The Profile of Soluble Amyloid β Protein in Cultured Cell Media

DETECTION AND QUANTIFICATION OF AMYLOID β PROTEIN AND VARIANTS BY IMMUNOPRECIPITATION-MASS SPECTROMETRY*

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To study the metabolism of amyloid β protein (Aβ) in Alzheimer’s disease, we have developed a new approach for analyzing the profile of soluble Aβ and its variants. In the present method, Aβ and its variants are immunoprecipitated with Aβ-specific monoclonal antibodies. The identities of the Aβ variants are determined by measuring their molecular masses using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The levels of Aβ variants are determined by their relative peak intensities in mass spectrometric measurements by comparison with internal standards of known identities and concentrations. We used this method to examine the Aβ species in conditioned media of mouse neuroblastoma cells transfected with cDNAs encoding wild type or mutant human amyloid precursor protein. In addition to human Aβ-(1–40) and Aβ-(1–42), more than 40 different human Aβ variants were identified. Endogenous murine Aβ and its variants were also identified by this approach. The present approach is a new and sensitive method to characterize the profile of soluble Aβ in conditioned media and biological fluids. Furthermore, it allows direct measurement of each individual peptide in a peptide mixture and provides comprehensive information on the identity and concentration of Aβ and Aβ variants.

Alzheimer’s disease (AD)1 is a progressive neurodegenerative disorder and the most common form of dementia (1). One of the neuropathological features of AD is the presence of amyloid deposits in senile plaques and in blood vessel walls (2, 3). These amyloid deposits are mainly composed of a 4-kDa protein, amyloid β protein (Aβ), which contains 39–43 amino acid residues (4–6). Aβ is derived from a 695–770-amino acid precursor, amyloid precursor protein (APP), through proteolytic processing (7–9). Since the initial isolation of Aβ from amyloid deposits (4), various forms of Aβ peptides have been reported. Both NH2-terminally (5, 10–13) and COOH-terminally (9, 14) truncated Aβ peptides have been isolated and identified either from plaque cores (11), from neurofibrillary tangles (15), or from cerebrovascular amyloid fibrils (11, 16) from patients with AD or with Down’s syndrome (5). The major Aβ peptide in aqueous cerebral cortical extracts from AD brains has been reported as Aβ-(1–40) (10). However, recent reports indicate that the insoluble amyloid in senile plaque cores is primarily Aβ-(1–42) (11, 12) and that diffuse senile plaques are primarily Aβ-(17–42) (17). These findings have been verified by measuring Aβ in a 70% formic acid brain extract using sandwich enzyme-linked immunosorbent assay (18). In comparison, vascular amyloid is reported to be a mixture of Aβ-(1–40) and Aβ-(1–42) (11, 12).

The discovery that soluble Aβ (sAβ) is a constituent of cerebrospinal fluid (CSF) (19–21) and cultured cell media (20, 22) indicates that Aβ is a normal product of cellular metabolism of APP. The major form of Aβ in biological fluids is Aβ-(1–40). Both NH2-terminally and COOH-terminally truncated sAβs have been identified in pooled CSF specimens (21). Aβ-(17-X) has also been detected in cultured cell media (22). The hypothesis that APP metabolism and Aβ production play central roles in the pathogenesis of AD is supported by the observation that certain familial AD mutations cluster within or immediately adjacent to the Aβ domain and these mutations influence APP processing and Aβ production. For example, the APPF717VA double mutations produce elevated Aβ-(1–40) and Aβ-(1–42) in vitro and in vivo (23, 24). Further, APPF717 mutations increase the relative amount of highly amyloidogenic Aβ-(1–42/1–43) (27, 28), which may, in turn, recruit Aβ-(1–40) to be deposited into amyloid plaques (29).

Numerous Aβ species, in various concentrations, have been described in amyloid deposits from AD and control brain tissues (30). However, no significant difference that distinguishes AD patients and controls has been described in CSF studies of either sAβ peptide species (30) or concentration (20, 31, 32). We consider it likely that earlier methodologies used for Aβ analysis may have failed to detect subtle variations in Aβ levels between samples.

