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Generation of β-amyloid in the secretory pathway in neuronal and nonneuronal cells

(amyloid precursor protein/Golgi complex/lysosome/astrocyte/Alzheimer disease)

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ABSTRACT **The cellular mechanism underlying the generation of β-amyloid in Alzheimer disease and its relationship to the normal metabolism of the amyloid precursor protein are unknown. In this report, we show that 3- and 4-kDa peptides derived from amyloid precursor protein are normally secreted. Epitope mapping and radiolabel sequence analysis suggest that the 4-kDa peptide is closely related to full-length β-amyloid and the 3-kDa species is a heterogeneous set of peptides truncated at the β-amyloid N terminus. The β-amyloid peptides are secreted in parallel with amyloid precursor protein. Inhibitors of Golgi processing inhibit secretion of β-amyloid peptides, whereas lysosomal inhibitors have no effect. The secretion of β-amyloid-related peptides occurs in a wide variety of cell types, but which peptides are produced and their absolute levels are dependent on cell type. Human astrocytes generated higher levels of β-amyloid than any other cell type examined. These results suggest that β-amyloid is generated in the secretory pathway and provide evidence that glial cells are a major source of β-amyloid production in the brain.**

The β-amyloid protein is a cleavage product of the amyloid precursor protein (APP) that accumulates at high levels in the brain in Alzheimer disease, Down syndrome, and some normal elderly persons (1–3). Two major pathways of APP processing have been identified. Normal processing of APP in the secretory pathway occurs by a proteolytic cleavage within the β-amyloid sequence generating a large secreted form of the protein (4) and a smaller membrane-associated C-terminal fragment (5, 6). A second pathway of APP metabolism has been identified in the endosomal–lysosomal system resulting in larger potentially amyloidogenic C-terminal fragments of APP (7–9).

Recent reports have shown that β-amyloid-related peptides are normally secreted by cultured cells and can be detected in human cerebrospinal fluid (10–12). However, the cellular mechanism of this processing event is unknown. Here we show that soluble β-amyloid peptides are generated in the APP secretory pathway rather than from the degradation of APP in lysosomes. The proteolytic cleavages that give rise to the secreted β-amyloid peptides are regulated in a cell-type-specific manner. Analysis of β-amyloid secretion in primary cortical cultures suggests that astrocytes may be a significant source of β-amyloid in the brain.

MATERIALS AND METHODS

Metabolic Labeling and Immunoprecipitation. An APP_{695} expression plasmid under the control of the cytomegalovirus promoter was constructed in the pcDNA1 vector (Invitrogen, San Diego). APP_{695} was overexpressed in COS-1 cells by transient transfection using the DEAE-dextran method (13). For steady-state experiments, 3×10^6 COS cells per 10-cm plate were labeled 48 hr after transfection with [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq) in methionine-free Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum for 16 hr at 37°C. For pulse–chase experiments, transfected COS cells were labeled with [^{35}S]methionine for 30 min, rinsed twice with phosphate-buffered saline, and then incubated with medium containing a 3-fold excess of unlabeled methionine. Labeled cells were harvested in RIPA lysis buffer (1% Triton X-100/1% sodium deoxycholate/0.1% SDS/150 mM NaCl/50 mM Tris·HCl, pH 7.2) in the presence of protease inhibitors [leupeptin (5 μg/ml)/aprotinin (10 μg/ml)/antipain (50 μg/ml)/pepstatin (5 μg/ml)/phenylmethylsulfonyl fluoride (100 μg/ml)] and phosphatase inhibitors (50 mM imidazole/50 mM potassium fluoride/25 mM β-glycerophosphate/100 μM sodium orthovanadate). Supernatants and lysates of transfected cells were clarified by centrifugation at 3000 × *g* for 20 min and 100,000 × *g* for 60 min, respectively, and then incubated with primary antibody for 4 hr at 4°C followed by immunoprecipitation with protein A-Sepharose (Pharmacia) for 12 hr at 4°C. The Sepharose beads were washed six times, resuspended in 2× reducing SDS sample buffer, and boiled for 3 min. The immunoprecipitated proteins were analyzed by SDS/PAGE on a 10% gel or by Tris–Tricine SDS/PAGE on a 10–20% gel (14) and fluorography with EN^{3}HANCE (New England Nuclear). The β1-40 antibody (B3) (1:70 dilution in lysates and 1:280 dilution in supernatants) is a rabbit antiserum raised to unconjugated synthetic β1-40 peptide corresponding to residues 597–636 of APP_{695}. The β28-40 antibody (1:125 dilution in lysates and 1:500 dilution in supernatants) is a rabbit antiserum raised against β28-40 peptide conjugated to keyhole limpet hemocyanin (15). The β8-17 antibody (1:40 dilution in lysates and 1:160 dilution in supernatants) is a mouse monoclonal antibody (16). The APP676-695 antibody (1:500 dilution in lysates and 1:2000 dilution in supernatants) is a rabbit antiserum raised against the synthetic APP676-695 peptide (17) conjugated to the purified protein derivative (PPD) of tubercle bacillus. The mouse monoclonal antibody α(APP511-608) (Alz-90; Boehringer Mannheim; 1:50 dilution in lysates and 1:200 dilution in supernatants) is immunoreactive with secreted and cellular APP (4). For peptide preabsorption controls, the antiserum was preincubated with 20 μg of the peptide to which the antibody was raised for 16 hr at 4°C.

