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Amyloidogenic Processing of the Human Amyloid Precursor Protein in Primary Cultures of Rat Hippocampal Neurons

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The aim of this study was to investigate the proteolytic processing of the amyloid precursor protein (APP) in polarized primary cultures of hippocampal neurons. We have used the Semliki Forest virus (SFV) vector to express human APP695 in hippocampal neurons, sympathetic ganglia, and glial cells. The latter two cells secrete little or no APP, whereas hippocampal neurons secrete two forms of APP695, which differ in sialic acid content and in their kinetic appearance in the culture medium. In addition, rat hippocampal neurons expressing human APP produced significant amounts of the 4 kDa peptide βA4. After 3 hr of metabolic labeling, the relative amount of βA4 peptide to total cellular APP was 5.3%. Fibroblasts expressing APP695 using the same SFV vector mainly produced a related 3 kDa p3 peptide, a nonamyloidogenic fragment. Remarkably, the hippocampal neurons also produced significant amounts of βA4-containing C-terminal fragments (10–12 kDa) intracellularly. Radiosequencing showed that these fragments were created at a previously described β-secretase cleavage site and at a cleavage site 12 residues from the N terminus of the βA4 domain (Thr^{584} of APP695), which we named δ-cleavage. Based on the observation that mature hippocampal neurons produce two potentially amyloidogenic fragments and secrete substantial amounts of βA4 when expressing human APP, our results strengthen the hypothesis that neurons play a central role in the process of βA4 deposition in cases of Alzheimer's disease and in aged primates.

Key words: Alzheimer's disease; hippocampal neurons; SFV vector; amyloid precursor protein; β-amyloid peptide; amyloidogenic processing

The amyloid precursor protein (APP) is a widely expressed 100–140 kDa integral membrane protein with a large N-terminal ectodomain and a small C-terminal cytoplasmic domain (Kang et al., 1987). Although its physiological function remains unknown, several findings suggest a central role for APP in the pathogenesis of Alzheimer's disease (AD) (Selkoe et al., 1994). APP is the parent molecule for a small, 39–43 residue amyloid peptide (βA4), which is the principal component of the senile plaques and vascular amyloid found in the brains of AD patients (Glenner and Wong, 1984; Masters et al., 1985). Furthermore, families with hereditary forms of AD, or familial AD (FAD), carrying missense mutations in the APP gene have been identified (Mullan and Crawford, 1993), and overexpression of such human APP mutants in transgenic mice causes amyloid deposition and plaque formation of similar tinctorial properties such as those occurring in AD (Games et al., 1995). Although APP is expressed ubiquitously, pathological manifestations of AD such as βA4 deposits are found exclusively in brain tissue, which suggests that there are mechanisms specific to the brain that are responsible for the pathogenesis. Among brain cells, neurons are strongly affected during AD by changes in their cytoarchitecture, giving rise to dystrophic neurites and neurofibrillary tangles. Neurons have been proposed as a potential source of βA4 in the amyloid plaques based on indirect evidence showing that neurons accumulate APP in the neurites surrounding the amyloid deposits (Cork et al., 1990; Cras et al., 1991). Moreover, βA4 depositions in the brain appear to spread along neuronal projections, supporting the idea that neurons play a causal role in the pathogenesis (Hyman et al., 1990).

Despite the great interest in neuronal APP processing in AD research, APP processing has been studied almost exclusively in non-neuronal cells. These studies have demonstrated that APP has three proteolytic cleavage sites. After transport from the Golgi apparatus, APP can be cleaved within the βA4 region (α-cleavage) (Esch et al., 1990; Sisodia et al., 1990), leading to the release of the large, 100 kDa ectodomain that is secreted. The amount of secreted protein varies from cell to cell but does not appear to exceed 30% of totally synthesized protein (Weidemann et al., 1989; Caporaso et al., 1992; Selkoe et al., 1994). The molecules escaping α-cleavage can be internalized from the cell surface, mediated via endocytosis signals in the cytoplasmic tails (Haass et al., 1992a,b; Koo and Squazzo, 1994), and then processed by β-cleavage at Met^{596} (numbering after APP695), creating the N terminus of βA4 (Asp^{597}). This scission is followed by an additional cleavage (γ-cleavage) in the region of Val^{636} to Thr^{640}, releasing βA4. APP processing is complicated further in cells that

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possess a polarized cell surface. In epithelial Madin–Darby canine kidney (MDCK) cells, APP is transported to the basolateral domain from which its proteolytic fragments, the 100 kDa ectodomain and the 4 kDa βA4 peptides, are secreted (Haass et al., 1994; Lo et al., 1994; De Strooper et al., 1995a).

Neurons have a polarized cell surface like epithelia and are differentiated into somatodendritic and axonal territories (Craig and Banker, 1994). Little is known about APP processing in polarized neurons; therefore, we decided to study the metabolism of human APP695 in the hippocampal neuron culture system. During culture *in vitro*, the hippocampal neurons undergo a sequence of differentiation steps that leads to maturation into fully polarized cells with axons and dendrites forming synaptic networks on the culture dish (Dotti et al., 1988). These neurons share many biological properties with their counterparts in the brain and, therefore, constitute an excellent *in vitro* model. It has been shown that human APP695 is expressed in these neurons and that it is delivered first to the axons and then transported to dendrites by a mechanism similar to what has been termed "transcytosis" in epithelial cells (Simons et al., 1995). Similar results have been obtained with endogenous APP in neuron culture, confirming that the overexpression of APP via the virus vector mimics the normally observed situation (Yamazaki et al., 1995).

To obtain sufficient levels of APP expression for biochemical analyses, we used recombinant Semliki Forest virus (SFV) coding for human APP695 to infect primary cultures of rat hippocampal neurons. This system has been used successfully to express heterologous proteins in neurons and offers the advantage of high protein expression without affecting the polarized organization of the cells during the early phase of infection (Ikonen et al., 1993; Olkkonen et al., 1993; De Hoop et al., 1994; Simons et al., 1995; De Strooper et al., 1995b).

MATERIALS AND METHODS

Cell culture. Hippocampal neuron cultures were prepared from 18-d-old fetal rats as described by Goslin and Banker (1991). Briefly, after the hippocampi were dissected and dissociated, the cells were plated on either poly-D-lysine-coated plastic dishes (at a density of 4×10^5 cells/6 cm dish) or glass coverslips (at a density of 1.5×10^5 cells/6 cm dish) and kept in minimum essential medium (MEM) supplemented with 10% horse serum. Cells were maintained in 5.0% CO_2 at 36.5°C in MEM with N2 supplement ("maintenance medium") (Bottenstein and Sato, 1979). Proliferation of non-neuronal cells was prevented by adding 5 μM cytosine arabinoside.