To investigate the production and metabolism of Aβ in tissue, biological fluids, and cultured mammalian cell conditioned medium, we have developed a sensitive method for measuring sAβ and its variants using the strategy of microscale immuno-
affinity capture (immunoprecipitation) and mass-specific identification (33, 34). The method relied on the combined approaches of immunoprecipitation and mass spectrometric analysis (IP/MS). The sAβ and its variants were selectively isolated by immunoprecipitation with anti-Aβ mAbs. The identities of these isolated Aβ peptides were determined by measuring their molecular masses using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (35). The relative signal intensities were used to estimate the concentrations of Aβ. Using this approach, we have detected several novel Aβ variants and have successfully quantified sAβs in the conditioned media of cultured mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse neuroblastoma cells (N2a), and cells stably transfected with Myc-epitope tagged wild type human APP695 cDNA (N2a/APP695) or APP695 cDNA encoding Swedish mutant (N2a/APP695.Swedish)(36) were cultured in Dulbecco’s modified Eagle’s medium (high glucose) (Hyclone, Logan, UT) containing 10% horse serum and 1% penicillin/streptomycin. All cell culture media were obtained from Life Technologies, Inc., Gaithersburg, MD. N2a cells were plated in multiwell cell culture plates (12-well plates, Becton Dickinson Labware, Franklin Lakes, NJ) and grown in 1.5 ml of growth medium containing 5% CO2 in a 37 °C incubator. Dulbecco’s modified Eagle’s medium (high glucose) and 0.2% fetal bovine serum, was added and cells were continually incubated in 5% CO2 in a 37 °C incubator.

Preparation of Serum-reduced Conditioned Media—N2a cells were plated in multiwell cell culture plates (12-well plates, Becton Dickinson Labware, Franklin Lakes, NJ) and grown in 1.5 ml of growth medium until approximately 90% confluent. The growth medium was discarded, and cells were washed with phosphate-buffered saline (Life Technologies, Inc.). After washing, a serum-reduced medium (1 ml), consisting of Dulbecco’s modified Eagle’s medium (high glucose) and 0.2% fetal bovine serum, was added and cells were continually incubated in 5% CO2 at 37 °C for 1 to 24 h. The conditioned media were harvested and centrifuged in a Beckman GS-6R centrifuge at 10,000 rpm (equal to 13,776 relative centrifugal force) for 5 min at 4 °C. The supernatants of centrifuged conditioned media were collected. Protease inhibitors (50 μg/ml antipain, 40 μg/ml bestatin, 2 μM EDTA-Na2, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM l-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone- HCl, 0.2 mM l-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone and 0.01% NaN3, were added immediately to prevent proteolytic degradation. Protease inhibitors were obtained from Sigma. The conditioned media supernatants were subjected to immunoprecipitation and mass spectrometric analysis.

Immunoprecipitation—Conditioned media supernatants (0.5–1.0 ml) were incubated with 1.0 μl of mAb 4G8 (2.5 mg/ml, anti-Aβ-(17–24) (37) or 6E10 (3.3 mg/ml, anti-Aβ-(1–16) (38)) in a rotator at 4 °C for 5–18 h. Protein G/A-agarose (3 μl, Oncogene Science, Inc., Cambridge, MA) was added, and rotational incubation was continued for another 3 h. The immunoprecipitated complex was collected by centrifugation in an Eppendorf Micro 5415C centrifuge at 10,000 rpm (equal to 8160 relative centrifugal force) for 2 min, and the supernatant was aspirated. The agarose beads were washed twice with ice-cold immunoprecipitation buffer (0.1% n-octylglucoside, 140 mM NaCl, 0.025% sodium azide, and 10 mM Tris-HCl, pH 8.0; Ref. 39) and once with 10 mM Tris-HCl, pH 8.0, containing 0.025% sodium azide. The samples were kept at 4 °C during the washing and centrifugation steps.

Mass Spectrometric Analysis of Immunoprecipitated Aβ—Immunoprecipitated Aβs were extracted with 3 μl of trifluoroacetic acid/water/acetonic acid (1:20:20, v/v/v) or formic acid/water/isopropanol (1:4:4, v/v/v), containing saturated α-cyano-4-hydroxycinnamic acid (UV-laser desorption matrix) (40) and 200 nM bovine insulin (internal mass calibration standard) (33, 34). The method relied on the combined approach of affinity capture (immunoprecipitation) and mass-specific identification (33, 34). The method relied on the combined approaches of affinity capture (immunoprecipitation) and mass spectrometric analysis (IP/MS). The sAβ and its variants were selectively isolated by immunoprecipitation with anti-Aβ mAbs. The identities of these isolated Aβ peptides were determined by measuring their molecular masses using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (35). The relative signal intensities were used to estimate the concentrations of Aβ. Using this approach, we have detected several novel Aβ variants and have successfully quantified sAβs in the conditioned media of cultured mammalian cells.

