The insoluble amyloid deposited extracellularly in the brains of patients with Alzheimer’s disease (AD) is composed of amyloid β protein, a ~4-kDa secreted protein that is derived from a set of large proteins collectively referred to as the amyloid β protein precursor (βAPP). During normal processing the βAPP is cleaved by β secretase, producing a large NH2-terminal secreted derivative (sAPPβ) and a COOH-terminal fragment beginning at βA1, which is subsequently cleaved by γ secretase releasing secreted Aβ. Most secreted Aβ is Aβ1–40, but ~10% of secreted Aβ is Aβ1–42. Alternative βAPP cleavage by α secretase produces a slightly longer NH2-terminal secreted derivative (sAPPα) and a COOH-terminal fragment beginning at βA17, which is subsequently cleaved by γ secretase releasing a ~3-kDa secreted form of Aβ (P3). Several of the βAPP isoforms that are produced by alternative splicing contain a 56-amino acid Kunitz protease inhibitor (KPI) domain known to inhibit proteases such as trypsin and chymotrypsin. To determine whether the KPI domain influences the proteolytic cleavages that generate Aβ, we compared Aβ production in transfected cells expressing human KPI-containing βAPP751 or KPI-free βAPP695. We focused on Aβ6 at Aβ42 because these forms appear to be most relevant to AD. Using specific sandwich enzyme-linked immunosorbent assays, we analyzed full-length Aβ1–42 and total Aβ ending at Aβ42 (Aβ1–42 and P3(42)). In addition, we analyzed the large secreted derivatives produced by α secretase (sAPPα) and β secretase (sAPPβ). In mouse teratocarcinoma (P19) cells expressing βAPP695 or βAPP751, expression of the KPI-containing βAPP751 resulted in the secretion of a lower percentage of P3(42) and sAPPα and a correspondingly higher percentage of Aβ1–42 and sAPPβ. Similar results were obtained in human embryonic kidney (293) cells. These results indicate that expression of the KPI domain reduces α secretase cleavage so that less P3 and relatively more full-length Aβ are produced. Thus, in human brain and in animal models of AD, the amount of KPI-containing βAPP produced may be an important factor influencing Aβ deposition.

In the brains of patients with Alzheimer’s disease (AD), one characteristic pathological feature is the deposition of amyloid in senile plaques. In many patients, amyloid is also deposited in the walls of cerebral and meningeal blood vessels. AD amyloid is composed of amyloid β protein (Aβ), a ~4-kDa secreted protein released from an ~120-kDa amyloid β protein precursor (βAPP) through cleavage by proteases referred to as secretases. Soluble, secreted Aβ is normally present in human cerebrospinal fluid (1, 2) and plasma (3). It is present in high concentration in medium conditioned by mixed human fetal brain cells (2) or a variety of transfected cells expressing βAPP (1, 4), and it can be detected at lower concentration in the medium of cultured cells expressing only endogenous βAPP (5, 6). Recent studies have established that the βAPP is normally cleaved by β secretase to produce a large NH2-terminal secreted derivative (sAPPβ) (7) and a cell-associated COOH-terminal derivative beginning at Aβ1 (8). Subsequent cleavage of this COOH-terminal fragment by γ secretase releases 4-kDa Aβ for secretion. Alternative cleavage of the βAPP by α secretase produces a slightly longer NH2-terminal secreted derivative (sAPPα) (9) and a COOH-terminal fragment beginning at Aβ17 (9), which is cleaved by γ secretase releasing a truncated ~3-kDa form of Aβ (P3) for secretion. None of the three secretases (α, β, or γ) has been isolated or cloned, so the specific protease(s) responsible for each secretase activity are currently unknown.

The βAPP is encoded by a single gene (APP) on chromosome 21 (10–13) which has 18 (14, 15) exons. Alternative splicing of exons 7, 8, and 15 gives rise to multiple βAPP isoforms that contain the entire Aβ domain, which is encoded by nucleotides comprising parts of exons 16 and 17. Initial studies identified βAPP695 (lacking exon 7 and 8) (11), βAPP714 (lacking exon 7) (16), βAPP751 (lacking exon 8) (17–19), and βAPP770 (all exons present) (17). Subsequent studies by König et al. (20) identified a comparable set of βAPPs lacking exon 15 which are referred to as L-βAPPs because they were first identified in leukocytes.