Glial cultures were prepared as described by Goslin and Banker (1991). The cerebral hemispheres were removed from newborn rats and mechanically and chemically (trypsin) homogenized. Cells were cultured in MEM supplemented with 10% horse serum. To prevent neuronal contamination, only glial cultures that were passaged two to three times were used.

Superior cervical ganglia were dissected from 19- or 20-d-old fetal rats and prepared as described by Peng et al. (1986). Explants were cultured for 14 d in N2-MEM supplemented with 10 μM fluorodeoxyuridine, 10 μM uridine, 20 μg/ml tri-iodo-L-threonine, and 10 ng/ml 2.5S nerve growth factor before use.

Baby hamster kidney cells (BHK; CLL10) were cultured in Glasgow's modified Eagle's medium supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 10% tryptose phosphate broth, 100 U/ml penicillin, and 100 gm/ml streptomycin.

Primary fibroblasts from 13.5-d-old mouse embryos were isolated as described previously (Robertson, 1987) and maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics.

Antibodies. Antibodies (Abs) used in the experiments included polyclonal anti-FdAPP raised against purified *Escherichia coli* FdAPP fusion protein consisting of the following: APP695 and the Fd fragment of murine IgM heavy chain (Weidemann et al., 1989); 22C11, a monoclonal Ab (mAb) that recognizes a region between amino acids 66 and 81 of APP695 (Hilbich et al., 1993); Ab 692, a polyclonal Ab raised against human βA4 synthetic peptide 1–40; B12/4, a polyclonal Ab against the 20 C-terminal amino acids of APP (De Strooper et al., 1995a); B7/6, a polyclonal Ab raised against synthetic human 1–40 βA4 peptide, which only recognizes the human 1–16 sequence (B. De Strooper, unpublished observations); R 217, made against APPs (Lowery et al., 1991); R1282, raised against human synthetic 1–40 βA4 peptide (Haass et al., 1992a,b); R48, raised against human synthetic βA4, 1–16 (Anderson et al., 1992); and 4G8, an mAb against residues 17–24 of βA4 (Kim et al., 1988). For immunoprecipitation, each Ab was used at 1:100 dilution except R217, which was used at 1:500, and B12/4, which was used at 1:1000. Ab 22C11 was used at 1:10,000 for immunoblotting.

DNA constructs. From the plasmid PreA4-pSP65 (Dyrks et al., 1988) containing the APP695 sequence, the *SpeI* site in the noncoding region of APP695 was removed by using the Klenow fragment. The 3 kb *SmaI* fragment containing the complete coding region of the APP695 cDNA was subcloned into the *SmaI* site of pSFV 1 (Liljestrom and Garoff, 1991).

Preparation of recombinant SFV. The pSFV-APP695 (human) and the pSFV-helper 1 were linearized with *SpeI*, and runoff transcription was performed with SP6 RNA polymerase. Each APP transcription mix was cotransfected with the helper-transcription mix to BHK cells using electroporation as described by Olkkonen et al. (1993). The culture supernatant was collected after 20–24 hr incubation (5% CO_2, 37°C). The virus-containing supernatant was titrated on BHK cells as described by Olkkonen et al. (1993).

Infection of the neurons and metabolic labeling. Recombinant SFV diluted in conditioned maintenance medium was placed on 7- to 14-d-old neurons. After allowing viral entry for 1 hr (5% CO_2, 37°C), the virus solution was replaced by maintenance medium and the infection continued for 2–3 hr. The medium then was exchanged by methionine-free MEM with 1/10 N2 supplement containing 15 mg/l methionine and 1% egg albumin. Metabolic labeling was performed for 3 hr with 200 μCi/ml [^{35}S]methionine. In time-course experiments 3 hr after infection, cells were pulsed for 10 min with methionine-free MEM containing 300 μCi/ml [^{35}S]methionine. Next, cells were washed twice with maintenance medium, and fresh medium was added at 0, 30, 60, and 120 min after radioactive pulse. Culture medium from each time interval was processed immediately for immunoprecipitation. For *in vivo* labeling with [^{32}P]orthophosphate, neurons were incubated 3 hr after infection in phosphate-free MEM supplemented with 1/10 N2 supplement containing 158 mg/l NaH_2PO_4 for 15 min and then labeled with 1 mCi/ml $H_3{}^{32}PO_4$ for 3 hr. Extracellular medium was processed as described, except that orthovanadate (100 μM) and sodium fluoride (50 mM) were added.

Immunoprecipitation, immunoblotting, gel electrophoresis, and quantification. After metabolic labeling, culture medium was harvested and cell extracts were prepared in 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS with 10 μg/ml chymostatin, leupeptin, anti-papain, and pepstatin. Both culture medium and cell extracts were cleared first from precipitations, cell debris, and nuclei via 15 min centrifugation. The supernatant was incubated with Ab overnight at 4°C. Immune complexes were recovered with protein A-Sepharose or protein G-agarose and processed as described previously (De Strooper et al., 1993). In the case of mAbs, polyclonal rabbit anti-mouse was used as capture Ab. For immunoblotting, cell extracts and extracellular medium were precipitated in 10% trichloroacetic acid (TCA), washed with acetone, and solubilized in SDS sample buffer. TCA precipitates and immunoprecipitated proteins were separated by 6% Tris-glycine, 4–20% Tris-glycine, or 10–20% Tris-tricine polyacrylamide gels (Novex, San Diego, CA). We used low-range See Blue prestained standard (Novex) and high-range prestained SDS-PAGE standard (Bio-Rad, Richmond, CA) as molecular weight standards. Gels were enhanced by Intensify (DuPont NEN, Boston, MA), dried, and exposed to Kodak XAR film (Rochester, NY). For immunoblotting, membranes were blocked in PBS/5% milk and 0.2% Triton X-100 and incubated for 1 hr at room temperature with primary Ab diluted in blocking buffer. Appropriate horseradish peroxidase-labeled secondary Ab (Bio-Rad) was added for 30 min. Blots were developed by enhanced chemiluminescence (Amersham Buchler, Braunschweig, Germany). The intensity of radiolabeled bands was calculated using densitometric scanning (Molecular Dynamics, Sunnyvale, CA).

Enzymatic deglycosylation. For digestion with neuraminidase (Boehringer Mannheim, Indianapolis, IN), immunoprecipitates were made with 1% SDS and 1% β-mercaptoethanol, boiled for 5 min, and diluted 10-fold to final concentrations of 50 mM sodium acetate and 4 mM calcium chloride, pH 5.5, 1% Nonidet P-40, 0.1% SDS, 10 μg/ml chymostatin, leupeptin, anti-papain, pepstatin, and 10 mU/ml neuraminidase. After incubation at 37°C for 16 hr, samples were analyzed by SDS-PAGE.

