Relative Increase in Alzheimer's Disease of Soluble Forms of Cerebral Aβ Amyloid Protein Precursor Containing the Kunitz Protease Inhibitory Domain*

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Although a number of studies have examined amyloid precursor protein (APP) mRNA levels in Alzheimer's disease (AD), no clear consensus has emerged as to whether the levels of transcripts for isoforms containing a Kunitz protease inhibitory (KPI)-encoded region are increased or decreased in AD. Here we compare AD and control brain for the relative amounts of APP protein containing KPI to APP protein lacking this domain. APP protein was purified from the soluble subcellular fraction and Triton X-100 membrane pellet extract of one hemisphere of AD (n = 10), normal (n = 7), and neurological control (n = 5) brains. The amount of KPI-containing APP in the purified protein samples was determined using two independent assay methods. The first assay exploited the inhibitory action of KPI-containing APP on trypsin. The second assay employed reflectance analysis of Western blots. The proportion of KPI-containing forms of APP in the soluble subcellular fraction of AD brains is significantly elevated (p < 0.01) compared with controls. Species containing a KPI domain comprise 32–41 and 76–77% of purified soluble APP from control and AD brains, respectively. For purified membrane-associated APP, 72–77 and 65–82% of control and AD samples, respectively, contain a KPI domain. Since KPI-containing species of APP may be more amyloidogenic (Ho, L., Fukuchi, K., and Yonkin, S. G. (1996) J. Biol. Chem. 271, 30929–30934), our findings support an imbalance of isoforms as one possible mechanism for amyloid deposition in sporadic AD.

The pathological hallmark of Alzheimer's disease (AD) is the deposition of amyloid as cerebrovascular, diffuse and neuritic plaques (within the brain extracellular space), and neurofibrillary tangles (within neurons). The principal component of extracellular amyloid is a 4-kDa peptide, the Aβ protein (1, 2) (also called the βA4 protein). The Aβ peptide is not expressed as a functional protein entity (3) but is released by the processing of a much larger transmembrane protein, the amyloid protein precursor (APP). The pathogenesis of AD is thought to involve the disregulated expression or abnormal processing of APP.

APP is encoded by a single 18-exon gene on chromosome 21 (4–6). Exons 7, 8, and 15 of the APP gene can be alternatively spliced to produce multiple isoforms. In brain the predominant isoform transcripts demonstrated to date are APP695, APP751, and APP770 (7–9). These transcripts code for species containing 695, 751, and 770 amino acids, respectively. The isoforms APP751 and APP770 both contain a Kunitz protease inhibitory (KPI) motif that APP695 lacks. APP770 contains an additional OX2 domain (7, 10). The secreted form of APP751 is identical to protease nexin II, a plasma serine protease inhibitor (11). In addition to KPI and OX2 domains several other structural features have been identified on APP, including binding domains for heparin (12), zinc (13, 14) and copper (15), and N-linked carbohydrate attachment sites (10).

Normal catabolism of APP involves proteolytic cleavage of full-length membrane-associated forms within the extracellular domain of the Aβ region and release of soluble COOH-terminal truncated species (sAPP) (16, 17). The proteases that release sAPP have yet to be identified but have been named the α- and β-secretases. The α-secretase cleaves within the Aβ sequence of APP and its products are non-amyloidogenic. The β-secretase cleavage site is the NH2 terminus of the Aβ domain. The proteolytic activities that release intact Aβ from the transmembrane domain of APP (COOH terminus of Aβ) have been designated γ-secretases. The catabolic pathway for Aβ generation is unclear but probably involves internalization of full-length APP from the cell surface and degradation in endosomal-lysosomal complexes (18–20). In cultured hamster cells one route for Aβ production involves a coated pit-mediated endocytic pathway (21). Low density lipoprotein receptor-related protein (LRP) has recently been shown to also mediate the internalization and degradation of KPI-containing (KPI⁺) APP forms (22, 23). Internalization via LRP has been shown to increase if APP is complexed to the binding protein for epidermal growth factor (EGFBP) (23, 24). EGFBP is specific for APP forms that contain a KPI domain and does not form complexes with APP695. At present it is unclear if LRP mediated internalization of APP leads to Aβ production.

