# Release of Excess Amyloid β Protein from a Mutant Amyloid β Protein Precursor

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**The 4-kilodalton amyloid β protein (Aβ), which forms fibrillar deposits in Alzheimer's disease (AD), is derived from a large protein referred to as the amyloid β protein precursor (βAPP). Human neuroblastoma (M17) cells transfected with constructs expressing wild-type βAPP or a mutant, $βAPP_{ΔNL}$, recently linked to familial AD were compared. After continuous metabolic labeling for 8 hours, cells expressing $βAPP_{ΔNL}$ had five times more of an Aβ-bearing, carboxyl terminal, βAPP derivative than cells expressing wild-type βAPP and they released six times more Aβ into the medium. Thus this mutant βAPP may cause AD because its processing is altered in a way that releases increased amounts of Aβ.**

One of the hallmarks of Alzheimer's disease (AD), is the deposition of amyloid in senile plaques (*1, 2*) and in the walls of cerebral blood vessels (*3*). AD amyloid is composed of a 39- to 43-residue amyloid β protein (Aβ) (*4–8*) that is derived from a set of precursor proteins 695 to 770 amino acids long (*7, 9–11*) collectively referred to as the amyloid β protein precursor (βAPP). In rare families, AD is inherited as an autosomal dominant trait. Strong evidence that amyloid deposition is critically important in the development of AD has come from the identification of familial AD (FAD) kindreds in which the AD phenotype cosegregates with mutations in the βAPP gene (Fig. 1). Three of the FAD-linked βAPP mutations convert the valine located three residues COOH to $Aβ_{43}$ ($val^{717}$ in $βAPP_{770}$), to isoleucine (ΔI) (*12*), phenylalanine (ΔF) (*13*), or glycine (ΔG) (*14*). A fourth double mutation (ΔNL) alters the lysine and methionine located, immediately amino to $Aβ_1$ ($lys^{670}$–$met^{671}$ in $βAPP_{770}$) to asparagine and leucine (*15*). The location of these mutations in close proximity to Aβ immediately suggests that they may cause AD by altering βAPP processing in a way that is amyloidogenic.

The βAPP is normally processed in a constitutive secretory pathway (*16*) in which cleavage occurs at $Aβ_{16}$ (*17*) to produce a large secreted derivative and an ~8.7-kD membrane-associated fragment, neither of which can produce amyloid because they do not contain the entire Aβ. Alternative processing, which occurs at least in part in the endosomal and lysosomal system, produces a complex set of COOH-terminal derivatives that includes potentially amyloidogenic forms with the entire Aβ at or near their $NH_2$-terminus (*18, 19*). Recently, we (*20*) and others (*21, 22*) have shown that, in addition to the large secreted derivative and the complex set of COOH-terminal derivatives, normal βAPP processing produces and releases a 4-kD Aβ that is essentially identical to the Aβ deposited as amyloid in AD. Aβ may be a βAPP fragment that is produced by lysosomal processing of Aβ-bearing COOH derivatives, and subsequently released, but the precise mechanism of Aβ production and release is currently uncertain.

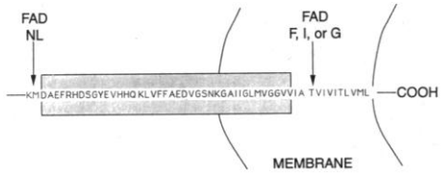


**Fig. 1.** Schematic diagram of the FAD-linked βAPP mutations. The box encloses $Aβ_{1-43}$. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

To evaluate production of Aβ and Aβ-bearing COOH-terminal derivatives in cells expressing the FAD-linked mutant βAPPs, we compared human neuroblastoma (M17) cells stably transfected with mutant or wild-type (WT) $βAPP_{695}$. In our initial experiment (Fig. 2), two ΔNL lines, a ΔI line, a WT line, and a line transfected with vector alone (CEP4β) were metabolically labeled with [$^{35}$S]methionine for 20 min to assess βAPP synthesis (*23*), and for 12 hours to analyze the COOH-terminal βAPP derivatives accumulating in cells and the Aβ released into the medium. After 12 hours of continuous labeling, the WT and ΔI lines were similar with respect to the COOH-terminal βAPP derivatives that accumulated and the Aβ that was released. In contrast, the 8- to 12-kD COOH-terminal derivatives accumulating in the two ΔNL lines were completely different (Fig. 2A, bracket) showing a marked increase in the relative amount of the 11.4-kD derivative (Fig. 2A, largest bracketed protein), a derivative that has Aβ at its $NH_2$-terminus. In addition, the medium conditioned by the two ΔNL lines contained, on average, 15-fold more 4-kD Aβ (Fig. 2B, arrow) than the medium conditioned by the WT and ΔI lines. After pulse labeling for 20 min, the two 695ΔNL lines contained five-fold more full-length βAPP than the WT and ΔI lines (Fig. 2A), but this increased expression did not account for the 15-fold increase in Aβ.