Samples treated with *N*-glycosidase F (10 U/ml, Boehringer Mannheim) were buffered in 50 mM sodium phosphate buffer, pH 7. For *O*-glycosidase (Boehringer Mannheim) treatment, immunoprecipitates were preincubated with 1 mU of neuraminidase at 37°C for 3 hr; 3 mU of *O*-glycosidase was used in sodium phosphate buffer, pH 7. As a control for unspecific degradation, parallel samples were incubated in the absence of enzymes.

Radiosequencing. After SDS-PAGE, [^{35}S]methionine- and [^{3}H]phenylalanine-labeled proteins were blotted into ProBlott membranes (Applied Biosystems, Foster City, CA). Membranes were subjected first to autoradiography, and strips were excised corresponding to the bands visualized by autoradiography. A modified cycle was used for protein sequencing on the Model 477A Protein Sequencer (Applied Biosystems). In the solvent-wash step, the S1 (*n*-heptane) delivery time was reduced from 100 to 25 sec. The S2 (ethyl acetate) delivery time was reduced to a total of 105 sec divided into three 35 sec deliveries alternated with two 5 sec pause steps. In the anilinothiazolinone extraction step, the S3 transfer step (52 sec) was split into three 25 sec transfers. Each transfer was followed by a 5 sec pause step and a 5 sec transfer with argon. The solvent was directed entirely to the fraction collector, and each fraction was mixed with 3.5 ml of liquid scintillator (Ready Protein Cocktail, Beckman, Fullerton, CA) before counting for 10 min (Beckman LS 6000IC).

Immunofluorescence. Immunofluorescence was performed as described previously (Simons et al., 1995). Cells were fixed with 4% *p*-formaldehyde in PBS, pH 7.4, quenched with 50 mM NH_4Cl in PBS for 20 min, permeabilized with increasing concentrations of ethanol, and blocked in 2% bovine serum albumin, 2% FCS, and 0.2% gelatin in PBS. The cells were incubated in the presence of primary Ab (anti-FdAPP and anti-MAP2) and fluoroscein- or rhodamine isothiocyanate-conjugated secondary Abs, as described by Simons et al. (1995). The coverslips were mounted in Moviol (Hoechst, Frankfurt, Germany), and the cells were viewed using an Axiophot (Zeiss, Jena, Germany). Photographs were taken using high-sensitivity film (Tmax 3200, Kodak).

RESULTS

To analyze processing and secretion of endogenous APP, neuronal cultures were labeled in the presence of [^{35}S]methionine for 3 hr, and cell lysate and extracellular medium were subjected to immunoprecipitation by a panel of different Abs against C- and N-terminal epitopes of APP (see Materials and Methods). The Abs revealed a prominent band at 115 kDa in the cell homogenate and a doublet of 120 and 100 kDa in the culture medium (Fig. 1*A*). A C-terminal Ab recognized the 115 kDa band from the cell lysate but not the APP cleavage products in the culture medium (data not shown), indicating that the latter forms had lost their cytoplasmic tails. The possibility that one of the bands in the culture medium represents the APP-like protein (APLP 1 or 2) was ruled out because both bands reacted with Ab R48 against epitope 1–16 of βA4 (not present in APLP) in immunoblots (Fig. 2*A*) and immunoprecipitations (data not shown).

To analyze the processing and secretion of APP in more detail, we used the SFV-based protein expression system (Liljestrom and Garoff, 1991). Neuronal cultures were infected by recombinant SFV carrying human APP695 and metabolically labeled with [^{35}S]methionine for 3 hr. Expression of human APP695 in hippocampal neurons led to secretion of the same 100 and 120 kDa bands (Fig. 1*A*), demonstrating that the use of this vector system led to processing similar to that observed for the endogenous APP. Also, the fact that the distribution of the endogenous marker protein MAP2 remained polarized during virus infection supports the use of this expression system for a detailed analysis of APP processing (Fig. 3*B*).

Primary neuronal cultures always contain a low amount of glial cells (5–20%). To investigate the possible contribution of infected glial cells to the APP produced by the neurons, a subconfluent homogenous culture of glial cells was infected and processed in parallel, and levels of APP expression were compared in both the hippocampal and the glial cultures. In addition, an explant of

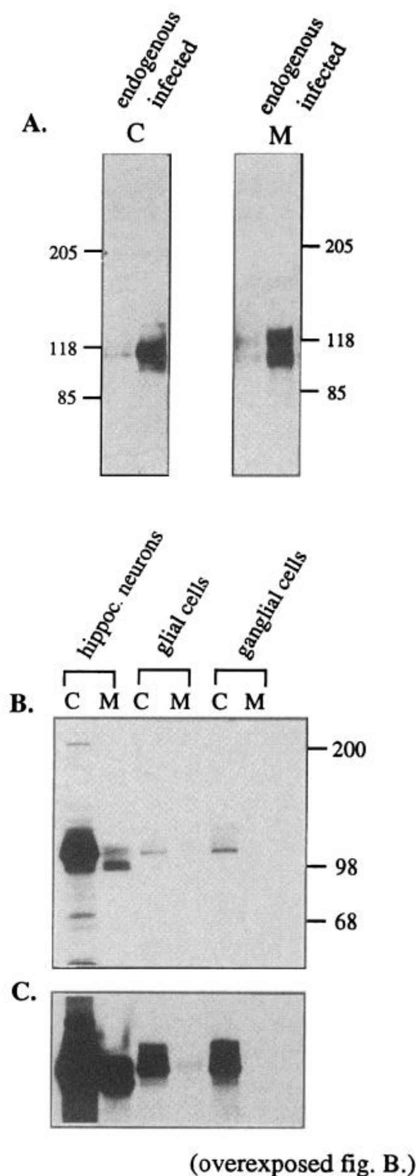

Figure 1. Immunoprecipitation of [^{35}S]methionine-labeled APP from cell homogenate and extracellular medium. *A*, *Endogenous* APP- and SFV-expressed APP695 (*infected*) were immunoprecipitated with Ab R217 from cell homogenate (*C*) and extracellular medium (*M*) of mature primary hippocampal neuron cultures after a 3 hr metabolic labeling with [^{35}S]methionine. Note the comigration of endogenous and SFV-expressed APP695. *B*, A hippocampal neuron (*hippoc. neurons*) culture (4×10^5 cells) and a glial culture (10^6 cells) (*glial cells*) were infected with SFV-carrying APP695 and labeled for 3 hr with [^{35}S]methionine. Immunoprecipitations of cell homogenate (*C*) and extracellular medium (*M*) were performed with Ab R217. Glial cells show little APP expression compared with hippocampal neuron cultures. Six cervical ganglion explants (*ganglial cells*) were infected with SFV-containing APP695 and metabolically labeled for 8 hr. Resulting cell homogenate (*C*) and conditioned medium (*M*) were pooled and processed as above. *C*, Overexposed version of *B*. Fluorogram was exposed for 3 weeks. Note the reduced secretion of glial cell cultures compared with neuronal cultures in *B* and the absence of detectable secretory APP in culture media of cervical ganglions.