βAPP751 and βAPP770, which contain a 56-amino acid Kunitz protease inhibitor (KPI) domain encoded by exon 7, are expressed both in the central nervous system and in peripheral tissues, whereas the KPI-free βAPP695 is expressed almost exclusively in brain, where it is more abundant than the KPI-containing isoforms. The secreted form of the KPI-containing βAPP751 is identical to protease nexin II, a plasma serine protease inhibitor (21, 22), and it has been shown to inhibit proteases such as trypsin (23–25), chymotrypsin (22, 24, 25), and...
and human coagulation factor XIs (22, 23).

To determine whether the KPI domain influences the proteolytic cleavages that generate Aβ, we compared Aβ production and sAPP secretion in transfected cells expressing human KPI-containing βAPP751 or KPI-free βAPP695. Aβ production was examined by sandwich ELISAs developed previously in our laboratory (26, 27) and by immunoprecipitation with Aβ-specific antibodies. The secretion of sAPP was evaluated by immunoprecipitating α or β secretase-derived sAPP (sAPPα or sAPPβ) with specific antibodies.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse teratocarcinoma P19 cells and human embryonic kidney 293 cells expressing βAPP695 and βAPP751 under the control of a cytomegalovirus enhancer and chick β-actin promoter were prepared by transfection with modified pCXN2 vectors (28) constructed as described by Fukuchi et al.1 P19 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin in the presence of G418. To select for high expression and clonal copy number, the P19 clones designated α were maintained in 0.8 mg/ml G418, and the P19 clones expressing at a lower level (β clones) were maintained in 0.4 mg/ml G418. P19 cells were split every other day, and the medium was changed every day. After transfection with Lipofectin (Life Technologies, Inc.), polyclonal 293 cells stably expressing βAPP695 or βAPP751 were selected with G418. The stable polyclonal 293 lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin in the presence of 0.5 mg/ml G418. For time course and synthesis rate measurements, P19 cells were seeded into six-well plates at 2 × 10^5/well 1 day prior to the experiment, and 293 cells were seeded into six-well plates at 0.2–6 × 10^5/well 1–3 days prior to experimentation. In some experiments, 293 cells were plated into 10-cm plates, and Aβ levels were determined at 48–96 h postplating.

Sandwich ELISAs for Aβ—Aβ-specific sandwich ELISAs were performed as described previously (26, 27). The monoclonal antibodies used in these assays were: BAN-50 (anti-Aβ1–16), 4G8 (anti-Aβ17–24), BA-27 (specific for Aβ ending at Aβ40), and BC-05 (specific for Aβ ending at Aβ42/43). The assays performed were BAN-50/BA-27 (specific for Aβ1–40), BAN-50/BC-05 (specific for Aβ1–42), BC-05/4G8 (specific for total Aβ ending at Aβ42/43)). Standards of increasing concentration, prepared using synthetic Aβ1–40 and Aβ1–42 peptides (Bachem) in growth media, were employed to calibrate the amount of Aβ measured on every ELISA plate.

Radio-labeling Experiments—Subconfluent cells on 10-cm plates were labeled for 5 or 8 h with Tran35S-label (250 μCi/ml) in 5 ml of minimum essential medium deficient for methionine/cysteine. Radiolabeled Aβ released into the medium was immunoprecipitated with Aβ-specific antibodies: R1280 (rabbit polyclonal anti-Aβ1–40) provided by D. J. Selkoe, 4G8, BA-27, or BC-05 conjugated to trisyl-activated agarose beads. Following separation by 10–20% Tris-Tricine SDS-polyacrylamide gel electrophoresis, the immunoprecipitated Aβ was visualized and quantitated by PhosphoImaging (1, 30). For measurement of the βAPP synthesis rate, cells seeded at 2–4 × 10^5/well in six-well plates on the day prior to experimentation were labeled for 20 min with Tran35S-label (250 μCi/ml) in 0.5 ml of minimum essential medium deficient for methionine/cysteine. Using antibody to the βAPP COOH terminus, the radiolabeled βAPP was immunoprecipitated from lysates, separated on 10% Tris-Tricine gels, and quantitated by PhosphoImaging as described above. To assess sAPPα and sAPPβ, cells seeded at 2–4 × 10^5/well in six-well plates on the day prior to experimentation were labeled for 2.5–10 h as described above. BAN-50 (anti-Aβ1–16) was used to immunoprecipitate sAPPα, and Ab53 (provided by B. Greenberg), which is specific for the COOH terminus of sAPP derived from β secretase cleavage, was used to immunoprecipitate sAPPβ. The immunoprecipitated sAPPα were processed and quantitated as described above.

