Intracellular Accumulation of Insoluble, Newly Synthesized Aβn-42 in Amyloid Precursor Protein-transfected Cells That Have Been Treated with Aβ1–42*

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Our early study indicates that intracellular Aβ1–42 aggregates are resistant to degradation and accumulate as an insoluble residue in lysosomes, where they alter the normal catabolism of amyloid precursor protein (APP) to cause the accumulation of insoluble APP and amyloidogenic fragments. In this study, we examined whether the addition of exogenous Aβ1–42 also leads to the accumulation of newly synthesized intracellular Aβ. Here we describe that newly synthesized Aβ, especially Aβn-42, is generated from metabolically labeled APP and accumulates in the insoluble fraction of cell lysates after Aβ1–42 treatment. These results suggest that intracellular Aβ may derive from a solid phase, intracellular pathway. In contrast to the pathway that primarily produces secreted Aβ1–40, the solid-phase intracellular pathway preferentially produces Aβn-42 with ragged amino termini. Biochemical studies and amino acid sequencing analyses indicate that these intracellular Aβ also share the same types of Aβ structures that accumulate in the brain of Alzheimer's disease patients, suggesting that a significant fraction of the amyloid deposits in Alzheimer's disease may arise by this solid-phase pathway.

The major protein component of amyloid deposits associated with Alzheimer's disease (AD) is a 39–42-amino acid, self-assembling peptide known as the amyloid Aβ peptide. Although significant progress has been made in our understanding of the proteolytic processing of amyloid precursor protein (APP) and the secretion of soluble amyloid Aβ peptide, the mechanisms for the accumulation of insoluble amyloid deposits and their role in AD pathogenesis remains a matter of speculation. It is clear that at least two pathways for APP processing give rise to fragments bearing Aβ sequences at their amino termini: processing by α-secretase, which cleaves within the Aβ sequence, thereby precluding amyloid accumulation, and β-secretase processing, which generates carboxyl-terminal APP fragments containing the Aβ sequence. Amyloidogenic, β-secretase processing events may take place within several intracellular organelles, including the rough endoplasmic reticulum, trans-Golgi network, and lysosomes (1–7). Further processing of APP within the transmembrane domain by γ-secretase releases soluble 3- and 4-kDa fragments containing all or part of the Aβ sequence (8). Recent evidence indicates that the familial AD amino acid substitutions within the APP transmembrane domain and presenilin favor the production of Aβ1–42 form of Aβ, which is preferentially localized within diffuse plaques and senile plaques in AD brain. This suggests that Aβ1–42 is more closely associated with AD pathogenesis than shorter Aβ isoforms (9, 10).

Biochemical studies of synthetic amyloid peptides have elucidated several important properties regarding their ability to assemble into the amyloid fibrils that characteristically accumulate in AD. Peptides that end at residue 42 aggregate much more rapidly than those ending at residue 39 or 40 (11, 12). The pH optimum for β-sheet formation and aggregation is between pH 4.0 and 5.5 (11, 13, 14). Perhaps because of the fact that it aggregates much more rapidly, Aβ1–42 is resistant to degradation once it has been internalized by endocytosis. It accumulates as an insoluble residue in late endosomes or secondary lysosomes, whereas Aβ1–40 and shorter peptides are degraded and eliminated (15, 16). The amyloid that accumulates in AD is structurally heterogeneous with ragged amino termini, but most of the Aβ peptides end at residue 42 (17, 18). The amyloid that accumulates in AD brain has the hallmarks of a long-lived protein, such as d-amino acids and isopeptide bonds (19).

The selective resistance of aggregated Aβ1–42 to degradation provides a simple and direct mechanism for why Aβ1–42 preferentially accumulates in the brain (17, 18). The accumulation of insoluble Aβ1–42 in lysosomes also alters the catabolism of APP and causes the accumulation of APP and a series of potentially APP amyloidogenic fragments (20, 21). Like the internalized Aβ1–42, these fragments accumulate in the insoluble fraction of the cell and display very long half-lives (20). Several features of this accumulation are analogous to prion replication (22). The prion model postulates a conformation change in the precursor protein preceding their proteolytic conversion to more prions. The APP amyloidogenic fragments appear to undergo such a conformation change, since they display an epitope that is specifically associated with Aβ aggregators (20). This suggests that they may have the same shape as aggregated Aβ and are therefore capable of adding on to the fibril lattice established by the internalized exogenous Aβ1–42. The fact that they co-purify in the insoluble fraction of the cell is consistent with the suggestion that they co-aggregate (20). The accumulation of amyloid is autocatalytic as predicted for prion replication, and once amyloid core is seeded, the continued presence of exogenous Aβ1–42 is not required for...
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further accumulation of APP and amyloidogenic fragments (20). To complete the prion-like cycle, the APP amyloidogenic fragments would need to be further proteolytically processed to Aβ.