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dium from non-transfected N2a cells was used as a control to examine the specificity of IP/MS for human Aβ. 1 ml of conditioned medium was immunoprecipitated by mAbs 4G8 and 6E10 (separately) with Protein G/A-agarose beads and the immunoprecipitated peptides were analyzed by MS. Using mAb 4G8, we observed a series of peaks in the spectrum (Fig. 1, A and B). The molecular masses of the observed peaks indicated that they correspond to the amino acid sequences of murine Aβ peptides (42). A summary of molecular masses of the observed peaks and the calculated molecular masses and amino acid sequences of their corresponding murine Aβ peptides are provided in Table I. Although mAb 4G8 was raised against the amino acid sequence of human Aβ, we have successfully detected murine Aβ. The mAb 4G8 detects epitope between amino acids 17–24 of Aβ, a sequence conserved between murine and human Aβs (Fig. 2). In contrast, mAb 6E10 is human-specific and fails to recognize murine Aβs (Fig. 1C). The amino acid sequences of murine Aβ and human Aβ differ at amino acids 5, 10, and 13 (Fig. 2). The differences in amino acid sequence also contribute to the differences in their molecular masses and make it possible to differentiate human and mouse Aβ species by mass spectrometry.

**Human Aβ and Its Variants Can Be Detected and Distinguished in Conditioned Medium of Cells Expressing Human APP—Cultured mammalian cells constitutively produce and secrete Aβ. The profile of total soluble Aβ expressed by cultured cells was characterized using mouse neuroblastoma (N2a) cells, which were stably transfected with human APP cDNAs encoding either wild type (N2a/APP695) or Swedish familial AD mutant APP (N2a/APP695,595/596NL). These mouse neuroblastoma cells were incubated in serum-reduced media (0.2% fetal bovine serum) for 24 h. The sAβ peptides secreted into the cultured media (0.5 ml) were analyzed by immunoprecipitation with anti-Aβ mAbs and MALDI-TOF-MS. The mass spectrum resulting from IP/MS analysis of Aβ peptides from N2a/APP695,595/596NL cell-conditioned medium using mAb 4G8 is shown in Fig. 3. The identities of peaks were assigned according to the measured molecular masses and several criteria (see below) and are identified in the figure according to human Aβ amino acid sequence numbers. In addition to the species containing Aβ amino acid sequences 1–40 (Aβ-(1–40)) and 1–42 (Aβ-(1–42)) (the major components found in senile plaque of AD), we observed several short forms of Aβ-related peptides. A summary of these Aβ-related peptides is given in Table II. A similar Aβ profile was observed for cells expressing wild type human APP695 (N2a/APP695) (data not shown).**

The molecular masses of individual peptides (observed as peaks in the mass spectrum) were measured and used to determine the identities of the these peptides. Several criteria were used for determining the identities of Aβ peptide variants.

(i) The peak observed in the spectrum of cell culture medium conditioned with N2a/APP695,595/596NL cells (or N2a/APP695 cells) should not exist in the control spectrum, which resulted from assay of medium conditioned by non-transfected N2a cells.

(ii) The measured molecular mass of that peak should match the calculated molecular mass of Aβ peptide(s) based on the human Aβ amino acid sequence.

(iii) The tentative matched Aβ peptide should contain the epitope site for the mAb used in the immunoprecipitation.

(iv) If the predicted Aβ peptide contains a methionine residue, then its mass should increase by 16 Da when formic acid/water/isopropanol is used as elution buffer, since the methionine residue in Aβ peptide at position 35 is readily oxidized under these conditions (42). The results from IP/MS analysis with mAb 4G8 indicated that Aβ peptides containing the 4G8 epitope can be identified. To obtain a more complete peptide profile for sAβ in cultured neuroblastoma cells expressing human APP, IP/MS analysis was also carried out using a human Aβ specific antibody, mAb 6E10. Again, Aβ-(1–42), Aβ-(1–40), Aβ-(1–34), Aβ-(1–28), and other long forms of Aβ-related peptides were detected, similar to the results obtained with mAb 4G8. A series of peaks with different masses from those in Fig. 3 were observed in the mass range of 1000 to 2500 Da (Fig. 4). These peaks corresponded to Aβ related peptides with further truncated carboxyl termini (Aβ-(1–13) to Aβ-(1–20), or with both carboxyl- and aminoterminal truncations (Aβ-(2–13) to Aβ-(2–20), Aβ-(4–14), Aβ-(4–18), and Aβ-(4–19)) (Table II). In addition to identifying Aβ-related peptides, our results indicated directly that the epitope site of mAb 6E10 is within amino acid sequence of 4–13 of Aβ.2 These Aβ species were also observed from cell culture media conditioned with N2a/APP695 cells (data not shown). Surprisingly, we identified a total of 44 different secreted human Aβ-related peptides (Table II).
Fig. 2. Amino acid sequence alignment of human and murine Aβ. The vertical dotted line indicates the variations between these two sequences. The black bars below the sequences indicate the epitope sites for mAbs 6E10 (Aβ 4–13) and 4G8 (Aβ 17–24), respectively.

