Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9439–9443, October 1992
Neurobiology

Increased expression of β-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage

(Alzheimer disease/brain development/membrane proteins/neurofilament/tubulin)

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Communicated by Walle J. H. Nauta, June 17, 1992 (received for review April 17, 1992)

ABSTRACT **Despite increasing evidence for a pathogenetic role for the β-amyloid precursor protein (βAPP) in Alzheimer disease, the physiological function of the protein remains unclear. The expression of the neural-specific isoform containing 695 amino acids, βAPP695, is consistent with a role for the protein in neuronal development. In this study, we analyzed the expression of βAPP during the retinoic acid-induced neuronal differentiation of P19 murine embryonal carcinoma cells. Northern blot and RNase protection analyses show a selective increase in βAPP695 expression, concomitant with the morphologic differentiation of P19-derived neurons. Moreover, the time course of increase observed for the βAPP695 mRNA is paralleled by other neuronal-specific transcripts. A similar increase in βAPP695 is observed at the protein level. Furthermore, we show that levels of βAPP695 protein progressively increase during the *in vitro* differentiation of primary hippocampal neurons. The finding that βAPP695 increases selectively and progressively during neuronal differentiation in two different cell culture systems suggests that this isoform has an important cellular function during this process in the brain. Unlike βAPP in most peripheral cell types, the increased levels of βAPP found in terminally differentiated neuronal cells are not processed in significant amounts by secretory cleavage. Thus, differentiation of neurons is accompanied by increased βAPP695 expression and membrane retention of the protein as intact, full-length molecules that could serve as potential substrates for amyloidogenesis.**

One of the pathologic hallmarks of Alzheimer disease is the deposition of fibrillar aggregates of a 40- to 43-amino acid polypeptide, the β/A4 peptide, within the brain parenchyma and the walls of the cerebral vasculature (1–3). This ≈4-kDa protein is derived by proteolytic cleavage from a larger glycosylated membrane-spanning precursor molecule, the β-amyloid precursor protein (βAPP) (4), and is comprised of the 28 amino acids immediately outside the membrane plus the first 12–15 amino acids of the predicted transmembrane domain.

Although βAPP transcripts are expressed ubiquitously in all tissues, there is increasing evidence for a unique role for the protein within the nervous system. βAPP mRNA levels are highest within the brain (5–7). In addition, the distribution of one alternatively spliced message encoding a protein of 695 amino acids (βAPP695) is largely restricted to neural cells (7, 8). Unlike the 751- and 770-amino acid isoforms of the protein (βAPP751 and βAPP770), βAPP695 lacks the active Kunitz protease inhibitor (KPI) domain, suggesting this isoform must be functionally different. Furthermore, proteolytic processing of βAPP may vary between peripheral and neural cells. In several peripheral cell types examined, constitutive cleavage of the precursor molecule gives rise to a large, soluble amino-terminal fragment that is released into the medium (9–11), accompanied by intracellular accumulation of a membrane-retained ≈10-kDa fragment (12). However, recent studies in primary neural cells show that little of this secretory cleavage event takes place in cultured astrocytes and microglia (13).

Functionally, little is known about the role of βAPP695 in the nervous system. The molecule is known to undergo fast axonal transport in peripheral neurons *in vivo* (14). However, the role that βAPP might play in the nerve terminal remains unknown. Cloning and characterization of an amyloid precursor protein-like (APPL) molecule in *Drosophila* has provided intriguing evidence that βAPP695 might indeed have a nervous system-specific function (15). The 886-amino acid APPL protein lacks a KPI domain but shares three distinct regions of homology with mammalian βAPP. *In situ* hybridization reveals neuronal-specific expression of the *Drosophila* transcript. During development, transcripts are absent in dividing neuroblasts; expression is observed exclusively in postmitotic differentiated neurons (16). Similarities in expression patterns between mammalian βAPP695 (17) and *Drosophila* APPL suggest that they might be functional homologs.