RESULTS

Aβ Accumulation in the Medium of Transfected P19 Cells Expressing βAPP695 or βAPP751—To investigate the effect of the KPI domain on the processing events that determine ex-  

\[ \text{K.-I. Fukuchi, F. Ohman, N. Deng, A. C. Smith, C. E. Furlong, and G. M. Martin, submitted for publication.} \]
far more abundant than Aβ1–42, and (ii) concentrations of AβX–42 measured by the BC-05/4G8 ELISA which consistently exceeded the corresponding concentrations of Aβ1–42 measured by BAN-50/BC-05 ELISA (Fig. 1B).

Previously published reports have shown that full-length Aβ and P3 (α-secretase-derived) are the major forms of Aβ produced by cultured cells (4, 5, 31). Thus, the increased signal seen with the BC-05/4G8 ELISA compared with the BAN-50/BC-05 ELISA for Aβ1–42(43) is presumably due primarily to P3 ending at Aβ42(43), P3(42). The concentration of this putative P3(42), calculated by subtracting the BAN-50/BC-05 signal from the BC-05/4G8 signal, is shown in Table I. In medium conditioned by P19-βAPP695 cells for 10 h, 55 ± 4% of Aβ ending at Aβ42 was P3(42), whereas in P19-βAPP751 cells, the percentage of P3(42) was substantially lower at 26 ± 8% (p < 0.03 by nonparametric rank sum Mann-Whitney test). Essentially identical results were obtained in two experiments analyzing a second pair of P19 clones in which βAPP synthesis in the P19-βAPP695 line was 0.9 times that in the P19-βAPP751 line (Table I). In the second P19-βAPP695 clone, 63 ± 28% of Aβ ending at Aβ42 was P3(42), whereas in the second P19-βAPP751 clone, the percentage of P3(42) was substantially lower at 17 ± 25%. To pursue these observations, we analyzed P3(42) twice more in the b clones and five more times in the α clones. When the data from all 14 measurements were pooled, 70 ± 5% of Aβ ending at Aβ42 was P3(42) in cells expressing βAPP695, whereas the percentage of P3(42) was only 35 ± 5% in cells expressing βAPP751 (Table I, p < 0.01 by nonparametric rank sum Mann-Whitney test). This effect was significant for both the b clones (75 ± 12 for βAPP695 versus 28 ± 12 for βAPP751, p < 0.04 by rank sum Mann-Whitney test) and the α clones (68 ± 5 for βAPP695 versus 38 ± 6 for βAPP75, p < 0.01 by rank sum Mann-Whitney t test).

To confirm that P3(42) accounts for the difference between BAN-50/BC-05 and BC-05/4G8 assays, we labeled cells from the initial P19-βAPP695 clone for 8 h with Tran35S-label, used BC-05 to immunoprecipitate the Aβ ending at Aβ42 which was secreted into the medium, separated 4-kDa Aβ and 3-kDa P3 by Tris-Tricine SDS-polyacrylamide gel electrophoresis, and quantitated the relative amounts of Aβ and P3 by PhosphorImaging. This clone was chosen for quantitative analysis of the percentage of P3(42) by immunoprecipitation because it produced far more Aβ than the other clones. For comparison, we analyzed the Aβ ending at Aβ40 which is produced by this clone by immunoprecipitation with BA-27. As expected from previous reports (4, 5, 31) and the results of our sandwich ELISAs, P19-βAPP695 cells secreted substantial amounts of P3 (Fig. 2A). Quantitation showed that P3(42) comprised 68% of the total Aβ immunoprecipitated by BC-05, a result in excellent agreement with the percentages (68 ± 5%) obtained in the 10 experiments where sandwich ELISA data were used to determine the percentage of P3(42) (Fig. 2B). P3(40) comprised 55% of the total Aβ immunoprecipitated by BA-27. Thus, the percentage of P3 was similar for the major secreted Aβ ending at Aβ40 and the minor Aβ ending at Aβ42(43). We could not analyze Aβ ending at Aβ40 with a BC-27/4G8 ELISA assay.

| Clone | Relative βAPP synthesis | Aβ1–40 | Aβ1–42 | AβX–42 | P3(42) | P3(42)/AβX–42 |
|-------|-------------------------|--------|--------|--------|--------|----------------|
| P19–695a(5) | 6 | 2,851 ± 523 | 260 ± 52 | 627 ± 180 | 367 ± 136 | 0.55 ± 0.041 |
| P19–751a(5) | 1 | 443 ± 84 | 36 ± 6 | 47 ± 8 | 13 ± 4 | 0.26 ± 0.08 |
| P19–695b(2) | 0.9 | 359 ± 142 | 18 ± 16 | 91 ± 111 | 73 ± 95 | 0.63 ± 0.28 |
| P19–751b(2) | 1 | 315 ± 180 | 17 ± 3 | 21 ± 3 | 4 ± 6 | 0.17 ± 0.25 |
| Total | | | | | | |
| P19–695 (14) | | | | | | 0.70 ± 0.05 |
| P19–751 (14) | | | | | | 0.35 ± 0.05 |

*The synthesis rates were determined by the incorporation of [35S]methionine/cysteine into the full-length βAPP during a 20-min pulse.