superior cervical ganglia, as a source for peripheral neurons, also was investigated for secretion (Fig. 1*B,C*). It is clear from Figure 1*B* that glial cells were infected much less efficiently with recombinant SFV than were the hippocampal neurons under the exper-

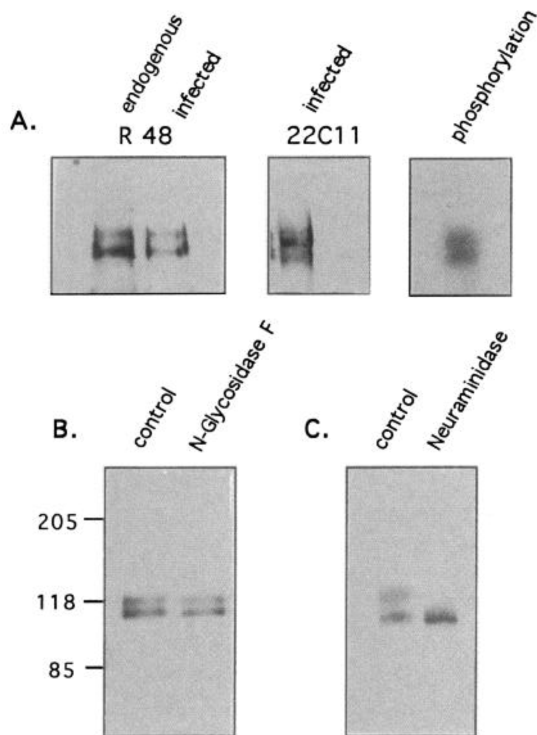

Figure 2. Characterization of secreted APP695. *A*: *Left,* Immunoblotting of endogenous secreted APP (*endogenous*) and SFV-expressed APP695 (*infected*) with Ab R48 specific for βA4 1–16 from conditioned media of cultured hippocampal neurons (*R 48*; 10-fold more medium was used from noninfected neuronal cultures). *Middle,* Immunoblotting of culture medium from SFV/APP695 neurons *infected* with Ab *22C11*. *Right,* Immunoprecipitation of cultured media from infected SFV/695 and [^{32}P]phosphate-labeled neurons (*phosphorylation*) with Ab R217. *B*, *C*, Endoglycosidase F and C (*N-Glycosidase F*) and *neuraminidase* treatment of immunoprecipitated SFV-expressed secretory APP695 from culture medium of hippocampal neurons. In *B* and *C*, the *left lanes* contain control samples that were incubated in the absence of enzyme.

imental conditions. Interestingly, glial cells secreted only minor amounts of APPs, detectable only after overexposure of the gels (Fig. 1*C*), which confirms previous investigations (Haass et al., 1991). Because the glial cultures contained at least 10-fold more glial cells than the hippocampal cultures routinely used, we concluded that contaminating glial cells did not contribute significantly to the pool of APP observed in our experiments. Although these experiments clearly demonstrate secretory processing of APP by hippocampal neurons, significant secretory processing of APP in the ganglion explant was not observed, even though the cells were infected by the virus and expressed APP695 (Fig. 1*B,C*).

To determine the time course of APP secretion, one dish (4 × 10^5 cells) of infected hippocampal neurons was pulse-labeled for 10 min, and medium was assayed for secreted APP after 30, 60, 120, and 180 min of chase (Fig. 4). At each time point, the complete medium was collected and replaced with an equal amount of fresh medium. Figure 4 represents the fractional accumulation of the pulse-labeled APP into the culture medium. Secretion of APP in hippocampal neurons was slow, and even after 3 hr APP from the pool synthesized during the 10 min pulse labeling continued to appear in the culture medium. Moreover, the overall kinetics of this process was not only somewhat different from that of other cells (Weidemann et al., 1989; Oltersdorf et al., 1990), but intriguing kinetic differences in the appearance of the two secretory APP products also were observed. The 120 kDa form appeared first, and its secretion was almost complete 120 min after the pulse, whereas the 100 kDa form was observed only after 60 min and was still being secreted after 180 min of chase. This was confirmed in four independent experiments with two different Abs. Quantitation of the kinetic differences is shown in Figure 4*B*.

Because in other cells APP695 usually yields one single secreted APP with M_r ~ 100 kDa (Weidemann et al., 1989; Esch et al., 1990; Oltersdorf et al., 1990; Golde et al., 1992), the nature of two soluble APPs in hippocampal cultures was explored further. Proteolysis of the higher molecular weight form by proteases present in the extracellular medium was excluded, because incubation of culture supernatant at 37°C during 3 hr in the absence of proteinase inhibitors did not change the ratio between the two forms (data not shown). Furthermore, both proteins reacted in immunoblots with Abs that recognize the C- and N-terminal ends of secretory APP (Fig. 2*A*), confirming that proteolysis in the culture medium was not involved in the generation of the two bands. Therefore, other post-translational modifications were investigated. Both proteins were weakly phosphorylated to a similar level (Fig. 2*A*), excluding the possibility that one band is phosphorylated and the other is not. Glycosylation was explored using carbohydrate-cleaving enzymes. Endoglycosidase F decreased the molecular weight of both bands by ~2 kDa, indicating similar levels of N-glycosylation (Fig. 2*B*). Treatment with neuraminidase caused the disappearance of the upper band and comigration of the upper band with the lower band (Fig. 2*C*). Treatment with *O*-glycosidase and neuraminidase led to the same result (data not shown). These results demonstrate that the heterogeneity of secreted APP695 in hippocampal neurons is caused by a higher content of sialic acids in the upper band, causing an increased M_r (~20 kDa).

One of the issues in AD that is still unresolved is to what extent neurons are able to produce βA4 and other low molecular weight peptides, including p3. The p3 peptide arises from cleavage by α-secretase within the βA4 domain and comprises βA4 residues 17–40.

One dish containing ~4 × 10^5 hippocampal neurons was infected with recombinant SFV/APP695 and labeled for 3 hr with 200 μCi/ml [^{35}S]methionine to analyze whether βA4 was secreted. Immunoprecipitation was carried out on the culture medium with the well characterized antiserum R1280 (Haass et al., 1992b), which precipitates both βA4 and p3 fragments in other cell cultures. A major 4 kDa protein was observed after 24 hr exposure of the gel (Fig. 5*A*), whereas a very weak p3 signal was observed only after longer exposures (data not shown). For comparison, we expressed APP695 in primary mouse fibroblasts via the SFV vector (Fig. 5*B*). The ratio of βA4 to p3 was reversed, demonstrating that hippocampal neurons differ in their processing of APP695. The 4 kDa peptide secreted from the neurons was identified as authentic βA4 peptide because it reacted with both mAb 4G8 against amino acids 17–24 and polyclonal Ab B7/6, which is specific for amino acids 1–16 of the βA4 sequence (Fig. 5*A*). Radiosequencing of the protein confirmed that the peptide was indeed βA4, starting at Asp[1] of the β-amyloid sequence (Fig. 5*C*). Densitometric scanning analysis showed that the relative amount of secreted βA4 peptide to total cellular APP (corrected for methionine content) was already 5.3 ± 1.9% (n = 6) after 3 hr of labeling.