To examine the expression of βAPP during neuronal differentiation, we have taken advantage of the P19 mouse embryonal carcinoma cell line (18, 19). When these cells are grown under normal conditions, they proliferate as undifferentiated cells. However, in the presence of retinoic acid (RA), they are induced to differentiate into large numbers of neurons and astrocytes as well as a smaller population of fibroblast-like cells (20). Furthermore, the antimetabolite cytosine arabinonucleoside (araC) can be added to the RA-induced cultures to select for the postmitotic neuronal cells (21). In contrast, P19 cells undergo differentiation into a muscle-like phenotype when exposed to dimethyl sulfoxide (DMSO) (19). This system provides the opportunity to examine how levels and processing of βAPP may be regulated during neuronal differentiation and maturation. Our results show that levels of βAPP695 mRNA and protein selectively increase upon RA treatment of P19 cells, in parallel with the morphologic differentiation of neurons. Furthermore, *in vitro* differentiation of primary rat hippocampal neurons is also paralleled by an increase in cellular levels of βAPP695 protein. The selective increase of one βAPP isoform during neuronal differentiation in two different cell culture systems indicates that βAPP695 may play an important role during brain development. In addition, we demonstrate that in both of these paradigms, terminally differentiated neuronal cells, like primary glial cells (13), appear to process only small amounts of membrane-bound βAPP into a soluble derivative,

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Abbreviations: βAPP, β-amyloid precursor protein; KPI, Kunitz protease inhibitor; RA, retinoic acid; DMSO, dimethyl sulfoxide; araC, cytosine arabinonucleoside.

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retaining most of the molecules as full-length proteins that could serve as a potentially amyloidogenic substrate.

METHODS

Cell Culture. P19 mouse embryonal carcinoma cells (ATCC CRL 1825) (18) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. To induce differentiation, cells were aggregated in bacterial-grade Petri dishes in the presence of 0.5 μM RA or 1% DMSO as described (21). After 4 days, cells were dispersed with trypsin and plated onto tissue-culture-grade dishes in the absence of inducing agent. To enrich for the postmitotic neuronal cells, the antimetabolite araC (Sigma) was added to RA-treated cells to a final concentration of 5 μg/ml 1 day after plating onto tissue culture dishes. Primary hippocampal neurons were prepared from the brains of 18-day-old Sprague–Dawley rat embryos (Taconic Farms) as described (22, 23). Dissociated cells were plated onto 35-mm polylysine-coated dishes at a density of 1–2 × 10^6 cells per dish in DMEM supplemented with 10% (vol/vol) horse serum. After 3 hr at 37°C, the medium was removed, and cultures were maintained in serum-free medium containing N2 supplements (24).

RNA Isolation and Analysis. Total cellular RNA was isolated and prepared by the guanidinium isothiocyanate method (25). Equal amounts of total RNA (15 μg) were fractionated on denaturing 2.2 M formaldehyde/agarose gels and transferred to nylon membranes. To probe for βAPP695 transcripts, a 30-nucleotide antisense oligonucleotide encompassing 15 bases on either side of the KPI domain (base pairs 851–880) was used. Specificity of the probe for βAPP695 under identical hybridization conditions has been demonstrated (26). As markers of neuronal differentiation, probes for the mouse neurofilament 68-kDa protein (NF-L) (27) and the 3′ untranslated region of the neuron-specific class III β-tubulin isoform (Mβ6) (28) were used for Northern blot hybridization. To control for equal RNA loading, blots were hybridized with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. All cDNA probes were labeled by random primed extension, while the oligonucleotide was 3′-end-labeled by terminal deoxynucleotide transferase with [^{32}P]dCTP.

For RNase protection analysis, cDNA constructs specific for human and murine βAPP were generated by PCR after reverse transcriptase-mediated cDNA synthesis from RNA isolated from human embryonic kidney 293 cells or DMSO-induced P19 cells, respectively. The sense primer corresponds to nucleotides 640–659 of βAPP770 preceded by a *Bam*HI restriction site. The antisense primer corresponds to nucleotides 1200–1219 of βAPP770 preceded by a *Hin*dIII site. The fragment corresponding to the PCR product derived from βAPP770 was subcloned into the *Bam*HI/*Hin*dIII sites of pGEM-3 (Promega). Radiolabeled antisense RNA probes were generated by *in vitro* transcription from *Bam*HI-linearized templates using SP6 polymerase (Boehringer Mannheim) and hybridized to 5 μg of total RNA per sample in 80% deionized formamide/40 mM Pipes, pH 6.4/400 mM NaOAc, pH 6.4/1 mM EDTA for 12–16 hr at 45°C. Samples were then digested with RNase A/RNase T_1 (Ambion, Austin, TX), according to the manufacturer's instructions.

