The Alzheimer Amyloid Precursor Protein

IDENTIFICATION OF A STABLE INTERMEDIATE IN THE BIOSYNTHETIC/DEGRADATIVE PATHWAY*

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The amyloid forming β-peptide of Alzheimer’s disease is synthesized as part of a larger integral membrane precursor protein (βAPP) of which three alternatively spliced versions of 695, 751, and 770 amino acids have been described. A fourth βAPP form of 563 amino acids does not contain the β-peptide region. Recent experiments using transient expression in HeLa cells (Weidemann et al., 1989; Masters et al., 1988) indicate that the βAPP undergoes several posttranslational modifications including the cleavage and secretion of a large portion of its extracellular domain. The nature and fate of the fragment that remains cell-associated following this cleavage has not heretofore been described. The metabolism of this fragment may have particular significance in Alzheimer’s disease since it must contain at least part of the β-peptide. To study the metabolic fate of this fragment, we have established cell lines over-expressing the 695- and 751-amino acid versions of βAPP. Pulse-chase studies show that this system is similar to the HeLa cell system in that both proteins are synthesized first as membrane-bound proteins of approximately 98 and 108 kDa carrying asparagine-linked sugar side chains and are subsequently processed into higher molecular mass forms by the attachment of sulfate, phosphate, and further sugar groups including sialic acid, adding approximately 20 kDa in apparent molecular mass. The mature form of βAPP is cleaved and rapidly secreted, leaving an 11.5-kDa fragment with the transmembrane region and the cytoplasmic domain behind in the cell. This fragment is stable with a half-life of at least 4 h.

Alzheimer’s disease is the most common neurodegenerative disorder, affecting over 2 million people in the United States alone (for a review see Katzman, 1986). It is characterized by progressive dementia together with the presence of defining neuropathological features: extracellular deposits in the form of amyloid plaques and vascular amyloid, as well as intracellular deposits in the form of neurofibrillary tangles. Amyloid plaques have also been found in Down’s syndrome and, in smaller numbers, in normal aged human brain and in the brains of some aged nonhuman primates (for a review see Selkoe, 1989). Ultrastructurally, amyloid plaques and cerebrovascular amyloid contain 6–10-nm straight filaments, which are comprised of a 42–43-amino acid subunit, the so-called β- or Aβ-peptide (Glenner and Wong, 1984a, 1984b; Masters et al., 1985). cDNA cloning has suggested that the β-peptide is synthesized as part of a much larger precursor molecule, the gene for which is located on chromosome 21 (Kang et al., 1987; Tanzi et al., 1987; Goldgaber et al., 1987; Robakis et al., 1987). The sequence together with cell fractionation studies (Dyrks et al., 1988; Selkoe et al., 1988) suggest that the βAPP is a membrane-bound protein with the β-peptide spanning the border between the extracellular domain and the transmembrane domain. To date, four differentially spliced versions of this β-amylloid precursor protein molecule have been described comprising 695 amino acids (βAPP 695, Kang et al., 1987), 751 amino acids (βAPP 751, Ponte et al., 1988; Tanzi et al., 1988), 770 amino acids (βAPP 770, Kitaguchi et al., 1988), and 563 amino acids (de Sauvage and Octave, 1989), respectively. The latter form does not contain the β-peptide region. The two larger forms carry a Kunitz-type protease inhibitor domain. The secreted forms of βAPP with this protease inhibitor have been shown to be identical to protease nexin II (Oltersdorf et al., 1989; Van Nostrand et al., 1989). A key question regarding the molecular and cellular events that lead to amyloid deposition is how the membrane-associated β-peptide is cleaved from its precursor and deposited extracellularly. It has recently been reported (Weidemann et al., 1989) that βAPP in HeLa cells is rapidly processed from a membrane-bound form to a secreted form that lacks the C terminus of the precursor. Weidemann et al. (1989) indicate that the secreted forms derive from full length βAPP by cleavage in the extracellular domain. This would suggest the existence of a C-terminal membrane-associated fragment that remains behind in the cell following the cleavage event. Such a fragment has indeed been seen in extracts of human and rat brain (Selkoe et al., 1988). However, the relationship of this fragment to the pathway of βAPP metabolism has not been investigated. Here, we describe the establishment of stably transfected 293 cells (transformed human embryonic kidney cells) expressing βAPP cDNAs. Using these cell lines we have analyzed the βAPP metabolic pathway including synthesis, complex posttranslational modifications, secretion, and ultimate formation of a long-lived, membrane-bound 11.5-kDa fragment.

