Release of Nontransmembrane Full-Length Alzheimer's Amyloid Precursor Protein from the Lumenar Surface of Chromaffin Granule Membranes†

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ABSTRACT: We previously demonstrated the presence of a soluble form of full-length Alzheimer's amyloid precursor protein (APP) in the lumen of adrenal medullary chromaffin granules (CG). Furthermore, full-length APP is released from CG membranes in vitro at pH 9.0 by an enzymatic mechanism, sensitive to protease inhibitors [Vassilacopoulou et al. (1995) *J. Neurochem. 64*, 2140−2146]. In this study, we found that when intact CG were subjected to exogenous trypsin, a fraction of APP was not digested, consistent with an intragranular population of APP. To examine the substrate−product relationship between membrane and soluble full-length APP, we labeled CG transmembrane APP with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), a lipophilic probe, specific for membrane-spanning domains of proteins. APP released from the membranes at pH 9.0 was not labeled with [^{125}I]TID. In addition, this APP was not biotinylated in intact CG. Combined, the results indicate that APP released from CG membranes derives from a unique nontransmembrane population of membrane-associated APP, located in the lumenal side of CG membranes. Dithiobis(succinimidylpropionate) (DSP) cross-linking indicated that APP in CG is situated in close proximity with other proteins, possibly with APP itself. APP complexes were also detected under nonreducing conditions, without DSP cross-linking. These results, combined with our previous studies, indicate that full-length APP within CG exists as three different populations: (I) transmembrane, (II) membrane-associated/nontransmembrane, and (III) soluble. The existence of nontransmembrane populations suggests that putative γ-secretase cleavage sites of APP, assumed to be buried within the lipid bilayer, could be accessible to proteolysis in a soluble intravesicular environment.

Amyloid β-peptide (Aβ)[1] (see Figure 1), derived proteolytically from Alzheimer's amyloid precursor protein (APP) (*1*−*4*), is deposited in neuritic plaques and cerebral blood vessels of Alzheimer's disease (AD) patients (*5*, *6*). APP represents a group of transmembrane proteins derived from a single gene in human chromosome 21 by differential splicing (for review, see ref 7). The predominant isoforms, APP770, APP751, and APP695 (numbers indicate the number of amino acids in each isoform), are widely expressed with some tissue-specific patterns (*8*, *9*). Aβ can be generated by normal cellular metabolism since it is secreted into the media from cell lines of both neuronal and

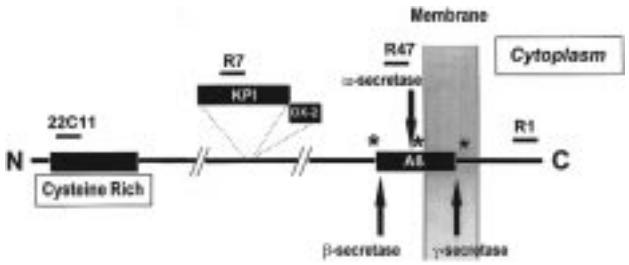

FIGURE 1: Diagramatic representation of major APP isoforms and epitopes of antibodies. The amyloid precursor protein (APP) gene undergoes alterrnative RNA splicing that synthesizes several APP isoforms. The most commonly expressed isoforms contain 695, 751, and 770 amino acids, respectively. APP751 and APP770 contain the KPI (Kunitz protease inhibitor) domain, and APP 770 contains the additional OX-2 domain. There is a cysteine-rich region near the N-terminus of all APP isoforms. The 22C11 monoclonal antibody recognizes that region (recognizes 60−100 residues of APP). The R1 antibody recognizes the cytoplasmic C-terminal 22 amino acids of APP and R47 antibody was generated against Aβ1−16. In transmembrane APP the carboxy-terminal region of Aβ is buried within the lipid bilayer. APP metabolism occurs at the predicted cleavage sites of α-, β- and γ-secretases, shown by arrows. Asterisks indicate the sites of mutations linked to familial AD; interestingly all of these mutations occur at or near putative cleavage sites of the secretases, known to affect the production of Aβ.

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[1] Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; APPm, transmembrane APP; APPma, nontransmembrane, membrane-associated APP; APPsol, soluble APP; CG, chromaffin granule(s); DSP, dithiobis(succinimidylpropionate); FAD, familial AD; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine.

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nonneuronal origin (*10*, *11*) and can be detected in biological fluids from healthy individuals (*12*). Thus far, five different point mutations within the APP gene are associated with early-onset familial AD (FAD), indicating a significant role for APP or its proteolytic derivatives in the development of this disease.

The precise subcellular location(s) of Aβ production has not been clearly defined. Likewise, the proteases responsible for cleavage of APP at the N- and C-termini of Aβ (Figure 1), known as β- and γ-secretases, respectively, have not yet been definitively characterized. Another proteolytic activity, by α-secretase, cleaves APP within the Aβ peptide sequence, thus, precluding formation of Aβ (Figure 1). There is growing evidence that, in neurons, APP is processed in the Golgi apparatus and in post-Golgi vesicles (*11*, *13*, *14*) and that Aβ is produced in these organelles (*15*−*17*). This is consistent with evidence for axonal transport and metabolism of APP (*18*) that generates amyloidogenic C-terminal fragments (*19*) and regulation of APP metabolism by neuronal activity (*20*). Furthermore, detection of full-length APP and an intermediate amyloidogenic fragment (*21*) within post-Golgi secretory vesicles of the adrenal medulla, known as chromaffin granules (CG), provide direct evidence supporting the hypothesis of APP processing in the neurosecretory vesicle pathway.

One mechanistic aspect of the amyloidogenic pathway of APP metabolism, which has puzzled many researchers, is the liberation of Aβ from APP at its C-terminus. This site is assumed to be buried within the lipid bilayer, rendering no access to the so-called γ-secretase. However, this site can be found in the aqueous phase, raising the possibility of a soluble precursor/substrate for γ-secretase. The detection of soluble full-length APP has been reported by a number of laboratories (refs 21−25 and this study). Interestingly, Bhasin et al. (*22*) found that Sf9 cells infected with APP mutated at the Val717-to-Ile (numbering on APP751) and fibroblast cultures derived from individuals carrying the same mutation secreted more soluble full-length APP in the conditioned media. This suggests that there may be a causal relationship between increased production of soluble full-length APP and Aβ.