In this report, we examined whether the addition of exogenous Aβ1–42 causes the accumulation of newly synthesized intracellular Aβ. Here we describe that 4-kDa Aβ, especially Aβn-42, is produced from metabolically labeled APP molecules and accumulates in the detergent-insoluble fraction of cells that have been incubated with synthetic Aβ1–42. Most of the newly synthesized Aβ peptides that accumulate have ragged amino termini and end at residue 42. The structure of these peptides is remarkably similar to the structure described for amyloid Aβ isolated from Alzheimer’s brain tissue (17, 18), suggesting that much of the brain amyloid may be derived from this solid-phase, intracellular pathway.

MATERIALS AND METHODS

Metabolic Labeling and Immunoprecipitation—Transfected cell cultures (1 × 10⁷ cells in a 10-cm plate) were incubated with methionine-deficient Dulbecco’s modified Eagle’s medium for 2 h before labeling. The cells were then incubated in 2 ml of methionine-deficient Dulbecco’s modified Eagle’s medium containing 25 μM radiolabeled amino acid and 1% bovine serum albumin and labeled with 100 μCi/ml [35S]methionine/cysteine (1000 Ci/mmol; Trans−3S-label, ICN) for 16 h. At the end of incubation time, the cells were washed twice with cold phosphate-buffered saline and lysed with Nonidet P-40 lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 2 mg/ml leupeptin, 0.2 unit/ml soybean trypsin inhibitor, 1 mg/ml aprotinin). The insoluble cell lysate was collected by centrifugation at 10,000 × g for 10 min, solubilized in 88% formic acid (v/v), and lyophilized. After lyophilization, the dried sample was resuspended with 2× radioimmune precipitation buffer, sonicated until clarified, diluted to 1× radioimmune precipitation buffer, and centrifuged at 10,000 × g for 10 min. The supernatant of sample was then subjected to immunoprecipitation analysis with various antibodies and analyzed by SDS-polyacrylamide gel electrophoresis.

Isolation of [35S]Met-labeled Aβ from the Insoluble Fractions of Cell Lysates—APP751-overexpressing cells were preincubated with methionine-deficient Dulbecco’s modified Eagle’s medium for 2 h before labeling. The cells were then incubated in 2 ml of methionine-deficient Dulbecco’s modified Eagle’s medium containing 25 μM Aβ1–42 and 1% bovine serum albumin and labeled with 100 μCi/ml [35S]methionine/cysteine (1000 Ci/mmol; Trans−3S-label, ICN) for 16 h. At the end of labeling period, the cells were then washed twice with cold phosphate-buffered saline and lysed with Nonidet P-40 lysis buffer. The insoluble cell pellet was then collected by centrifugation at 10,000 × g for 10 min and solubilized in 88% formic acid. The formic acid-solubilized cell fraction was then injected over a Superdex 75 gel filtration column that had been previously equilibrated in 60% ACN, 10% formic acid. The sample was eluted from the gel filtration column in 60% formic acid (v/v), and lyophilized. The fractions containing Aβ were then pooled and lyophilized. The lyophilized material was then redissolved in 60% formic acid and subjected to a second round of reverse-phase HPLC chromatography on a Water C-4 column. The sample was eluted in a 5×95% ACN gradient, and fractions coeluted with synthetic Aβ1–42 were collected for further analysis.