Comparing the results from cell culture medium of N2a/APP<sub>695,595,596NL</sub> cells (Figs. 3 and 4 and Table II) with results from cell culture medium of non-transfected N2a cells (Fig. 1 and Table I), peptides corresponding to human Aβ sequence were uniquely observed only in N2a/APP<sub>695,595,596NL</sub> cells (Figs. 3 and 4) and were absent in the control (Fig. 1). Finally, in contrast to the results from mAb 4G8 IP/MS, no murine Aβ or variants were detected in the spectrum resulted from the IP/MS analysis of cell culture medium conditioned with non-transfected N2a cells when mAb 6E10 was used (Fig. 3c). These results confirm the specificity of the mAb 6E10 for human Aβ.

The Sensitivity and Dynamic Range of IP/MS for Measuring Aβ Peptides—Four synthetic human Aβ peptides, Aβ(1–42) (4514.1 Da), Aβ(1–40) (4329.9 Da), Aβ(1–28) (3262.5 Da), and Aβ(12–28) (1555.2 Da), were chemically synthesized, and their concentrations were determined by amino acid analysis. Aβ(1–28) and Aβ(12–28) were obtained commercially (Sigma). A series of dilutions of each peptide was prepared ranging from 100 to 0.01 nM for peptides Aβ(1–42) (450 ng/ml to 45 pg/ml), Aβ(1–40) (430 ng/ml to 43 pg/ml), and Aβ(1–28) (320 ng/ml to 32 pg/ml) using serum-reduced media conditioned by non-transfected N2a cells for 12 h and immediately treated with protease inhibitors upon harvest. Conditioned medium of N2a cells was used to mimic the conditions used in a time-course study (see below). Only a small amount of endogenous murine Aβ was produced within 12 h and did not interfere with the measurement of the human Aβ peptides. Aβ(12–28), as internal standard, was added to each of the diluted solutions with a constant concentration of 20 nM prior to immunoprecipitation. We used Aβ(12–28) peptide as the internal standard because it appears not to be produced naturally (we did not detect it in our analysis of secreted peptides from N2a cells). The effects of experimental variations (e.g., signal variations due to the absolute amount of Protein G/A-agarose beads or to the laser intensity used during the MS measurement) on the resulting MS peak height was corrected by normalization to the internal standard, i.e., the peak height of Aβ(12–28). The peak heights of Aβ(1–42), Aβ(1–40), and Aβ(1–28) to Aβ(12–28) were calculated. These relative peak heights were used to evaluate the reproducibility and sensitivity of IP/MS in measuring Aβ peptides.

Fig. 5 depicts a series of mass spectra resulting from immunoprecipitation of five different concentrations of Aβ peptides using mAb 4G8. The peaks corresponding to Aβ(1–42), Aβ(1–40), Aβ(1–28), and Aβ(12–28) are labeled and also indicated by dotted lines. Other peaks appear in the spectra, especially in spectra of low Aβ concentrations. These peaks correspond to endogenous murine Aβ peptides. The spectra in Fig. 5 clearly show that Aβs can be detected at levels as low as 0.1 nM (0.01 nM for Aβ1–28), with a signal-to-noise level greater than 2. This
Profile of Aβ Protein and Its Variants in Cultured Cell Media