Transcripts for APP695 are expressed almost exclusively in...
brain where they are a major neuronal APP mRNA species (25, 26). The KPI+ isoforms APP751 and APP770 are expressed in both the central nervous system and in peripheral tissues. Several lines of evidence suggest that expression levels of KPI+ isoforms of APP may be important in AD pathogenesis. There is evidence that the cerebral APP751/APP695 mRNA ratio increases with age (27, 28). Although reports are conflicting, mRNA levels for KPI+ APP751 and APP770 species may be elevated in the brains (6, 8, 29–36) and fibroblasts (37, 38) of AD patients. The cerebrospinal fluid of AD patients may also be elevated in response to trauma (42–45) and transgenic mice over-expressing KPI+ species of APP show age-related learning deficits (46). A recently developed transgenic mouse that exhibits extensive cerebral Aβ amyloid deposition (47) has been reported to overexpress human APP-KPI+ mRNA and have suppressed endogenous APP-KPI+ message levels (38). Finally, it has recently been confirmed that KPI+ isoforms of APP are more amyloidoigenic, at least in transfected cells (48).

At present it is unclear if an increase in KPI+ isoforms of APP occurs at the protein level in the brains of AD patients. In this study, APP was purified from the soluble subcellular fraction and Triton X-100 membrane pellet extract of AD and normal brains and then compared for KPI+ isoforms by two different assay methods. We find that the level of APP-KPI+ in purified material from the soluble subcellular fraction of AD brains was significantly elevated (doubled) relative to control patients.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lyophilized purified trypsin from bovine pancreas and carbobenzoxy-valyl-glycyl-arginine-4-nitranilide acetate (chromozym TRY) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Electrophoretic molecular weight markers were purchased from Amersham (Buckinghamshire, United Kingdom). Electrophorosis reagents were from Bio-Rad and polynylidene difluoride membrane from Millipore Corp. (Bedford, MA). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL). Protein A-Sepharose and other chromatographic resins were from Bio-Rad and 100- to 120-kDa chromatographic resins were from Pharmacia (Uppsala, Sweden). The monoclonal antibody (mAb) 22C11 available from Boehringer Mannheim GmbH and polyclonal (pAb) R45 rabbit antiserum recognize an epitope near the amino terminus of APP (10, 49). MAb 7H5 was raised against a bacterial construct encompassing the KPI domain (residues 289–347 of APP751) and was kindly provided by Dr. Dale Schenck, Athens Neurosciences. Secondary antibodies used in Western blots were purified IgG fractions of anti-mouse antibodies conjugated to alkaline phosphatase (Promega, Sydney, Australia).

**Case Selection**

Human brains were obtained 12–24 h post mortem. At the time of autopsy one cerebral hemisphere was frozen at –70 °C and the other hemisphere was fixed in formalin for histological examination. The clinical diagnosis of Alzheimer’s disease was confirmed by subsequent histopathological evidence of amyloid plaques or neurofibrillary tangles. Neurological control patients were clinically diagnosed as having Parkinson’s disease or Huntington’s disease with subsequent pathological confirmation. All clinical groups were age matched.

**Purification of APP**

APP was purified to homogeneity from the ultracentrifuged soluble fraction and Triton X-100 membrane pellets of human brain homogenate according to the method of Moir et al. (50). Briefly, isolated human brain cortex was homogenized and centrifuged. The supernatant (soluble subcellular fraction) was removed and the pellet incubated with Triton X-100. Insoluble material was pelleted by a second centrifugation and the supernatant (membrane extract) removed. Purification of APP from the crude brain subcellular fractions involves a sequential series of chromatographic steps: anion exchange (MacroPrep high Q resin), affinity chromatography (heparin-Sepharose gel), a second anion exchange step (Mono Q column) and hydrophobic interaction chromatography (phenyl-Superose column). Peak APP fractions from the final phenyl-Sepharose elute were concentrated and desalted into 20 mM Tris, pH 7.4, and 150 mM NaCl (TBS) on Centricon 30 Protein Concentrators (Amicon, Beverly, MA). APP was monitored at each purification step by mAb 22C11 immunoblots. Total APP protein yields were determined by amino acid analysis and BCA assay.