Soluble holo-APP may be generated through different possible mechanisms. For example, membrane leakage could be involved. In support, increased membrane fluidity is a prominent feature of AD (*26*, *27*) and plasma membranes of dystrophic neurites in senile plaques of AD patients appear markedly fragile (*28*). However, we have evidence to suggest that release of APP from membranes may occur under normal, non-disease-related conditions.

We previously demonstrated the presence of full-length APP in the soluble fraction of CG (*21*). Additionally, it was found that an enzymatic mechanism, optimal at pH 9, sensitive to protease inhibitors, divalent cations, and heat inactivation is involved in the release of APP from a specific PC12 microsomal membrane fraction and from CG membranes (*21*, *24*). In this report we show that the released full-length APP derives from a unique nontransmembrane subpopulation of membrane-associated APP. We also provide evidence that APP is associated with another CG membrane protein(s) which could serve as a possible "anchor(s)".

MATERIALS AND METHODS

Cell Fractionation and CG Purification. CG were prepared from fresh bovine adrenal medulla by homogenization and differential centrifugation followed by discontinuous sucrose density centrigugation (through a 1.8 M sucrose cushion), essentially as described previously (*29*). CG were lysed by freezing and thawing in a hypotonic buffer [20-fold diluted phosphate-buffered saline (PBS)]. In some experiments, a CG-enriched fraction was obtained by homogenization and differential centrifugation in 0.32 M sucrose (*30*).

Trypsin Protection Experiments. A CG-enriched fraction, obtained from 20 adrenals, was washed three times in an equal volume of 0.32 M sucrose and finally resuspended in 60 mL of 0.32 M sucrose. Aliquots (50 μL) of intact CG and deoxycholate-solubilized CG (in 1% deoxycholate) were incubated with trypsin at final concentrations of 20 and 4 μg/mL, at room temperature (25 °C) for 20 min. CG samples were immediately placed on ice, phenylmethanesulfonyl fluoride (PMSF) and leupeptin protease inhibitors were added (at final concentrations of 0.2 and 2 mM, respectively), and samples were heated (95 °C for 10 min) in Laemmli sample buffer (LSB) and subjected to Western blots utilizing the R1 antibody which was raised against the 22 C-terminal amino acids of APP (*8*). To assess the integrity of CG (intact and deoxycholate-solubilized) after incubation with trypsin, CG were pelleted by centrifugation at 15000*g* for 5 min at 4 °C; (Met)enkephalin in the resultant supernatant was measured by radioimmunoassay as described previously (*31*).

In Vitro Release of APP from CG Membranes. This was performed as described in detail by Ripellino et al. (*24*) and Vassilacopoulou et al. (*21*). Briefly, membranes were isolated from purified CG extracts by centrifugation at 150000*g* for 30 min and stripped of peripheral proteins by treatment with 0.1 M sodium carbonate. After two washes with water, membranes were resuspended in buffers of pH 7 or 9 (50 mM HEPES/NaOH, pH 7, or glycine/NaOH, pH 9). Following incubation for 30 min at 37 °C, samples were centrifuged at 150000*g* for 30 min at 4 °C to separate membranes from the solubilized (released) molecules.

[^{125}I]TID Labeling of CG Membranes. (3-Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) labeling of CG transmembrane proteins was performed essentially as described by Blanton and Cohen (*32*). Briefly, CG membranes (1 mg/mL) (after stripping of peripheral proteins as described in the previous section) in 100 mL of a buffer containing 50 mM sodium phosphate, pH 7, were allowed to equilibrate at room temperature for 30 min in the dark with 10 μCi of [^{125}I]TID (Amersham, 10 Ci/mmol). Samples were then irradiated for 15 min at a distance of 1 cm with a 365-nm lamp (EN-Spectroline) under constant shaking at room temperature. Noncovalently bound radioactivity was removed from membranes by washing (15000*g* for 15 min) three times in 50 mM sodium phosphate, pH 7, containing 1% BSA and once with the same buffer without BSA.

The final membrane pellets were resuspended directly in buffers for APP release, as described above. Resultant membranes and solubilized molecules were separated by centrifugation (150000*g* for 30 min), and both fractions were resuspended in 0.35% SDS and boiled for 10 min. APP was immunoprecipitated as described previously (*13*) using R1

antibodies (1:1000 final dilution). The immunoprecipitates were applied to SDS−PAGE and analyzed by autoradiography. Aliquots from these samples were also analyzed by Western blotting using R1 antibodies as described previously (*13*). As a control, synaptotagmin (p65) (*33*), another type I transmembrane calmodulin-binding protein found in CG, was also analyzed by Western blotting using monoclonal antibody CI.41.1 (Dr. R. Jahn, Yale University). To ensure that the probe labels only transmembrane, lipid-associated protein sequences, CG soluble fraction (5 mg/mL) or purified soluble APP or nexin II (400 μg/mL) were also subjected to the labeling procedure and analyzed similarly by immunoprecipitation using R1 or R7 (directed against amino acids 296−315 of APP751, corresponding to the KPI domain; ref 34) antibodies. Other controls included nonphotolyzed samples and irradiation in the presence or absence of 25 mM glutathione, which binds free [^{125}I]TID in the aqueous phase (*32*).

Biotinylation of Intact and Lysed CG. The CG pellet obtained from the discontinuous sucrose density gradient was resuspended in ice-cold PBS following a washing step with PBS. An aliquot was lysed in a hypotonic solution (20-fold diluted PBS) by freezing and thawing. Both samples, intact and permeablized (lysed) granules (10 mg/mL protein) were biotinylated as described by Shioi et al. (*35*) using sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin, Pierce). NHS-SS-biotin is a water-soluble ester of biotin which forms amide bonds with primary amines. Briefly, CG were incubated in PBS with NHS-SS-biotin (0.5 mg/mL) at 4 °C for 30 min, and quencing was performed with 50 mM ammonium chloride. Intact CG were lysed by freezing and thawing following 20-fold dilution in water. Biotinylated membranes from intact or permeablized granules were treated to remove peripheral proteins and then used for the in vitro release of APP. Following the release, samples were centrifuged for the separation of the soluble molecules from those in membranes and then samples were brought to 0.35% SDS before the streptavidin purification step.

