Novel Amyloid Precursor Protein Gene Mutation (Codon 665^{Asp}) in a Patient with Late-Onset Alzheimer's Disease

M. L. Peacock, BS,* D. L. Murman, MD,* A. A. F. Sima, MD, PhD,†‡ J. T. Warren, Jr, PhD,*
A. D. Roses, MD,§ and J. K. Fink, MD*

Amyloid plaques in Alzheimer's disease contain β-amyloid, encoded by portions of exons 16 and 17 of the amyloid precursor protein. The specific association of rare amyloid precursor protein mutations with some kindreds with early-onset familial Alzheimer's disease suggests that specific abnormalities in amyloid precursor protein may contribute to the pathogenesis of Alzheimer's disease. Until now, there has been no evidence suggesting that amyloid precursor protein mutations could be involved in late-onset or sporadic Alzheimer's disease. We used reverse transcription–polymerase chain reaction, denaturing gradient gel electrophoresis, and direct DNA sequencing to analyze amyloid precursor protein exons 16 and 17 from postmortem cerebellar samples from patients with histologically confirmed Alzheimer's disease and control subjects. We found a novel point mutation, substitution of cytosine for guanine, at nucleotide 2119 (amyloid precursor protein 770 messenger RNA transcript) in a patient with late-onset Alzheimer's disease. This substitution deletes a BglII site and substitutes aspartate for glutamine at codon 665. Denaturing gradient gel electrophoresis analysis showed that this mutation was absent in 40 control subjects and 127 dementia patients. Whether this mutation is a rare but normal variant or contributes to the development of Alzheimer's disease is not known. The BglII restriction fragment length polymorphism enables investigators to determine the frequency of this polymorphism in normal subjects and Alzheimer's disease patients.

Senile plaques in Alzheimer's disease (AD) contain a variety of substances including β-amyloid (βA4), apolipoprotein E, chymotrypsin, and immunoglobulins [1, 2], as recently reviewed [3]. There is strong evidence that βA4, a major plaque component, may be pathogenic in some kindreds in which early-onset AD is transmitted as an autosomal dominant disorder [3]. βA4 is a 4-kd fragment encoded by portions of exons 16 and 17 of the 110-kd amyloid precursor protein (APP) [4, 5]. Rare mutations in the APP coding sequence have been found in affected members of ethnically diverse kindreds with early-onset familial AD. Three of these mutations altered precisely the same codon (APP codon 717$^{Val \rightarrow Ile}$, 717$^{Val \rightarrow Phe}$, and 717$^{Val \rightarrow Gly}$) [6–9]. In addition, mutations of two adjacent codons (APP codon 670$^{Lys \rightarrow Asn}$, 671$^{Met \rightarrow Leu}$) segregated with early-onset familial probable AD [10, 11]. The disease-specific association of these rare APP mutations suggests that APP abnormalities may contribute to the pathogenesis of early-onset familial AD. In addition to rare APP mutations that segregate with AD, a missense mutation at codon 713$^{Ala \rightarrow Thr}$ and a silent mutation at codon 715 [12] were found in a 64-year-old patient who developed AD at age 59. These mutations did not segregate with AD in her family. Another mutation at codon 713$^{Ala \rightarrow Val}$ was found in a patient with schizophrenia and cognitive deficits [13]. Whether or not APP is an important etiological factor for the majority of AD patients, particularly those with late-onset disease, is not known.