Protein Analysis. Protein extracts were prepared by Nonidet P-40 lysis as described (13). Equal amounts of total protein were separated by SDS/PAGE (7% polyacrylamide gels) and immunoblotted with affinity-purified antibody αC7, a polyclonal antiserum raised against a synthetic peptide comprising the last 20 amino acids of βAPP (29). Affinity purification of antibodies was performed as described (13). For analysis of βAPP processing, cells were metabolically labeled with 150 μCi (1 μCi = 37 kBq) of [^{35}S]methionine for 6 hr in methionine-free DMEM containing 10% fetal calf serum (P19 cells) or methionine-free N2-containing medium (primary hippocampal neurons). βAPP molecules containing the C terminus were immunoprecipitated with affinity-purified αC7 (13) and analyzed by separation on 5–20% gradient SDS/PAGE gels. Immunoprecipitations of conditioned medium were performed with antibody αR1285, raised against βAPP amino acids 527–540 as described (13), except that the medium was precleared with preimmune serum (60 min) and protein A-Sepharose (45 min) prior to addition of specific antibody.

RESULTS

βAPP695 Transcripts Are Upregulated During RA-Induced Differentiation of P19 Cells. To investigate the regulation of the neural-specific βAPP695 isoform in P19 embryonal carcinoma cells after induction of neuronal differentiation with RA, RNA isolated on consecutive days after RA treatment was analyzed by Northern blotting. By using an oligonucleotide probe specific for this transcript, levels of βAPP message progressively increased, with the largest change occurring at the time of plating between days 4 and 5 (Fig. 1). At this time, cell aggregates were dissociated and allowed to adhere, initiating morphologic differentiation of the cells into process-bearing neurons (20). To correlate the expression of this isoform with other neuronal-specific markers, we compared the time course of βAPP695 upregulation with the appearance of transcripts encoding the neurofilament 68-kDa protein (27) and the neuron-specific class III β-tubulin isoform (28). Similar to βAPP695, the levels of these transcripts were also increased at days 4 and 5 during P19 neuronal differentiation, strikingly parallel to the rise in the neural-specific βAPP695 message (Fig. 1).

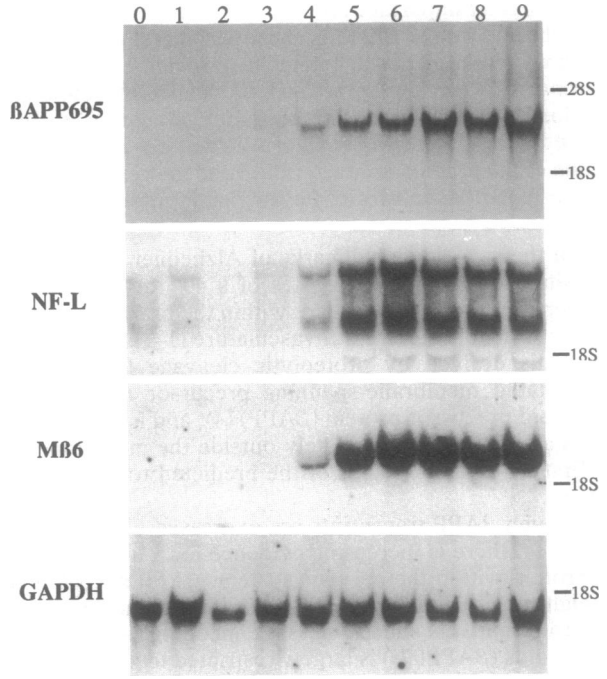

FIG. 1. Northern blot analysis of RNA isolated from P19 cells on consecutive days after RA induction as indicated at the top of the lanes. Total RNA (15 μg) was hybridized to an antisense oligonucleotide probe specific to βAPP695 (a 30-mer derived from βAPP695, base pairs 851–880). To compare the time course of βAPP expression to other neuronal markers, the blot was also hybridized to cDNAs encoding the neurofilament 68-kDa subunit (NF-L) [note two alternative transcripts as described (28)] and the neuron-specific class III β-tubulin subunit (Mβ6). As controls for equal RNA loading, a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. Day 0 indicates uninduced P19 cells.