| Table II | Soluble Aβ and its variants detected from cell culture medium conditioned with N2a/APP770:670/671NL cells by IP/MS |
|----------|-------------------------------------------------------------------------------------------------------------------|
| Aβ (observed) | M<sub>r</sub> (calculated) | Amino acid sequence |
|------------|-----------------------------|---------------------|
| 4513.8<sup>a,b</sup> | 1–42 | 4514.1 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 4330.0<sup>a</sup> | 1–40 | 4329.9 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 4231.6<sup>a</sup> | 1–39 | 4230.7 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 4131.9<sup>a</sup> | 1–38 | 4131.6 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 4074.7<sup>a</sup> | 1–37 | 4074.5 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 3978.0<sup>a</sup> | 1–36 | 3978.2 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAIIGLMVQGIA |
| 3875.4<sup>a</sup> | 1–33 | 3870.4 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAII |
| 3615.8<sup>a</sup> | 1–32 | 3616.9 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSNKGAII |
| 3596.0<sup>a</sup> | 1–30 | 3599.6 | DAEFRHDGSEYHVHQQKLVFQAEEDVGSN |
| 3262.4<sup>a</sup> | 1–28 | 3262.5 | DAEFRHDGSEYHVHQQKLVFQAEEDVSN |
| 3123.4<sup>a</sup> | 1–27 | 3134.1 | DAEFRHDGSEYHVHQQKLVFQAEEDVG |
| 2461.8<sup>a</sup> | 1–20 | 2461.7 | DAEFRHDGSEYHVHQQKLHF |
| 2315.0<sup>a</sup> | 1–19 | 2314.5 | DAEFRHDGSEYHVHQQKL |
| 2167.3<sup>a</sup> | 1–18 | 2167.3 | DAEFRHDGSEYHVHQQL |
| 2068.2<sup>a</sup> | 1–17 | 2068.2 | DAEFRHDGSEYHVHQ |
| 1955.0<sup>a</sup> | 1–16 | 1955.0 | DAEFRHDGSEYHQQ |
| 1827.0<sup>a</sup> | 1–15 | 1826.9 | DAEFRHDGSEYH |
| 1699.0<sup>a</sup> | 1–14 | 1698.7 | DAEFRHDGSEY |
| 1561.8<sup>a</sup> | 1–13 | 1561.6 | DAEFRHDG |
| 2972.9<sup>a</sup> | 1–28 | 2970.5 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 2786.0<sup>b</sup> | 1–40 | 2786.3 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 2649.8<sup>b</sup> | 1–40 | 2651.9 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 2393.2<sup>b</sup> | 1–40 | 2392.8 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 2588.5<sup>b</sup> | 1–38 | 2588.0 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 2542.5<sup>b</sup> | 1–38 | 2550.9 | HQLVFQAEEDVGSNKGAIIGLMVQGIA |
| 3170.9<sup>b</sup> | 6–34 | 3168.5 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 2916.8<sup>b</sup> | 6–34 | 2916.3 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 2243.7<sup>b</sup> | 14–34 | 2243.6 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 2104.8<sup>b</sup> | 15–34 | 2104.6 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 1978.2<sup>b</sup> | 16–34 | 1978.3 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 1848.5<sup>b</sup> | 17–34 | 1850.1 | HDGSYHVHQQKLVFQAEEDVGSNKGAII |
| 2858.4<sup>a</sup> | 5–29 | 2857.1 | RHDSYHVHQQKLVFQAEEDVGSN |
| 2672.0<sup>a</sup> | 5–27 | 2671.9 | RHDSYHVHQQKLVFQAEEDVGSN |
| 2515.1<sup>a</sup> | 6–27 | 2515.7 | RHDSYHVHQQKLVFQAEEDVGSN |
| 2346.8<sup>a</sup> | 2–20 | 2346.6 | AEFRHDGSEYHVHQQKLVF |
| 2199.5<sup>a</sup> | 2–19 | 2199.4 | AEFRHDGSEYHVHQQKLVF |
| 2053.5<sup>a</sup> | 2–18 | 2052.2 | AEFRHDGSEYHVHQQKL |
| 1712.2<sup>a</sup> | 2–15 | 1711.8 | AEFRHDGSEYHVH |
| 1583.8<sup>a</sup> | 2–14 | 1583.6 | AEFRHDGSEYH |
| 1446.5<sup>a</sup> | 2–13 | 1446.5 | AEFRHDGSEY |
| 1512.4<sup>a</sup> | 3–14 | 1512.6 | AEFRHDGSEY |
| 1999.4<sup>a</sup> | 4–19 | 1999.2 | FRHDGSEYHVHQQKLVF |
| 1851.9<sup>a</sup> | 4–18 | 1852.0 | FRHDGSEYHVHQQKL |
| 1383.4<sup>a</sup> | 4–14 | 1383.4 | FRHDGSEYH |

* Detected by both mAbs 4G8 and 6E10.
*<sup>b</sup> 16 Da mass increase was observed when formic acid/water/isopropanol were used for elution.
*<sup>c</sup> Detected by mAb E10 only.
*<sup>d</sup> Detected by mAb 4G8 only.