We then retransfected M17 cells to produce new, stably transfected 695WT, 695ΔI, and 695ΔNL lines. During pulse labeling for 20 min, the three new ΔI and

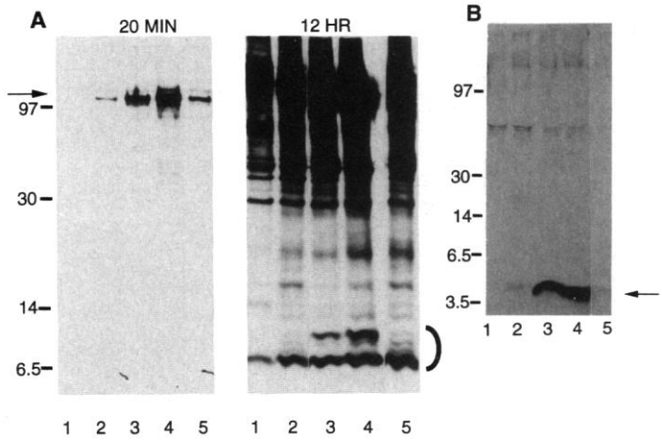


**Fig. 2.** Processing of FAD-linked mutant βAPPs. Human neuroblastoma (M17) cells stably transfected with vector alone (lanes 1), WT-1 (lanes 2), ΔNL-1 (lanes 3), ΔNL-2 (lanes 4) or ΔI-1 (lanes 5) were grown to confluence in wells 35 mm in diameter. Cells were labeled in 1 ml of medium containing 0.2 mCi of [$^{35}$S]methionine. Immunoprecipitates were separated by 10/16% tris-tricine SDS–polyacrylamide gel electrophoresis (PAGE). Assembly of the CEP4β-695ΔNL and CEP4β-695ΔI expression constructs was as described (*26*). All other methods were as described (*19, 20*). (**A**) Synthesis and accumulation of full-length βAPP and COOH-terminal βAPP derivatives. Lysates of cells labeled for 20 min or 12 hours were immunoprecipitated with an antiserum to the COOH-terminus of the βAPP. Arrow shows position of full-length βAPP; bracket shows COOH-terminal derivatives. (**B**) Release of Aβ. The conditioned media from cells labeled for 12 hours was immunoprecipitated with anti-$Aβ_{1-40}$ (SGY2134). Arrow shows position of Aβ.

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ΔNL lines accumulated comparable amounts of full-length βAPP, but the three new WT lines accumulated 3.3-fold more βAPP than the mutants indicating that expression was 3.3-fold higher (*23*) in these lines (Fig. 3, A and D). During 8 hours of continuous labeling, the three ΔI and WT lines showed similar accumulation of 8- to 12-kD COOH-terminal derivatives (Fig. 3B) and release of 4-kD Aβ (Fig. 3C) with reduced amounts produced by the ΔI lines as expected from their reduced expression of βAPP. In contrast, the 8- to 12-kD COOH-terminal derivatives produced by the ΔNL lines (Fig. 3B) were considerably different, again showing the marked increase in the 11.4- as compared to the 8.7-kD derivative observed in our initial experiment (Fig. 2A). Despite βAPP expression less than one-third that of the WT lines, the ΔNL lines accumulated considerably more of the Aβ-bearing, COOH-terminal derivative of 11.4 kD after 8 hours of continuous labeling (Fig. 3B), and medium conditioned with the ΔNL lines contained considerably more 4-kD Aβ (Fig. 3C). Quantitative analysis of these results with phosphorimaging technology (Fig. 3D) confirmed the striking differences observed in the autoradiograms. In cells pulse-labeled for 8 hours, the ratio of the 11.4- to 8.7-kD cell derivatives was more than five times higher in the ΔNL than in the WT or ΔI lines, and, when the amount of Aβ in medium was normalized to the full-length βAPP present after pulse labeling for 20 min, Aβ was more than six times higher in the ΔNL than in the ΔI or WT lines.