Biotinylated released and detergent-solubilized membrane proteins were isolated by incubation with streptavidin-agarose resin (Sigma), at 4 °C for 1 h. Samples were centrifuged in a microfuge (15000*g*) for 1 min, and the supernatants were collected representing nonbiotinylated proteins. The biotinylated proteins were dissociated from the streptavidin resin by boiling in LSB containing β-mercaptoethanol. Both biotinylated and nonbiotinylated proteins were subjected to SDS−PAGE and analyzed for APP and p65 by Western blotting.

Chromaffin Cell Metabolic Labeling. Chromaffin cells were dispersed from fresh bovine adrenal medulla by digestion with collagenase I (Worthington) as described previously (*31*) and cells were plated at 5×10^5 cells/well in 24-well plates (Costar). After 3 days in culture, media were replaced with DMEM lacking methionine and supplemented with [^{35}S]-methionine (150 μCi/mL). Cells from 6 plates were labeled for 12−16 h and then harvested, homogenized and fractionated by differential centrifugation (*29*) to obtain an enriched preparation of CG.

Chemical Cross-Linking of CG APP: Analyses by Western Blotting, Sucrose Gradients, and Immunoprecipitation. Chemical cross-linking was performed with dithiobis(succinimidyl propionate) (DSP) (Pierce), a lipophilic homobifunctional lysine-specific cross-linker with a spacer arm of 12 Å. For the cross-linking reaction, CG membranes (0.25−1 mg/mL protein) in the presence or absence of 1% Triton X-100 or lysed CG (0.5−5 mg/mL protein) were incubated for up to 2 h at 4 °C or room temperature with 0.25−5 mM DSP in PBS. The reaction was stopped by adding 1.0 M Tris-HCl, pH 7.0, to a final concentration of 50 mM Tris-HCl and was kept at 4 °C for an additional 15 min. Cross-linked samples were solubilized in LSB, with or without β-mercaptoethanol, and analyzed by Western blots with R1 antiserum.

DSP cross-linked CG membranes were also analyzed by sucrose gradients to analyze APP complexes formed with other CG membrane proteins. After DSP cross-linking, CG membranes were solubilized in 1% Triton X-100 and subsequently applied onto a 5−30% sucrose gradient, layered on a 30% sucrose cushion. Sucrose gradients (3.3 mL) were centrifuged at 105000*g* for 16 h. Fractions (200 μL) were collected from the bottom of the tube with a peristaltic pump. Aliquots (30 μL) were applied directly to 5% SDS−PAGE gels in the presence or absence of β-mercaptoethanol and were analyzed by Western blots with R1 antiserum.

When CG membranes from metabolically labeled ([^{35}S]-methionine-labeled) chromaffin cells were cross-linked with DSP, cross-linked membranes were solubilized in 0.35% SDS, and then APP and/or APP complexes were immunoprecipitated with R1 antibodies, as described previously (*13*). Immunocomplexes were dissociated in LSB and analyzed by SDS−PAGE and autoradiography.

RESULTS

Much evidence suggests cellular trafficking of APP through the secretory pathway (*13*, *14*, *18*, *20*) that routes APP to the plasma membrane where APP has a transmembrane orientation with a cytoplasmic C-terminal domain and an extracellular N-terminal region (Figure 1). Within the secretory vesicle, these data suggest that APP traverses the membrane with its C-terminal domain located in the cytoplasm and the N-terminal region located within the lumen of the vesicles.

Previous studies indicate that soluble full-length APP is present within the lumen of secretory vesicles of adrenal medulla (known as chromaffin granules). In addition, it was demonstrated that a fraction of membrane APP can be released enzymatically upon incubation of CG membranes at pH 9 (*21*). In this study we examined whether this APP could be representing a distinct population with a different type of attachment to the membranes.

Protection of APP in Intact CG from Degradation by Trypsin. When intact CG were incubated with trypsin, under conditions where there was no lysis of CG (Table 1), it was revealed that a portion of APP, as detected by Western blots with R1, remained resistant to degradation by the protease (Figure 2A, lanes 3 and 4). The integrity of the CG was monitored by the presence of the intragranular marker (Met)-enkephalin in the incubation buffer (Table 1). In contrast, APP immunoreactivity (detected by R1) in deoxycholate-treated CG was abolished under the same conditions (Figure 2B, lanes 3 and 4). This is consistent with the existence of a population of APP that is transmembrane, with the C-terminal region extruding the granule and an additional

Table 1: Integrity of Intact Chromaffin Granules Monitored with the Intragranular Marker (Met)Enkephalin[a]

	(Met)enkephalin (pg/mL) in buffer after centrifugation of	
treatment	intact CG	deoxycholate-solubilized CG
4 °C, no trypsin (control)	46	333
25 °C, no trypsin (control)	36	425
25 °C, trypsin, 20 mg/mL	93	758
25 °C, trypsin, 4 mg/mL	86	566

[a] Intact CG or deoxycholate-solubilized CG were incubated at 25 °C with or without trypsin for 20 min (50 μL total volumne, as described in Materials and Methods). Samples were centrifuged at 15000*g* for 5 min at 4 °C to pellet intact CG; deoxycholate-solubilized CG samples were centrifuged similarly The resultant supernatants (with intact CG removed) were assayed for (Met)enkephalin, an intragranular CG marker, by radioimmunoassay, as described previously (*31*). (Met)enkephalin values represent averages from two experiments that varied by no more than 10%. Results show that CG remained intact during incubation with trypsin.

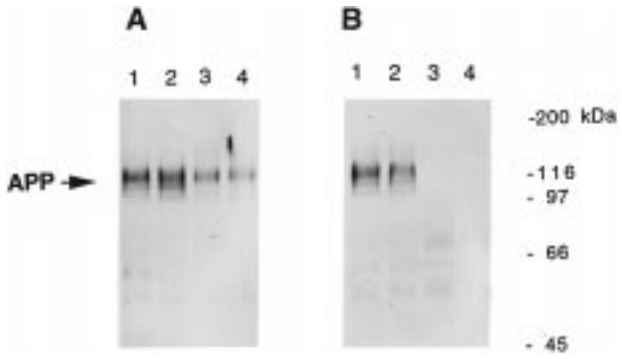

FIGURE 2: Protection of APP in intact CG from degradation by exogenous trypsin. Panel A: intact CG incubated with trypsin. Intact CG (in 0.32 M sucrose) were incubated at 4 or 25 °C alone (without trypsin, lanes 1 and 2, respectively), or with 20 and 4 μg/mL trypsin at 25 °C for 20 min (lanes 3 and 4, respectively). Incubates were subjected to Western blotting for APP using R1 antibody. Panel B: deoxycholate-solubilized CG incubated with trypsin. CG were solubilized in 1% deoxycholate (in the presence of 0.32 M sucrose) and incubated at 4 or 25 °C alone (without trypsin, lanes 1 and 2, respectively), or with 20 and 4 μg/mL trypsin at 25 °C for 20 min (lanes 3 and 4, respectively). Incubates were subjected to Western blotting for APP using R1 antibody.

population of APP residing within the interior of CG that is inaccessible to the action of the protease when CG are intact. This was also consistent with our previous findings of a population of soluble full-length APP in the lumen of CG.