Determining the extent of APP mutations in normal subjects and AD patients with analyzing the effects of these mutations on APP βA4 structure and processing will provide insight into the processes responsible for amyloid accumulation. We developed a denaturing gradient gel electrophoresis (DGGE) [14, 15] strategy that expedites discovery of APP mutations. Recently, we used this technique to find a mutation in APP codon 673^{Thr} (adjacent to one of the sites at which APP is cleaved to generate βA4), in a subject who did not
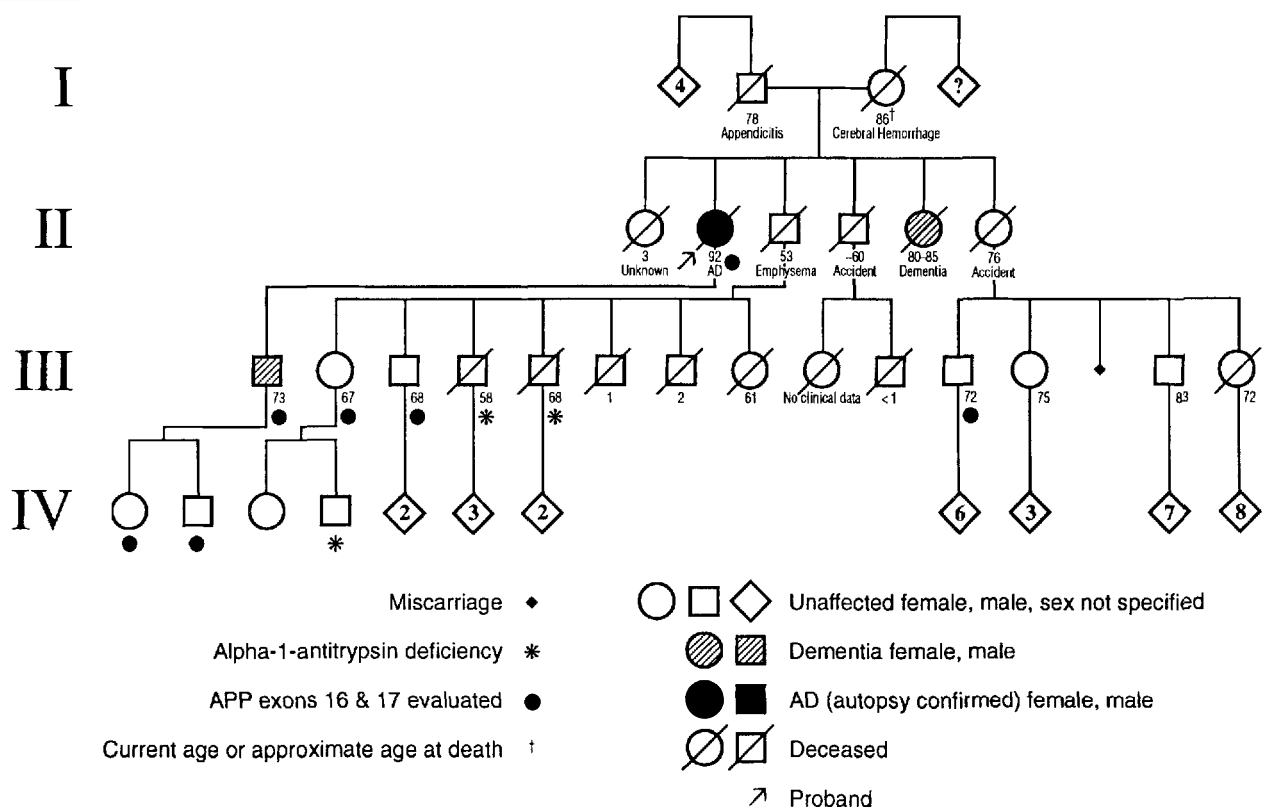
Fig 1. Pedigree.

have AD [16]. We now report the identification of an amino acid–substituting mutation in the APP coding sequence in a patient with late-onset AD [17].

Methods and Materials

Clinical Analysis

The proband (II.2, Fig 1) was ascertained by finding a novel polymorphism in the APP coding region through DGGE analysis of postmortem brain samples from 40 control subjects and 128 dementia patients including AD (84), AD and Parkinson's disease or Parkinson's disease with AD changes (16), Parkinson's disease and dementia (1), AD and cerebrovascular disease (4), AD and gliosis (3), AD and Lewy bodies (2), Pick's disease (1), AD and progressive supranuclear palsy (PSP) (2), dementia and PSP (4), dementia not otherwise classified (10), and diffuse subcortical gliosis (1). These samples were obtained from the Michigan Alzheimer's Disease Research Center and the Joseph and Kathleen Bryant Alzheimer's Disease Research Center at Duke University. Clinical information regarding the proband was obtained by reviewing medical records and from interviewing 2 relatives (III.11 and III.13; see Fig 1). Pedigree information was obtained from 3 relatives (III.2, III.11, and III.13; see Fig 1).