Corresponds to about 0.4 ng/ml for Aβ-(1–40). The average relative peak height for each Aβ peptide obtained by three independent measurements are plotted versus concentration in Fig. 6. This plot indicates that IP/MS has a very wide dynamic range (linearity between the relative peak height and the Aβ concentration) for Aβ measurement. The correlation coefficients between the relative peak heights and peptide concentrations are 0.9993 for Aβ-(1–28) in the concentration range of 0.01 nM to 100 nM, 0.9992 and 0.9997 for Aβ-(1–40) and Aβ-(1–42), respectively, over the concentration range of 0.1 nM to 100 nM. The standard deviations (S.D.) based on three measurements are plotted in Fig. 6 with error bars, which indicate standard errors of the means. The coefficient of variation (the percentage of standard deviation to the measured mean) is 21.8%, averaging all data in this study.

A Time-course Measurement of Aβ Protein and Its Variants Secreted by Cultured Cells—The accumulation of Aβ-(1–40), Aβ-(1–42), and several Aβ fragments produced by cultured cells were carefully monitored by IP/MS as a function of time. N2a/APP<sub>770:670/671NL</sub> cells were propagated in 1.5 ml of growth medium in 12-well tissue culture plates. To prepare conditioned media, cell monolayers were washed three times with phosphate-buffered saline and then placed in 1 ml of serum-reduced medium and incubated at 37°C, 5% CO<sub>2</sub> for times between 1 h and 30 h. The harvested media were treated immediately with a protease inhibitor mixture (see above) to prevent proteolytic degradation. Aβs were immunoprecipitated together with an internal standard, Aβ-(12–28), by mAb 4G8 and analyzed by MS. The mass spectra from four time points are shown in Fig. 7. In order to reduce the fluctuation in the Aβ measurements that is caused by the variation in the cell number in each measurement, two separate measurements were performed for each time point. The average peak heights of Aβ peptides relative to standard (Aβ-(12–28)) and measured Aβ peptide concentrations using standard curve (Fig. 6) were plotted versus time to view the relationship between the truncated Aβ and Aβ-(1–40/1–42) (Fig. 8). We observed that the total level of Aβs increased during the 20-h incubation time. The data shown in Fig. 8 represent the integrated values of sAβ peptides in cell culture media. In other words, these results represent net Aβ levels, which are presumably determined by the balance of production and degradation of Aβ in the cell.
culture media. The rate of Aβ turnover cannot be determined from our current data due to the continuous production of Aβ by cells.

Proteases in Conditioned Medium Degrade Exogenous Aβ to an Insignificant Degree—To examine the origin of the short Aβ peptides observed in cell culture media, we studied Aβ proteolysis in conditioned medium of non-transfected N2a cells. Conditioned medium was freshly prepared by conditioning serum-reduced medium with non-transfected N2a cells. Synthetic human Aβ-(1–40) (50 nM) was incubated at 37 °C for 22 h in the medium of cell conditioned for 18 h. The degraded Aβ peptide fragments were analyzed by IP/MS with mAbs 4G8 and 6E10. The representative mass spectra resulting from 0-h and 22-h samples of Aβ-(1–40) are shown in Fig. 9. We observed very low levels of truncated Aβ-(1–40) after a 22-h incubation (Fig. 9, B and D). These degraded Aβ peptides, primarily generated by proteolytic cleavages after lysine 28, histidine 13/14, and hydrophobic amino acids (phenylalanine 19/20, glycine 33, and leucine 34), are present at low levels in comparison to full-length Aβ-(1–40).

**DISCUSSION**

Amyloid β Protein and Its Variants Are Readily Detected by Immunoprecipitation/Mass Spectrometric Analysis—The present study reports a comprehensive profile of sAβ secreted by cultured cell lines using a novel IP/MS method. Sixty-four Aβ-related peptides (44 from human and 20 from murine Aβ sequences) have been identified from immunoprecipitated conditioned cell culture media without further purification. Compared to other methodologies used to measure sAβ, the IP/MS has several significant advantages. High affinity antibodies were utilized to specifically purify and enrich the peptides of interest. This enrichment enabled us to analyze the peptides of interest directly from a small volume of biological fluid with low Aβ concentrations. This technique should be very useful in the analysis of sAβ from human specimens (e.g. CSF). Accurate molecular masses of Aβ peptides were determined in the present measurement. Because highly specific Aβ monoclonal antibodies were utilized, these accurate molecular masses provide essential information regarding the identities, amino acid composition, and sequence length of Aβ and its related variant peptides. Another important feature of this mass-specific detection assay is the ability to simultaneously analyze complex mixtures of Aβs in biological samples in a single assay. Hence, both the molecular masses and the concentrations of sAβs can be determined in a single measurement using appropriate Aβ peptide analogs as internal standards with appropriate standard curves (see below).