**Absorption of APP by MacroPrep High Q Resin**—MacroPrep high Q column effluent was titrated against corresponding starting material (crude brain subcellular fractions) on mAb 22C11 Western blots. Crude starting material and MacroPrep high Q column effluent is unsuited to analysis by direct immunoblotting on our Western blotting system because of severe band distortion and poor resolution on SDS-PAGE caused by the relatively large sample volumes and high protein loading required for a detectable APP signal. Therefore, all samples were concentrated by immunoprecipitation with pAb R45 (raised against the same epitope as mAb 22C11) before electrophoresis. PAb R45 immunoprecipitates prepared from increasing volumes of MacroPrep high Q effluent (500–1500 µl in 100-µl increments) and 75 µl of corresponding starting material were compared on mAb 22C11 Western blots by reflectance analysis. Purification efficiency was calculated from the volume of column effluent observed to give an equivalent signal (sum of all bands) to the sample of starting material. Calculations included an adjustment for the change in sample volume during chromatography. Samples were also analyzed by mAb 22C11 immunoblots. Since APP signal in chromatographic eluates is readily detected without prior concentration, these samples were not immunoprecipitated with pAb R45 before electrophoresis. The ratio of 100–110- to 120–130-kDa bands were determined for the selected lanes. The ratio of the upper band density to lower band density (density of the upper band/density of the lower band) was then calculated for starting material and corresponding column eluates.

**Recovery of APP in Chromatographic Eluates**—MacroPrep high Q and desalted phenyl-Sepharose eluates were also compared on mAb 22C11 immunoblots. Since APP signal in chromatographic eluates is readily detected without prior concentration, these samples were not immunoprecipitated with pAb R45 before electrophoresis. The ratio of 100–110- to 120–130-kDa immunoreactive material in the eluates was calculated as described previously for immunoprecipitates of crude brain subcellular fractions and MacroPrep high Q effluents.

**Trypsin Activity Assay**

Trypsin (30 ng) was incubated with or without APP in TBS (175 µl) for 20 min at 20 °C in 96-well microtiter plates. Trypsin activity was then determined in each well by the addition of 25 µl of the synthetic trypsin substrate Coumarin-4-Nitroanilide (chromozym TRY) (43) followed by incubation at 37 °C. Absorbance (415 nm) over 20 min. Under the conditions used, assay linearity was maintained for at least 40 min after the addition of substrate and no increase in absorbance was detected in the presence of APP alone.

**Immunoprecipitation**

Samples for immunoprecipitation were incubated overnight at 4 °C with pAb R45 or mAb 7H5 in TBS containing 1% bovine serum albumin and 0.1% Tween 20. R45-antigen complexes were precipitated by addition of protein A-Sepharose (10 µl of swollen beads). For precipitation of 7H5-antigen complexes, protein A-Sepharose was first precipitated with rabbit anti-mouse IgG to increase capture efficiency. The Sepharose beads were washed three times prior to release of immunoprecipitated material from the resin by addition of 100 µl of SDS-PAGE sample buffer.