EXPERIMENTAL PROCEDURES

Cell Lines—293 (transformed human embryonic kidney) and HS 683 (human glioma) cells were obtained from ATCC. Cell lines were grown as suggested by the supplier.

Expression of cDNAs for Human βAPP in Human Cells—The
establishment of 293 cells overexpressing βAPP695 from a cDNA driven by the human cytomegalovirus promoter/enhancer has been described earlier (Selkoe et al., 1988). 293 cells overexpressing βAPP751 and HS 683 cells overexpressing βAPP695 were obtained using essentially identical methods. The βAPP cDNAs were derived from人HL-60 promyelocytic leukemia cell line library (Tanzi et al., 1988).

Expression of βAPP Fragments in Escherichia coli and Preparation of Antibodies—βAPP fragments coding for amino acids 444-592 (re-striiction sites BamH I to BgII I) and 592-695 (restriction sites BgII I to SpeI) were inserted in the BamH I restriction site of plasmid pBS5. In addition the endothionine-free cDNA coding for the extracellular domain (anti-BX5) or against a fragment extending from the β-peptide through the C-terminal end of the molecule (anti-BX6). βAPP695 and βAPP751 were each represented by two major bands in these cells and by a single band in conditioned medium (Fig. 1A). The apparent molecular masses of these bands estimated on the basis of a "C-labeled marker proteins (Amersham Corp.) were for βAPP695, 98 and 115 kDa in cells and 102 kDa in medium; for βAPP751, 108 and 130 kDa in cells and 115 kDa in medium. Other marker proteins (Bio-Rad) led to approximately 10 kDa higher estimates. While the cell-associated βAPP forms were recognized by both anti-BX5 and anti-BX6, the secreted forms in medium were not detected by anti-BX6 (data not shown), indicating that they had lost the cytoplasmic part of the molecule. This was confirmed by use of the previously described antiserum C1 directed against a peptide encompassing the C-terminal 20 residues of βAPP (Selkoe et al., 1988), which similarly recognized the cell-associated but not the secreted βAPP forms (data not shown). In addition to the high molecular mass bands of 98-130 kDa, anti-BX6 and anti-C1 also detected a band of strong immunoreactivity at approximately 11.5 kDa (Fig. 1B) present in cell extracts which was not seen using anti-BX5. This 11.5-kDa band was seen in extracts of cells overexpressing either βAPP695 or βAPP751 but was not found in the medium. Its

### Metabolic Labeling and Immunoprecipitation

For metabolic labeling, 5 x 10⁵ to 10⁶ cells were incubated with 0.2 μCi of [³⁵S]methionine (100 Ci/mmol, Amersham Corp.) in 2 ml of methionine-free Dulbecco's modified Eagle's medium for 10 min. After two washing steps, cells were incubated for chase times from 20 min to 8 h in serum-free medium. Then the medium was collected and cells were lysed in TBSI (10 mM Tris, pH 7.5, 150 mM NaCl) containing 1% Nonidet P-40, 5 mM EDTA, and 2 μg/ml leupeptin. Samples were preabsorbed with 15 μg of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) and 20 μg of rabbit antisera against the fusion proteins (anti-BX5 and anti-BX6). Precipitates were washed in incubation buffer and run over another column containing the partially purified fusion protein, which had served as the antigen, coupled to activated Sepharose. Immunoprecipitated medium was concentrated by precipitation in 4 volumes of cold methanol. Precipitates were reprecipitated with methanol prior to loading on SDS gels.