Radiochemical Sequencing of Amyloid Peptide—APP751-transfected cells were metabolically labeled with either [35S]Met or [3H]Phe, and the secreted or intracellular Aβ were then purified as described above. The amyloid peptides were then subjected to automated Edman degradation amino acid sequencing analysis, and the amount of [3H]Phe radioactivity eluted from each sequencing cycle was then determined by liquid scintillation counting. In the case of [35S]Met-labeled amyloid peptide sequencing reaction, 4,000 cpm of purified labeled Aβ was mixed with 10 μg of synthetic Aβ1–42 in 0.2 mM ammonium bicarbonate buffer (pH 8.0) and digested with 0.1 μg/ml of 1-to-tyrosine-2-phenethyl chloromethyl ketone-trypsin (Sigma) at 37 °C for 16 h. The [35S]Met-containing tryptic fragments (Aβ29–42) were then collected by centrifugation on a tabletop centrifuge at 14,000 rpm for 30 min. The pellet was then washed twice with water and dissolved in 40% ACN, 60% formic acid right before the radiochemical sequencing analysis.

RESULTS

Immunoprecipitation of Newly Synthesized Aβ from the Detergent-insoluble Fraction of Aβ1–42-treated Cells—Our previous work indicates that Aβ1–42 preferentially accumulates in late endosomes and lysosomes of both cultured human fibroblast and PC12 cells and is resistant to degradation. We examined whether the presence of intracellular Aβ affects the catabolism of APP and Aβ in APP-overexpressing human embryonic kidney 293 cells since both nonamyloidogenic and amyloidogenic APP-processing pathways have been demonstrated in this cell line. Previous studies on the uptake of 125I-labeled Aβ1–42 demonstrated that most of the internalized 125I-labeled Aβ is sedimentable at 10,000 × g (15, 16). Because the amyloidogenic fragments of APP also accumulate in the nonionic detergent-insoluble fraction of cells treated with Aβ1–42 (20), we investigated whether some of these fragments are ultimately converted to 4-kDa Aβ.

APP-overexpressing human embryonic kidney 293 cells were treated with Aβ1–42, metabolically labeled with [35S]Met for 16 h, and then lysed with Nonidet P-40 lysis buffer as described under “Materials and Methods.” The Nonidet P-40-insoluble fraction was then dissolved in 88% formic acid, lyophilized, and resuspended in radioimmune precipitation buffer, and the 100,000 × g-soluble supernatant was immunoprecipitated with antibodies raised against Aβ1–42, Aβ1–28, or the carboxyl terminus of APP (13G8). All three antibodies immunoprecipitate a broad size range of labeled products from Aβ1–42-treated cells that are absent in untreated cells (Fig. LA). Because of the low efficiency of immunoprecipitation with anti-Aβ antibodies due to the presence of the exogenously supplied, unlabeled Aβ1–42, we devised a more specific method of immunoprecipitating Aβ using cells that were treated with synthetic Aβ4–42 instead of Aβ1–42. We verified that Aβ4–42 is not recognized by an anti-Aβ monoclonal antibody, 3D6, that recognizes the first 5 residues of Aβ (Fig. 1B). When the detergent-insoluble fraction of cells treated with Aβ4–42 is immunoprecipitated with this antibody, a small amount of 4-kDa Aβ is detected (lane 5, Fig. 1A indicated by arrow). To further demonstrate that the accumulation of intracellular newly synthesized Aβ is because of the Aβ treatment, a time course analysis was performed. APP-overexpressing cells were treated with 25 μM Aβ, and the amount of [35S]Met-labeled Aβ was then detected by immunoprecipitation at indicated times. Our previously published data (20) and the results from this study (Fig. LA) indicate that there is a large amount of APP carboxy-terminal fragments in this insoluble fraction after Aβ treatment. To improve the sensitivity of Aβ detection by eliminating the cross-reactivity between the APP carboxy-terminal fragments and the anti-Aβ antibody, cell extracts were preabsorbed with anti-APP carboxy-terminal antibodies (13G8) before immunoprecipitation with the anti-Aβ antibody, 3D6. As shown in Fig. 1C, there is no detectable newly synthesized [35S]Met-labeled Aβ in the first 4 h of Aβ incubation. Newly synthesized Aβ is first observed after 6 h of Aβ treatment, suggesting that the [35S]Met-labeled Aβ is derived from the amyloidogenic APP fragments that accumulate in the insoluble fraction that have internalized Aβ1–42. This observation is consistent with the recent findings that Aβ1–42 can be produced intracellularly in the detergent-insoluble fraction of both cultured human NT2 neurons and APP-overexpressing human embryonic kidney cells (1–3), suggesting that a common amyloidogenic pathway exists in these cells.