**Electrophoresis and Immunoblotting**

Samples were electrophoresed on SDS-PAGE (10% acrylamide gels) and blotted to polyvinylidene difluoride membrane. Membranes were blocked with bovine serum albumin (3%) before incubation with mAb 22C11 (1:3000) or mAb 7H5 (1:1000) for 3 h at room temperature. Following a 2-h incubation at room temperature with anti-mouse IgG coupled to alkaline phosphatase (1:10000), blots were developed for 15 min with Fast Red (3 mg/ml) and naphthol (0.2 mg/ml) in 100 mM NaCl, 5 mM MgCl2, and 100 mM Tris-HCl at pH 8.0. Quantitation of APP on protein blots were determined by reflectance analysis as described by Bush et al. (51).
TABLE I

| Soluble fraction | Membrane fraction |
|------------------|-------------------|
| Control AD      | >92%              |
| Control AD      | >92%              |
| AD              | >91%              |
| AD              | >90%              |

* % of total APP captured from brain subcellular fractions by MacroPrep high Q column (first chromatographic step).

RESULTS

Purification of APP from Control and AD Brains

APP was purified to homogeneity from the soluble subcellular fraction and Triton X-100 membrane pellet extract of cerebral cortex isolated from AD (n = 10), normal (n = 7), and neurological control (three Huntington’s disease and two Parkinson’s disease cases) brains. Purity was confirmed by protein staining of SDS-PAGE gels using silver and by NH2-terminal sequencing. Purified APP samples contained one minor (80–90 kDa) and two major (100–110 and 120–130 kDa) bands on mAb 22C11 Western blots and silver-stained SDS-PAGE gels. Identity of each band was confirmed by amino-terminal sequencing.

Starting material, chromatographic fractions, and purified protein from AD and control brains were compared and monitored for selective loss or concentration of APP species during purification. APP-KPI levels in the test brains used in these experiments are typical for their respective clinical groups. Initial experiments compared crude brains subcellular fractions and corresponding column effluents from the first chromatographic step (MacroPrep high Q column). Under the conditions used, positive absorption of mAb 22C11 immunoreactive material by MacroPrep high Q resin is greater than 90% for both soluble and detergent extract fractions of control and AD brains (Table I). Addition of eluates back to the effluents restored APP signal to that of corresponding starting material (data not shown). Consistent with uniform absorption of APP forms by the MacroPrep high Q resin, immunoprecipitates of starting material and chromatographic effluents contain similar relative amounts of 100–110- and 120–130-kDa species (Table II). Eluates from the MacroPrep high Q column and the last purification step also have similar relative amounts of the two major APP bands (Table II). In addition, Western blot analysis at each chromatographic step found no evidence of proteolytic degradation of APP during purification. These results are concordant with close conservation of the relative amounts of different APP forms during purification of both control and AD subcellular fractions.

Data showing the average yield of cortex and purified APP is presented in Table III. AD brains yield significantly less cortex (p = 0.0011 by t test) per hemisphere than pooled controls. The yield of purified APP protein is also significantly less for AD brains (p = 0.0023).

Table III also shows the yield of APP for each clinical group after normalizing for the reduced mass of cortex recovered from AD brains (APP yield per g of cortex). Recovery of purified APP from AD brains remains significantly less than controls after normalizing for the different yields in cortex, although the difference is barely significant at the p = 0.05 level (p = 0.048).

The subcellular distribution of APP is similar for all clinical groups, with equivalent soluble and membrane extract fractions yielding similar amounts of purified material. These results are consistent with the widespread neuronal loss associated with AD.

KPI-containing Isoforms in Purified APP

Two methods were used to determine the amount of KPI isoforms in the purified APP samples. The first assay exploits the inhibitory action of KPI isoforms on trypsin. The second KPI isoform assay employs immunoblotting techniques and anti-NH2-terminal (mAb 22C11) and -KPI domain (mAb 7H5) antibodies.