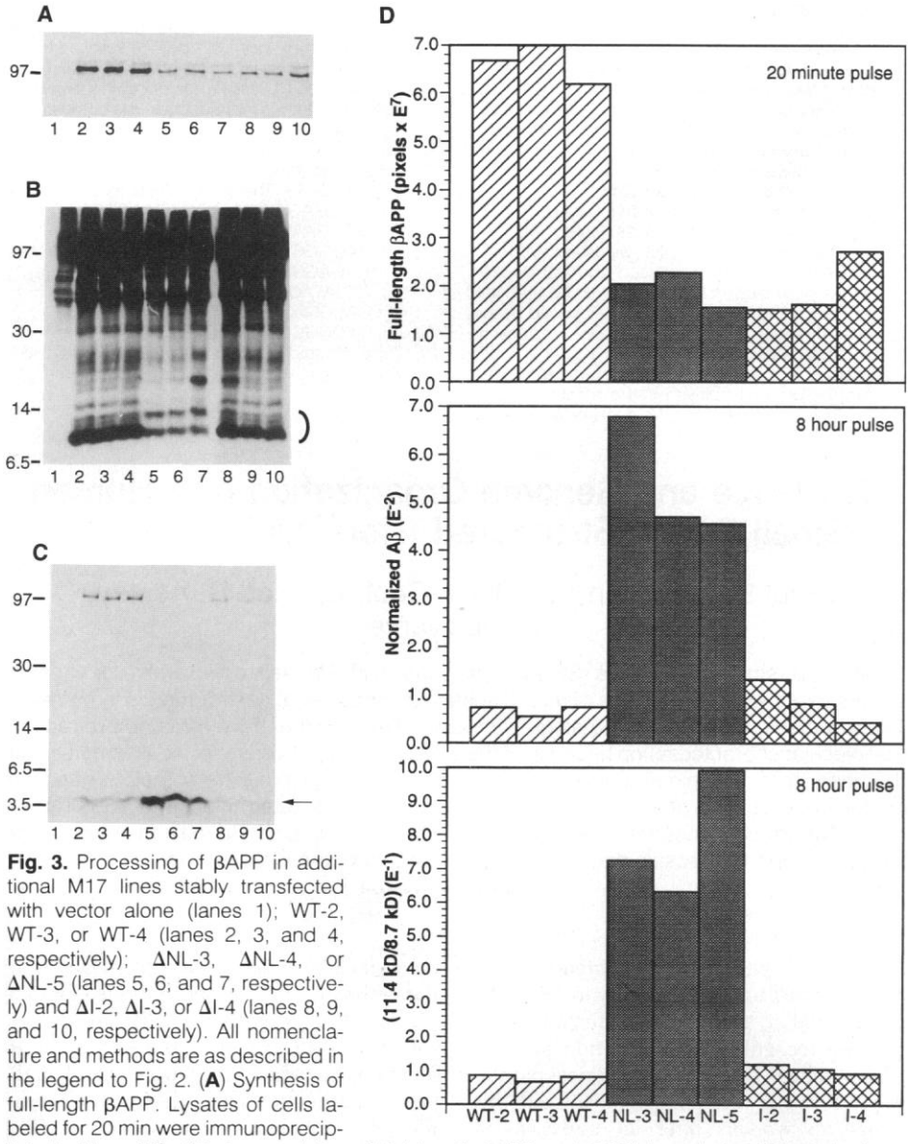


**Fig. 3.** Processing of βAPP in additional M17 lines stably transfected with vector alone (lanes 1); WT-2, WT-3, or WT-4 (lanes 2, 3, and 4, respectively); ΔNL-3, ΔNL-4, or ΔNL-5 (lanes 5, 6, and 7, respectively) and ΔI-2, ΔI-3, or ΔI-4 (lanes 8, 9, and 10, respectively). All nomenclature and methods are as described in the legend to Fig. 2. (**A**) Synthesis of full-length βAPP. Lysates of cells labeled for 20 min were immunoprecipitated with anti-C. (**B**) Accumulation of full-length βAPP and COOH-terminal βAPP derivatives. Lysates of cells labeled for 8 hours were immunoprecipitated with anti-C. Bracket indicates COOH-terminal derivatives. (**C**) Release of Aβ. The conditioned media from cells labeled for 8 hours was immunoprecipitated with anti-$Aβ_{1-40}$ (SGY2134). Arrow indicates Aβ. (**D**) Quantitation of changes in βAPP processing. Individual proteins were quantitated by phosphorimaging. Expression level is compared by showing the signal for total βAPP produced by each line during 20 min of pulse labeling. COOH-terminal derivatives accumulating in cells during 8 hours of labeling are compared by determining the ratio of the 11.4-kD Aβ-bearing derivative that begins at $Aβ_1$ (uppermost protein within the bracket in 3B) to the secretase-derived 8.7-kD βAPP derivative that begins at $Aβ_{17}$ (lowermost protein within the bracket in 3B). To normalize for differences in βAPP expression, the amount of Aβ in conditioned media after 8 hours of labeling for each sample was divided by the signal for total full-length βAPP accumulating during a 20-min pulse.

The observation that M17 cells expressing $βAPP_{ΔNL}$ show a marked increase in Aβ-bearing, COOH-terminal derivatives and release increased amounts of 4-kD Aβ provides strong evidence that $βAPP_{ΔNL}$ causes AD because its processing is altered in a way that releases increased amounts of Aβ, thereby promoting amyloid deposition. More generally, the linkage of this form of FAD to a $βAPP_{ΔNL}$ mutation now demonstrated to increase Aβ production greatly strengthens the hypothesis that amyloid deposition plays a central role in the development of all forms of AD.