Neuropathological Analysis

At time of autopsy the brain was divided in half, the left hemisphere being fixed in 10% formalin and the right hemisphere immediately being frozen. From the formalin-fixed hemisphere, samples were taken from various cortical areas, hippocampus, basal ganglia, hypothalamus, thalamus, midbrain, and the cerebellum. Formalin-embedded sections were stained with Luxol fast blue, cresyl violet and eosin. Bielschowsky's silver stain, and Congo red.

Molecular Genetic Methods

Total messenger RNA (mRNA) was extracted [18] from postmortem cerebellar samples from the proband, 127 other dementia patients, and 43 control subjects. APP complementary DNA (cDNA) encoding exons 16 and 17 was prepared by reverse transcription followed by the polymerase chain reaction (RT-PCR). Amplification products were denatured by heating to 95°C for 10 minutes, reannealed at room temperature, and then electrophoresed on a 6.5% polyacrylamide gel (acrylamide-bisacrylamide = 29:1) containing a vertical gradient of 36 to 79% denaturant (100% denaturant = 40% formamide/6 M urea). Direct DNA sequencing of RT-PCR–amplified APP cDNA was performed as previously described [19]. Restriction enzyme analysis was used to determine whether the proband's APP mutation was present in her available relatives (III.1–3, III.11, IV.1, and IV.2; see Fig 1). For these analyses, a fragment of APP cDNA was prepared from leukocyte and postmortem cerebellum mRNA by RT-PCR. The major APP cDNA fragment prepared from postmortem brain samples was 465 bp. Owing to alternative mRNA splicing (variable inclusion of APP exon 15 [20]), the APP cDNA fragment prepared from leukocyte mRNA showed relatively less of the exon 15–

containing 465-bp fragment and relatively more of a shorter (exon 15-minus) 411-bp fragment. Amplification products were digested with $BglII$ and then electrophoresed on a 1.8% agarose gel and visualized with ethidium bromide staining.

Results

Clinical Analysis

The proband (Subject II.2, see Fig 1) was a white female who lived alone following the death of her husband. She was in good health with the exception of arthritis affecting her hips, shoulders, elbows, and hands. She was able to manage her daily affairs until the age of 86. After age 86 she developed intermittent periods of confusion and required home nursing care (and a housekeeper) to help her with her activities of daily living. Periods of confusion and disorientation became more frequent and severe over the next 2 years. At age 88 she refused to eat and was hospitalized for dehydration and malnutrition. She was disoriented to place and time and had significant memory impairment. During hospitalization, her cognitive deficits did not change despite improvement in her medical condition. She was discharged to a nursing home where she required complete nursing care assistance with activities of daily living. She died 4 years later at age 92.

The proband's 73-year-old son (III.1, see Fig 1) was well until approximately age 50 when he developed acute left hemiparesis as a result of an ischemic stroke. He continued to practice dentistry after the stroke. Several years later he experienced a myocardial infarction and subsequently underwent coronary artery bypass grafting. Progressive memory and other cognitive impairment were first evident between ages 55 to 60. At age 61 he had an unexplained fall (and may have had a brief loss of consciousness) and was hospitalized. He was transferred to a nursing home because of confusion and need for supervision. Neurological examination at age 73 showed that he was oriented to the season, but not the year. He had mild memory impairment and had to rely on family members for details of past events. Proverb interpretation was concrete. Simple calculations were performed relatively well and there was no apraxia or language disturbance. Speech was fluent and without aphasia. Cranial nerve examination was normal with the exception of moderate hearing impairment. He had right hemiparesis and right-sided hyperreflexia.

The proband's sister (II.5) had progressive memory disturbance. The precise age of onset is not known with certainty. Cognitive impairment became progressively severe during the 7 to 8 years she was in a nursing home. Prior to her death at approximately age 85, she was unable to recognize family members. Significant tremor and gait disturbance were apparently absent.

Pedigree

Family history was significant for late-onset dementia affecting the proband, her son, and her sister (II.2, III.1, II.5; see Fig 1). In addition, family history was significant for α_1-antitrypsin deficiency and emphysema in several relatives (see Fig 1).