Radiolabel Microsequencing. Immunoprecipitated [^{35}S]methionine-labeled proteins were resolved by 10–20% Tris–Tricine SDS/PAGE and then electroblotted to poly(vinylidene difluoride) membrane (Millipore) (18). The radiolabeled

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Abbreviation: APP, amyloid precursor protein.

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band was identified by autoradiography, excised from the blot, and placed in an Applied Biosystems model 475 gas-phase sequencer. The anilinothiozolinone amino acids were collected at each cycle into a fraction collector and the radioactivity was determined by liquid scintillation counting. Placement of the labeled methionine residue was made on the basis of the known primary sequence of APP_{695} (17) and epitope mapping data.

Primary Cell Culture. Primary cultures were established as described (19) from the cortex or hippocampus of embryonic day 18 rat embryos or from the cortical tissue of human fetal abortuses of 16–17 weeks of gestation. The protocol for tissue procurement complied with Federal and institutional guidelines for fetal research. Neuronal cultures without astrocytes were obtained by treatment of mixed cultures with 5 μM cytosine arabinoside for 24 hr on days 2 and 6 after plating. Astrocyte cultures were obtained as described (20) by plating cells without polylysine and removal of nonadherent cells 12 hr after plating. Immunocytochemical staining showed that neuronal cultures were >99% positive for neuronal microtubule-associated protein 2 and astrocyte cultures were positive for glial fibrillary acidic protein. Primary cells were used after 12 days in culture and were metabolically labeled for 16 hr at 37°C.

RESULTS

Secretion of Soluble β-Amyloid. The normal cleavage products of APP were analyzed in COS cells, which express high levels of APP after transfection with an APP expression plasmid. Immunoprecipitation of cell lysates and analysis by Tris–Tricine SDS/PAGE in 10–20% gels (14) to resolve low molecular weight proteins revealed the major 9-kDa C-terminal fragment derived from constitutive cleavage of APP (Fig. 1*A*) (6). A less-abundant C-terminal fragment of 11.5 kDa was also detected that was immunoreactive with antibodies to β-amyloid, including antibodies to the N-terminal and C-terminal domains of β-amyloid (Fig. 1*A*), suggesting that this fragment is potentially amyloidogenic.

Immunoprecipitation of proteins secreted by transfected COS cells revealed two unique APP-derived peptides of ≈3 and ≈4 kDa that were not detectable in cell lysates (Fig. 1*A*). These peptides were substantially enriched in the medium of cells transfected with either APP_{695} (Fig. 1*A*) or APP_{751} (data not shown) expression constructs relative to nontransfected controls. The 3- and 4-kDa secreted peptides were immunoprecipitated by antibodies to full-length β-amyloid [$\alpha(\beta 1\text{-}40)$] and the β-amyloid C terminus [$\alpha(\beta 28\text{-}40)$] corresponding to part of the transmembrane domain of APP (Fig. 1*A*). Preabsorption of these antibodies with synthetic β-amyloid (β1-40) abolished the immunoprecipitation of the 3- and 4-kDa peptides. A monoclonal antibody to the domain of β-amyloid N-terminal to the APP constitutive cleavage site [$\alpha\beta(8\text{-}17)$] selectively immunoprecipitated the 4-kDa peptide but not the 3-kDa peptide (Fig. 1*A*). Thus, the 4-kDa peptide contains the N-terminal and C-terminal domains of β-amyloid whereas the 3-kDa peptide is truncated at the N terminus. Synthetic β1-40 peptide resolved in the same gel system comigrated with the 4-kDa secreted peptide (Fig. 1*B*). The 3- and 4-kDa peptides were not immunoreactive with an antibody to the APP C-terminal domain (Fig. 1*A*).