[^{125}I]TID Labeling of CG APP. To further asses the topology of CG membrane APP and the origin of soluble APP, we labeled CG membranes with [^{125}I]TID. [^{125}I]TID is a reagent that partitions strongly in favor of the lipid phase of membranes, and the photogenerated carbene labels intrinsic membrane proteins in a highly selective manner. It has been used successfully to label the transmembrane domains of several proteins. These include Na−K ATPase (*36*), and the acetylcholine receptor (*32*, *37*).

Following [^{125}I]TID labeling of CG membranes, APP was released (solubilized) from membranes by incubation at pH 9. The distribution of APP between the released fraction and remaining membrane fraction was analyzed, by both immunoprecipitation/autoradiography (Figure 3A) and Western blotting (Figure 3B) using R1 antiserum. As shown in Figure 3A (lanes 1 and 2), CG membrane-anchored APP

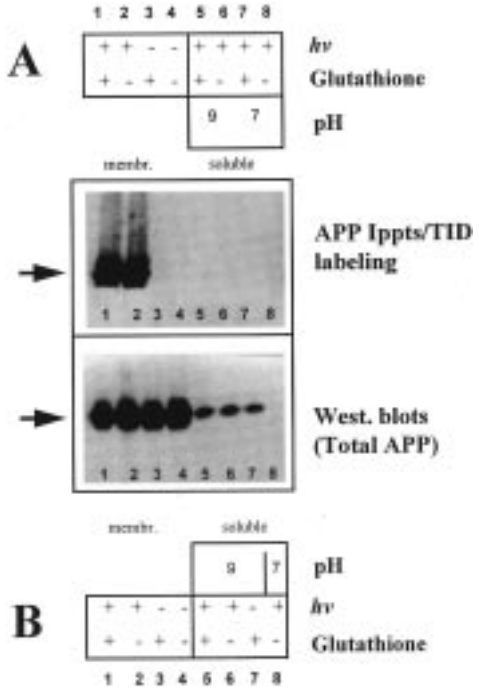

FIGURE 3: [^{125}I]TID-labeling demonstrating that released APP is not derived from transmembrane APP. Panel A: [^{125}I]TID labeling of transmembrane APP. CG membranes were equilibrated with [^{125}I]-TID in the absence (lanes 2, 4, 6, and 8) or presence (lanes 1, 3, 5, and 7) of 25 mM glutathione. Samples (except those in lanes 3 and 4) were irradiated at 365 nm. APP was immunoprecipitated by R1 antibodies and analyzed by SDS−PAGE and autoradiography. Following labeling of CG membranes with [^{125}I]TID, in vitro solubilization of APP from membranes was conducted (lanes 5−8). Lanes 1−4, CG membranes; lanes 5 and 6, solubilized proteins at pH 9; lanes 7 and 8, solubilized proteins at pH 7. The arrow shows full-length APP of approximately 130 kDa. Panel B: Release of APP from CG membranes is not affected by [^{125}I]TID labeling. Aliquots from the [^{125}I]TID-labeling experiment were also analyzed by Western blotting using R1. Samples were irradiated at 365 nm (except those in lanes 3, 4, and 7) in the absence (lanes 2, 4, 6, and 8) or presence (lanes 1, 3, 5, and 7) of 25 mM glutathione. APP was detected using R1 antibodies. Lanes 1−4, CG membranes; lanes 5−7, solubilized proteins from CG membranes at pH 9; lane 8, solubilized proteins from CG membranes at pH 7. Arrow shows full-length APP.

was labeled by [^{125}I]TID. Labeling was not reduced in the presence of 25 mM reduced glutathione (lane 1), which would scavenge [^{125}I]TID in the aqueous phase, indicating that the labeling was specific for intrinsic membrane proteins. Omission of photolysis, prevented [^{125}I]TID labeling (Figure 3A, lanes 3 and 4). In addition, the probe did not label soluble full-length APP as judged by the failure to detect any [^{125}I]TID-labeled APP in the CG soluble fraction by immunoprecipitation with R1 antibodies (data not shown), even though this fraction contains 20−25% of the total CG full-length APP (ref 21; also see Figure 8). Specificity of the probe for intrinsic membrane APP was also confirmed by its inability to label nexin II (N-terminal fragment generated from the action of α-secretase on APP, data not shown), purified from conditioned media from PC12 cells (Tezapsidis and Pangalos, unpublished observations). Further, [^{125}I]TID label was present in the CG membranes after release (not shown).

In agreement with previous observations, APP was released from CG membranes in vitro following incubation at pH 9. Densitometric analysis determined that this repre-