Neuropathology

The brain weighed 1,040 gm at the time of autopsy. It showed marked gyral atrophy of the frontal and temporal lobes. On sectioning of the formalin-fixed left hemisphere, there was marked atrophy of the centrum semiovale with dilation of the lateral ventricle. The hippocampus was small. Microscopic examination revealed neuronal loss of the pyramidal cell layer of the hippocampus and temporal and frontal cortices with diffuse astrogliosis. There was marked neuronal loss of the basal nucleus of Meynert. The midbrain showed moderate loss of pigmented neurons in the substantia nigra. Bielschowsky's silver-stained sections revealed high counts of neuritic plaques and neurofibrillar tangles in the hippocampus, entorhinal cortex, amygdala, superior temporal gyrus, and the insular cortex, confirming the diagnosis of AD. Congo red-stained sections revealed amyloid deposition in meningeal arteries of the occipital and parietal cortices. Only occasional intraparenchymal vessels displayed amyloid deposition.

Molecular Genetic Analysis

DGGE analysis of APP cDNA encoding exons 16 and 17 from the proband (Fig 2A, lane 7) revealed a single heteroduplex band and a single homoduplex band consistent with the presence of a single heterozygous point mutation (principles of DGGE analysis are reviewed in [15]). The fact that only a single homoduplex and a single heteroduplex band were evident (as opposed to two homoduplex and two heteroduplex bands) indicated that the polymorphism created only minor changes in the fragment's melting temperature and was most likely to be either a cytosine ↔ guanine or adenine ↔ thymidine replacement. Direct DNA sequencing of the amplified product identified the heterozygous substitution of cytosine for guanine at nucleotide 2119 (APP 770 transcript) (Fig 2B). This polymorphism predicts the substitution of aspartate for glutamate at codon 665 (Fig 3) and deletes a $BglII$ restriction enzyme site.

We used DGGE and restriction enzyme analysis to determine whether or not the proband's available relatives (see Fig 2C, samples A–G) had this mutation. The mutation was present in a nondemented relative older than 65. Figure 1 does not identify the subject with this mutation in order to preserve this person's anonymity within the family. Neither the proband's son (with mild, slowly progressive memory impair-
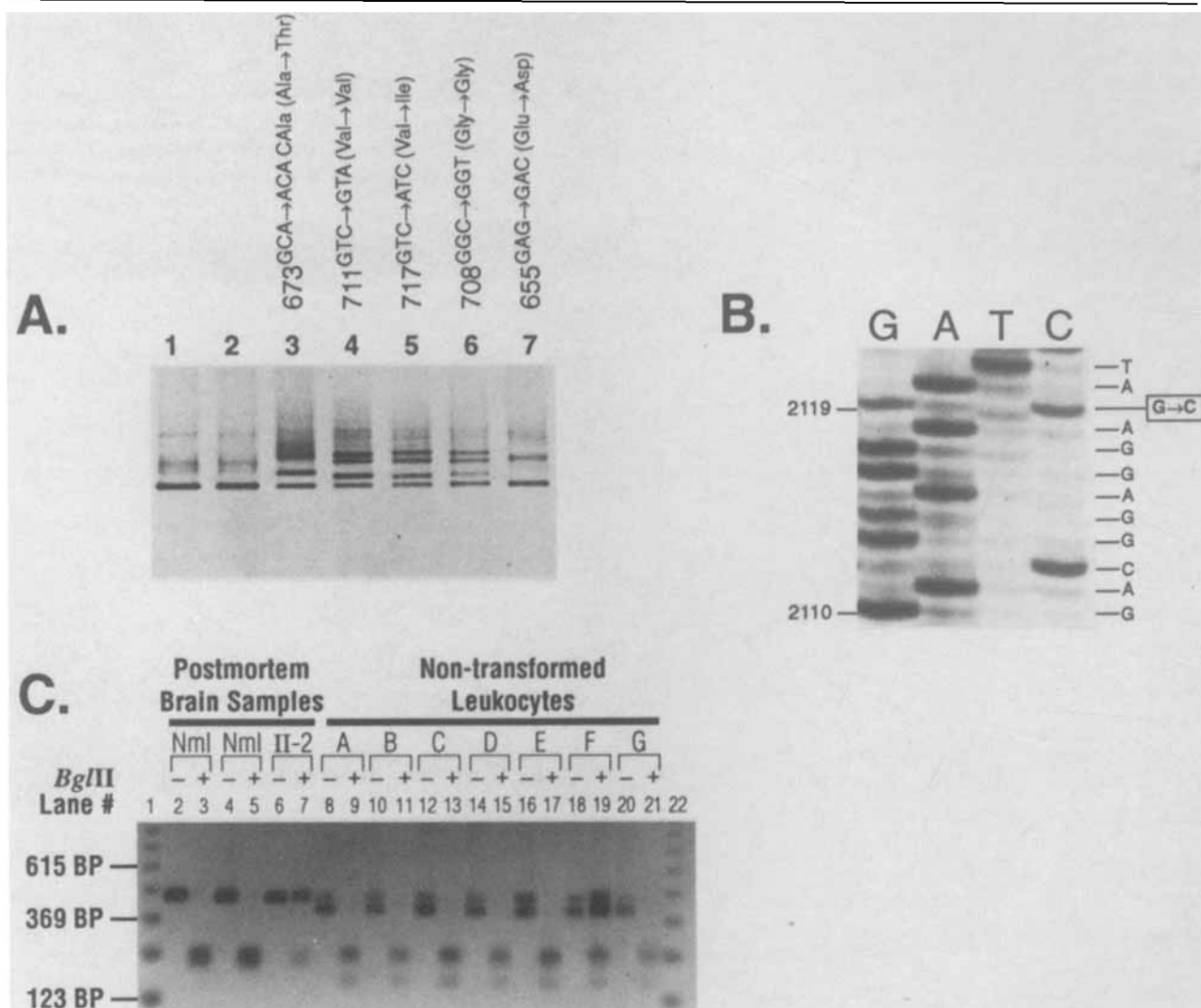
A.