The identities of the 3- and 4-kDa peptides were confirmed by microsequence analysis of the radiolabeled immunoprecipitated peptides (8). The secreted 4-kDa peptide showed a primary labeled methionine peak at residue 3 and a secondary peak at residue 6 (Fig. 2*A*), suggesting a major 4-kDa species with an N terminus at Val^{-3} (APP_{695} residue 594) and a minor species with an N terminus at Ile^{-6} (APP_{695} residue 591). The major 3-kDa species had an N terminus at Leu^{17} (APP_{695} residue 613; Fig. 2*B*) corresponding to the APP constitutive cleavage site (6). Several distinct secondary 3-kDa species were also detected as well as a staggered N terminus extending from Gly^{9} to Val^{24}, consistent with the broad electropho-

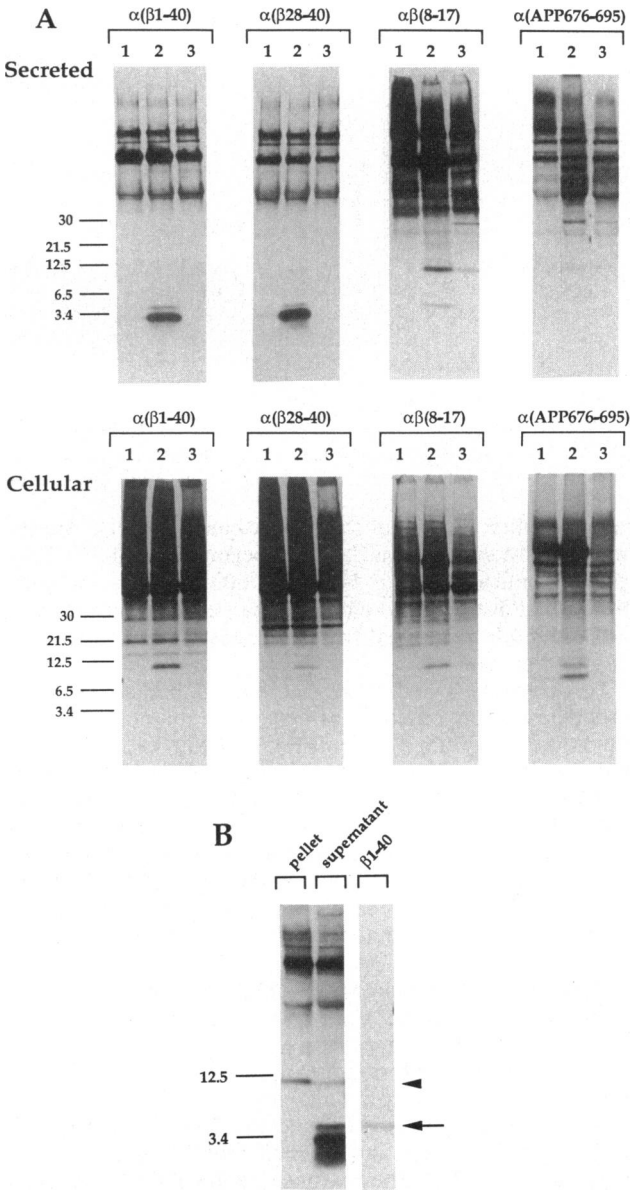

FIG. 1. Secreted and cellular peptides derived from APP. (*A*) Immunoprecipitation of cell-conditioned medium and cell lysates. Lanes: 1, nontransfected COS cells; 2, COS cells overexpressing βAPP_{695} after transfection; 3, transfected COS cells immunoprecipitated with antibody preabsorbed with synthetic β1-40 peptide [$\alpha(\beta 1\text{-}40)$, $\alpha(\beta 28\text{-}40)$, and $\alpha\beta(8\text{-}17)$ antibodies] or APP676-695 peptide [α(APP676-695) antibody], as indicated. Preabsorption of β-amyloid antibodies with β1-40 synthetic peptide abolishes immunoprecipitation of the 3- and 4-kDa secreted peptides. Preabsorption of αAPP676-695 antibody with APP676-695 peptide abolishes immunoprecipitation of the cellular 9- and 11.5-kDa proteins. (*B*) The 3- and 4-kDa β-amyloid peptides are soluble. Synthetic β1-40 peptide resolved in the same gel system and stained by Coomassie blue comigrates with the 4-kDa secreted peptide (arrow). The 11.5-kDa C-terminal fragment (arrowhead) is recovered primarily in the insoluble (pellet) fraction. Centrifugation of labeled medium from transfected COS cells was performed at 100,000 × *g* for 6 hr at 4°C. The pellet was resuspended in RIPA lysis buffer and the pellet and supernatant fractions were immunoprecipitated with the β1-40 antibody. Solubilization of the pellet in 35% (vol/vol) acetonitrile followed by dilution gave the same result. (*A* and *B*) Molecular masses in kDa are shown.