Finally, we investigated the C-terminal APP fragments produced by hippocampal neurons. After infection and metabolic labeling, the

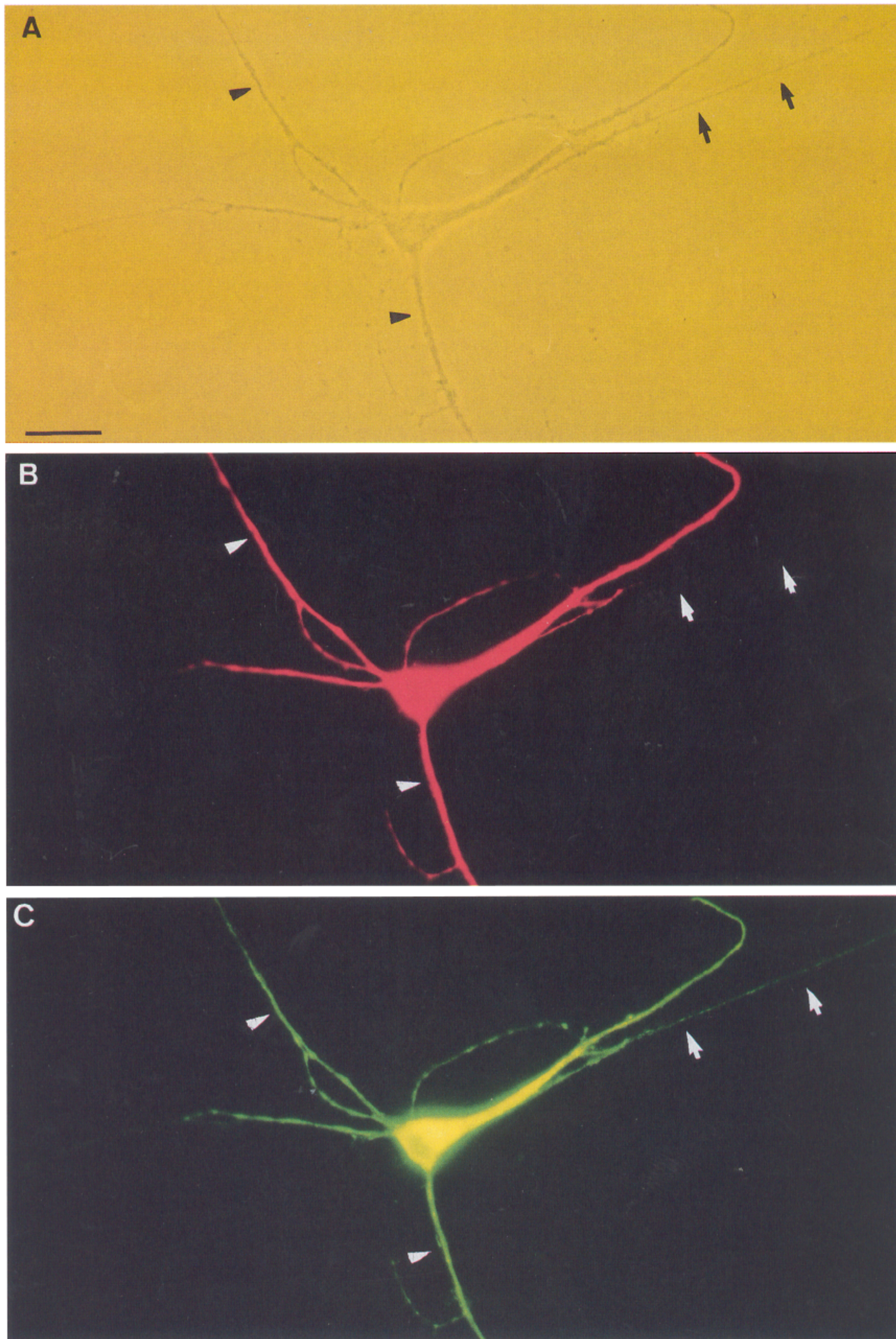

Figure 3. Expression of APP695 via the SFV vector does not change the polarity of the endogenous marker MAP2. A hippocampal neuron infected with recombinant SFV is shown 6 hr after infection in phase-contrast (*A*), with MAP2 labeling (*B*), and with APP695 labeling (*C*). Note the absence of axonal MAP2, whereas the axon is labeled with anti-APP. The axon is marked with *arrows*, and the dendrites are marked with *arrowheads*.

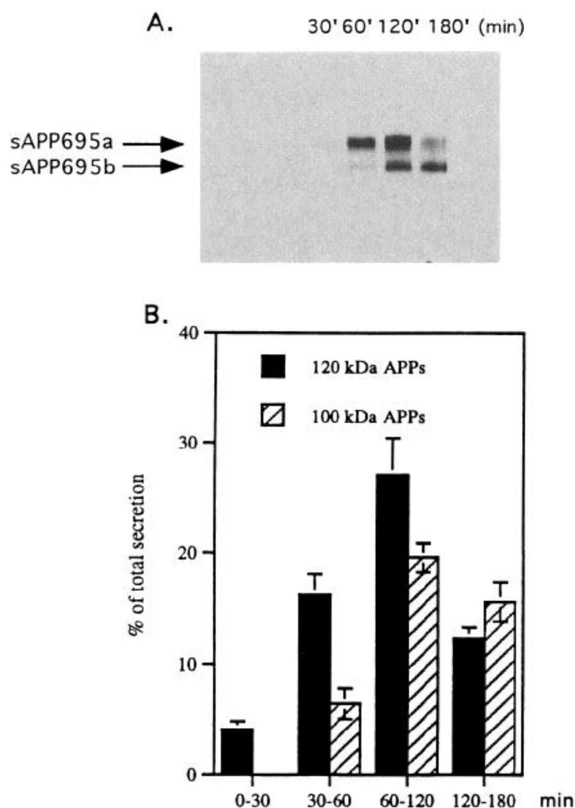

Figure 4. Time course of APP695 secretion in hippocampal neuron cultures. *A*, SFV/APP695-infected hippocampal neurons were pulse-labeled for 10 min with [^{35}S]methionine, and culture media were collected at the times indicated (see also Materials and Methods). Secreted APP695 was immunoprecipitated with Ab R217 at each time interval. *B*, Quantitative analysis of four independent experiments performed as in *A*. *Filled columns* indicate 120 kDa APPs; *hashed columns* indicate 100 kDa APPs.

cell lysates were immunoprecipitated with Ab B12/4 against the 20 C-terminal amino acid residues of the cytoplasmic tail of APP. In 10–20% of the Tris-tricine polyacrylamide gels, four C-terminal fragments appeared between 8 and 12 kDa (Fig. 6*A*). The two upper bands reacted with Ab B7/6, indicating that they contained the entire βA4 peptide (data not shown). By densitometric scanning analysis, the relative amount (corrected for methionine content) of the C-terminal fragments compared with that of full-length APP was shown to be 13.4 ± 5.25% (n = 8) after 3 hr of labeling. The βA4-containing (potentially) amyloidogenic fragments represented ~67% of the C-terminal fragments.