Using RNase protection analysis of total RNA isolated before and after RA induction, we observed the selective increase of βAPP695 mRNA. As controls, we used RNA isolated from human embryonic kidney 293 cells, either untransfected (Fig. 2*A*, lane 1) or stably transfected with the human βAPP695 or βAPP751 cDNAs (11, 12) (Fig. 2*A*, lanes 2 and 3). Exposure to RA resulted in a specific increase in the levels of the βAPP695 protected fragment, with little change in the amounts of βAPP751 and βAPP770 transcripts (Fig. 2*A*, compare lanes 4 and 6). In contrast, little change in βAPP expression was detected during DMSO-stimulated differentiation (Fig. 2*A*, lane 5). Analysis of the time course of increasing βAPP expression during the differentiation process by RNase protection confirmed the results shown by RNA blot analysis (Fig. 2*B*). Furthermore, there is relatively little change in the levels of the KPI-containing βAPP751 and βAPP770 transcripts.

Selective Increase in βAPP695 Protein Levels During Neuronal Differentiation in Two Culture Systems. To examine expression of βAPP at the protein level, total P19 cell extracts of undifferentiated and RA-induced P19 cells were prepared and examined by immunoblotting with an affinity-purified antiserum (αC7) raised against the C terminus of βAPP (29). Whereas cells cultured in the absence of inducing agent contain only trace amounts of proteins recognized by αC7, the antibody strongly identified a set of proteins of ≈105–120 kDa in the RA-treated cells (Fig. 3*A*). The electrophoretic mobility of these bands is consistent with those reported for βAPP in other cells and tissues (10, 12); the induced proteins comigrate specifically with βAPP695 derived from stably transfected 293 cells (Fig. 3*A*; compare lanes 1 and 5). In fact, unlike the 293 cells, which contain roughly equal amounts of both the immature N-glycosylated form and the mature N+O-glycosylated form (10, 11), the induced P19 cultures contain a greater proportion of the N-glycosylated species. Neuronal enrichment with araC increased the relative intensity of the αC7 immunoreactivity detected in the same amount of total cell protein (Fig. 3*A*, lane 6), suggesting that the increased βAPP protein derives in large part from postmitotic neuronal cells. In addition, following two subcultures of the RA-induced cells during which most neurons are lost (21), cell extracts contained substantially less αC7-immunoreactive material (data not shown). As suggested by the mRNA analysis (above), exposure to DMSO did not alter the pattern of the βAPP proteins (Fig. 3*A*, lane 4).

To compare these results in P19 cells to βAPP regulation during differentiation of primary neurons, we cultured hippocampal neurons isolated from 18-day-old rat embryos. These cells have been shown to undergo an intrinsic stereotyped pattern of differentiation in culture (30), with coincident expression of neuronal-specific proteins such as GAP-43 (31). Cell extracts prepared after various times in culture were analyzed by immunoblotting with αC7. During neuronal

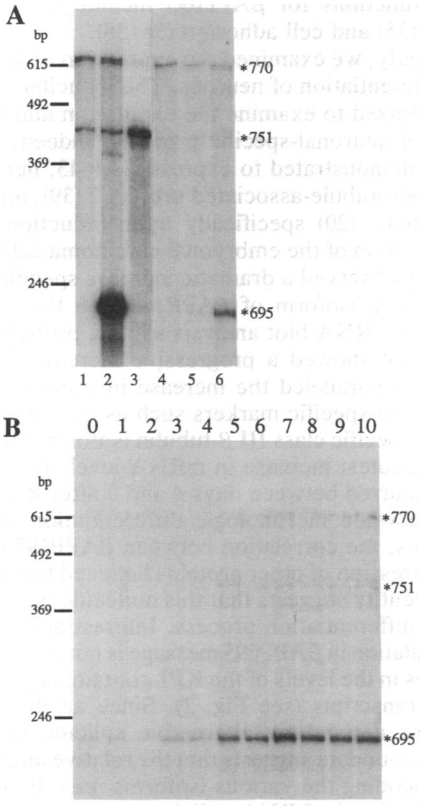

FIG. 2. (*A*) RNase protection analysis indicates a selective increase in the βAPP695 transcript after RA induction. Lanes: 1, untransfected 293 cells (note low endogenous level of primarily βAPP751 and βAPP770 transcripts); 2, 293 cells stably transfected with human βAPP695 cDNA; 3, 293 cells stably transfected with βAPP751; 4, uninduced P19 cells; 5, P19 cells 10 days after induction with DMSO; 6, P19 cells 10 days after RA induction. (*B*) Consecutive days after RA induction of βAPP695, as indicated at the top of lanes. Note that the levels of βAPP751 and βAPP770 transcripts are largely unchanged during the 10 days after exposure to RA. Five micrograms of total RNA were used per lane. Asterisks indicate the protected fragments corresponding to the three major transcripts.