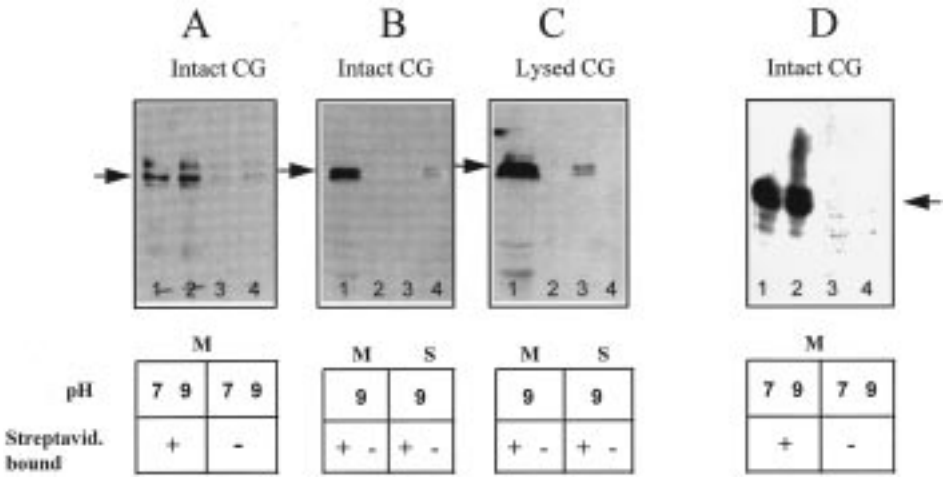

FIGURE 4: Biotin labeling of APP in intact CG. The labeling that APP released from membranes does not derive from transmembrane APP. Panel A: biotinylated and nonbiotinylated APP in intact CG. Intact CG (prepared as described in Materials and Methods) were biotinylated and then membranes were isolated. Detergent-solubilized biotinylated membrane proteins were captured with streptavidin-agarose and analyzed by Western blotting using R1 antibodies following their dissociation in LSB (lanes 1 and 2). Unbound to streptavidin fractions representing nonbiotinylated proteins were also analyzed as above (lanes 3 and 4). Before the purification with streptavidin, membranes were resuspended in buffers of pH 7 (lanes 1 and 3) or pH 9 (lanes 2 and 4). Arrow shows full-length APP. M, CG membranes. Panel B: in vitro released APP from CG membranes is not biotinylated in intact CG. Intact CG were biotinylated and then membranes were isolated and incubated at pH 9 for the release of APP, as described in Materials and Methods. Released proteins (lanes 3 and 4) and remaining membranes (lanes 1 and 2) were subjected to purification by streptavidin-agarose to obtain streptavidin-bound (+) and unbound (−) fractions. Samples were applied to 8% SDS−PAGE and probed by Western blotting using R1 antibodies. Lane 1 and 2, membranes after release; lanes 3 and 4, released proteins; lanes 1 and 3, streptavidin-bound fraction; lanes 2 and 4, streptavidin-unbound fraction. M, membranes after release; S, proteins released in vitro from CG membranes. Panel C: In vitro released APP from CG membranes is biotinylated in lysed (permeablized) CG. Lysed CG were biotinylated and then processed as described in panel B. Samples were applied to 8% SDS−PAGE and probed by Western blotting using R1 antibodies. Lanes 1 and 2, membranes after release; lanes 3 and 4, released proteins; lanes 1 and 3, streptavidin-bound fraction; lanes 2 and 4, streptavidin-unbound fraction. M, membranes after release; S, proteins release in vitro from membranes at pH 9. Panel D: membrane p65 is completely biotinylated in intact CG. Intact CG were biotinylated and processed as in panel B. Streptavidin-bound (lanes 1 and 2) and streptavidin-unbound (lanes 3 and 4) proteins were analyzed by Western blotting using anti-p65 antibodies Membranes were resuspended prior to the biotinylation in a pH 7 buffer (lanes 1 and 3) or a pH 9 buffer (lanes 2 and 4). M, membranes.

sented approximately 10% of membrane APP (Figure 3B, lanes 5−7), in agreement with previous results (*21*). There was no difference in the amount of APP released at pH 9 from membranes that were subjected to the labeling and photoactivation step (lanes 5 and 6 in Figure 3B), when compared to nonphotolyzed (unlabeled) controls (lane 7 in Figure 3B). This indicated that the labeling procedure itself, i.e., the binding of [^{125}I]TID to the transmembrane domain of APP, does not interfere with the mechanism of the in vitro solubilization of APP from CG membranes. Another CG type I membrane protein, p65 (*33*), was not released from the membranes under the same conditions (not shown), in agreement with Vassilacopoulou et al. (*21*), indicating that not all integral membrane proteins were subject to this release phenomenon.

Most interestingly, we were unable to detect release of [^{125}I]APP (representing transmembrane APP) at pH 9 (Figure 3A, lanes 5 and 6). This indicates the existence of a distinct population of APP that is not anchored via a transmembrane domain but is strongly associated to CG membranes.

Biotin Labeling of CG APP. To validate our conclusions from these findings, a different approach was employed. Transmembrane APP was labeled in intact CG using NHS-SS-biotin. This method has been successfully used in this laboratory for the labeling of cell-surface APP (*35*). Biotinylation of intact CG, successfully labeled transmembrane APP, as demonstrated in Figure 4A (lanes 1 and 2). The granules remained intact and impermeable to the probe during the procedure, as judged by the inability to detect any biotinylated APP (as detected by Western blotting using R1 antibody) in the CG soluble fraction (results not shown). This further supported the conclusion that only transmembrane APP was labeled during the procedure. Lanes 3 and 4 in Figure 4A illustrate that a fraction of membrane APP was not bound to streptavidin (this fraction was found in the flowthrough). However, when permeablized CG were used, all of the membrane APP was bound to the resin and none could be detected in the flowthrough (results not shown). These results indicate that the majority of membrane-associated APP in CG is transmembrane and that a small fraction is contained within the vesicle, representing membrane-associated, nontransmembrane APP, in agreement with the [^{125}I]TID labeling. In contrast, all of p65, which is a type I transmembrane protein, was biotinylated in intact granules (Figure 4D, lanes 1−2). Following biotinylation of intact or permeablized granules, membranes were prepared and then incubated at pH 7 or 9 for the in vitro release of full-length APP. Incubation of CG membranes at pH 9 resulted in the release of APP. The released APP was biotinylated (was bound to streptavidin) when membranes were derived from biotinylated permeablized CG (Figure 4C, lane 3) but was not biotinylated (streptavidin unbound fraction) when membranes were derived from biotinylated intact CG (Figure 4B, lane 4). These results confirmed those obtained with the [^{125}I]TID-labeling of CG membranes which suggest that the released population of APP is derived from membrane associated, nevertheless nontransmembrane APP.