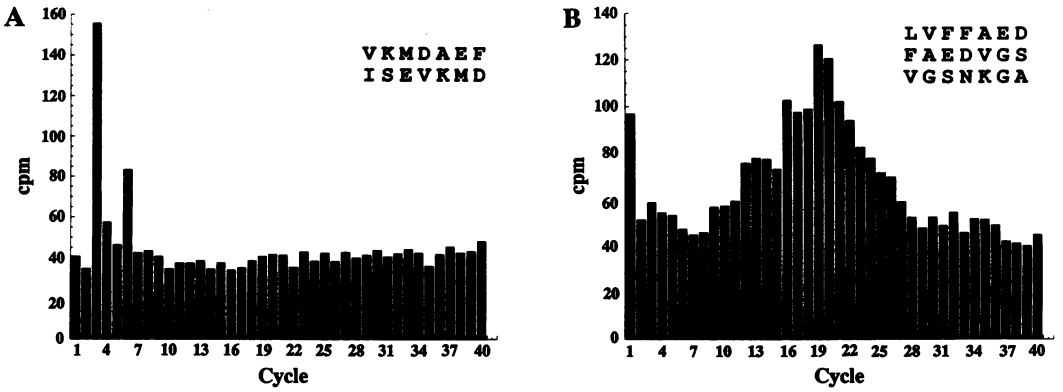

FIG. 2. Radiolabel sequence analysis of the secreted β-amyloid-related peptides. (*A*) The 4-kDa peptide. (*B*) The 3-kDa peptide. Immunoprecipitated protein labeled with [^{35}S]methionine was isolated on poly(vinylidene difluoride) membranes and sequenced. Shown is [^{35}S]methionine radioactivity (cpm) at each sequencer cycle. Predicted major and minor N-terminal sequences are shown.

retic mobility pattern of the 3-kDa band (Fig. 1). Another minor 3-kDa species was detected beginning at Met^{35}. These results confirm that the 4-kDa peptide contains the entire N-terminal domain of β-amyloid and suggest that the major 3-kDa peptide is derived from the constitutive "secretase" cleavage of APP.

To determine whether the secreted 3- and 4-kDa βAPP peptides were released from cells in a soluble form, cell-conditioned medium was centrifuged at 100,000 × *g* for 6 hr, and the pellet and supernatant were analyzed by immunoprecipitation with the β1-40 antibody. The 3- and 4-kDa peptides were recovered entirely in the supernatant fraction, suggesting that the released peptides were in a soluble form (Fig. 1*B*). The 11.5-kDa C-terminal fragment of APP detected in cells was also detected in conditioned medium and was predominantly associated with the insoluble fraction (Fig. 1*B*).

Generation of β-Amyloid in the APP Secretory Pathway. To determine whether the 3- and 4-kDa amyloid peptides are derived from the secretory or endosomal–lysosomal pathways, the kinetics of formation of the peptides and effects of metabolic inhibitors were examined. Pulse–chase kinetic analysis showed that the secreted peptides rapidly appeared in the culture medium and reached peak levels after 1 hr of chase time (Fig. 3*A*). The rate of secretion of the 3- and 4-kDa peptides closely paralleled the rate of secretion of the 93- to 97-kDa secreted forms of APP_{695} and the formation of the cell-associated 9-kDa C-terminal APP fragment (Fig. 3 *A* and *B*). Thus, the kinetics of generation of the 3- and 4-kDa peptides are similar to that of the secreted form of APP.