Trypsin Inhibition Assay—Trypsin was preincubated for 20 min with purified APP and then assayed for activity using the synthetic trypsin substrate Chromozym TRY. Initial experiments confirmed that trypsin activity decreased linearly with increasing concentrations of both soluble and membrane-associated APP (Fig. 1). The purified APP samples from normal, AD, and neurological disease control brains were then compared for trypsin inhibition. The proportion of KPI isoforms in each sample was calculated from the inhibition of trypsin (30 mg) after preincubation with 50 mg of purified protein (Table IV). The calculations assume that trypsin and KPI forms of APP interact with a 1:1 stoichiometry (52, 53), that total binding approaches 100% and that the average molecular mass of APP is 80 kDa and trypsin 24 kDa (based on the amino acid sequences of these proteins). Soluble and membrane-associated APP purified from control brain (pooled) have significantly different levels (p < 0.0001) of KPI isoforms. Membrane-associated material is predominantly KPI+, while most APP purified from the soluble fraction is KPI+. Both normal and neurological disease control groups display similar subcellular distributions of KPI isoforms. However, for AD brains, soluble and membrane-associated APP contain similar levels of KPI material. Furthermore, KPI isoforms in APP from the soluble subcellular fraction of AD brains are significantly elevated (p = 0.0014) compared with corresponding material from pooled controls. The level of KPI isoforms in membrane-associated APP from AD-affected patients is not significantly different from control subjects.

Immunoblot Assay—Western blots of the purified APP samples were probed with anti-NH2-terminal (mAb 22C11) or -KPI domain (mAb 7H5) antibodies and the percentage of KPI isoforms determined from the relative staining intensity of each immunoreactive band. Fig. 2 shows a typical Western blot of APP from human brain subcellular fractions and culture media from cells transfected with APP695 or APP751. Initial experiments determined the difference between mAb 7H5 and mAb 22C11 immunoreactivity per unit protein for KPI forms of human brain APP. Purified membrane-associated APP (triplicate samples) was immunoprecipitated with mAb 7H5. Increasing amounts of precipitated material were electrophoresed on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and the blots probed with mAb 22C11 or mAb 7H5. Relative band densities were determined by reflectance analysis of the blots. Immunoreactivity increased linearly with APP concentration for both antibodies. However, mAb 22C11 was found to react with the immunoprecipitate with only 66 ± 7% of the intensity of mAb 7H5 under the conditions used.

The purified APP samples were assayed for KPI isoforms in duplicate (40 ng of protein/lane). Only the 100–110- and 120–130-kDa species were analyzed. An initial analysis indicated that there are no differences between the levels of the minor species.
Recovery of purified APP from AD and control brain

APP was purified from the soluble and membrane extract fractions of cerebral cortex isolated from AD, non-demented, and neurological disease (three Huntington’s chorea and two Parkinson’s disease cases) control patients. Purified APP was judged to be homogeneous by silver staining of SDS-PAGE gels and was assayed for total protein by BCA and amino acid analysis. Data collected during the purification procedure is summarized in the table. Values are averages for each clinical group ± S.E. The pooled control group includes both normal and neurological disease patients.

Weights of cerebral cortex isolated and total APP yields are per cerebral hemisphere. APP protein yields for soluble and membrane extract fractions in the table. Values are averages for each clinical group ± S.E. The pooled control group includes both normal and neurological disease patients. Weights of cerebral cortex isolated and total APP yields are per cerebral hemisphere. APP protein yields for soluble and membrane extract fractions are normalized for the mass of cerebral cortex (wet weight). Comparisons between groups use a t test (two-tailed, heteroscedastic) significance level of p < 0.01.

### Table II

| Chromatographic sample                        | Ratio of major bands (upper band/lower band) |
|-----------------------------------------------|---------------------------------------------|
| Immonoprecipitated ppt of starting material   | Control brain                              |
| Immunoprecipitated ppt of eluate from first step | Membrane                                  |
| Eluate from MacroPrep high Q (first step)     | 0.49 ± 0.07                                 |
| Eluate from phenyl-Superose (last step)       | 0.44 ± 0.04                                 |
| Eluate from MacroPrep high Q (first step)     | 0.49 ± 0.07                                 |
| Eluate from phenyl-Superose (last step)       | 0.44 ± 0.04                                 |

### Table III

Yields of cerebral cortex and purified APP protein from control and AD brains

APP was purified from the soluble and membrane extract fractions of cerebral cortex isolated from AD, non-demented, and neurological disease (three Huntington’s chorea and two Parkinson’s disease cases) control patients. Purified APP was judged to be homogeneous by silver staining of SDS-PAGE gels and was assayed for total protein by BCA and amino acid analysis. Data collected during the purification procedure is summarized in the table. Values are averages for each clinical group ± S.E. The pooled control group includes both normal and neurological disease patients. Weights of cerebral cortex isolated and total APP yields are per cerebral hemisphere. APP protein yields for soluble and membrane extract fractions are normalized for the mass of cerebral cortex (wet weight). Comparisons between groups use a t test (two-tailed, heteroscedastic) significance level of p < 0.01.