Purification of Intracellular Amyloid Peptide by Gel Filtration and HPLC Column Chromatography—To further characterize the newly synthesized 4-kDa Aβ generated by cells treated with Aβ, we size-selected the 4-kDa fraction using gel filtration methods that were developed to isolate and characterize 4-kDa Aβ from insoluble brain amyloid deposits (19). APP-overexpressing cells were treated with Aβ1–42 and met-
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Characterization of the Amino Terminus of Newly Synthesized Aβ by Radiochemical Sequencing—We radiochemically sequenced the purified 4-kDa newly synthesized Aβ from Aβ1–42-treated cells to determine the amino terminus of newly synthesized intracellular Aβ. APP-overexpressing cells were metabolically labeled with [35S]Met for 6–12 h and then lysed with Nonidet P-40 lysis buffer as described under “Materials and Methods.” The Nonidet P-40-insoluble fraction was then dissolved in 60% formic acid and then loaded onto a Superdex 75HR size exclusion column and eluted in 60% formic acid as described (19, 24). A peak elutes between fractions 27 and 30 that co-migrates with authentic Aβ1–42 and is absent from control cells that were not treated with Aβ1–42. (Fig. 2A). The Aβ42-containing fractions (fractions 27–30, as indicated on Fig. 2A) were pooled and further purified by a semi-preparative reverse-phase HPLC. As shown in Fig. 2B, the majority of the [35S]Met-labeled material in the 4-kDa peak from the gel filtration column elutes as a broad peak from fractions 31–51, and the peak is coincident with the elution profile of synthetic Aβ1–42. Therefore, most of the newly synthesized, [35S]Met-labeled Aβ elutes with a profile that is identical to authentic Aβ1–42. As we reported earlier and shown in Fig. 2B (inset), Aβ1–40 elutes as a sharp peak on an analytical reverse-phase HPLC that precedes the broad elution profile of Aβ1–42 (11). The sharp peak of [35S]Met-labeled material elutes at fraction 31, and this elution position is identical to synthetic Aβ1–40. This suggests that the [35S]Met-labeled insoluble fraction contains a mixture of Aβ1–42 and Aβ1–40, and this question is addressed in greater detail below.

Characterization of the Amino Terminus of Newly Synthesized Aβ by Radiochemical Sequencing—We radiochemically sequenced the purified 4-kDa newly synthesized Aβ from Aβ1–42-treated cells to determine the amino terminus of newly synthesized intracellular Aβ. APP-overexpressing cells were metabolically labeled with [35S]Met in the presence of 25 μM Aβ1–42 for 16 h. The insoluble fraction of cell lysates was then immunoprecipitated with antisera against Aβ1–28 (lane 2), Aβ1–42 (lane 3), and anti-APP carboxyl-terminal monoclonal antibodies (13G8) (lane 4). To increase the efficiency of immunoprecipitation, the insoluble fraction of cell lysate from cells that were treated with 25 μM Aβ1–42 for 6 h was immunoprecipitated with anti-Aβ1–5 monoclonal antibodies (3D6), and the accumulation of newly synthesized Aβ (indicated by the arrow, lane 5) was determined by gel electrophoresis and autoradiography. The immunoprecipitated products were resolved on a 15% Tris-glycine gel and visualized by autoradiography. The amount of APP and APP fragments were increased dramatically in cells treated with Aβ compared with cells that have not been exposed to Aβ (lane 1). B, the specificity of anti-Aβ1–5 antibody, 3D6, is indicated by Western blot analysis of synthetic Aβ4–42 (lane 1), 8–42 (lane 2), and 1–42 (lane 3). 3D6 recognizes a 4-kDa full-length Aβ1–42 and a 16-kDa Aβ aggregate but fails to detect any amino-terminal Aβ deletion peptides. C, cultured APP-overexpressing cells were metabolically labeled with [35S]Met in the presence of 25 μM Aβ1–42 for indicated times. The accumulation of newly synthesized Aβ from the insoluble fraction of cell lysate was then determined first by preclearing with anti-APP carboxyterminal antibodies (13G8) and then immunoprecipitating with anti-Aβ antibodies (3D6). The immunoprecipitated Aβ was then resolved on a 15% Tris-glycine gel and visualized by autoradiography. Hr, hours; Ab, antibody.