The effects of lysosomal inhibitors were examined to determine whether lysosomal processing of APP is required for generation of the secreted β-amyloid peptides. Pulse–chase labeling was used to examine the effects of inhibitors after a 2-hr incubation, the time of maximal β-amyloid secretion (Fig. 3*A*). The lysosomal inhibitors chloroquine, ammonium chloride, and leupeptin did not inhibit the secretion of the 3- and 4-kDa peptides (Fig. 3*C*). This result was confirmed by quantitation of the secreted 4-kDa peptide by using a PhosphorImager. Pulse–chase analysis demonstrated that chloroquine and ammonium chloride did not significantly affect the initial or later stages of secretion of β-amyloid (Fig. 4). Analysis of the cell lysates showed that chloroquine, ammonium chloride, and leupeptin inhibited lysosomal degradation of C-terminal fragments of APP, indicating that the inhibitors were active (Fig. 3*D*).

We examined the effect of monensin, an ionophore that inhibits the processing of proteins in the Golgi complex. Monensin almost completely blocked the secretion of the 3- and 4-kDa peptides (Fig. 3*C*). Pretreatment with brefeldin A, which prevents transport of secretory and membrane proteins in the Golgi, completely blocked the secretion of the 3- and 4-kDa peptides (data not shown). Thus, inhibition of the processing of APP in the secretory pathway prevents the generation of β-amyloid peptides, whereas inhibition of lysosomal processing has no effect. These results with the parallel kinetics of secretion of APP and β-amyloid peptides suggest that soluble β-amyloid peptides are generated in the secretory pathway and not in lysosomes.

Regulation of β-Amyloid Secretion in Neurons and Astrocytes. Primary cultures of neurons and astrocytes were established from fetal rat and human cerebral cortex. Neurons, astrocytes, and mixed cortical cultures secreted both the 4- and 3-kDa amyloid peptides (Fig. 5*A*). Neurons derived from rat cortex and hippocampus secreted significantly higher levels of the 3-kDa truncated β-amyloid peptide than astrocytes. PhosphorImager analysis showed that the molar ratio of the 3-kDa to 4-kDa peptides was 4.7-fold greater for rat cortical neurons than for rat cortical astrocytes ($P < 0.001$; Fig. 5*A*). Human astrocytes secreted very high levels of the 4-kDa β-amyloid peptide relative to the other cell types examined (Fig. 5*A*). In addition, human astrocytes secreted 3- to 4-fold higher levels of 4-kDa β-amyloid than rat astrocytes.

Secretion of the amyloid peptides was also examined in continuous cell lines of neuronal and glial origin. The M17 human neuroblastoma and PC12 rat pheochromocytoma cell lines exhibited a pattern of secretion similar to that of primary neurons with secretion of higher levels of the 3-kDa peptide than 4-kDa peptide (Fig. 5*B*). Two cell lines established from human gliomas showed a pattern similar to that of primary astrocytes with predominant secretion of the 4-kDa peptide. Other cell types including rat and human skin fibroblasts (Fig. 5*A*), COS cells (Fig. 1*A*), and hepatoma and olfactory epithelial cells (data not shown) generated lower levels of β-amyloid. These results suggest that the processing of APP to β-amyloid peptides is cell-type-dependent and also indicate that astrocytes may be a major source of β-amyloid in the brain.

DISCUSSION

These results suggest that β-amyloid is a normal secretory product of cells that express APP consistent with other recent reports (10–12). Our findings suggest that β-amyloid is generated in the APP secretory pathway in the Golgi complex, at the cell surface, or from a secreted precursor. The inability to inhibit β-amyloid secretion with lysosomal inhibitors and the parallel kinetics of secretion of β-amyloid and APP suggest that lysosomal processing is not required for the