Characterization of Amyloid β Protein and Its Variants—Aβ-(1–40) has been detected in all cell lines studied, including cells transfected with human APP and non-transfected mouse cells. Aβ-(1–40) is the predominant form isolated from cerebral cortical deposits (9), vascular deposits (10, 15), cerebrospinal fluid (18), human mixed-brain cell culture (18), and other cultured cell lines. Aβ-(1–42) on the other hand, has only been detected in the medium of cells transfected with human APP cDNAs. In addition, more than 40 truncated Aβ peptides have been identified from cell culture media conditioned with N2a/AIP695/595/596NL or N2a/APP695 Cells (Table II). These Aβ peptide fragments can be classified into five different groups according to the patterns of the peptides: i) peptides with truncated carboxyl termini (these range from Aβ-(1–13) to Aβ-(1–39)); ii) peptides with truncated amino termini (Aβ-(14–
42), Aβ-(14–40), Aβ-(15–40), and Aβ-(17–40) have been observed; (iii) peptides resulting from ragged NH₂ termini and new COOH termini, possibly resulting from internal cleavage of Aβ (these include Aβ-related peptides ending with Gly³⁸, Leu³⁴, and Asn²⁷ at their COOH termini); (iv) peptides with ragged COOH termini and a new NH₂ termini, possibly resulting from internal cleavage of Aβ (these include Aβ-related peptides beginning with Ala², Phe⁴, Arg⁵, and His⁶ at their NH₂ termini); (v) other internal peptide fragments of Aβ (for example, peptide Aβ-(3–14)). Several peaks that correspond to Aβ-(1–38), Aβ-(1–34), Aβ-(1–28), Aβ-(1–20), Aβ-(1–19), and Aβ-(1–14) were observed as prominent peaks in the mass spectra (see Figs. 3 and 4).

In contrast to the Aβ-related peptides observed from cell culture media conditioned with N2a/APP⁶⁹⁵/⁵⁹⁵NL and N2a/APP⁶⁹⁵ cells, a different proteolytic digestion pattern was observed from cell culture media conditioned by non-transfected N2a cells. The predominant murine Aβ peptide fragments observed in this study apparently resulted from cleavages around amino acid residue Glu¹¹. These peptides start at either Glu¹¹ or Val¹² at their NH₂ termini. The different termini in Aβ species of human versus mouse Aβs may be due to alternative proteolytic events that are directed by the differences in human and mouse Aβ peptide sequences (Fig. 2).

We have observed a complex set of Aβ peptides from cultured cell media. We are quite confident that these peptides are not artificially produced by proteolysis of full-length 1–40/42 peptides in the medium after harvest, or due to our antibody incubation steps. In support of this view, we have demonstrated that synthetic Aβ-(1–40) and Aβ-(1–42) peptides are not degraded upon addition to the medium of non-transfected N2a cells conditioned for 12 h and subsequently subject to IP/MS (Fig. 5), in the existing of protease inhibitors. Furthermore, these Aβ peptide fragments are also not generated extracellularly by proteolytic degradation as consequence of proteolytic activity in fetal bovine serum that was used in the conditioning medium (data not shown). The mechanisms by which these Aβ-related peptides are generated is presently not clear. Nevertheless, clarification of the relationship of these short Aβ forms and longer Aβ-(1–40/1–42) forms may provide some insight into the production, metabolism and deposition of Aβ in AD.

**Fig. 7.** Mass spectra of Aβ and variants obtained during a 30-h time course of N2a/APP⁶⁹⁵/⁵⁹⁵NL cell conditioned media. The resulting spectra from four time points (2, 4, 10, and 21 h, as indicated) were normalized to the internal standard, Aβ-(12–28), and plotted. Aβ peptides were measured by IP/MS (see "Experimental Procedures").