673 GCA→ACA CAla (Ala→Thr)
711 GTC→GTA (Val→Val)
717 GTC→ATC (Val→Ile)
708 GGC→GGT (Gly→Gly)
655 GAG→GAC (Glu→Asp)

1 2 3 4 5 6 7

B.

G A T C

2119 —
—T
—A
G→C
—A
—G
—G
—A
—G
—G
—C
—A
2110 —
—G

C.

Postmortem Brain Samples   **Non-transformed Leukocytes**

Nml Nml II-2   A   B   C   D   E   F   G

*Bgl*II   − + − + − + − + − + − + − + − + − + − +
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

615 BP —
369 BP —
123 BP —

Fig 2. (A) *Denaturing gradient gel electrophoresis of amyloid precursor protein (APP) cDNA (exons 14–18) prepared from postmortem cerebellar samples from patients with Alzheimer's disease (AD). Lanes 1 and 2 display single homoduplex bands, indicating that each of these samples is homozygous throughout this region. Lanes 3 through 7 display homoduplex and heteroduplex bands, indicating that for each of these samples, this APP region is heterozygous at at least one position. Lanes 3 through 6 are from AD patients with known APP mutations at codons 673*[Ala→Thr] *(lane 3), 711*[Val→Val] *(lane 4), 717*[Val→Ile] *(lane 5), and 708*[Gly→Gly] *(lane 6). Lane 7 contains the sample from Subject II.2 (see Fig 1). (B) Direct DNA sequencing of reverse transcription–polymerase chain reaction (RT-PCR)– amplified exon 16 reveals heterozygous substitution of cytosine for guanine at nucleotide 2119 (APP 770 transcript). (C) Detecting APP codon 665*[Asp] *mutation by* Bgl II *restriction enzyme digestion. Total cellular mRNA was extracted from postmortem brain samples (lanes 2–7) and nontransformed leukocyte samples (lanes 8–21) and a portion of APP amplified by RT-PCR. Amplification products were analyzed on a 1.8% agarose gel prior to (−) and following (+) digestion with* Bgl II. *APP was amplified from postmortem brain samples from 2 normal subjects (*Nml*) (lanes 2–5) and the AD proband (Subject II.2 in Fig 1) (lanes 6 and 7) and from nontransformed leukocytes from 7 relatives (*A–G*) of the proband. Lanes 1 and 22 contain molecular weight markers (123-bp ladder).*
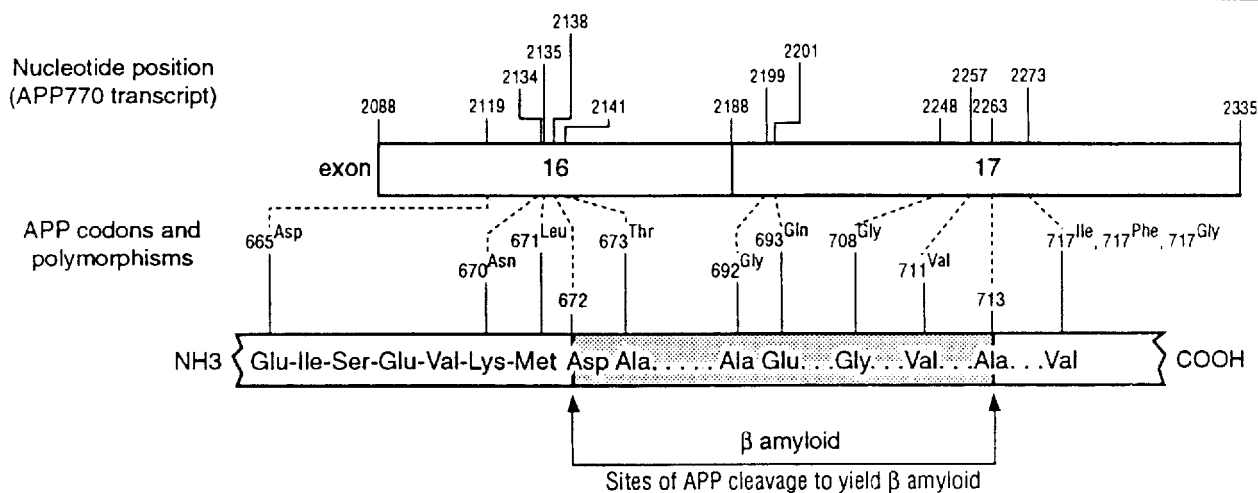