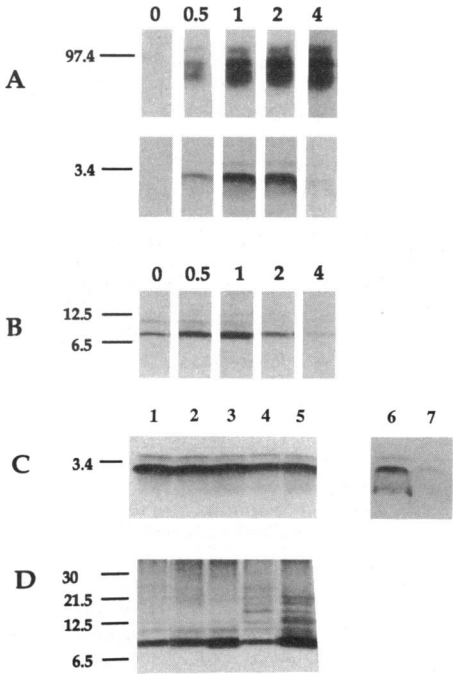

FIG. 3. Kinetics of secretion of amyloid peptides and effects of inhibitors of lysosomal and Golgi processing. (*A*) Pulse–chase kinetic analysis of secreted APP (*Upper*) (10% gel, SDS/PAGE; 3-day exposure) and secreted 3- and 4-kDa peptides (*Lower*) (10–20% gel, Tris–Tricine SDS/PAGE; 3-day exposure). (*B*) Cellular 9-kDa C-terminal fragment (10–20% gel, Tris–Tricine SDS/PAGE; 5-day exposure). Note that the rates of secretion of APP and the 3- and 4-kDa peptides are similar. Immunoprecipitation was performed with APP511-608 antibody to resolve secreted APP, β1-40 antibody to resolve the secreted 3- and 4-kDa peptides, and APP676-695 antibody to resolve the cellular 9-kDa APP fragment. Lane labels in *A* and *B* are chase time in hours. (*C*) Lysosomal inhibitors do not inhibit the generation of the 3- and 4-kDa peptides. COS cells were pulse-labeled with [^{35}S]methionine for 30 min and then incubated in unlabeled medium for 2 hr in the absence (control; lanes 1 and 6) or presence of either 1 or 10 mM ammonium chloride (lanes 2 and 3, respectively), leupeptin (20 μg/ml; lane 4), chloroquine (80 μg/ml; lane 5), or 10 μM monensin (lane 7). Shown are the 3- and 4-kDa peptides immunoprecipitated from 2 ml of conditioned medium with the β1-40 antibody. (*D*) Lysosomal inhibitors prevent the degradation of cellular C-terminal fragments of APP. Shown are immunoprecipitates of cell lysates with the α(APP676-695) antibody after the treatments described in *C*. Note that the level of the 9-kDa fragment is increased by chloroquine and ammonium chloride. Additional higher molecular weight C-terminal fragments are increased by chloroquine and to a lesser extent by leupeptin. (*A*–D) Molecular masses in kDa are shown.

generation of soluble β-amyloid. It has been reported that ammonium chloride inhibits β-amyloid secretion when used at higher concentrations and for longer incubation periods than used in this study (12). However, this may have been due to an inhibitory effect of ammonium chloride on Golgi processing after long incubations (21). Potentially amyloidogenic fragments of APP have been detected in lysosomes (7–9). It remains to be determined whether under some circumstances these fragments can be processed to β-amyloid.

The 4-kDa and 3-kDa β-amyloid peptides represent a heterogeneous set of secreted peptides based on radiolabel sequencing. The 4-kDa peptide includes at least three closely related species beginning at Asp^{1} (10–12), Val^{-3} (10), and Ile^{-6}. The 3-kDa species is a more heterogeneous array of peptides with the major peptide beginning at the APP constitutive cleavage site. Our inability to detect the particular 4-kDa species that begins at Asp^{1} may reflect a limitation of

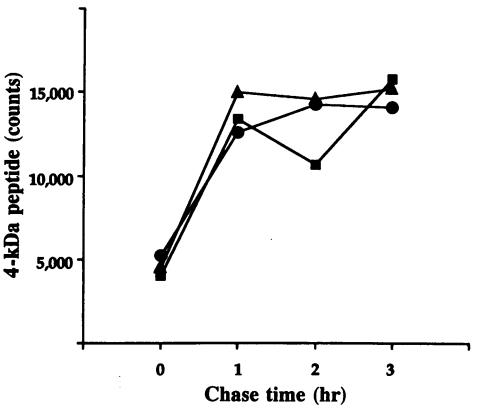

FIG. 4. Quantitative analysis of the secretion of β-amyloid in the presence of lysosomal inhibitors. COS cells were pulse-labeled with [^{35}S]methionine for 1 hr in the absence (control; ●) or presence of chloroquine (80 μg/ml; ■) or 10 mM ammonium chloride (▲) and then chased in unlabeled medium in the presence or absence of the inhibitors for the indicated times at 37°C. Values shown represent the 4-kDa β-amyloid peptide immunoprecipitated with β1-40 antibody and quantitated using a PhosphorImager (Molecular Dynamics). PhosphorImager counts represent an arbitrary unit measured under standardized conditions.