**Fig. 8.** A, the relative peak height versus time resulting from two independent time-course experiments were plotted to show the relationship between the truncated Aβ peptide and Aβ-(1–40/1–42). B, the peptide concentrations for Aβ-(1–40), Aβ-(1–42) and Aβ-(1–28) were measured using the standard curve (Fig. 6) and plotted to correct the experimental effect on the peak intensity. The highest concentrations of Aβ-(1–40), Aβ-(1–42), and Aβ-(1–28) obtained from this study were 11.0, 2.9, and 0.34 nM at the 21 h time point. Since the lack of synthetic Aβ-(1–34) peptide, the concentration of Aβ-(1–34) was not determined in this experiment. The relative peak height correlation coefficients between Aβ-(1–42), Aβ-(1–34), and Aβ-(1–28) to Aβ-(1–40) were 0.988, 0.979, and 0.988, respectively, during the first 21 h.

**Broad Concentration Ranges of Aβ Peptide Can Be Measured by IP/MS**—In conventional immunoprecipitation, quantitative recovery of a specific protein (antigen) is the primary goal. This is achieved by using excess antibody and excess precipitation reagents. Normally, 10 μl of mAb and 15 μl of Protein G/AG-agarose beads are used for immunoprecipitation from 1 ml of cellular extraction or cell culture media (according to the manufacturer’s protocol, Oncogene Science, Inc). In comparison, our present approach uses only 1 μl of mAb and 3 μl of Protein G/AG-agarose beads for immunoprecipitation. The small amount of immunoprecipitation reagent used here is intended to reduce the final elution volume and allow direct mass spectrometric measurement of antigens. We have attempted to achieve quantitative sampling of the antigens rather than 100% immunoprecipitation of them. For quantitative sampling (using small amount of precipitation reagent), a proper ratio of mAb and precipitation reagent is essential to provide high binding capacity and high detection sensitivity for the peptide (antigen) of interest. If the ratio is too low, the peptide binding capacity will be reduced and only the most abundant species and some minor species (possibly certain polymeric Aβ peptides) with substantially higher avidity to the mAbs will be detected. If the ratio is too high, the sensitivity will be reduced by the increased occupation of free antibodies on the binding site of the precipitating reagent. Therefore, the amount of mAb and Protein G/AG-agarose bead and the ratio of these two reagents was carefully optimized. Using a mAb/precipitation reagent ratio of 1:3 (v/v), we have been able to measure a very broad concentration range of Aβ peptides (from 0.01 nM to 100 nM) (Fig. 6). The concen-
The results of our time-course experiments indicate that the concentration of Aβ in the CHO conditioned medium reaches a peak level about 6 h into the time course, and diminishes over the next 18 h. The inhibition of Aβ degradation in CHO conditioned medium required the presence of all four major classes of protease inhibitors. Rapid degradation of Aβ in COS conditioned medium has also been reported (44). A serine protease-α2-macroglobulin complex was described in the latter report as being responsible for Aβ degradation in COS conditioned medium. This novel serine protease cleaves Aβ primarily after hydrophobic amino acid residues (Aβ residues 17–19 and 32–34) and degrades synthetic Aβ-(1–40) almost completely in 16 h. The protease activity can be inhibited by certain types of serine protease inhibitors (26). In the present report, we demonstrated Aβ degradation in N2a conditioned medium. However, the cleavage sites and degradation rates were different from those shown in CHO and COS cells. These differences most likely indicate that cell line-dependent processes are operative. However, in view of our demonstration that the shortened Aβ-related species appear at the earliest time points and accumulate in proportion to Aβ-(1–40) over the time course (Figs. 7 and 8) and our observation that only a small portion of exogenously added Aβ peptide was degraded in conditioned medium (Fig. 9), we conclude that the vast majority of shortened Aβ-related species are generated during intracellular processing of APP.

Here, we have shown that the combination of immunoprecipitation and mass spectrometry provides a specific and sensitive approach for studying amyloid β protein and its variants in conditioned media. This method should greatly facilitate studies of the metabolism of Aβ in vitro and in vivo.

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Fig. 9. Aβ peptide fragments generated by proteolytic degradation of synthetic human Aβ-(1–40) peptide in non-transfected N2a cell conditioned medium were identified by IP/MS analysis. N2a cell conditioned medium (18 h) was freshly harvested and synthetic Aβ-(1–40) peptide (50 nM) was incubated in the conditioned medium at 37 °C for 0 h (A and C) and 22 h (B and D) (see “Experimental Procedures”). A and B are the resulting spectra of IP/MS analysis using mAb 4G8, and C and D are the resulting spectra from mAb 6E10. The identities of peaks observed in these spectra were labeled with human Aβ sequence numbers (see Fig. 1 legend).