FIG. 1. Aβ1–42 causes the accumulation of Aβ and APP fragments in APP overexpressing cells. A, APP751-transfected human embryonic 293 cells were metabolically labeled with [35S]Met in the presence of 25 μM Aβ1–42 for 16 h. The insoluble fraction of cell lysates was then immunoprecipitated with antisera against Aβ1–28 (lane 2), Aβ1–42 (lane 3), and anti-APP carboxyl-terminal monoclonal antibodies (13G8) (lane 4). To increase the efficiency of immunoprecipitation, the insoluble fraction of cell lysate from cells that were treated with 25 μM Aβ1–42 for 6 h was immunoprecipitated with anti-Aβ1–5 monoclonal antibodies (3D6), and the accumulation of newly synthesized Aβ (indicated by the arrow, lane 5) was determined by gel electrophoresis and autoradiography. The immunoprecipitated products were resolved on a 15% Tris-glycine gel and visualized by autoradiography. The amount of APP and APP fragments were increased dramatically in cells treated with Aβ compared with cells that have not been exposed to Aβ (lane 1). B, the specificity of anti-Aβ1–5 antibody, 3D6, is indicated by Western blot analysis of synthetic Aβ4–42 (lane 1), 8–42 (lane 2), and 1–42 (lane 3). 3D6 recognizes a 4-kDa full-length Aβ1–42 and a 16-kDa Aβ aggregate but fails to detect any amino-terminal Aβ deletion peptides. C, cultured APP-overexpressing cells were metabolically labeled with [35S]Met in the presence of 25 μM Aβ1–42 for indicated times. The accumulation of newly synthesized Aβ from the insoluble fraction of cell lysate was then determined first by preclearing with anti-APP carboxyterminal antibodies (13G8) and then immunoprecipitating with anti-Aβ antibodies (3D6). The immunoprecipitated Aβ was then resolved on a 15% Tris-glycine gel and visualized by autoradiography. Hr, hours; Ab, antibody.
A sequence of Aβ isolated from cultured media is much more homogeneous, and [3H]Phe radioactivity can only be detected in fractions 4, 19, and 20, suggesting the amino terminus of soluble Aβ is generated by a more specific mechanism (25). Interestingly, Aβ isolated from the plaques of the brains of Alzheimer’s patients also contained such amino-terminal truncation as we describe here (18, 24, 26, 27).

Characterization of the Carboxyl Terminus of the Newly Synthesized, Intracellular Aβ—Our early study from the biochemical properties of synthetic Aβ indicated that Aβ1–42 rather than Aβ1–40 is able to form SDS-resistant aggregates and migrates as a 16-kDa band on SDS-polyacrylamide gel electrophoresis at high concentrations (11, 28). The specificity of SDS-resistant Aβ aggregates is demonstrated in Fig. 4A, where trace amounts of [3H]Phe-labeled Aβ1–42 are mixed with increasing amounts of unlabeled Aβ1–40 or Aβ1–42 individually. A 16-kDa SDS-resistant aggregate is only observed at higher concentrations of radiolabeled Aβ1–42 with unlabeled Aβ1–42, whereas the radiolabeled Aβ1–42 does not co-assemble into 16-kDa SDS-resistant aggregates with unlabeled Aβ1–40. Therefore, this property can be used to distinguish between the