Fig 3. Location of polymorphisms in amyloid precursor protein (APP) exons 16 and 17 and sites of APP cleavage to produce β-amyloid deposited in Alzheimer's disease. Nucleotide positions were derived from the published APP 751 transcript (39) into which the sequence for exon 8 (57 bp) was inserted.

ment) nor 3 other unaffected relatives older than 65 had this mutation.

DGGE analysis showed that this mutation was absent in samples from 127 unrelated AD patients and 43 control subjects.

Discussion

We used DGGE and direct DNA sequencing to identify an amino acid–substituting mutation in the APP gene in a patient with late-onset AD. These mutation scanning techniques are extremely sensitive. Previously [16] we used these techniques to identify a nonconserved APP mutation at codon $673^{Ala \rightarrow Thr}$, as well as conserved mutations at codons $708^{Gly \rightarrow Gly}$ and codon $711^{Val \rightarrow Val}$ (see Fig 2A). In addition to their sensitivity, the extreme efficiency of these techniques permits rapid analysis of many samples from patients and control subjects.

It is difficult to determine whether or not a novel APP mutation is a benign variant or contributes to the development of AD. The functional consequences of APP mutations are difficult to assess since APP's functions are not well understood. APP may be involved in cell-to-cell and cell-to-matrix interactions [21–24], regulation of neuritic branching [25], growth, cell division [26], lymphocyte activation, and signal transduction through G-proteins [27], and may have an autocrinelike function on the regulation of cell growth [28]. In addition, APP may play a significant role in hemostasis. APP (in the form of protease nexin-II [29, 30]) released from platelets is a potent inhibitor of factor XIa [31]. Although any disturbance of these functions could be pathogenic, it is not clear which, if any, of

APP's putative functions are actually abnormal in AD patients. Currently, there are no in vivo or in vitro models in which the pathogenicity of APP mutations can be directly tested. The following criteria by which a mutation may be considered "possibly pathogenic" are difficult to apply for late-onset familial AD.