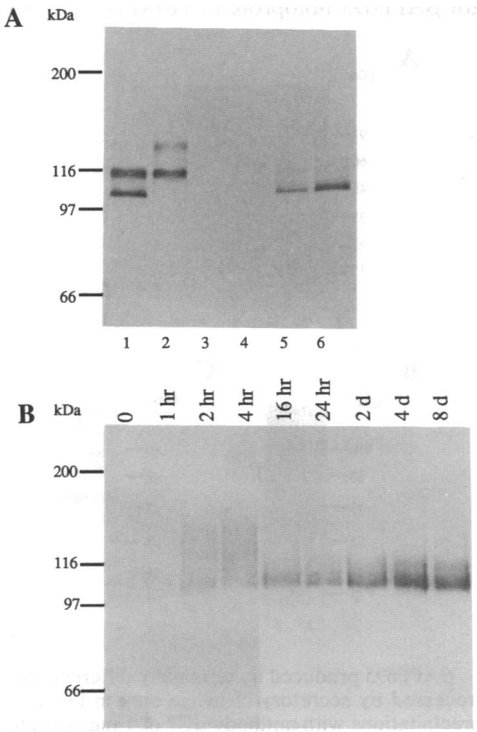

FIG. 3. Analysis of βAPP protein expression during neuronal differentiation. (*A*) Immunoblot analysis of protein extracts from uninduced and induced P19 cultures with antibody αC7, raised against a synthetic peptide corresponding to the last 20 amino acids of βAPP. Lanes: 1, 293 cells transfected with βAPP695; 2, 293 cells transfected with βAPP751; 3, uninduced P19 cells; 4, P19 cells 10 days after exposure to DMSO; 5, P19 cells 10 days after RA treatment; 6, RA-induced P19 cells treated with araC to enrich for postmitotic neuronal cells. One hundred micrograms of protein extracts were loaded per lane. (*B*) Immunoblot analysis of βAPP expression during *in vitro* differentiation of primary rat hippocampal neurons. Primary hippocampal neurons prepared from the brains of 18-day-old rat embryos were cultured for the time indicated at the top of each lane (d = days) and then lysed in Nonidet P-40 lysis buffer. Total protein (65 μg per lane) was loaded and immunoblotted with antibody αC7.

differentiation, we detected steadily increasing protein levels of βAPP695 (Fig. 3*B*). In addition, most of this protein was present as the immature N-glycosylated species. Thus, protein expression of βAPP in the RA-induced P19 system closely parallels that observed in this paradigm for *in vitro* neuronal differentiation.

βAPP695 Expression in Terminally Differentiated Neuronal Cells Is Characterized by Little Secretory Cleavage. To analyze the proteolytic processing of βAPP in RA-induced P19 cells, the cells were metabolically labeled with [^{35}S]methionine 10 days after induction, when they are fully differentiated. Using αC7 to immunoprecipitate from equal amounts of total cell protein, we detected similar amounts of full-length proteins in RA/araC-treated P19 cells and transfected 293 cells (Fig. 4*A*, lanes 1 and 4). DMSO-induced P19 cells showed very low amounts of specifically immunoprecipitated proteins (Fig. 4*A*, lane 2). Strikingly, in comparison to the transfected 293 cells, the RA-induced neuronal cultures produced much less of the membrane-retained ≈10-kDa fragment that remains after constitutive cleavage and secretion of the extracellular domain. This is paralleled by a decrease in the amount of βAPP soluble forms immunoprecipitated from the culture supernatant (data not shown).