**p < 0.03 by nonparametric Mann-Whitney test.

*p < 0.01 by nonparametric Mann-Whitney test.
equivalent to the BC-05/4G8 assay for αβ ending at Aβ42 because the sensitivity of the BA-27/4G8 ELISA is very low.

We were unable to detect the radiolabeled full-length Aβ(42) and P3(42) secreted by our P19-βAPP695 clones after BC-05 immunoprecipitation, presumably because the efficiency of BC-05 immunoprecipitation was insufficient to detect the smaller amount of αβ secreted by the P19-βAPP751 clones. Thus, to compare full-length αβ and P3 in P19-βAPP695 and P19-βAPP751 α clones, we immunoprecipitated radiolabeled Aβ with R1280, an efficient polyclonal rabbit antiserum that recognizes both 4-kDa αβ and 3-kDa P3 (Fig. 2C), although it is not selective for forms ending at Aβ40 or Aβ42. After R1280 immunoprecipitation, the percent P3 (P3/αβ + P3) × 100% in P19-βAPP695 and P19-βAPP751 cells was 57 and 26%, respectively. Thus, immunoprecipitation with this antibody (i) showed a reduced percent P3 in P19-βAPP751 cells as expected from our sandwich ELISA data and (ii) gave P3 percentages for the P19 α clones which were in reasonable agreement with the percentages obtained by sandwich ELISA.

To assess the generality of the phenomenon observed in the P19 cell lines, we analyzed the relative abundance of Aβ1–42 and P3(42) in conditioned media of polyclonal transfected human embryonic kidney (293) cells expressing βAPP695 or βAPP751. In 293-βAPP695 cells, 87 ± 1% of Aβ ending at Aβ42 was P3(42), whereas in the 293-βAPP751 cells, the percentage of P3(42) was significantly (p < 0.0001) lower at 74 ± 2% (Table II). Thus, 293 cells make relatively more P3(42) than P19 cells, but in both cell types the percentage of P3(42) is reduced significantly in cells expressing βAPP751 compared with those expressing βAPP695.

sAPPα and sAPPβ in the Medium of Transfected P19 Cells Expressing βAPP695 or βAPP751—There is good evidence that P3 is derived from COOH-terminal βAPP derivatives produced by a secretase (4, 5, 31, 32). Thus, a reduction of α secretase activity could account for the reduced percentage of P3 observed in transfected P19 cells expressing βAPP751. If a secretase activity is reduced, then the secretion of a secretase-derived sAPP (sAPPα) should be decreased. To determine whether sAPPα is decreased in P19-βAPP751 cells, we radio-labeled P19 cells expressing βAPP751 or βAPP695 for 2.5–10 h with Tran35S-label, used antibodies specific for sAPPα or sAPPβ to immunoprecipitate these derivatives from the conditioned medium, separated the immunoprecipitated, radiolabeled sAPP by SDS-polyacrylamide gel electrophoresis, and quantitated the sAPP by PhosphorImaging. As in our previous experiments, βAPP synthesis in each transfected cell line was analyzed in sister cultures by quantitating the newly synthesized βAPP radio-labeled during a 20-min pulse of Tran35S-label. Cells from two pairs of P19-βAPP695 and P19-βAPP751 clones were examined. Analysis of the synthesis rates of these lines showed overlapping levels of βAPP expression as illustrated in Fig. 3A.

We used BAN-50 (anti-αβ1–16) and Ab53 (specific for the COOH terminus of sAPPβ) to immunoprecipitate specifically sAPPα and sAPPβ, respectively. Both sAPPα and sAPPβ were detected readily in medium conditioned by P19-βAPP695 or P19-βAPP751 cells (Fig. 3B). To determine whether the relative amounts of sAPPα and sAPPβ are different in cells expressing βAPP695 or βAPP751, we compared sAPPα/sAPPβ and sAPPβ/sAPPα in three experiments (Table III). The relative amounts of sAPPα and sAPPβ were highly reproducible in lines expressing the relevant βAPPs at widely varying levels (Fig. 3A). Overall, the relative amount of sAPPα was significantly (p < 0.001 by both nonparametric rank sum Mann-Whitney test and unpaired t test) lower for P19–751 cells than for P19–695 cells with means of 63 ± 1% and 83 ± 2%, respectively.