### Table IV

Trypsin inhibition by purified APP from AD and control brains

APP was purified from the soluble and membrane extract fractions of normal (n = 7), diseased control (three Huntington’s chorea and two Parkinson’s disease cases), and AD (n = 10) brains. Trypsin (30 ng) and APP (50 ng) were preincubated before the addition of a synthetic trypsin substrate (Chromozym TRY). Cleavage of Chromozym TRY was determined by monitoring absorbance at 415 nm. The percentage of KPI-containing species in each sample was calculated assuming a 1:1 binding stoichiometry between trypsin and APP and a molecular mass of 80 kDa for APP and 24 kDa for trypsin. All assays were performed in triplicate, and average values are expressed ± S.E. Comparisons between groups use a t test (two-tailed, heteroscedastic) significance level of p < 0.01.

### Discussion

Purified APP from the soluble subcellular fraction of Alzheimer’s disease brains show a significant elevation in the proportion of KPI-containing isoforms compared with equivalent material from control subjects (Tables IV and V). Two independent
Immunoreactivity of purified APP to antibodies directed at the KPI-domain. Purified human brain APP from soluble (Soluble) or membrane extract (Membrane) fractions and MacroPrep high Q eluates of media from cells transfected with APP751 (APP-APP) or APP751 (APP-APP) were electrophoresed and transferred to polyvinylidene difluoride membrane. The membrane was probed with monoclonal antibodies directed against the NH2 terminus (22C11) or KPI-domain (7H5) of APP.

Electrophoresis on SDS-PAGE resolves purified human brain APP into three bands with apparent molecular masses of 80–90, 100–110 and 120–130 kDa (Fig. 2). The 80–90-kDa band is a minor band (<11% of total APP) and is not immunoreactive to mAb 7H5. We have previously reported that in human brain the relative intensity of the 80–90-kDa band increases with post-mortem time and that these species are probably proteolytic degradation products of the higher molecular mass APP forms (50). The 120–130-kDa band is strongly immunoreactive to mAb 7H5, and co-migrates with immunoreactive material from cells transfected with APP751. This material probably contains mature APP751 and APP770 isoforms. The 100–110-kDa band contains most of the mAb 22C11 immunoreactive material in human brain. While electrophoretic migration of this band seems most consistent with identity as APP695, a portion of this material is also immunoreactive to mAb 7H5. The identity of the 100–110-kDa KPI+ species is unclear. The apparent molecular mass of APP on SDS-PAGE has been observed to change with maturation (10) and the 100–110-kDa mAb 7H5 immunoreactive material may be immature KPI+ species. The 100–110-kDa band may also contain mature KPI+ species that have been NH2 terminally truncated or have modified groups attached to their polypeptide backbone that change the electrophoretic mobility of the protein. Immunoreactivity of the 100–110-kDa form of human brain APP to anti-KPI antibodies has also been reported by other investigators (54).