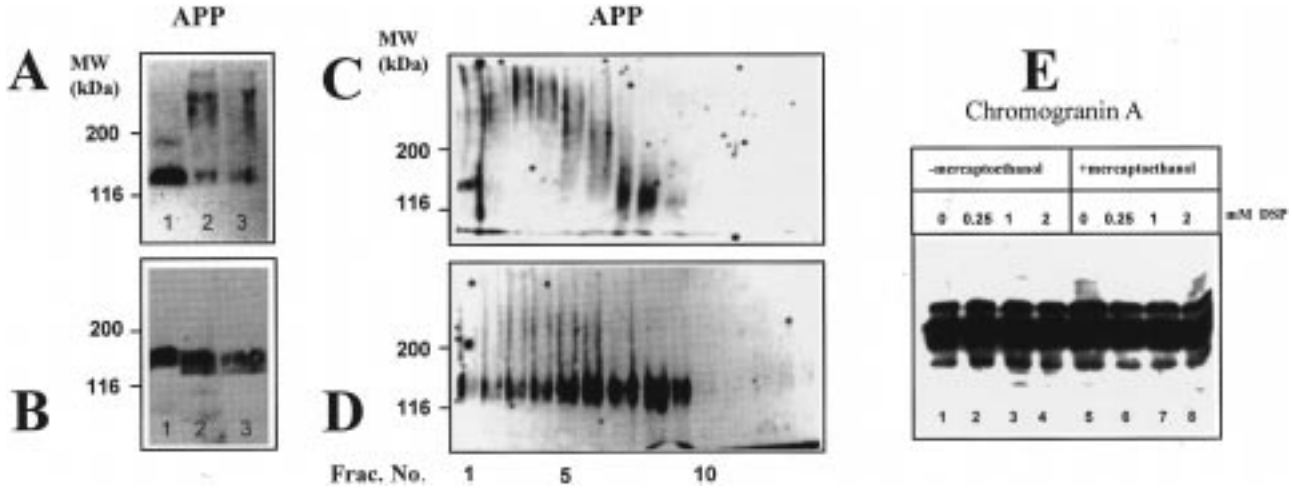

FIGURE 5: DSP cross-linking, sucrose gradient fractionation of CG APP complexes. Panel A: formation of high-molecular-weight APP complexes is DSP concentration dependent. CG membranes were cross-linked with 0.25, 1, and 2.5 mM DSP (lanes 1, 2, and 3, respectively) for 2 h at 4 °C after which the reaction was stopped by the addition of Tris to a final concentration of 50 mM and applied to PAGE in the absence of β-mercaptoethanol. Western blotting was performed with R1 antibodies. Panel B: cross-linking with DSP is reversible. As in panel A, but samples contained β-mercaptoethanol. Panel C: fractionation of DSP cross-linked APP complexes by sucrose gradient centrifugation, analysis by anti-APP western blots in the absence of β-mercaptoethanol. CG membranes were cross-linked with 1 mM DSP, solubilized in 1% Triton X-100, and subjected to ultracentrifugation on a 5−30% sucrose gradient. Fractions were collected from the bottom of the gradient tube (represented by fraction 1), and equal aliquots from each fraction were analyzed by SDS−PAGE in the absence of β-mercaptoethanol, followed by anti-APP western blots with the R1 antiserum. Panel D: fractionation of DSP cross-linked APP complexes by sucrose gradient centrifugation, analysis by anti-APP western blots in the presence of β-mercaptoethanol. Aliquots from the sucrose gradient fractions, described in panel C, were treated with β-mercaptoethanol, subjected to SDS−PAGE, and analyzed by anti-APP Western blots with the R1 antiserum. Panel E: chromogranin A does not become cross-linked with DSP. CG were cross-linked with 0 (lanes 1 and 5), 0.25 (lanes 2 and 6), 1 (lanes 3 and 7), and 2 mM (lanes 4 and 8) DSP. Samples were subjected to SDS−PAGE in the absence (lanes 1−4) or presence (lanes 5−8) of β-mercaptoethanol. Western blotting was performed with an anti-chromogranin A antibody (Incstar, Stillwater, MN).

In addition, they establish that this population resides within the lumenar surface of CG.

Chemical Cross-Linking. We subsequently investigated whether other proteins may be tightly associated with APP in CG serving as a membrane anchor for this unique population of APP. Chemical cross-linking of APP in CG was performed with the homobifunctional lipophilic DSP. This reagent can be cleaved by β-mercaptoethanol, liberating the individual complex components. Cross-linking resulted in the disappearance of the APP monomeric form in a DSP concentration-dependent manner (Figure 5A, lanes 1−3) concomitant with the formation of high-molecular-weight APP complexes. Inclusion of β-mercaptoethanol in the sample buffer results in the reappearance of monomeric APP (Figure 5B). Specificity of this probe for APP was demonstrated by the inability of DSP to cross-link chromogranin A (Figure 5E), a major CG protein with an apparent molecular weight of 45 000 (*46*). To partially purify the APP complexes, these were solubilized in 1% Triton X-100 and applied onto a 5−30% sucrose gradient. The high-molecular-weight complexes migrate at lower, more dense fractions of the gradient (Figure 5C, fractions 1−6). The monomeric APP band, migrating as a 130-kDa protein on SDS−PAGE, eluted at a position near the top of the sucrose gradient (Figure 5C, fractions 7−10). Addition of β-mercaptoethanol prior to electrophoresis converted the high-molecular-weight APP complexes to monomeric 130 000 APP (Figure 5D).

To visualize the molecular sizes of protein(s) complexed with APP by DSP, CG membranes from [^{35}S]methione metabolically labeled chromaffin cells were prepared. Cross-linking of these CG was performed with 0−2.5 mM DSP, followed by immunoprecipitation with R1 antibodies and

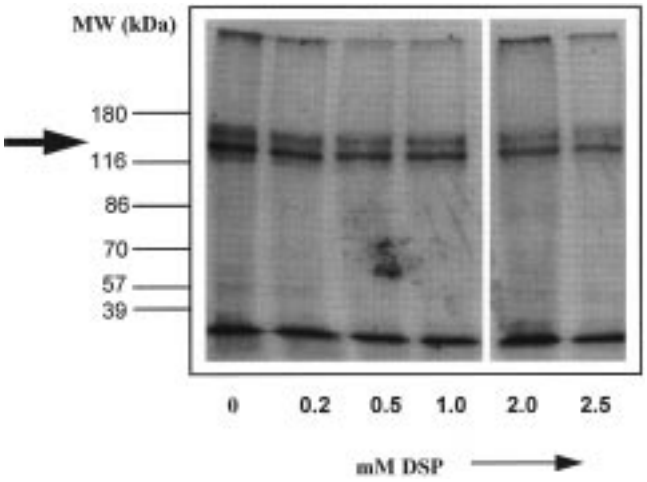

FIGURE 6: APP cross-linking in CG membranes from metabolically labeled chromaffin cells. CG membranes were prepared from metabolically labeled chromaffin cells as described in Materials and Methods. These membranes were then cross-linked with 0.25−2.50 mM DSP. Following solubilization in SDS, APP and APP complexes were immunoprecipitated using R1 antibodies. Immunoprecipitates were applied to 7% SDS gels in the presence of β-mercaptoethanol and analyzed by autoradiography. The arrow indicates full-length APP as a 130-kDa protein.

