP695R/K) was deviated from the default secretion pathway. Because APP is not sorted towards the regulated secretion pathway (Overly et al,. 1991), the alternative pathway must enter the endosomal-lysosomal compartment. Lysosomal glycoprotein 120 is an example of a glycoprotein that follows such a direct pathway from the late Golgi towards the endosomes. This pathway is saturable and the sorting signal is Gly-Tyr (Harter and Mellman, 1992). Interestingly, the NPTY sequence in APP is preceded by Gly-Tyr. Protein phosphorylation in PC12 cells stimulates the secretion of APP into the culture medium (Buxbaum et al., 1990; Caporaso et al., 1992). If phosphorylation occurs on one of the two tyrosines or on the threonine present in the GYENPTY sequence of APP, this could render the signal inactive, a situation mimicked by the truncated mutant APP695TRUNC.

The sorting signals targeting APP away from the secretase pathway towards a potential amyloidogenic pathway (Haass et al., 1992*a*,*b*; Estus et al., 1992; Golde et al., 1992; Shoji et al., 1992), explain at a molecular level the paradox of APP following two different pathways in the cell.

Until now, it has been found very difficult to interfere with APP-secretase activity (Maruyama et al., 1991; De Strooper et al., 1992). It has recently been demonstrated that mutations which disrupt the α-helicoidal structure around the APP cleavage site decrease the cleavage of APP (Sisodia, 1992). We describe here a new secretion mutant in which the α-helicoidal propensity is not significantly altered. The two amino acid substitutions which resulted in a much less efficient secretion of APP, have mainly an effect on the charge distribution in that region, changing two positive residues towards two negative ones. The cell associated APP695R/KMUT displayed a slightly lower mobility than wild-type APP695. Whether the two amino acid substitutions, or whether differences in posttranslational modifications are responsible for this mobility shift remains to be investigated. It is anticipated however, that this, and the other slow secretion mutants (Sisodia et al., 1992), are excellent tools to investigate the metabolic and cellular pathways followed by APP once it has escaped APP-secretase activity. Because these pathways are potentially amyloidogenic, this will help our understanding of β-amyloid formation and hopefully Alzheimer's disease.

The authors thank Dr. C. Abraham, Dr. U. Mönnig and Dr. K. Beyreuther for providing us with carboxyl terminal specific antibodies against APP.

The expert technical assistance of Lucie Raymakers and Marleen Willems is gratefully acknowledged. This work was supported by the National Fund for Scientific Research, Belgium; by a grant "Geconcerteerde Acties" from the Belgian Government and by the Inter-University Network for Fundamental Research sponsored by the Belgian Government (1987–1991). We thank the action "Levenslijn-NFWO" for financial support. B. De Strooper is an "aangesteld navorser" of the National Fund for Scientific Research (Belgium).

Received for publication 17 August 1992 and in revised form 17 December 1992.

References

Anderson, J. P., F. S. Esch, P. S. Keim, K. Sambamurti, I. Lieberburg, and N. K. Robakis. 1991. Exact cleavage site of Alzheimer amyloid precursor in neuronal PC-12 Cells. *Neurosci. Lett.* 128:126–128.

Benowitz, L., W. Rodriguez, P. Paskevich, E. Mufson, D. Schenk, and R. Neve. 1989. The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer Disease subjects. *Exp. Neurol.* 106: 237–250.

Braun, M., A. Waheed, and K. von Figura. 1989. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3633–3640.

Buxbaum, J., S. Gandy, P. Cicchetti, M. Ehrlich, A. Czernik, R. P. Fracasso, T. V. Ramabhadran, A. Unterbeck, and P. Greengard. 1990. Processing of Alzheimer βA4 amyloid precursor protein: modulation by agents that regulate protein phosphorylation. *Proc. Natl. Acad. Sci. USA.* 87:6003–6006.

Caporaso, G., S. Gandy, J. Buxbaum, T. Ramabhadran, and P. Greengard. 1992. Protein phosphorylation regulates secretion of Alzheimer β/A4 amyloid precursor protein. *Proc. Natl. Acad. Sci. USA.* 89:3055–3059.

Chartier-Harlin, M. C., F. Crawford, H. Houlden, A. Warren, D. Hughes, L. Fidani, A. Goate, M. Rossor, P. Roques, J. Hardy, and M. Mullan. 1991. Early-onset of Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene. *Nature (Lond.).* 353:844–846.

Chen, W., J. Goldstein, and M. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116–3123.

De Sauvage, F., and J. N. Octave. 1989. A novel mRNA of the A4 Amyloid Precursor Gene coding for a possibly secreted protein. *Science (Wash. DC).* 245:651–653.

De Strooper, B., F. Van Leuven, G. Carmeliet, H. Van den Berghe, and J. J. Cassiman. 1991*a*. Cultured human fibroblasts contain a large pool of precursor β1-integrin but lack an intracellular pool of mature subunit. *Eur. J. Biochem.* 199:25–33.