### Immunoblotting

Cells were lysed in TBS, 1% Nonidet P-40, 5 mM EDTA, 2 μg/ml leupeptin, boiled in reducing SDS sample buffer, and loaded on standard Laemmli SDS gels containing either 6.25% acrylamide or linear gradients of 7.5–20% acrylamide. After running, gels were fixed, incubated with AmplifyTM (Amersham Corp.), dried, and autoradiographed at -80 °C. For metabolic labeling with [³²P]sulfate (25–40 Ci/mg, Amersham Corp.) or [³²P]phosphate (1 μCi/ml, Amersham Corp.), 10⁶ cells in a 75-cm² dish were grown in the presence of 1 μCi of the label for 3 h in 3 ml of serum-free Dulbecco's modified Eagle's medium without additional sulfate or phosphate, respectively. Immunoprecipitation was done as described above.

#### Immunoblotting—Cells were lysed in TBS, 1% Nonidet P-40, 5 mM EDTA, 2 μg/ml leupeptin. Protein from serum-free 48-h conditioned medium was concentrated by precipitation in 4 volumes of cold methanol. Proteins were separated on gels as described above. Transfer onto Immobilon P membranes (Millipore) was performed according to standard procedures. Filters were blocked in 10% skim milk and incubated with primary antibodies at a concentration of approximately 2 μg/ml. An alkaline phosphatase-coupled goat anti-rabbit antibody (1:3000, Bio-Rad) served as second antibody. Bound alkaline phosphatase activity was detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). Washing steps were carried out with TBS (10 mM Tris, pH 7.5, 150 mM NaCl) and 0.05% Tween 20.

### Treatment with Endoglycosidase F and Neuraminidase

For digestion with endoglycosidase F (Boehringer Mannheim), immunoprecipitates, obtained as described above, were washed in incubation buffer (50 mM sodium citrate, pH 5.0) and incubated overnight at 37 °C in 25 μl with 0.25 unit of enzyme. To control for nonspecific degradation, parallel samples were incubated in the absence of enzyme. For digestion with neuraminidase (Genzyme, Boston, MA), aliquots of 1 μl

1. The abbreviations used are: TBS, Tris-buffered saline; SDS, sodium dodeyl sulfate; HPLC, high pressure liquid chromatography.
reactivity with anti-BX6 indicates that it is derived from the C terminus of βAPP. Three antisera raised against synthetic peptides containing amino acids 1–38 and 1–43 of the β-peptide and two antisera raised against HPLC-purified amyloid plaques from Alzheimer brain recognized neither the 11.5-kDa fragment nor full length or secreted βAPP in Western blot (data not shown), although these antisera react strongly with amyloid plaque protein in immunohistochemistry.

**Time Course of βAPP Synthesis**—To investigate the time course of appearance of the various βAPP immunoreactive bands in the 293 cell transfectants, cells were pulse-labeled for 5 min with [35S]methionine and chased with cold methionine-containing medium for various lengths of time, after which cell extracts and conditioned medium were harvested and immunoprecipitated with anti-BX6. When cells were harvested immediately after pulse labeling without a chase period, cell extracts contained a single βAPP-specific band corresponding to the lower molecular weight band of the two seen in Western blots (Fig. 2). After 40 min of chase the higher molecular weight form appears, and after 120 min nearly all of the radioactivity was in the higher molecular weight form. Experiments with longer chase periods (Fig. 3) demonstrated that by 4 hr both the higher and lower molecular weight forms were virtually gone. Time courses did not differ significantly between βAPP695 and βAPP751. Identical results were obtained when anti-BX5 was used for immunoprecipitation (data not shown). In conditioned medium from pulse-chase experiments, a single band was detected by anti-BX5 after 40–60 min of chase. The intensity of this band reached a maximum after approximately 2 hr and was markedly decreased after 4 hr (Fig. 2). The upper band (Fig. 2, arrow) which was immunoprecipitated from conditioned medium of βAPP695 overexpressing cells at the 120-min time point represents the endogenous form of βAPP751/770 (Tanaka et al., 1989; Weidemann et al., 1989), indicating that its rate of secretion is similar to that of βAPP695. This was confirmed with cells transfected with a βAPP751 expression construct (data not shown). Immunoprecipitation was specific since the relevant bands could not be immunoprecipitated after preabsorption of the antibodies with the appropriate bacterial fusion protein (Fig. 3, ctrl.).