Similarly, we examined βAPP processing in primary hippocampal cultures. Immunoprecipitates from metabolically labeled neurons again showed relatively high levels of the full-length βAPP695 holoprotein. However, as observed in the P19-derived neurons, very little of the membrane-retained 10-kDa fragment was present in cell lysates (Fig. 4*B*). The amount was considerably less than that observed in 293 cells transfected with βAPP695. Immunoprecipitation of conditioned medium from the same cells confirmed these data, as soluble βAPP695 was identified only in the medium of the transfected 293 cells (Fig. 4*C*). These data indicate that while terminally differentiated neurons—both those derived from retinoic acid induction of embryonal carcinoma cells and those isolated from rat brain—synthesize substantial amounts of βAPP695, they process only small amounts into secreted forms, retaining most of the precursor as a full-length, membrane-bound molecule.

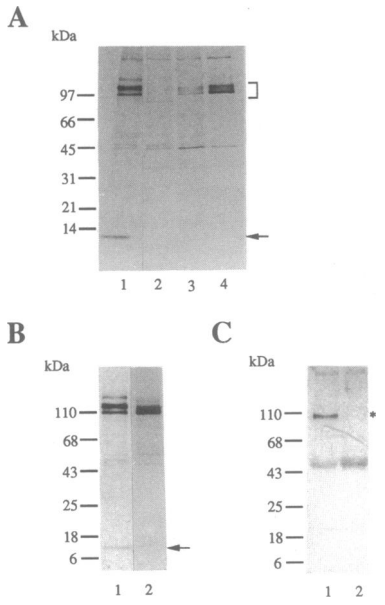

FIG. 4. βAPP695 produced by terminally differentiated neuronal cells is processed by secretory cleavage only in low amounts. (*A*) Immunoprecipitations with antibody αC7 of 1 mg of protein extract from uninduced and induced cells metabolically labeled with [^{35}S]methionine. Lanes: 1, 293 cells transfected with βAPP695; 2, P19 cells 10 days after exposure to DMSO; 3, P19 cells 10 days after RA treatment; 4, RA-induced P19 cells treated with araC. Note that, unlike the transfected 293 cells, only very small amounts of the membrane-retained 10-kDa fragment (indicated by arrow) can be detected in the lysates of P19 neurons. The bracket indicates full-length βAPP. (*B*) Immunoprecipitation of total cell extracts from [^{35}S]methionine-labeled primary hippocampal neurons. Extracts from 293 cells transfected with βAPP695 (lane 1) and primary hippocampal neurons (lane 2) were immunoprecipitated with αC7. The arrow indicates the 10-kDa fragment retained after secretory cleavage. (*C*) Immunoprecipitation of the corresponding conditioned media from cells in *B* with antibody αR1285. The asterisk denotes soluble βAPP695 detected in the medium of stably transfected 293 cells but not in the medium of primary neurons. The band at ≈45 kDa is nonspecific.

DISCUSSION

Recent genetic studies have identified a number of families with familial Alzheimer disease (FAD) that have missense mutations at amino acid 717 of βAPP770 that segregate with the disease (32). These findings implicate βAPP as a primary factor in the pathogenesis of the disease. However, despite the interest in the effect of the amyloid β-protein fragment (β/A4) on neurons, the normal physiologic role of the precursor molecule, especially the 695-amino acid isoform, in neuronal cells remains unknown. Indeed, the lack of a functional protease inhibitor domain suggests that βAPP695 does not act as a regulator of extracellular serine proteases, as has been suggested for βAPP751 and βAPP770. Instead, proposed functions for βAPP695 include roles in growth regulation (35) and cell adhesion (36–38).