1. Pathogenic mutations should be associated specifically with AD and should be rare in unaffected subjects. In other words, pathogenic mutations should be present in unrelated AD patients and absent (or quite rare) in a large number of clinically and pathogenically normal subjects. Given the probably incomplete genetic penetrance and variable age at which late-onset familial AD begins, it is possible that a pathogenic mutation could be present in some subjects who are currently unaffected.

2. The mutation should segregate with the disorder if it is found in a subject for whom the disorder is familial. For reasons just mentioned, pathogenic mutations may be present in some currently asymptomatic members of a late-onset familial AD kindred. In addition, sporadic and familial forms of AD may coexist in the same kindred. The absence of an APP mutation in an affected member of a late-onset familial AD kindred could be taken as evidence of genetic recombination between the disease locus and the APP locus and suggest that the mutation was not etiologically significant.

3. The mutation should change some aspect of APP (or βA4) mRNA (including transcriptional rate, splicing, or stability) or protein (including function or stability). As noted above, APP has diverse effects in vitro. The consequences of mutations on these parameters can be tested directly. For example, the double mutation at codons $670^{Lys \rightarrow Asn}$ and $671^{Met \rightarrow Leu}$ has been shown to cause increased βA4 production in transfected cells [32, 33]. AD patho-

genesis in these patients could be related to excess synthesis or secretion of βA4. Other mutations could be functionally relevant if they occurred in the Kunitz protease inhibitor domain or at the sites at which APP is cleaved to yield βA4, or if they altered regional hydrophobicity in the membrane-spanning domain.

Nonetheless, mutations could be pathogenic even if they occurred in a domain of unknown functional significance. For example, APP codon 717 mutations that are present only in early-onset familial AD patients and that segregate with the disorder in these kindreds do not occur in a domain of known functional significance.

How does the novel APP codon 665^{Asp} fare according to these criteria? This mutation was found in an elderly subject with late-onset dementia who fulfilled the neuropathological criteria of AD. It cannot be concluded that the mutation is necessarily benign because it was absent in her son who had an ischemic stroke and who subsequently had insidiously progressive memory impairment. AD is genetically heterogeneous and undoubtedly of multifactorial etiology. The codon 665^{Asp} mutation could contribute to dementia in the proband. Her son could have sporadic senile dementia of the Alzheimer's type from another cause. In the context of his early-onset dementia (decades before disease onset in his mother) and his history of ischemic stroke, the possibility of multiinfarct dementia must be considered. The presence of this mutation in the proband's nondemented relative (older than 65) does not indicate that the mutation is benign. Although this mutation replaces a neutral amino acid (glutamine) with a charged amino acid (aspartate), it is not known whether this causes any functional disturbance. At this point, we conclude that this novel APP mutation, although found in an elderly AD patient, is of uncertain clinical significance. The restriction fragment length polymorphism (deletion of a *Bgl*II site) created by this mutation will help investigators determine its frequency in normal subjects and AD patients.

Identifying APP as a major component of amyloid plaques in AD and finding rare APP mutations in patients with early-onset familial AD have greatly advanced AD research. It is extremely important to determine the extent of APP polymorphisms in normal subjects and AD patients. Mutations that are uniquely present in similarly affected patients and that segregate with the disorder in familial AD kindreds may be useful for confirmation of diagnosis and genetic counseling. It is important to analyze the effects of each mutation not only on such parameters as APP mRNA processing, protein turnover, posttranslational modification, secretion, and each of APP's known in vitro effects but also on the consequences of these mutations on APP's and (βA4's) interaction with with factors that

could contribute to AD. For example, apolipoprotein E, the major apolipoprotein in the brain [34], was shown recently to bind to βA4 in vitro and to be deposited with βA4 in plaques in AD [1, 2, 35]. In addition to binding to βA4, there is intriguing genetic evidence that apolipoprotein E is associated with AD [36–38]. It will be important to determine systematically whether novel APP mutations affect the binding of APP to apolipoprotein E, fibronectin, and other factors with which it interacts. Such analyses will provide valuable insight into the mechanisms responsible for βA4 deposition and the role of amyloid plaques in the molecular cascade that ultimately leads to neuronal dysfunction and degeneration in AD.

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