De Strooper, B., F. Van Leuven, and H. Van Den Berghe. 1991*b*. The amyloid β protein precursor or proteinase nexin II from mouse is closer related to its human homolog than previously reported. *Biochim. Biophys. Acta.* 1129: 141–143.

De Strooper, B., F. Van Leuven, and H. Van Den Berghe. 1992. α2-macroglobulin and other proteinase inhibitors do not interfere with the secretion of Amyloid Precursor Protein in mouse neuroblastoma cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 308:50–53.

De Strooper, B., and F. Van Leuven. 1993. Inhibition of Amyloid Precursor Protein secretion by proteinase inhibitors suggests an intracellular localization of secretase. *Alzheimer Dis. Assoc. Disord.* In press.

Eberle, W., C. Sander, W. Klaus, B. Schmidt, K. von Figura, and C. Peters. 1991. The essential Tyrosine of the internalization signal in Lysosomal Acid Phosphatase is part of a β-turn. *Cell.* 67:1203–1209.

Esch, F. S., P. S. Keim, E. C. Beattle, R. W. Blacher, A. R. Culwell, T. Oltersdorf, D. McClure, and P. J. Ward. 1990. Cleavage of Amyloid β peptide during constitutive processing of its precursor. *Science (Wash. DC).* 248: 1122–1124.

Estus, S., T. Golde, T. Kunishita, D. Blades, D. Lowery, M. Eisen, M. Usiak, X. Qu, T. Tabira, B. Greenberg, and S. Younkin. 1992. Potentially amyloidogenic, carboxyl-terminal derivatives of the amyloid protein precursor. *Science (Wash. DC).* 255:726–728.

Fernandez-Madrid, I., E. Levy, K. Marder, and B. Frangione. 1991. Codon 618 variant of Alzheimer amyloid gene associated with inherited cerebral haemorrhage. *Ann. Neurol.* 30:730–733.

Glenner, G., and C. Wong. 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885–890.

Goate, A., M. C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, L. James, et al. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature (Lond.).* 349:704–706.

Golde, T., S. Estus, L. Younkin, D. Selkoe, and S. Younkin. 1992. Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science (Wash. DC).* 255:728–730.

Haass, C., E. Koo, A. Mellon, A. Hung, and D. Selkoe. 1992*a*. Targeting of cell-surface β-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature (Lond.).* 357:500–502.

Haass, C., M. G. Schlossmacher, A. Hung, C. Vigo-Pelfrey, A. Mellon, B. Ostaszewski, I. Lieberburg, E. Koo, D. Schenk, D. Teplow, and D. Selkoe. 1992*b*. Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature (Lond.).* 359:322–325.

Harter, C., and I. Mellman. 1992. Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol.* 117:311–325.

Hendriks, L., C. M. van Duijn, P. Cras, M. Cruts, W. Van Hul, F. van Harskamp, A. Warren, M. G. McInnis, S. E. Antonorakis, J. J. Martin, A. Hofman, and C. Van Broekhoven. 1992. Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the β-amyloid precursor protein gene. *Nature Genetics.* 1:218–222.

Kang, J., H. G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K. H. Grzeschik, G. Multhaup, K. Beyreuther, and B. Müller-Hill. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature (Lond.).* 325:733–736.

Kitaguchi, N., Y. Takahashi, Y. Tokushima, S. Shoijiri, and H. Ito. 1988. Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature (Lond.).* 331:530–532.

Knauer, D., and D. Cunningham. 1984. Protease nexins: cell-secreted proteins which regulate extracellular serine proteases. *TIBS (Trends Biochem. Sci.)* May:231–233.

König, G., U. Mönning, C. Czech, R. Prior, R. Banati, U. Schreiter-Gasser, J. Bauer, C. L. Masters, and K. Beyreuther. 1992. Identification and differential expression of a novel alternative splice isoform of the βA4 amyloid precursor protein (APP) mRNA in leukocytes and brain microglial cells. *J. Biol. Chem.* 267:10804–10809.

Lammers, G., and J. C. Jamieson. 1989. Studies on the effect of lysosomotropic agents on the release of Galβ1-4GlcNac α-2,6-sialyltransferase from rat liver slices during the acute-phase response. *Biochem. J.* 261:389–393.

Levy, E., M. D. Carman, I. J. Fernandez-Madrid, M. D. Power, I. Lieberburg, S. G. Van Duynen, G. TH. A. M. Bots, W. Luyendijk, and B. Frangione. 1990. Mutation of the Alzheimer's Disease Amyloid gene in hereditary cerebral haemorrhage, Dutch type. *Science (Wash. DC).* 248:1124–1126.

Luthman, H., and G. Magnusson. 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucl. Acids Res.* 11:1295–1308.

Maruyama, K., F. Kametani, M. Usami, W. Yamao-Harigaya, and K. Tanaka. 1991. Secretase, Alzheimer amyloid protein precursor secreting enzyme is not sequence-specific. *Biochem. Biophys. Res. Commun.* 179:1670–1676.

Masters, C., G. Simms, N. Weinman, G. Multhaup, B. McDonald, and K. Beyreuther. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. USA.* 82:4245–4249.