The sequence of the 8, 8.5, 10, and 12 kDa C-terminal derivatives was identified by radiosequencing. [^{35}S]Methionine or [^{3}H]phenylalanine metabolically radiolabeled C-terminal fragments were isolated from 10–20% of the Tris-tricine gels and subjected to N-terminal radiosequence analysis (Fig. 6*B–D*). The 12 kDa [^{35}S]methionine-labeled fragment revealed a radioactive signal at cycle 12. Alignment with the known sequence of APP indicated that cleavage of APP had occurred at the 12 amino acids N-terminal to βA4 (Fig. 6*B*). The results show that neuronal cells are capable of generating a fourth cleavage at the N-terminal side of the β-cleavage site, which we propose to name the δ-cleavage site, which is generated in neurons by an unidentified δ-secretase.

Radiosequencing of the [^{35}S]methionine-labeled 10 kDa fragment caused ^{35}S peaks in cycle 4 and cycles 19–20, indicating that this band was generated by β-cleavage (Fig. 6*C*). The 8 and 8.5 kDa fragments could not be separated from each other and, therefore, were radiosequenced as a mixture. [^{3}H]Phenylalanine radioactivity peaks were obtained at cycles 3, 4, 5, 6, 9, and 10 (Fig. 6*D*). These results confirm that the fragments in these bands are heterogeneous, likely consisting of C-terminal fragments starting at Leu^{17} of the classical α-secretase site and at two other positions, i.e., Glu^{11} and Gln^{15} of the βA4 sequence. For simplicity, we will call the two additional sites α′-secretase sites, although the site at position Gln^{11} likely is also amyloidogenic, because an important fraction of peptides in amyloid plaques of AD patients starts exactly at that position (Masters et al., 1985; Naslund et al., 1994).

DISCUSSION

Although the metabolism of APP has been studied in detail in many cell types, information about the processing of APP in the cells that are affected in AD, i.e., hippocampal neurons, is absent. The reason for this is the difficulty in obtaining sufficient amounts of material from cultured neurons for biochemical analyses. We circumvented this problem by expressing human APP695 via the SFV-expression system. This vector is ideally suited for this purpose because a wave of newly synthesized APP is transported through the biosynthetic pathway after infection; therefore, post-translational modifications can be monitored easily. All of our controls in this study (Figs. 1, 3) and in previous studies (Simons et al., 1995; Yamazaki et al., 1995) suggest that the membrane-trafficking systems in these neurons perform normally during the time course of the study.

Particularly important was the observation that rat hippocampal neurons are able to produce βA4 peptide in relatively high amounts compared with the p3 peptide when expressing human APP695. We evaluated this by using the widely used Ab R1282. This Ab usually precipitates more p3 (residues 17–40 of βA4) than βA4 from a variety of cells, including stably transfected human 293 kidney cells and MDCK cells, Chinese hamster ovary cells, and human umbilical vein endothelial cells (Haass et al., 1992b, 1994). In contrast, we found that rat hippocampal neurons produce substantial amounts of βA4, whereas p3 was visible only faintly, even after prolonged exposure of the fluorographs from R1282 immunoprecipitations. A reverse ratio was observed in fibroblasts (Fig. 5*B*). Because the small amounts of contaminating glial cells in the cultures did not contribute significantly to APP production after SFV infection (Fig. 1*B,C*), our conclusion is that the hippocampal neurons are the source of the βA4 peptide.

Previously, βA4 production has been studied in a number of brain-derived cell types, including rat astrocytes, human astrocytes, rat cortical and hippocampal neuronal cultures, and human mixed-brain cultures (Haass et al., 1992b; Busciglio et al., 1993). These studies found that mostly p3 was produced from rodent neuronal cultures, whereas human astrocytes and human mixed-brain cultures produced the highest levels of βA4. The discrepancy with our findings could be attributable to the difficulty in detecting significant amounts of βA4 in rodent neurons because of reduced affinity of the anti-βA4 Abs to the rodent βA4 peptide. Another possibility is that rodent neurons indeed produce only small amounts of endogenous βA4. Only after human APP was introduced into these cells would they start secreting βA4. Recent results from our laboratories demonstrate that rodent APP expressed via the SFV vector produces much smaller amounts of βA4 compared with the amounts observed after expressing human APP. Several familial APP mutants, however, produced more βA4 wild-type APP695 (De Strooper et al., 1995b). These data are in agreement with the results obtained with the recent transgenic mouse model expressing human APP with