**Posttranslational Modifications**—The existence of two bands derived from each βAPP cDNA expression construct suggested that the protein is subject to posttranslational modification. The early appearance of a lower molecular weight band followed by the appearance of a higher molecular weight band further suggested that these were immature and mature forms of full length βAPP, respectively. We investigated this directly by use of sugar-cleaving enzymes and biosynthetic labeling. Treatment of immunoprecipitated βAPP695 and βAPP751 from cell extracts with endoglycosidase F decreased the molecular mass of the immature (98 and 108 kDa) bands by approximately 2 kDa (Fig. 4A), indicating the presence of asparagine-linked carbohydrate. Endoglycosidase H treatment led to similar results (data not shown). The mature (115 and 130 kDa) bands, however, showed no detectable decrease in molecular mass upon endoglycosidase F treatment. They were markedly reduced in size, however, when treated with neuraminidase, as were the secreted forms of βAPP (Fig. 4B). This indicates the presence of a large number of sialic acid residues in the mature β-APP molecules. Treatment with fucosidase, N-acetyl-β-D-glucosaminidase, endo-α-N-acetylgalactosaminidase and combinations of these enzymes were without detectable effect (data not shown). Biosynthetic labeling with [32P]phosphate and [35S]sulfate demonstrated that the βAPP incorporates both labels (Fig. 4, C and D). These labels were only seen in the mature forms of βAPP. This shows that βAPP is a phosphoprotein and is also in accord with reports of the presence of tyrosine sulfate in the molecule (Schubert et al., 1989; Weidemann et al., 1989).

**Detection of a Stable 11.5-kDa Intermediate in the Processing of βAPP**—The 11.5-kDa fragment seen in Western blots of 293 cell extracts with anti-BX6 (Fig. 1B) was also detected in pulse-chase experiments. It appeared in cell extracts after 20 min, increased in intensity over 2 hr, and was still present...
in considerable amounts after 8 h, when full length βAPP had disappeared completely from the cell (Fig. 5). No significant differences in the rate of formation of this fragment were seen between βAPP695 and βAPP751 overexpressing cells (Fig. 5). This fragment was not detected by anti-BX5, and preabsorption of anti-BX6 with BX6 fusion protein abolished immunoprecipitation of the fragment, demonstrating the specificity of the anti-BX6 reaction. The molecular mass of approximately 11.5 kDa, which corresponds to about 100 amino acids, and the fact that the fragment is recognized by antibodies against the C terminus of βAPP but not by antibodies against a βAPP fragment directly N-terminal of the β-peptide region, suggest that the N terminus of this fragment lies in the vicinity of the N terminus of the β-peptide. One should expect that the location of this cleavage site relative to the β-peptide could be clarified by antibodies directed against the β-peptide. A total of six such antisera has been tested in immunoprecipitation: two peptide antisera raised against amino acids 1-38 of the β-peptide derived from two different laboratories, two antisera raised against amino acids 1–43, and two antisera raised against HPLC-purified amyloid plaque protein from Alzheimer post-mortem brain. All of these antibodies recognize amyloid plaques in immunohistochemistry. However, as in the Western blot experiments described above none of them recognized full length βAPP, secreted βAPP, or the 11.5-kDa fragment even when protein extracts were boiled in 0.5% SDS before immunoprecipitation to uncover masked epitopes (data not shown).