autoradiography (Figure 6). At all concentrations, only the ^{35}S band of 130-kDa APP was detected without the gradual appearance (with increasing DSP concentration) of new radiolabeled protein(s). Increasing concentrations of the cross-linker did not alter the levels of the immunoprecipitated material, indicating that the antibodies recognize both the cross-linked APP complexes and monomeric APP with the same affinity. This was the case even at the highest cross-

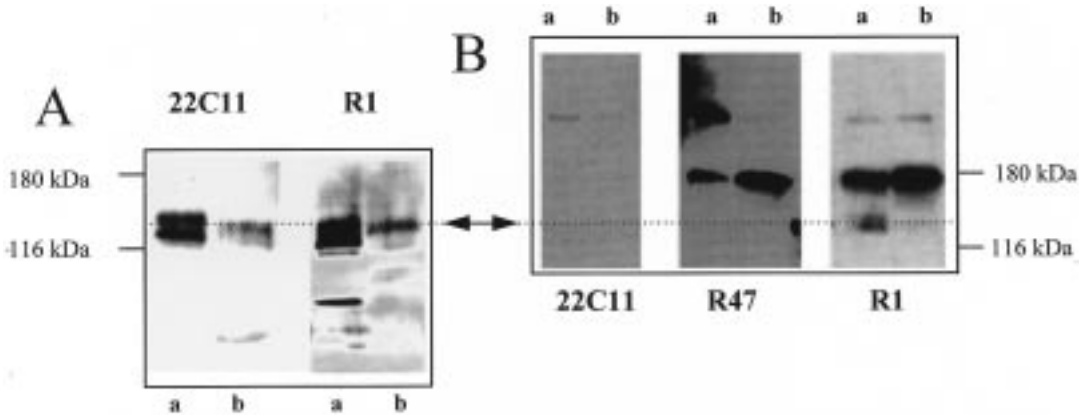

FIGURE 7: APP presence in the lumen (soluble component) and membranes of CG as a complex, sensitive to β-mercaptoethanol. CG membranes (lanes a) and CG lumen (lanes b), prepared as described in Materials and Methods, were subjected to 6% PAGE in the presence (panel A) or the absence (panel B) of β-mercaptoethanol. Western blotting was performed with antibodies 22C11, R47, or R1. Membranes were resuspended in half the volume of the original CG extract, and equal volumes were applied from the membrane and soluble fractions (25 μL). The protein content was 250 μg for the soluble and 125 μg for the membrane fraction. Double arrowhead and dotted line indicate the migration of monomeric APP on both Western blots.

linker concentration used (2.5 mM DSP) at which no monomeric APP exists. A decrease in monomeric APP recovery by immunoprecipitation from samples with increasing cross-linker concentration would have suggested a weaker interaction of the antibodies with the complex, either due to cross-linking of APP at its C-terminus (the epitope for R1) or general steric hindrance. This result suggests that APP forms DSP complexes with itself.

Interestingly, during the cross-linking experiments it was observed that in the control samples, which did not contain either DSP or β-mercaptoethanol, R1-reactive high-molecular-weight complexes could be detected (Figure 7). These complexes were disrupted totally under reducing conditions, indicating that these complexes were formed through disulfide bridges. This was further investigated with two additional anti-APP antibodies, 22C11, directed against the N-terminus of APP (amino acids 60−100), and R47, directed against Aβ1−16 (Figure 7; see also Figure 1). Monomeric APP was recognized by 22C11, R1, and R47 (the later not shown) as a 130-kDa protein when CG fractions (membrane or soluble) were applied to SDS−PAGE in the presence of β-mercaptoethanol. Under nonreducing conditions, 22C11 and R47 antibodies recognize on Western blots a high-molecular-mass complex of APP migrating near 180 kDa. However, 22C11 antibody, could not recognize the complex (Figure 7B) found in both membrane and soluble fractions (Figure 7A). This may be the result of epitope masking, a conformational change created by the formation of disulfide bridges within the cysteine-rich region of APP (Figure 1), which contains the epitope recognized by the 22C11 antibody. The APP immunoreactivity found in CG, both in the membranes and the soluble fraction corresponding to this high-molecular-weight complex is converted in its entirety to monomeric APP in the presence of β-mercaptoethanol, implicating solely the involvement of disulfide bridges in its formation (Figure 7A). It is noted that in several experiments R1 and R47 antibodies also recognized monomeric APP when CG extracts were subjected to SDS−PAGE in the absence of β-mercaptoethanol. Overall, however, the majority of immunoreactivity was associated with high-molecular-weight complexes.

DISCUSSION

The results presented in this report strongly support and extend our original observation of the existence of a unique, soluble form of full-length APP in the lumen of CG (*21*). Full-length APP has also been detected as a soluble protein in a specific microsomal fraction from PC12 cells (*24*). These soluble forms of APP are distinct from the more extensively characterized, α-secretase generated, C-terminal-truncated soluble APP (*38*, *39*). Of particular interest was our finding that, in CG, membrane-associated APP can be released (solubilized) at basic pH through an enzymatic mechanism, providing an insight in the mechanism of biosynthesis of soluble APP. In this study, we examined whether the immediate precursor of soluble APP in our in vitro studies maintained the widely accepted transmembrane topology of APP. Interestingly, our results indicate that soluble APP may derive from a novel population of membrane-associated, nevertheless nontransmembrane APP. It appears that the majority of APP in CG has the topology of a transmembrane protein, since trypsinization of intact granules results in the digestion of a large portion of R1 immunoreactivity (directed against the C-terminal of APP). This later result is also direct evidence for the orientation of transmembrane APP in CG with the C-terminal end extruding from CG and the large N-terminal domain residing within. Not all R1 immunoreactivity in intact CG was digested by trypsin, confirming the existence of an intragranular pool of full-length APP, inaccessible to the protease. There is now evidence to suggest that a certain population of membrane-associated APP does not traverse the membrane (see Figure 8 for a diagrammatic representation). Previous work (*21*, *24*) showed that the amount of APP released from the membranes at pH 9 was saturable; no further release was observed even after prolonged incubations. This is in perfect agreement with our present data: only a fraction of membrane-associated APP can serve as a precursor for the releasable form of APP, and this subpopulation is nontransmembrane. It cannot be labeled by [^{125}I]TID and it cannot be biotinylated in intact CG.

