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

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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., E 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 APE devoid of the cytoplasmic domain, is more efficiently cleaved and secreted than wild-type APE 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) 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 Broeckhoven 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.

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 (Olterdorph 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 (Olterdorph 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., 1992a,b). This intact APP is endocytosed (probably mediated by the same signal) and degraded in the lysosomes. This pathway is potentially amyloidogenic (Haass et al., 1992a,b; Estus et al., 1992; Golde et al., 1992; Shoiji 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 gp120 (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., 1992a; 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 Arg609 to Asp and Lys612 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, APP was isolated by double-immune precipitation as described below. Fluorography was done as described (De Strooper et al., 1991a). 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 PSG 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 experiments, antibiotics (ampicillin-streptomycin, GIBCO BRL) were included in the culture medium.

The pSVG plasmid contains the early SV40 promoter, a β-globin intron, the cloned APP or its mutants and a polycadenylation 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 pSVG 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 pBluescript II KS(−) (Stratagene Inc., La Jolla, CA). The full insert was sequenced and subcloned into pBluescript II KS(−) (Stratagene Inc., La Jolla, CA) for a second round of sequencing and subcloning.

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 COS-1 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.

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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., 1991b) 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.

TCGACGCGCATGCTCTGAACGCGGATCATGACAATTGCAGCT

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.

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 α2-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., 1991b). 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 5. Secretion of mutated forms of APP. COS-I 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.

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. 6). 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 antisera against cleaved and intact APP (rabbit antiserum B2/3) and with an antisera 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 antisera, 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 antisera 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 measure 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 APP695TRUNC (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-
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 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 into the medium (lane 7 compared with lane 5). Most of the cell associated APP is recognized by antiserum B2/3 (compare lane 3 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 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.
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 methyamine inhibited also the secretion, but not the cleavage of haptoglobin, which is proteolytic processed in the ER, the effect of methyamine 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 methyamine-treated transfected COS cells after 2 h continuous labeling (Fig. 4 b) and showed accumulation of APP with the mobility of mature APP in methylene treated cells after a 2-h chase (Fig. 9 a), it remains to be further investigated whether the methyamine 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 methyamine is mainly uncleaved. This led us to the conclusion that methyamine 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 methyamine 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., 1992a), 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 (Overy 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., 1992a,b; Estus et al., 1992; Golde et al., 1992; Shoji et al., 1992), explain at a molecular level the paradox of APP secretion (De Strooper et al., 1992). It has recently been demonstrated that mutations which disrupt the α-helical structure around the APP cleavage site decrease the cleavage of APP (Sisodia, 1992). We describe here a new secretion mutant in which the α-helical 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 Raymnkers 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-NPWO” for financial support. B. De Strooper is an “aangesteld navorser” of the National Fund for Scientific Research (Belgium).
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