**DISCUSSION**

The accumulation of the β-peptide in amyloid plaques suggests that the normal metabolism of βAPP has been altered in Alzheimer's disease brain. We have used cell lines stably transfected with βAPP cDNA expression constructs to analyze the normal metabolism of βAPP and to look for steps that may be intermediates in the amyloidogenic pathway. In pulse-chase experiments with human embryonic kidney 293 cells separately transfected with βAPP695 and βAPP751 cDNA expression plasmids, each transfected cDNA led to two major forms of βAPP detectable inside cells: an immature, endoglycosidase F-sensitive species which was synthesized early, and a higher molecular weight, mature neuraminidase-sensitive form which appeared over a 2-h period. Analysis of culture supernatants revealed that for each βAPP form there was an anti-BX5 immunoreactive protein that was lower in molecular weight than the respective mature membrane-associated form. This secreted material was first seen in the medium after 40 min of cold chase and peaked at approximately 2 h. A similar sequence of events has been established recently for βAPP metabolism in HeLa cells (Weidemann et al., 1989). In our experiments, we have identified an additional 11.5-kDa βAPP band that appeared during the chase period in the cell pellets, with a time course similar to that seen in the medium for secreted material. These results suggest that for each of βAPP695 and βAPP751, an immature N-glycosylated protein is rapidly synthesized, which is then processed to a higher molecular weight, mature, fully glycosylated form. This mature form is then cleaved to simultaneously yield a secreted extracellular domain and a long-lived, 11.5-kDa, membrane-bound fragment. This cleavage event probably occurs also in brain, since an approximately 11-kDa band has been seen in rat and human cerebral cortex with βAPP C-terminal antibodies (Selkoe et al., 1988) and bands of 91 and 112 kDa have been seen in human cerebrospinal fluid with antibodies against the extracellular part of βAPP (Palmert et al., 1989; Weidemann et al., 1989). The cleavage of membrane-bound precursors to yield secreted products has previously been described for several proteins including epidermal growth factor (Doolittle et al., 1984), transforming growth factor α (Bringman et al., 1987), and rat liver sialyltransferase (Paulson et al., 1987).
Fig. 5. 11.5-kDa fragment of βAPP. Immunoprecipitation after a 10-min pulse label with [35S]methionine and indicated chase periods. A, 293 cells transfected with βAPP751; the antibody used was anti-BX6. Two nonspecific bands are seen in each lane, including the preabsorbed control lane (ctrl). The specific 11.5-kDa band is the lowest of the three bands and is seen at all time points but absent in the control lane. B, 293 cells transfected with βAPP695; the antibody used was anti-BX6. As in A, the lowest band is the specific 11.5-kDa band. C, same as A but probed with anti-BX5. Note that anti-BX5 reacts only with the nonspecific bands also present in the control lane and does not recognize the 11.5-kDa band. D, 293 cells transfected with βAPP855 and chased for shorter periods of time; 11.5-kDa fragment visible in small amounts after 20 min. ctrl, control lanes where samples identical to those of the 1-h time point were immunoprecipitated with antibodies preabsorbed with corresponding bacterial fusion proteins BX5 and BX6.

Several considerations further support the conclusion that the fully glycosylated βAPP is the precursor to the cleaved 11.5-kDa and secreted forms. First, although each βAPP expression plasmid generated two cell-associated forms, each yielded only one secreted form. These secreted forms, which by immunological criteria have lost a C-terminal fragment, have an estimated molecular mass 10–15 kDa lower than the mature βAPP, in accord with the proposed precursor-product relationship. In contrast, the secreted forms are larger than their respective immature cell-associated βAPP forms. Second, the secreted forms show a molecular weight shift upon neuraminidase treatment that is comparable to that seen upon enzyme treatment of the mature βAPP (Fig. 5), whereas the immature form is unaffected by such treatment. And third, bands corresponding in mobility to those seen in the medium were never observed in immunoprecipitation of cell pellets. This suggests that the cleavage which generates the secreted form occurs either very late in the intracellular secretion pathway or on the cell surface. This is in accord with cleavage occurring after late posttranslational modifications have been made in the trans-Golgi compartment.

The metabolism of the long-lived 11.5-kDa fragment may have special relevance to the mechanism of amyloidosis, since its size suggests that it may contain the β-peptide. It must contain the transmembrane domain and thus at least part of the β-peptide and the C terminus since it is an integral membrane fragment and reacts with C-terminal antibodies. Antibodies directed against the β-peptide were not helpful in identifying exactly how much of the β-peptide is part of the 11.5-kDa fragment. A total of eight such antisera were tested in Western blot and in immunoprecipitation and were found not to react with full-length βAPP, secreted βAPP, or the 11.5-kDa fragment. This lack of reactivity may reflect specificity for conformational epitopes only present in aggregated amyloid as all of these antisera recognize amyloid plaques in brain. Clarification of the exact location of the cleavage site will require purification and sequencing of the fragment.