If amyloid deposition is invariably pivotal in the development of AD, then one would also expect the $βAPP_{717}$ mutations (ΔI, ΔF, and ΔG) to alter βAPP processing in a way that is amyloidogenic. In an extensive series of experiments examining the turnover of full-length $βAPP_{ΔI}$ and the secretion of its large $NH_2$-terminal derivative, we have observed significant differences in the processing of $βAPP_{ΔI}$ as compared to $βAPP_{WT}$ (*24*). However, neither these data, nor the data presented here provide evidence that the processing of $βAPP_{ΔI}$ in M17 cells is altered in a way that would obviously promote amyloidogenesis. In fact, both our unpublished data and the data presented here (Fig. 3D) suggest that, if anything, M17 cells expressing $βAPP_{ΔI}$ produce less secreted 4-kD Aβ than those producing WT βAPP.

It is possible that the aberrant processing of $βAPP_{ΔI}$ that produces amyloid in the brain does not occur in transfected M17 cells. This would occur, for example, if the processing occurs only in certain cell types or develops in association with age-related alterations in βAPP processing. Alternatively, it could be that amyloidogenic processing is occurring in M17 cells but has so far gone undetected. Isolation of Aβ from AD amyloid has revealed COOH-terminal heterogeneity, with Aβ's ranging in size from 39 to 43 residues in length (*5–8*). Although some secreted 4-kD Aβ is 40 residues long (*22*), this Aβ may well show similar COOH-terminal heterogeneity.

Our data indicate that the ΔNL mutation increases Aβ production by augmenting cleavage at or very near the site of mutation on the amino side of Aβ. Thus this mutation likely increases all Aβ in a way that does not alter the specific site (or sites) of COOH-terminal cleavage. Although the $\beta APP_{717}$ mutations do not appear to increase overall Aβ production, these mutations on the COOH-side of Aβ may shift cleavage to favor generation of the longer Aβ's (42 to 43 residues long) that are specifically associated with senile plaque amyloid. As these longer Aβ's have biophysical properties that favor amyloid deposition (*25*), shifting the site of cleavage could result in amyloid deposition without increasing the overall amount of Aβ. Such a shift would not be detectable with our current gel system, and the longer forms might more readily form insoluble aggregates that would impair detection. Whatever the precise mechanism proves to be, it is likely, given the positive results obtained with the ΔNL mutation, that continued analysis of the $\beta APP_{717}$ mutations will ultimately reveal altered processing that is highly informative with regard to the mechanism through which these mutations produce AD.