Murrell, J., M. Farlow, B. Ghetti, and M. D. Benson. 1991. A mutation in the Amyloid Precursor Protein Associated with hereditary Alzheimer's disease. *Science (Wash. DC).* 254:97–99.

Naruse, S., S. Igarashi, K. Aoki, K. Kaneko, K. Iihara, T. Miyatake, H. Kobayashi, T. Inuzuka, T. Shimizu, T. Kojima, and S. Tsuji. 1991. Missense mutation Val-Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. *Lancet.* 337:978–979.

Oda, K., and Y. Ikehara. 1985. Weakly basic amines inhibit the proteolytic conversion of proalbumin to serum albumin in cultured rat hepatocytes. *Eur. J. Biochem.* 152:605–609.

Oka, K., Y. Koriyama, Y. Yamada, and Y. Ikehara. 1986. Effects of weakly basic amines on proteolytic processing and terminal glycosylation of secretory proteins in cultured rat hepatocytes. *Biochem. J.* 240:739–745.

Oltersdorf, T., P. Ward, T. Henriksson, E. Beattie, R. Neve, I. Lieberburg, and L. Fritz. 1990. The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/degradative pathway. *J. Biol. Chem.* 265:4492–4497.

Overly, C., L. Fritz, I. Lieberburg, and L. McConlogue. 1991. The β-amyloid precursor protein is not processed by the regulated secretory pathway. *Biochem. Biophys. Res. Commun.* 181:513–519.

Ponte, P., P. Gonzalez-DeWhitt, J. Schilling, J. Miller, D. Hsu, B. Greenberg, K. Davis, W. Wallace, I. Lieberburg, F. Fuller, and B. Cordell. 1988. A new A4 amyloid mRNA contains a domain homologous to serine protease inhibitors. *Nature (Lond.).* 311:525–527.

Sambamurti, K., J. Shioi, J. P. Anderson, M. A. Pappolla, and N. Robakis. 1992. Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. *J. Neurosci. Res.* 33:319–329.

Selkoe, D. 1989. Amyloid β protein precursor and the pathogenesis of Alzheimer's disease. *Cell.* 58:611–612.

Selkoe, D. 1990. Deciphering Alzheimer's disease: the amyloid precursor protein yields new clues. *Science (Wash. DC).* 248:1058–1060.

Shoji, M., T. Golde, J. Ghiso, T. Cheung, S. Estus, L. Shaffer, X. D. Cai, D. McKay, R. Tintner, B. Frangione, and S. Younkin. 1992. Production of the Alzheimer amyloid β protein by normal proteolytic processing. *Science (Wash. DC).* 258:126–129.

Sisodia, S. 1992. β-Amyloid precursor protein cleavage by a membrane-bound protease. *Proc. Natl. Acad. Sci. USA.* 89:6075–6079.

Sisodia, S., E. Koo, K. Beyreuther, A. Unterbeck, and D. Price. 1990. Evidence that β-amyloid protein in Alzheimer's disease is not derived by normal processing. *Science (Wash. DC).* 248:492–495.

Tanzi, R. E., A. I. McClatchey, E. D. Lamperti, L. Villa-Komaroff, J. F. Gusella, and R. L. Neve. 1988. Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature (Lond.).* 331:528–530.

Van Broekhoven, C., J. Haan, E. Bakker, J. Hardy, W. Van Hul, A. Wehnert, M. Vegter-Van Der Vlis, and R. Roos. 1990. Amyloid β protein precursor gene and hereditary cerebral haemorrhage with amyloidosis (Dutch). *Science (Wash. DC).* 248:1120–1122.

Van Leuven, F. 1982. Human alpha-2-macroglobulin: structure and function. *Trends Biochem. Sci.* 7:185–187.

Van Leuven, F., J. J. Cassiman, and H. Van Den Berghe. 1980. Primary amines inhibit recycling of α2M-receptors in fibroblasts. *Cell.* 20:37–43.

Van Leuven, F., S. Torrekens, L. Overbergh, K. Lorent, B. De Strooper, and H. Van den Berghe. 1993. The primary sequence and the subunit structure of mouse alpha-2-macroglobulin, deduced from protein sequencing of the isolated subunits and from molecular cloning of the cDNA. *Eur. J. Biochem.* 210:319–327.

Wang, R., J. F. Meschia, R. J. Cotter, and S. S. Sisodia. 1991. Secretion of the β/A4 amyloid precursor protein. Identification of a cleavage site in cultured mammalian cells. *J. Biol. Chem.* 266:16960-16964.

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

Yamada, T., H. Sasaki, H. Furuya, T. Miyata, I. Goto, and Y. Sakaki. 1987. Complementary DNA for the mouse homolog of the human amyloid beta protein precursor. *Biochem. Biophys. Res. Commun.* 149:665–671.

Yoshioka, K., T. Miki, T. Katsuya, T. Ogihara, and Y. Sakaki. 1991. The 717Val to Ile substitution in amyloid precursor protein is associated with familial Alzheimer's disease regardless of ethnic groups. *Biochem. Biophys. Res. Commun.* 178:1141–1146.