We have shown that the 11.5-kDa fragment is quite stable in membranes. The pulse-chase experiments showed that it accumulated over a 2-h period and that substantial amounts remained after 8 h of chase (Fig. 5). Western blots of cell pellets, which reflect the steady state accumulation of protein in a cell, show substantial accumulation of this fragment (Fig. 1). Similarly, the 11.5-kDa fragment is readily detectable in postmortem brain and rat brain (Selkoe et al., 1988). The stability and accumulation of the 11.5-kDa fragment raises the possibility that it may be an intermediate in the process of amyloid deposition. Indeed, it has been shown that a similar sized fragment created by in vitro translation aggregates in the absence of membranes and that aggregates can be digested by addition of proteinase K to yield a 4-kDa monomer (Dyrks et al., 1988).

Biosynthetic labeling of βAPP695 and βAPP751 showed that both are sulfated and phosphorylated in HS 683 cells. These results are in accord with previous observations that βAPP undergoes a complex set of posttranslational modifications. Sulfate has been demonstrated in βAPP secreted from PC12 cells and HeLa cells and shown to be covalently attached to tyrosine in βAPP (Schubert et al., 1988; Weidemann et al., 1989). Regarding phosphorylation, previous work has shown that synthetic peptides derived from the βAPP sequence can serve as substrates for protein kinase C and Ca++/calmodulin-dependent protein kinase II (Gandy et al., 1988). Further work will be necessary to assess the relationship of these synthetic peptide results to our observations that βAPP is a phosphoprotein, since we do not know the identity of the kinase(s) which act on βAPP in cells. It is of interest to note, however, that a possible site for tyrosine phosphorylation similar to that seen in the epidermal growth factor receptor is present at residue 687 in the cytoplasmic domain of βAPP, but we do not know if this site is actually phosphorylated.

The susceptibility of βAPP to endoglycosidase F, endoglycosidase H, and neuraminidase treatments indicates that it contains covalently bound sugars. The immature cell-associated form showed an apparent molecular mass decrease of approximately 2 kDa upon treatment with either endoglycosidase F or H, indicating the presence of N-linked high mannose sugars. This confirms earlier work which showed that tunicamycin treatment caused a similar shift in mobility of βAPP (Weidemann et al., 1989) and is in accord with in vitro translation results (Dyrks et al., 1988). We could not observe any effect of endoglycosidase F on mature βAPP. This may be due to maturation of N-linked sugars to a complex form not cleaved by endoglycosidase F or H, indicating the presence of N-linked high mannose sugars. This confirms earlier work which showed that tunicamycin treatment caused a similar shift in mobility of βAPP (Weidemann et al., 1989) and is in accord with in vitro translation results (Dyrks et al., 1988). We could not observe any effect of endoglycosidase F on mature βAPP. This may be due to maturation of N-linked sugars to a complex form not cleaved by endoglycosidase F or H, indicating the presence of N-linked high mannose sugars. This confirms earlier work which showed that tunicamycin treatment caused a similar shift in mobility of βAPP (Weidemann et al., 1989) and is in accord with in vitro translation results (Dyrks et al., 1988). We could not observe any effect of endoglycosidase F on mature βAPP. This may be due to maturation of N-linked sugars to a complex form not cleaved by endoglycosidase F or H, indicating the presence of N-linked high mannose sugars. This confirms earlier work which showed that tunicamycin treatment caused a similar shift in mobility of βAPP (Weidemann et al., 1989) and is in accord with in vitro translation results (Dyrks et al., 1988).
et al., 1982). In N-CAM, however, these sugars can be removed completely by endoglycosidase F (Cunningham et al., 1983). The observation that tunicamycin does not block the shift in mobility from immature to mature forms (Weidemann et al., 1989) also argues that maturity involves addition of O-linked sugars. Further work will be necessary to assess the potential importance of posttranslational modifications in the control of βAPP turnover and in the processes that lead to β-peptide aggregation and amyloid deposition.

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