Currently, we can only speculate that this newly discovered membrane orientation of APP in neurosecretory vesicles may

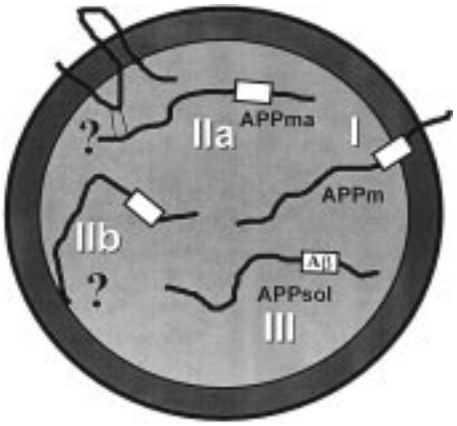

FIGURE 8: Proposed model of APP topology in CG. This study, combined with our previous investigations (*21*, *25*) demonstrate that in CG (the neurosecretory vesicles of chromaffin cells), full-length APP exists as three different populations: transmembrane (APPm, I), membrane-associated nontransmembrane (APPma, II), and soluble (APPsol, III). The majority of APP (70−80%) in CG exists as a transmembrane species (I, APPm) with the short C-terminal region exposed to the cytoplasmic surface and the N-terminus in the lumen. A population of membrane-associated APP (∼10%) does not traverse the membrane (APPma), yet it may be attached to membranes through an anchor (IIa, IIb). One possibility is that APPma is attached to another membrane protein through disulfide bridges (this protein may be transmembrane APP (APPm), IIa). Another possibility is that APPma is attached directly to the lumenar surface of the membranes through its N-terminus (IIb). A membrane-bound enzymatic activity, sensitive to thiol and serine protease inhibitors, possibly a protease, can release APPma from the membranes to produce APPsol (III). Both APPma and APPsol presumably do not have the C-terminal region of Aβ buried within the lipid bilayer. Secretion of granular contents in the extracellular space results in the release of APPsol.

be the result of differential sorting of APP early in the secretory pathway, similar to the mechanism of membrane translocation of prion protein during its biogenesis (*40*). The tight association of proteins to plasma or subcellular membranes other than through their intrinsic imbedding in the membranes is not an unusual phenomenon. For instance, proteins are known to be linked to membranes through a number of lipid anchors (*41*) including glycosyl phosphatidylinositol attachment, palmitoylation, and myristoylation. In combination, the protease inhibitor sensitivity of the enzymatic activity that we identified as being responsible for the release of APP from CG membranes and the slightly faster migration rate on SDS gels of the soluble APP (Figure 7; see also ref 25) are consistent with the following scheme: The solubilization of APP from the CG membranes may be due to a membrane-bound protease that cleaves nontransmembrane APP at a site near its N-terminus, C-terminally to an anchor. This hypothesis is currently being tested experimentally.

We decided to investigate whether other CG membrane proteins can serve as anchor(s). The chemical cross-linking experiments show that APP may be closely associated with other proteins in CG. The complexes formed by DSP were readily cleaved by treatment with β-mercaptoethanol and could be resolved on a sucrose gradient. However, the DSP cross-linked APP complexes in metabolically radiolabeled CG appeared to contain only APP. This suggested that APP may be cross-linked to itself. Other possibilities could be that APP is cross-linked to another protein which is indistinguishable from APP by SDS−PAGE. However, clear increase in the labeled immunoprecipitable material with increasing DSP concentration was not observed. Alternatively, it could be that APP is cross-linking to a protein with a very low metabolic rate not allowing its sufficient labeling or that APP is cross-linking to a number of proteins. However, longer exposures of the autoradiographs did not support such possibilities. Formation of APP aggregates has been reported to occur in vitro (*42*) and it is possible that APP does exist as a polymer. The mechanism by which this may take place in vivo has not been determined. One possibility for self-association of APP is the formation of disulfide bridges between APP molecules, connecting the cysteine-rich regions at their N-termini. Indeed, recently, a peptide corresponding to 139−149 aa of APP751, containing a single cysteine, could be dimerized following oxidation in vitro by Cu^{2+} (*43*). This implies that full-length APP has the potential to dimerize under certain conditions. Our finding of a high-molecular-weight, β-mercaptoethanol-sensitive APP immunoreactive species in the CG membrane and soluble fractions is additional proof that APP may be associated with another protein (maybe another APP) through disulfide bridges.

A strong implication of the existence of soluble APP is that this may be the direct precursor of Aβ and further that this conversion occurs within the neurosecretory vesicles. As soluble cytoplasmic tail-containing APP can be stored in normal CG (*21*) and released by regulated exocytosis (*25*), this may have physiological implications relevant to AD. The ability of cholinomimetics to stimulate the release of full-length APP (*25*) may be related to the observed decrease of Aβ levels under identical conditions (*44*). Stimulated secretion of full-length APP in neurons may result in reduced proteolytic processing of APP and production of Aβ. Inhibition of the stimulus (as would be the case with degenerating cholinergic neurons in AD) would prolong the processing of APP in the vesicular lumen and increase the production of Aβ. The hypothesis that Aβ is produced intracellularly rather than extracellularly is supported by the detection of this peptide or its intermediate-size precursors within cells and secretory vesicles (*15*, *16*, *21*).

So far, there is no direct evidence to show a substrate/product relationship between soluble full-length APP and Aβ. However, in support of this notion is the observation that the FAD-linked mutation Val717-to-Ile on APP751 causes both an increase in the levels of Aβ1−42 in the conditioned media of cells transfected with this mutant APP (*45*) and an increase in the levels of soluble full-length APP (*22*). If indeed soluble APP is the precursor of Aβ, inhibition of APP release from neurosecretory vesicle membranes into the lumen can be a possible novel target for preventative and/or therapeutic drugs for AD patients. On the other hand, if APP has a beneficial biological function as a holoprotein rather than as a precursor for neurotoxic Aβ, stimulation of its release from subcellular membranes and neurons (see above) may be of interest.

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