**DISCUSSION**

Our results show, for the first time, that βAPP751 is processed differently than βAPP695. Specifically, our comparisons of transfected cells expressing βAPP695 or βAPP751 at various levels indicate that, independent of their level of expression, cells expressing βAPP751 make relatively less sAPPα and relatively less P3(42) than cells expressing βAPP695. Thus, it appears that the expression of the KPI domain diminishes cleavage by α secretase. This diminution of α secretase cleavage could be due to inhibition of α secretase by protease nexin II released extracellularly and/or into an intracellular compartment, to a conformational change in the βAPP751 holoprotein which renders it relatively less susceptible to cleavage by α secretase or to some other mechanism such as altered trafficking which reduces the exposure of the βAPP751 holoprotein to α secretase. Our preliminary studies on P19 cells suggest that inhibition of a secretase by extracellular protease nexin II may not be the major mechanism by which βAPP751 exerts its effect, since medium conditioned by P19-βAPP751 cells had little effect on the production of P3(42) by P19-βAPP695 cells. Additional studies are clearly needed, however, to identify the mechanisms involved.

We wish to emphasize that the rather subtle differences identified were best demonstrated by carefully analyzing cells expressing βAPP695 or βAPP751 in parallel for the relative
amount of α-secretase-derived βAPP derivatives. The reason for this is that, unlike AβX-42(43), P3(42), or sAPPα concentration, P3(42)/AβX-42 and sAPPα/sAPPβ are not influenced substantially by methodological variance or by the differences in level of expression which occur in transfected cells. Our comparison of medium conditioned by P19 and 293 cells (Tables I and II) showed a much higher percentage of P3(42) in the medium of 293 cells, an observation in good agreement with the previous results of Seubert et al. (7), who showed that more than 90% of the sAPP produced by 293-βAPP695 cells is sAPPα. Thus, the relative amount of α-secretase activity is influenced by cell type; but in both of the cell types that were analyzed relatively less P3(42) and sAPPα were produced from βAPP715 than from βAPP695.

Studies of Aβ secretion by primary cultures indicate that there are substantial differences in the relative amount of 4-kDa Aβ and P3 which is secreted by neuronal and non-neuronal cells. Neurons, which express βAPP695, produce substantially higher levels of P3 (5); whereas astrocytes (5) and skin fibroblasts (33), which express predominantly βAPP715/770, produce mostly full-length Aβ. The results obtained in the present study suggest that the isoform expressed by these cell types may participate in determining the relative amount of P3 and full-length Aβ which is produced, although intrinsic differences in α-secretase activity obviously could also contribute to the differences observed.

Our findings also have important implications concerning the amyloid deposition process in aging, AD, and in experimental models of AD. There is evidence that the cerebral APP715/APP695 mRNA ratio increases with age (34, 35) as might be expected as neurons, which have relatively high expression of APP695 mRNA ratio increases with age (34, 35) as might be expected as neurons, which have relatively high expression of βAPP695, are lost during normal aging. In addition there is evidence that the neuronal APP715/APP695 mRNA ratio is correlated positively with plaque density in brains of AD and control patients (36). Finally, the available evidence suggests that efforts to model Aβ deposition in transgenic mice overexpressing βAPP are more effective when KPI-containing forms are expressed. Recently Games et al. (37) reported Alzheimer-like pathology in mice expressing human βAPP with the βAPPV717F mutation linked to familial AD. The βAPPV717F transgene in these mice was driven by platelet-derived growth factor promoter, and it contained portions of APP introns 6–8, allowing alternative splicing of exons 7 and 8 by the host. With this construct, this group achieved marked overexpression of human βAPP in the transgenic mouse brain, and they observed extracellular amyloid deposition, dystrophic neuritic components, and gliosis that resembled AD. In the brains of these transgenic mice, the predominant human mRNA expressed was APP751. In a series of studies on transgenic mice expressing human βAPP751 at lower levels than those in the transgenic mice of Games et al., Cordell and her colleagues have reported an age-related, subtle increase in Aβ deposition and tau staining (38–41) as well as age-dependent deficits in spatial learning in a water maze task and in spontaneous alternation in a Y maze (42). In their discussion of these animals with subtle histopathological changes (42), the authors indicate that transgenic mice expressing βAPP695 do not develop similar histopathological (38, 39) or behavioral (29) changes. Based on these suggestive findings in transgenic mice and the results that we report here, we speculate that the level of expression of the KPI domain may influence the development of AD by altering a secretase cleavage in a way that changes the specific species of Aβ that are secreted.

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