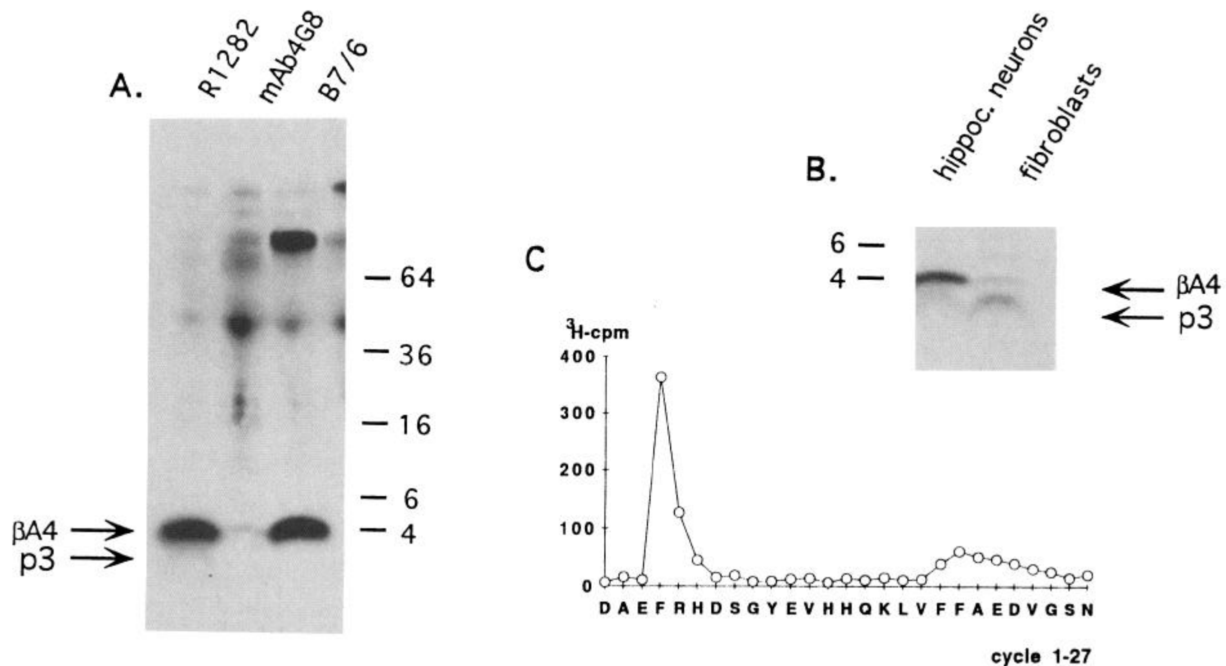

Figure 5. βA4 secretion from primary cultures of hippocampal neurons. *A*, Immunoprecipitation of βA4 from culture medium of SFV/APP695-infected neurons after a 3 hr [^{35}S]methionine labeling with the polyclonal Ab *R1282* (against βA4 1–40), the monoclonal Ab 4G8 (*mAb4G8*) (against βA4 17–24), and the polyclonal Ab *B7/6* (against βA4 1–16). *B*, Comparison of βA4/p3 ratio in hippocampal neurons (*hippoc. neurons*) and *fibroblasts*. APP695 was expressed via the SFV vector, and cells were labeled with [^{35}S]methionine for 3 hr. Medium was immunoprecipitated with Ab 692. A high βA4/p3 was observed in hippocampal neurons. The ratio was reversed in fibroblasts. *C*, Radiosequencing of a [^{3}H]phenylalanine-labeled 4 kDa peptide from culture medium of hippocampal neurons.

FAD 717 (FAD mutant; mutation in codon 717), which produced typical AD-associated amyloid plaques (Games et al., 1995).

In contrast to previous studies, which concluded that secretory processing of APP in neurons was less prominent (Haass et al., 1991; Hung et al., 1992) or nonexistent (Alliquant et al., 1994), we found appreciable α-secretase processing of APP695 in both uninfected and infected hippocampal neurons. In our opinion, the reasons for this apparent discrepancy between our findings and those of others most likely are to be found at the level of the neuronal culture systems that were used. The hippocampal neuron culture used here yields well differentiated neurons that elaborate many interneuronal contacts (Goslin and Banker, 1991). These interneuronal contacts may be important in this respect because we found that neurons derived from cervical sympathetic ganglion cells, which do not form synaptic contacts *in vitro*, did not secrete APP in detectable amounts (Fig. 1*B*,*C*). The alternative possibility that secretory processing of APP is a particular characteristic of hippocampal neurons [as opposed to cortical neurons used in the other study (Alliquant et al., 1994) and sympathetic neurons used here] cannot be ruled out at this time.

Secretory processing of APP in hippocampal neurons is somewhat slower than that of non-neuronal cells and, in contrast to other cells (Weidemann et al., 1989; Oltersdorf et al., 1990), it yielded two APP forms of ~100 and 120 kDa in the extracellular medium. Both forms are phosphorylated and N-glycosylated, but they contain different amounts of sialyl residues because they both migrate together as a single band in SDS-PAGE after treatment with neuraminidase (Fig. 2*C*). Intriguingly, a short pulse (10 min) of metabolic labeling caused secretion of labeled APPs for >3 hr. The sialic acid-rich form of 120 kDa started to appear in the medium after 30–60 min, whereas the 100 kDa form appeared only after 60 min and beyond (Fig. 4). The most likely interpretation of these results is that the secretory processing of APP in neurons is a complex process taking place at different stations during APP trafficking in the neurons. We do not know whether the latter form is produced by desialylation of the 120 kDa form, e.g., after endocytosis and subsequent release by recycling, or whether the 100 kDa is derived from a pool of APP following another transport route through the biosynthetic pathway. We have observed that there is a pool of APP in hippocampal neurons that never achieves full glycosylation within the time span during which we followed the cells (M. Simons and B. De Strooper, unpublished observations). That the desialylation occurs by secreted sialidases after secretion is virtually excluded, however, because prolonged incubation of the two proteins in conditioned medium at 37°C did not change the relative amounts of the two forms. The function of APP processing in neurons is still not known, but we assume that α-secretory processing of APP has an important physiological role in the central nervous system, which is in agreement with other studies (Nitsch et al., 1992).

The study of the intracellular C-terminal remnants of APP was particularly informative. Typically, the nonamyloidogenic fragments are the primary cleavage products in many cell types (Oltersdorf et al., 1990; Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992a; Lo et al., 1994). In neurons, however, we find high amounts of βA4-containing C-terminal fragments (Fig. 6) compared with nonamyloidogenic fragments, emphasizing the possibility that neurons have an intrinsic tendency to catabolize APP in an amyloidogenic manner. Another interesting finding in this study was the identification by radiosequencing of a C-terminal APP fragment starting at Asn^{585} of the APP695 sequence. This fragment was detected reproducibly in all of our experiments and is an authentic product of APP processing in neurons. Because this cleavage product is found in equal amounts