The possibility exists that the difference in KPI+ isomeric levels between subcellular fractions and between AD and control patients is an artifact of APP purification. To address this potential source of error, chromatographic samples from the purification of control and AD subcellular fractions were closely monitored by immunoprecipitation and Western blotting for selective loss or concentration of APP forms. If particular, APP species are selectively excluded or concentrated during chromatography then the intensity of upper 120–130 kDa forms relative to the lower 100–110 kDa material could be expected to change during purification. In initial experiments, starting material and column effluent from the first chromatographic step were compared. Since >90% of mAb 22C11 immunoreactive material in subcellular fractions loaded onto the MacroPrep high Q column is retained by the resin (Table I), the band ratio in chromatographic effluents from this purification step are especially sensitive to selective absorption or exclusion of particular APP species. Analysis indicates that the relative amounts of the different APP bands are closely conserved between starting material and effluents for both AD and control samples (Table II). Similarly, comparison of chromatographic eluates (first and last steps) show the change in the relative amounts of 100–110- to 120–130-kDa forms of APP during purification is insufficient to explain the large differences in APP-KPI+ levels observed between purified AD and control material. Data presented in Table III (APP recovery for AD and control brains) is also consistent with our purification procedure having no selective proclivity toward concentrating either KPI+ or KPI- isoforms. This conclusion is based upon the observation that yields of total APP are similar when comparing soluble and membrane extract fractions for both AD (~0.86 µg/g for both subcellular fractions) and control (~1.01 µg/g for both subcellular fractions) samples. If purification selectively concentrates APP-KPI+ in the AD-soluble subcellular fraction, then it could be expected that total APP yields for this subcellular fraction would increase relative to membrane-associated material. Conversely, if relative enrichment of KPI+ species is due to exclusion of KPI- forms of sAPP during purification of AD-soluble subcellular fractions, then a decrease in total yields of purified protein could be expected. Since the yield of purified sAPP was similar to that observed using membrane-associated material for both clinical groups, the data does not support selective enrichment of APP-KPI+ by either mechanism during purification. This data also indicates a nearly equal distribution of the total brain APP pool between soluble and full-length forms in both control and AD subjects. Finally, our laboratory routinely purifies APP695, APP751, or APP770 from the culture media of stably transfected cells using the same protocol as this

### Table V

| Subcellular fraction | % KPI+ APP; mAb 7H5/mAb 22C11 |
|----------------------|--------------------------------|
| Soluble              | Normal control: 41 ± 16 40 ± 12 41 ± 14\(^b\) 77 ± 32\(^a\) |
|                     | Diseased control: 73 ± 34 71 ± 32 72 ± 33\(^a\) 65 ± 21 |
| Membrane             | Normal control: 41 ± 16 40 ± 12 41 ± 14\(^b\) 77 ± 32\(^a\) |
|                     | Diseased control: 73 ± 34 71 ± 32 72 ± 33\(^a\) 65 ± 21 |

\(^a\) Pooled control soluble fraction versus pooled control membrane fraction; \(p = 0.0053\).

\(^b\) Pooled control soluble fraction versus AD-soluble fraction; \(p = 0.0051\).
study and all three isoforms purify with similar efficiencies (data not shown). Based on these results, it seems unlikely that our findings of elevated APP-KPI in AD brains is due to selective enrichment of these isoforms during purification.

The increase in the proportion of KPI isoforms in AD brains that we observe could reflect a shift in a major cell population that is synthesizing APP. For example, the increase in KPI APP protein may be due to loss of neurons and/or increases in glia or fibroblast cell types. However, our results are consistent with a predominant neuronal origin for the purified APP. As would be the case if most of the APP were neuronally derived, neuron-depleted AD brains yield less APP per gram of cortex than controls (Table III). In addition, in situ hybridization (32, 33, 55, 57–70) and immunohistochemical (71–74) studies support a predominantly neuronal origin of APP in both normal and AD brains. If these findings continue to be confirmed, then the differences we observed between AD and control brains could indeed reflect changes in neuronal expression or turnover of APP.