![Fig. 2. Purification of intracellular amyloid peptide by gel filtration and HPLC column chromatography. A, cells overexpressing APP751 incubated with 25 μM of Aβ1–42 for 6–12 h were metabolically labeled with [35S]Met and lysed with Nonidet P-40 lysis buffer. The insoluble cell lysate was then extracted with 60% formic acid. After centrifugation, the formic acid-soluble fraction was injected over a Superdex 75 gel filtration column. The sample was then eluted from the gel filtration column in 60% formic acid at 1 ml/min, and the absorbance was monitored at 254 nm. B, the intracellular [35S]Met-labeled Aβ was mixed with 20 μg of synthetic Aβ in 1 ml of 88% formic acid and subjected to reverse-phase HPLC in a semi-preparative Vydac C-4 column as described (15). The sample was then developed in a 5–95% ACN gradient, fractions were collected, and the amount of radioactivity was then determined by scintillation counting. As indicated, [35S]-labeled peptide (●) was coeluted with unlabeled synthetic Aβ1–42 and displays a broad elution profile that is a characteristic feature of Aβ1–42 peptide and is not a reflection of heterogeneity (see inset, 2 μg each of synthetic Aβ1–40 and Aβ1–42 were injected onto a Vydac C-4 analytical reverse-phase column. The sample was then eluted by a 5–95% ACN gradient).](image1)

![Fig. 3. Radiochemical sequencing of intracellular Aβ peptide. A, APP-overexpressing cells treated with Aβ1–42 were metabolically labeled with [3H]Phe, and Aβ purified from the reverse-phase HPLC were pooled and radiochemically sequenced by automated Edman degradation. The results indicates that the amino terminus of the intracellular Aβ was ragged and may have been subjected to limited proteolysis as previously observed for the exogenously added peptides. B, in the case of [35S]Met-labeled amyloid peptide-sequencing reaction, 4,000 cpm of purified, labeled Aβ was mixed with 10 μg of Aβ1–42 in 0.2 M ammonium bicarbonate buffer (pH 8.0) and digested with 0.1 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Sigma) at 37 °C for 16 h. The [35S]Met-containing tryptic fragments (Aβ29–42) were then collected by centrifugation on a tabletop centrifuge and subjected to Edman radiochemical sequence analysis. The majority of [35S]-Met was only detected in the cycle 7, which is consistent with the predicted Aβ sequence.](image2)
metabolically labeled Aβ1–42 and Aβ1–40 and shorter Aβ peptides. The HPLC-purified, [35S]Met-labeled intracellular Aβ was mixed with synthetic Aβ1–28, Aβ1–42, and Aβ1–40 at a concentration of 500 µg/ml at pH 5.0 for 24 h. The labeled peptide forms 16-kDa SDS-resistant aggregates only with synthetic Aβ1–42 but not Aβ1–40 (indicated by the black line, Fig. 4B), suggesting that a fraction of the newly synthesized [35S]Met Aβ from the insoluble fraction of cell lysate is Aβ1–42 or closely related products that are capable of co-assembling with synthetic Aβ1–42 into SDS-resistant aggregates.

Recently, an analytical, chromatographic method for separating Aβ1–42 from Aβ1–40 was described that employed reverse-phase separation at high temperature (29). As indicated in Fig. 5A, Aβ1–42 can be resolved as a single peak on a Zorbax C-18 reverse-phase column at 65 °C and is well separated from Aβ1–40 by almost 2 min. The purified 4-kDa radiolabeled peptides were dissolved in 1 ml of 70% formic acid and injected onto a Zorbax C-18 reverse-phase column. The radioactive sample was then eluted by a 20–45% ACN gradient at 65 °C, B, purified [35S]Met Aβ from the Aβ-treated cells was mixed with 2 µg each of synthetic Aβ1–40, and Aβ1–42 was injected over a Zorbax C-18 HPLC column. Using the same condition described above, the fractions were collected and subjected to scintillation counting. The radiolabeled peptides (●) eluted as two major peaks at the position of both synthetic Aβ40 and Aβ42 (solid line), indicating that a mixture of newly synthesized Aβn-42 and Aβn-40 were accumulated in cells responding to Aβ1–42 treatment.

**FIG. 4.** Purified [35S]Met intracellular Aβ peptides form SDS-resistant aggregates with synthetic Aβ1–42. A, 10 ng of [14C]-labeled Aβ1–42 was mixed with increasing concentrations (50–500 µg) of unlabeled Aβ1–42 and Aβ1–40. The [14C]-labeled Aβ formed high M, aggregates with unlabeled Aβ1–42 with an apparent M, of 16 kDa (indicated by an arrowhead), whereas [14C]-labeled Aβ1–42 does not aggregate with unlabeled Aβ1–40. B, approximately 2,000 cpm of purified [35S]Met-labeled 4-kDa Aβ peptides were incubated with various Aβ synthetic analogs at concentrations of 500 µg/ml for 24 h at pH 5.0 (0.1 M sodium acetate). The samples were then resolved on a 15% Tris-glycine polyacrylamide gel and visualized by autoradiography. The result shows that purified [35S]Met-labeled Aβ peptide is only able to form SDS-resistant aggregates with synthetic Aβ1–42 (lane 4, as indicated by arrow) but not with shorter isoforms such as Aβ1–28 (lane 2) and Aβ1–40 (lane 3).