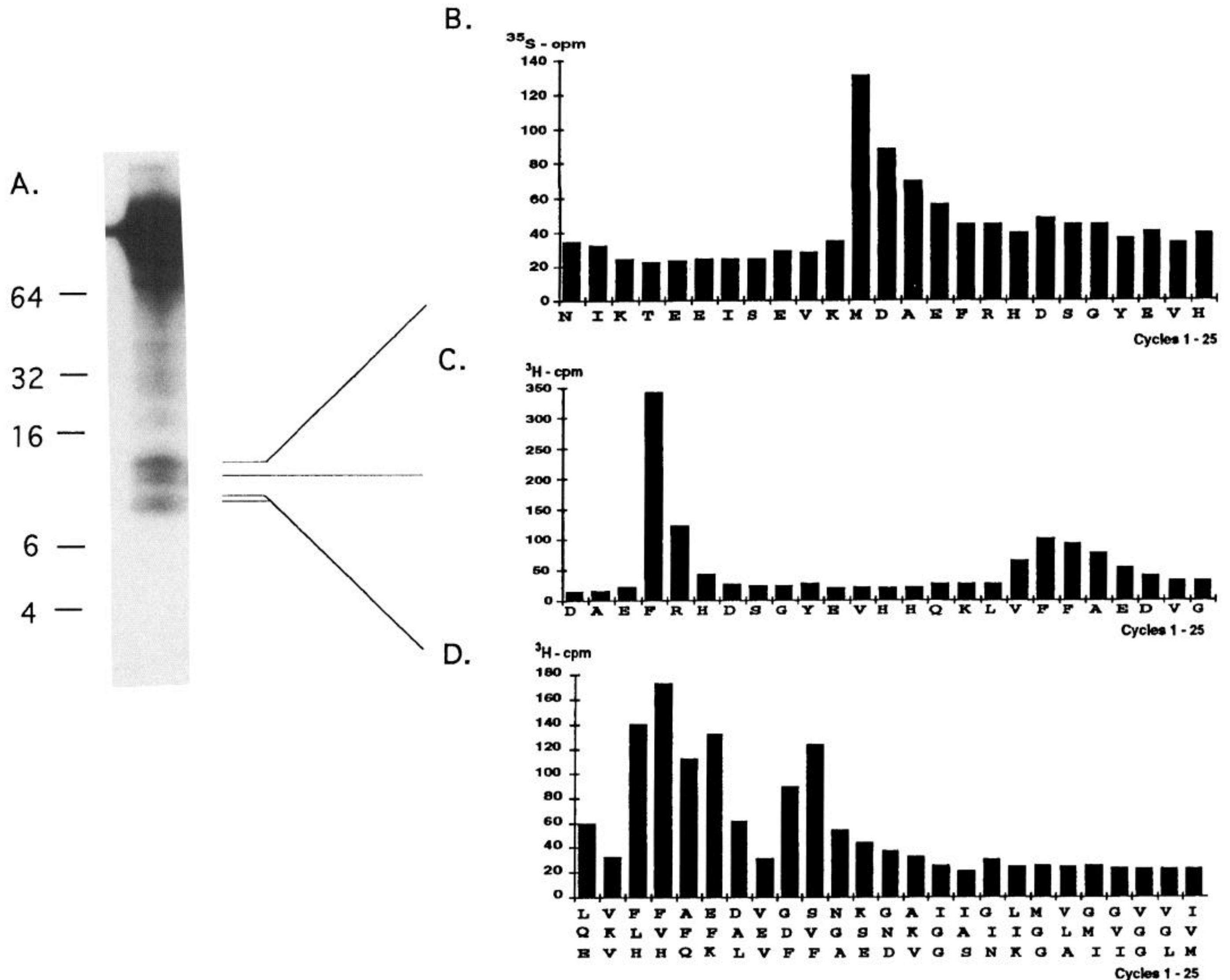

Figure 6. Analysis of APP695-derived C-terminal fragments in hippocampal neurons. *A*, Hippocampal neurons were infected with SFV/APP695 and metabolically labeled with [^{35}S]methionine or [^{3}H]phenylalanine. Cells were solubilized, and detergent-soluble lysates were immunoprecipitated with Ab B12/4. Immunoprecipitates were separated on a 10–20% Tris-tricine gel. Radiosequence analysis of the [^{35}S]methionine-labeled sample is shown for the 12 kDa fragment (*B*). Radioactivity obtained at each cycle for the [^{3}H]phenylalanine-labeled samples is graphed for the 10 kDa (*C*) and 8–8.5 kDa C-terminal fragments (*D*).

compared with those of the the α- and β-cleaved fragments, it represents a significant intermediate in APP metabolism in these cells. Whether this intermediate serves as a precursor for βA4 production is under further investigation. A complex set of 8–12 kDa C-terminal fragments also has been isolated from brain homogenates, which suggests that these fragments also are produced *in vivo* (Estus et al., 1992). Expression of βA4-bearing APP C-terminal fragments has shown that these fragments have a high tendency to aggregate both *in vitro* (Dyrks et al., 1988) and in cells (Wolf et al., 1990). Therefore, βA4-containing fragments may function not only as precursors for βA4, but they also could form intracellular aggregates during the pathogenesis of AD.

Although radiosequencing of the 10 kDa C-terminal fragment (Fig. 6*B*) showed that it was generated by a single type of β-cleavage yielding a fragment starting at Asp^1 of the βA4 sequence, the 8–8.5 kDa C-terminal fragments displayed N-terminal heterogeneity, yielding three major products. One fragment started at residue Leu^{17}, which is at the classical α-secretase site (Esch et al., 1990). The other two fragments are slightly longer, with N termini likely starting at positions Gln^{15} and Glu^{11} of the βA4 sequence, suggesting that α-secretase in neurons cleaves APP at three different positions. The alternative explanation, that α-secretase processing in neurons is performed by a set of different enzymes, is more likely. The latter possibility was advanced recently by other investigators (Zhong et al., 1994) based on studies in fibroblasts. Multiple α-secretases also would explain the extremely relaxed specificity of α-secretase (Sisodia et al., 1992; Maruyama et al., 1991; De Strooper et al., 1993) and the problem of inhibiting its activity by a panel of different protease inhibitors (De Strooper et al., 1992).

Of particular relevance for AD is that one of the alternative α-cleaved fragments started exactly at a position identified previ-

ously as the first residue of βA4 (11-42), a major component of AD plaques (Masters et al., 1985; Naslund et al., 1994). This finding, therefore, corroborates our conclusion that neurons are the primary producers of amyloidogenic peptides in AD.

When differences in APP processing in neuronal and non-neuronal cells are considered, it is also important to compare APP trafficking in various cell types. Recent studies have shown that APP is sorted to the basolateral side in epithelial MDCK cells (Haass et al., 1994; Lo et al., 1994; De Strooper et al., 1995a), where its secretory products are released. Less βA4 appears to be produced in MDCK cells (Haass et al., 1994) compared with hippocampal neurons. Usually, basolateral proteins are routed to the dendrites in neurons and apical proteins are transported to the axon (Craig and Banker, 1994). The reason APP does not follow this scheme is not yet known. It may be that the basolateral sorting signals in APP are inactivated by post-translational modifications in neurons. One such possible modification is phosphorylation of the basolateral signal (Mostov et al., 1992). Alternatively, the protein might interact with basolateral proteins in MDCK cells and be carried piggyback to the basolateral plasma membrane. Another possibility is that the sorting machinery in neurons and epithelial cells differs, and that this leads to different sorting behavior. Future work will have to address these important issues and pinpoint the subcellular localization of the processing events.

In conclusion, our results support the working hypothesis that the intracellular trafficking of APP is related to its metabolism, and our data emphasize the necessity to investigate the metabolism of APP in polarized neurons. The finding of several new proteolytic cleavage sites in neurons supports this contention and has encouraged us to analyze the metabolism of the familial mutants of APP in detail in this system as well (De Strooper et al., 1995b). We anticipate that the current approach of expressing heterologous proteins in fully differentiated neurons using the SFV expression system will prove useful for the study of many other central nervous diseases and will be widely applicable. In addition, this system might serve as an informative *in vitro* model for AD because of the high production of βA4 and amyloidogenic APP fragments.

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