The cause of the increase in the APP-KPI/APP-KPI ratio is, as yet, unclear. The increase may be the result of a change in mRNA levels or increased catabolism of KPI isoforms and/or a decreased catabolism of KPI species. A number of studies have compared APP transcript levels in normal and AD brains. However, findings to date have been contradictory with data reporting a selective reduction in APP695 (8, 29–31), an increase in APP655 (65, 70), an increase in the ratio of APP720/ APP695 (25, 27, 34, 35) or APP737/APP695 (32, 36, 38), and no change in the relative message levels of different isoforms (57, 58, 75–80). Message levels for APP511 and APP720 are also reported to be increased in fibroblasts carrying the FAD Swedish APP67071 mutation and in normal fibroblasts in response to stress (81). While a consensus on APP transcripts levels in AD patients has yet to be reached, studies using animal models have convincingly demonstrated that a selective induction of mRNA for KPI isoforms occurs in response to acute neuronal insults. Elevated levels of APP transcripts for KPI species have been observed in rat cerebral cortex following ischemia (42, 43) and neurotoxic damage (44) and in hippocampus following kainic acid induced seizures (45). In addition, the forebrain of behaviorally impaired aged rats have been reported to have elevated KPI levels (82). These previous findings are consistent with the elevation of KPI protein levels in AD affected brain tissue that we observed being mediated, at least in part, by changes in APP message levels. However, as a consequence of the progressive neurodegeneration of AD, other changes such as selective shifts in APP protein catabolism are also possible.

A number of investigators have attempted to reproduce the lesions of AD in rodents by creating mice transgenic for human APP (see review by Higgins and Cordell (83)). Of the transgenic animals developed to date, the most effective at modeling β deposition is a mouse line that overexpress human APP containing a mutation linked to familial AD (APPV717F) (47). Investigation of mRNA levels in the brains of these mice show overexpression of human KPI transcripts and a reduction in endogenous KPI mouse APP message (38). APP-KPI levels may also be decreased in the Alzheimer’s disease patients we investigated since the increase in KPI isoforms in the soluble subcellular fraction of AD brains was not accompanied by higher levels of total sAPP protein (Table III). Transgenic mice lines expressing lower levels of human APP-KPI transcripts than the APPV717F mice have also been reported to develop age-related pathologic changes, including subtle increases in β deposition and tau staining (84, 85) as well as age-dependent deficits in spatial learning in water maze tasks and spontaneous alternation in a Y maze (46). These findings are consistent with an important, perhaps central role for KPI isoforms in Aβ amyloidosis. However, Aβ deposition has also been reported in the brains of transgenic mice overexpressing APP95 (86) and in the pancreas of a mouse line overexpressing the last 99 COOH terminus residues of APP (87). Thus, while increased levels of KPI isoforms may promote amyloidogenesis, these species may not be essential for Aβ generation.

It therefore remains unclear if our observation of increased KPI forms of APP protein in human brain occurs in response to the disease process or actually contributes to amyloidogenesis. If the increase in KPI sAPP does promote amyloidosis, the most likely mechanism would be by driving the catabolism of full-length APP toward pathways that generate Aβ. Recent findings suggest that endocytosis and subsequent degradation of KPI-containing species of soluble and full-length APP occurs via LRP (22, 23). Internalization via LRP is enhanced if APP is complexed to a cognate protease such as EGFBB (23, 24). Higher concentrations of soluble APP-KPI may reduce the pool of LRP and/or EGFBB-like proteases available for binding full-length KPI species of APP. Thus, increased competition by KPI sAPP may decrease LRP-mediated internalization and degradation of membrane-associated APP and divert these full-length molecules to more amyloidogenic catabolic routes such as the coated pit-mediated endocytic pathway (21). Alternatively, elevated levels of KPI sAPP forms may increase the production of Aβ by inhibiting proteases that normally cleave within the Aβ domain (8). The α-secretase is a possible candidate for such inhibition, although other proteases not directly involved in cleavage of the Aβ region of APP may also be affected.

In summary, our results show that the brains of AD patients have a significantly higher proportion of soluble KPI species of APP than those of unaffected individuals. Furthermore, KPI species of APP are more abundant in human brain than expected from studies of the relative levels of APP transcripts. An understanding of the contribution of the increase in KPI species in AD brains to pathologic lesions is as yet unclear and awaits elucidation of the mechanisms that lead to Aβ production.

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