**FIG. 5.** Characterization of the carboxyl terminus of the newly synthesized, intracellular Aβ by reverse-phase HPLC. A, synthetic Aβ1–40 and Aβ1–42 were injected onto a Zorbax C-18 reverse-phase column. The sample was then eluted by a 20–45% ACN gradient at 65 °C. B, purified [35S]Met Aβ from the Aβ-treated cells was mixed with 2 µg each of synthetic Aβ1–40, and Aβ1–42 was injected over a Zorbax C-18 HPLC column. Using the same condition described above, the fractions were collected and subjected to scintillation counting. The radiolabeled peptides (●) eluted as two major peaks at the position of both synthetic Aβ40 and Aβ42 (solid line), indicating that a mixture of newly synthesized Aβn-42 and Aβn-40 were accumulated in cells responding to Aβ1–42 treatment.

**DISCUSSION**

Our results suggest that a solid-phase pathway for the simultaneous production and accumulation of amyloid Aβ exists within late endosomes or secondary lysosomes of cells that contain degradation-resistant Aβ1–42 aggregates. We have previously shown that the presence of intracellular Aβ1–42 aggregates alters the normal catabolism of APP to cause the accumulation of APP and potentially amyloidogenic APP fragments in lysosomes of cultured cells. In this report, we have demonstrated that newly synthesized 4-kDa Aβ also accumulates in the same insoluble fraction as the amyloidogenic APP fragments. Because of the requirement for exogenously added synthetic Aβ1–42 to initiate the accumulation of insoluble frag-
ments of APP, we must rely on radiochemical methods to unambiguously distinguish the newly synthesized Aβ that is derived from the transfected APP gene from the synthetic Aβ1–42 added to the cell cultures. A [35S]Met-labeled 4-kDa band is immunoprecipitated from the formic acid-soluble fraction with a variety of anti-Aβ antibodies. The efficiency of immunoprecipitation of 4-kDa Aβ is enhanced by using Aβ4–42 instead of Aβ1–42 to prime the cells and immunoprecipitating with 3D6, a monoclonal antibody specific for the first 5 residues of Aβ. Similarly, the efficiency of immunoprecipitation of 4-kDa Aβ is improved by immunodepletion of cross-reacting carboxy-terminal fragments of APP.

Like the amyloid deposits from AD brain, the detergent-insoluble fraction of Aβ1–42-treated cells is largely solubilized in formic acid; therefore, we have used the purification methods employed for purifying Aβ from AD brain to purify and characterize the 4-kDa peptides in the insoluble fraction of Aβ1–42-treated cells. The fractions that elute in formic acid from a Superdex HR75 column at the position of denatured 4-kDa Aβ1–42 were pooled and further purified by reverse-phase HPLC. Most of the radiolabeled material elutes from the reverse-phase HPLC at the same position as Aβ1–42. Radiochemical sequencing of this purified material indicates that the amino termini of these peptides are heterogeneous, with peptide ends both longer and shorter than those beginning at residue 1. The heterogeneity may also be because of non-Aβ contaminants, but after trypsin cleavage to create homogeneous cleavage sites, most of the [35S]Met label is observed at cycle 7 as expected for Aβ. The carboxyl terminus of the newly synthesized 4-kDa product was characterized by analytical reverse-phase HPLC under conditions that resolve Aβ1–42 and Aβ1–40. Although labeled material is recovered in both peaks, most of the radioactivity is associated with Aβ1–42. Perhaps the most convincing evidence that the newly synthesized 4-kDa peptides are Aβ1–42 and closely related structures is the ability to co-assemble into SDS-resistant aggregates with authentic Aβ1–42 at concentrations above the critical concentration for aggregation. This property is specific to Aβ1–42 and Aβ1–43, since no SDS-resistant aggregates are observed for Aβ1–40 even in the presence of a vast excess of Aβ1–42. Thus, the intracellular Aβ displays a number of similarities to the amyloid that accumulates in AD brain tissue. The intracellular amyloid is insoluble in nonionic detergents, but like amyloid isolated from brain, a substantial fraction of the amyloid is soluble in formic acid (17, 18). Both the intracellular amyloid and brain amyloid display considerable amino-terminal heterogeneity, and the majority of the amyloid peptides end at residue 42 (17, 18).