radiolabel sequencing using [^{35}S]methionine. This method would produce a peak at sequencer cycle 35 for the Asp^{1} species, which we may have been unable to detect due to loss of yield with successive sequencer cycles. Although the C-terminal sequence of the peptides was not defined, the size and N termini of the 3-kDa forms would be consistent with heterogeneous C termini, in some instances extending beyond residue 40 of β-amyloid in the transmembrane domain of APP. Thus, proteolytic cleavage of APP to β-amyloid peptides is somewhat heterogeneous. The relative abundance of particular species in amyloid plaques may reflect their propensity for aggregation and resistance to proteolytic degradation.

The relative abundance of the intact and truncated β-amyloid peptides was highly dependent on cell type. Although astrocytes and neurons produced both major species of β-amyloid peptides, neurons generated substantially higher levels of the truncated 3-kDa peptide. The role of the trun-

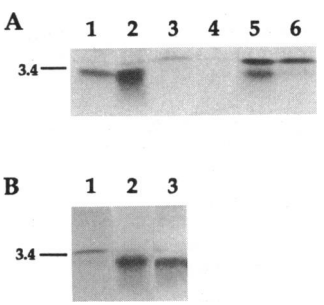

FIG. 5. Secretion of β-amyloid by neuronal and nonneuronal cells. (*A*) Primary cell cultures. Lanes: 1, rat cortical neurons; 2, rat hippocampal neurons; 3, rat astrocytes; 4, rat skin fibroblasts; 5, human mixed cortical culture; 6, human astrocytes. Shown are the 4-kDa and 3-kDa peptides immunoprecipitated from cell-conditioned medium after normalization for protein content in the cell lysate (Bio-Rad protein assay). PhosphorImager analysis showed 3-kDa/4-kDa peptide molar ratios of 1.85 ± 0.12 for rat cortical neurons and 0.39 ± 0.05 for rat astrocytes (mean ± SEM; $n = 3$; $P < 0.001$ by Student's t test). (*B*) Continuous cell lines. Lanes: 1, human glioma; 2, human M17 neuroblastoma; 3, rat PC12 pheochromocytoma. The glioma cell line HTB148 was obtained from the American Type Culture Collection and the M17 cell line was provided by J. Biedler. (*A* and *B*) Molecular mass in kDa is shown.

cated forms of β-amyloid in senile-plaque formation is unclear. Biophysical studies suggest that truncation of 10 or more residues from the N terminus of β-amyloid results in conformational changes that increase amyloid fibril formation (22, 23). Under some circumstances, the truncated β-amyloid peptides may initiate amyloid aggregation and plaque formation in the brain, which may then propagate by accretion of the intact 4-kDa peptide.

Our findings provide support for the idea that amyloid plaques are derived from local production of β-amyloid in the brain rather than from a peripheral source (24, 25). Human astrocytes produced higher levels of intact β-amyloid than any of the other cell types examined. Neurons also produced significant levels of amyloid peptides raising the possibility that both astrocytes and neurons may contribute to the development of senile plaques. Activated astrocytes and microglia are associated with the amyloid cores of senile plaques (26) and are the source of the plaque-associated protein α_1-antichymotrypsin (27). We found that human astrocytes produced much higher levels of β-amyloid than rat astrocytes, suggesting that the regulation of β-amyloid synthesis may be species-specific. The formation of β-amyloid deposits is characteristic of the aged brain in several primate species but not in rodents (28).

The generation of β-amyloid may not, by itself, be sufficient to cause amyloid-plaque formation and Alzheimer-type pathology. The association of Alzheimer disease with Down syndrome and inherited mutations in APP (29, 30) suggests that altered processing or overexpression of APP predisposes to β-amyloid deposition in the brain. However, β-amyloid is normally secreted without the formation of fibrillar deposits, even when APP is overexpressed in transfected cells or transgenic mice (31), suggesting that additional factors that promote the formation of fibrillar amyloid must be operative in the aged brain and in Alzheimer disease. Recent findings suggest that soluble β-amyloid must undergo a conversion to an aggregated or fibrillar form to become neurotoxic (19, 32, 33). The progression from normal secretion of β-amyloid to pathological deposition of amyloid fibrils may not involve the processing of APP but rather may be due to other plaque-associated proteins or postsynthetic modifications of β-amyloid that alter its state of aggregation.

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