In this study, we examined the expression of βAPP during *in vitro* differentiation of neurons. The inducible P19 system has been utilized to examine the expression and function of a number of neuronal-specific proteins. Indeed, these cells have been demonstrated to express GAP-43, nerve-specific enolase, microtubule-associated protein 2 (39), and neurofilament proteins (20) specifically upon induction with RA. During induction of the embryonal carcinoma cells with RA, we similarly observed a dramatic increase specifically in the 695-amino acid isoform of βAPP at both the mRNA and protein levels. RNA blot analysis using a probe specific for this transcript showed a progressive increase in βAPP695 message that paralleled the increase in transcript levels of other neuronal-specific markers such as neurofilaments and the neuron-specific class III β-tubulin isoform (Fig. 1). Strikingly, the greatest increase in mRNA levels for all of these proteins occurred between days 4 and 5 after RA induction, at the time when morphologic differentiation of the cells begins. Thus, the correlation between βAPP695 expression and the expression of other proteins believed to contribute to neuronal identity suggests that this molecule may play a role during the differentiation process. Interestingly, the significant upregulation in βAPP695 message is not accompanied by any changes in the levels of the KPI-containing βAPP751 and βAPP770 transcripts (see Fig. 2). Since all three principal mRNAs are derived by alternative splicing of the same pre-mRNA, our data suggests that the relative amounts of the mRNAs encoding the various isoforms must be tightly regulated at the level of RNA splicing during neuronal development. This result is consistent with findings that the ratio between βAPP695 and βAPP751/βAPP770 in different brain regions changes throughout development (7, 26, 40, 41) and suggests that specific splicing regulatory factors may play an important role in determining βAPP expression patterns both *in vivo* and *in vitro*. Our results are similar to that reported in a recent study which demonstrated an increase in βAPP RNA during induction of embryonal carcinoma cells by RNA blot analysis (42). However, that study observed an increase in all three primary transcripts of βAPP; our results with RNase protection analysis clearly differ in this respect.

In addition, we analyzed βAPP expression and processing in these cells at the protein level and observed a selective increase in protein levels of βAPP695 during differentiation of P19 cells (Fig. 3). This finding is in accord with the recent report of Fukuchi *et al.* (43), who have similarly reported an up-regulation of βAPP in P19 cells upon RA-induced differentiation. We extended this analysis to primary hippocampal neurons, demonstrating that βAPP695 expression is also correlated with neuronal differentiation in a different cell culture paradigm (Fig. 3). Furthermore, examination of precursor processing in these terminally differentiated cells indicates that very little of the synthesized βAPP species gives rise to a secreted, soluble form. Correspondingly, both P19-derived neurons (Fig. 4*A*, lanes 3 and 4) and primary hippocampal neurons (Fig. 4*B*) contain very low amounts of the membrane-retained ≈10-kDa fragment that remains after secretion. This result differs strikingly from the βAPP metabolism previously described in peripheral cell types (10, 11) including human embryonic kidney 293 cells stably transfected with βAPP cDNAs (Fig. 3*B*, lanes 1 and 2) and PC12 cells (9) that both secrete large amounts of soluble βAPP and retain significant levels of the ≈10-kDa fragment, but it is similar to that observed in primary glial cells (13). Indeed, the finding that neurons and glial cells retain the majority of βAPP molecules as full-length precursors indicates that processing of the protein is distinct between different cell types. Based on these data, we hypothesize that βAPP in brain, especially the 695-amino acid isoform in neurons, may function primarily as a membrane-bound holoprotein rather than as a precursor for a secreted molecule. Alternatively, the cleavage event in neuronal cells might not occur constitutively, as has been observed in peripheral cells. Instead, processing and secretion might be regulated in a cell type-specific manner or be dependent on the functional state of the neuron. Our studies indicate that the P19 embryonal carcinoma cell line provides a relevant system to further examine the regulation of βAPP synthesis and processing in neuronal cells.

Whether perturbation of normal βAPP functions might contribute to the pathogenesis of Alzheimer disease remains unknown. Nevertheless, analysis of βAPP expression and function during neuronal development may provide insight into the normal intracellular trafficking of the molecule in neurons. Recent evidence suggests that in all cell types examined to date, a proportion of βAPP molecules remains uncleaved and is rapidly reinternalized from the cell surface (44). Subsequently, these molecules are targeted to the lysosome (44), where proteolytic degradation generates a variety of low molecular weight fragments containing the intact β/A4 region (33, 34, 44). The data reported here suggest that fully differentiated neurons express high levels of βAPP with little secretory cleavage; thus, they might potentially contain a significant amount of substrate for this alternative processing pathway.

We thank Dr. N. Cowan for providing us with the NF-L and Mβ6 cDNA probes. We are grateful to A. Mellon for invaluable assistance in cell culture and to the other members of our laboratory for helpful suggestions and advice. This work was supported by National Institutes of Health Grants AG07911 and AG06173 (to D.J.S.), and fellowships from Merck Sharp and Dohme Research Laboratories (to A.Y.H.), the Alzheimer's Association (to E.H.K.), and the Deutsche Forschungsgemeinschaft (to C.H.).

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