The finding that Aβn-42 accumulates inside cells treated with amyloid Aβ1–42 provides further support for the hypothesis that amyloid accumulation is an autocatalytic process mechanismically related to prion replication. Like PrPSc, the core structure of aggregated Aβn-42 is resistant to proteolysis, both inside cells and in vitro (15, 16, 30). The fact that metabolically labeled, newly synthesized Aβ accumulates indicates that at least a fraction of the APP molecules are converted to more Aβ as the prion replication model predicts. Because the amyloid core is resistant to degradation, this conversion may be carried out by nonspecific proteolysis and exopeptidase activities. This may explain why the Aβ that accumulates in the insoluble fraction displays ragged amino and carboxyl termini. Misfolded APP molecules and amyloidogenic fragments appear to be intermediates in amyloid accumulation because they also accumulate in the nonionic detergent-insoluble fraction of the cell where they turn over very slowly (20). At least a subset of these amyloidogenic fragments appear to have adopted the same conformation as aggregated Aβ because they display an unique conformation-dependent epitope that is only detected in aggregated Aβ (20). This suggests that the misfolded amyloidogenic APP fragments may be capable of binding to aggregated Aβn-42 and extending the amyloid lattice. Because both amyloidogenic carboxy-terminal APP fragment precursors and the 4-kDa Aβ product accumulate in the insoluble fraction, the simplest hypothesis is that this conversion takes place in the solid phase.

Co-aggregation of poorly metabolized Aβ and amyloidogenic fragments of Aβ may explain why the intracellular Aβ immunoreactivity is not morphologically recognizable as amyloid fibrils. Because the insoluble aggregates are a heterogeneous collection of fragments, perhaps their underlying fibrillar lattice is not revealed until the fragments have been digested to their protease-resistant amyloid core. The fact that the intracellular amyloidogenic fragments themselves are poorly degraded suggests that this conversion process may be quite slow and may even occur extracellularly, after the insoluble residue has been externalized, either by exocytosis or the death of the cell. This accumulation appears to mimic the “granulovacuolar” pathophysiology of degenerating neurons and dystrophic neurites, where Aβ and APP immunoreactivity have been localized to granular or globular deposits (31–33) that are also positive for ubiquitin immunoreactivity (34, 35). This may represent the site at which the intracellular Aβ and APP-insoluble residue may be externalized, perhaps by breaking off of vesicles from neurite termini. How this insoluble residue is ultimately converted to Aβ is unknown, but the microglia surrounding the plaque may phagocytose the residue and digest it to the protease-resistant amyloid core. It has been proposed that microglia may play a role in depositing amyloid fibrils in senile plaques (36). Because the center of neuritic plaques and mature or cored plaques are not positive for the non-Aβ epitopes (31–33), these may represent regions of the amyloid deposits where the conversion process is complete.

Although the significance of intracellular Aβ immunoreactivity and the origin of extracellular Aβ deposits is still controversial, a recent report by Cataldo et al. (37) indicates that lysosomal hydrolases can be detected in extracellular amyloid deposits of AD and Down syndrome brains. This is consistent with the lysosomal origin of these deposits under pathological conditions (37–39). Aβ immunoreactivity has been detected in the vacuole of chloroquine-induced rat soleus muscle cells, and immunohistochemical studies indicate that most of Aβ in the vacuoles reacts with anti-Aβ1–42, and only a few react with Aβ1–40-specific antibodies (40). Our results are also consistent with several recent findings that there is an intraneuronal amyloid pool that accumulates with time in culture (1, 23). How the solid-phase pathway for Aβ1–42 accumulation relates to the intracellular Aβ immunoreactivity in human brain and AD pathogenesis remains a challenge for further experimentation.

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