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23. Each of our cell lines is polyclonal and was independently transfected using the episomal replicon CEP4β. Although polyclonal lines show less variation than monoclonal lines, it is nonetheless critically important to assess the level of expression (synthesis) of WT and mutant βAPPs before concluding that the processing of a mutant βAPP is altered in a way that increases Aβ release. Synthesis is best measured by quantitating the full-length βAPP that accumulates during a labeling interval sufficiently short that no appreciable degradation takes place. We labeled for 20 min, the shortest interval that permitted us to assess multiple lines in parallel in a way that was practical. Although some degradation may occur during this short interval, this method of assessing synthesis is nonetheless a reliable way to ensure that excess production of Aβ in lines expressing mutant βAPPs is due to altered processing and not to increased expression.
24. X.-D. Cai *et al.*, unpublished data.
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26. To assemble CEP4β-695ΔNL, we used a $\beta APP_{695}$ template and oligonucleotides 1 and 2 to amplify by the polymerase chain reaction (PCR) a cDNA extending from base 1762 to 2119 followed by an adaptor sequence containing an Xba I restriction site [numbering is according to Kang *et al.* (*7*)]. Oligonucleotide sequences are shown below. The forward primer employed in this reaction (oligonucleotide 1) altered the WT nucleotides at positions 1785 and 1786 from G to T and A to C, respectively, changing the encoded amino acids immediately amino to $A\beta_1$ from lysine to asparagine and methionine to leucine, respectively. The Bgl II–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of a $\beta APP_{695}$ cDNA (nucleotides −30 to 2119) in BSKS$^-$ (Stratagene). To assemble CEP4β-695ΔI, we used a $\beta APP_{695}$ template and oligonucleotides 3 and 2 to PCR amplify a cDNA extending from 1906 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The forward primer used in this reaction (oligonucleotide 3) altered the WT nucleotide at position 1924 from G to A, changing the encoded valine to isoleucine. This cDNA was gel-purified and used as a template along with a $\beta APP_{695}$ template and oligonucleotides 4 and 5 to produce a ΔI cDNA extending from base 1761 to 2119 followed by an adaptor sequence containing an Xba I restriction site. The Eco RI–Xba I fragment of this amplified cDNA was then used to replace the corresponding fragment of Bluescript $\beta APP_{695}$. Both the full-length 695ΔNL and 695ΔI sequences in Bluescript were then subcloned into Hind III–Not I sites of CEP4β. The oligonucleotides used were
(1) ACGGAGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCC
(2) GAGGGCCATGCCGGCCTCTAGAGTCCAACTTCAGAGGCTGCT
(3) GTCATAGCGACAGTGATCATCATCAC
(4) GACGGAGGAGATCTCTGAAGT, and
(5) GAGGGCCATGCCGGCCTCTAGA.


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# Sequence and Genome Organization of a Human Small Round-Structured (Norwalk-Like) Virus


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**Small round-structured viruses (SRSVs), also known as Norwalk or Norwalk-like viruses, are the major worldwide cause of acute, epidemic nonbacterial gastroenteritis in humans. These viruses, which contain a single-stranded RNA genome, have remained refractory to molecular characterization because of the small amounts of virus in clinical samples and the absence of an animal model and an in vitro culture system. The complete genomic nucleotide sequence of an SRSV, Southampton virus, was determined. The 7696-nucleotide RNA genome encodes three open reading frames whose sequence and organization strongly support proposals that SRSVs are members of the Caliciviridae.**


The term "epidemic winter vomiting disease" was first used by Zahorsky in 1929 (*1*) to describe a syndrome in humans now globally recognized as acute nonbacterial gastroenteritis (*2*). The prototypical pathogen, Norwalk virus, was identified by American workers after an outbreak of acute nonbacterial gastroenteritis in an elementary school in Norwalk, Ohio (*3*). Morphologically indistinguishable viruses have since been described in clinically similar outbreaks and have been termed Hawaii (*4*, *5*), Snow Mountain (*6*, *7*), and Taunton agents (*8*, *9*) after the geographical locations in which they were first found. Our electron microscopy (EM) studies, performed with human convalescent sera (*10*), of virus isolates collected from laboratory-confirmed outbreaks have established at least four serotypes in the United Kingdom and the United States. We designate the Norwalk and Hawaii viruses as serotypes 1 and 2, respectively, because of their historical precedence; the prototype strains for serotypes 3 and 4 are the Snow Mountain and Taunton viruses. Others also recognize at least four serotypes (*11*). Since February 1991, serotype 3 has been the most preva-


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