and bind DNA through the heterologous Gal4 peptide, the confounding effects of the stress-induced modification of the dimerization partners of CHOP is avoided. MMS treatment or overexpression of p38 markedly activated a reporter gene driven by Gal4 binding sites only when the CHOP-Gal4 chimeric protein was present (7.9 and 10 times as much, respectively); overexpression of SAPK-1β or ERK2 was without effect. The effect of MMS and p38 appeared to be dependent on CHOP phosphorylation because in cells expressing CHOP-Gal4 with an $Ala^{78,81}$ substitution, reporter gene expression was not enhanced by these stimuli (Fig. 3C).

Overexpression of CHOP leads to attenuated adipocytic differentiation of 3T3-L1 cells (6). This effect is dependent on the ability of the protein to dimerize and bind DNA. We compared the ability of wild-type and of $Ala^{78,81}$ CHOP to inhibit adipocytic differentiation in 3T3-L1 cells. Both proteins inhibited differentiation; however, cells expressing wild-type CHOP showed less lipid accumulation than cells expressing the $Ala^{78,81}$ substitution mutant (Fig. 3D). Thus, we conclude that Ser^{78} and Ser^{81} are necessary for the full biological activity of CHOP.

Under favorable conditions, CHOP is not present in cells. Stress leads to accumulation of CHOP in the nucleus. Our results indicate that stress also leads to phosphorylation of the protein and that this modification results in enhanced transcriptional activation by CHOP. The CHOP accumulation in response to stress is apparently dependent on the activity of cellular kinases because it can be inhibited by the broad-spectrum kinase inhibitors 2-aminopurine and H7 (*1*). However, the signaling pathway that activates CHOP phosphorylation is distinct from the one that leads to CHOP expression; an inhibitor of p38 does not block CHOP expression (Fig. 2B). Perhaps the potent ability of CHOP to modify cell growth and differentiation requires careful modulation beyond that provided by the mechanisms that control expression of the protein. The stress-activated p38 appears to serve a specific role in this fine-tuning of CHOP activity.

REFERENCES AND NOTES

1. A. J. Fornace *et al.*, *Mol. Cell. Biol.* **9**, 4196 (1989); S. G. Carlson *et al.*, *ibid.* **13**, 4736 (1993); J. D. Luethy and N. J. Holbrook, *Cancer Res.* **52**, 5 (1992); B. Price and S. Calderwood, *ibid.*, p. 3814; Q. Chen, K. Yu, N. J. Holbrook, J. L. Stevens, *J. Biol. Chem.* **267**, 8207 (1992); S. L. Sylvester, C. M. J. ap Rhys, J. D. Leuthy-Martindale, N. J. Holbrook, *ibid.* **269**, 20119 (1994).
2. D. Ron and J. F. Habener, *Genes Dev.* **6**, 439 (1992).
3. M. Ubeda *et al.*, *Mol. Cell. Biol.*, **16**, 1479 (1966).
4. M. V. Barone, A. Y. Crozat, A. Tabaee, L. Philipson, D. Ron, *Genes Dev.* **8**, 453 (1994).
5. H. Zinszner, R. Albalat, D. Ron, *ibid.*, p. 2513.
6. N. Batchvarova, X.-Z. Wang, D. Ron, *EMBO J.* **14**, 4654 (1995).
7. Treatment of cells with tunicamycin, culture in low concentrations of glucose, and culture in MMS have similar effects on CHOP. For reasons of convenience, MMS was used preferentially in subsequent experiments.
8. J. Kyriakis *et al.*, *Nature* **369**, 156 (1994).
9. R. Davis, *J. Biol. Chem.* **268**, 14553 (1993).
10. ———, *Trends Biochem. Sci.* **19**, 470 (1994).
11. D. Robbins *et al.*, *Adv. Cancer Res.* **63**, 93 (1994).
12. B. Dérijard *et al.*, *Cell* **76**, 1025 (1994).
13. J. Han, J.-D. Lee, L. Bibbs, R. Ulevitch, *Science* **265**, 808 (1994).
14. J. Rouse *et al.*, *Cell* **78**, 1027 (1994).
15. J. C. Lee *et al.*, *Nature* **372**, 739 (1994); A. Cuenda *et al.*, *FEBS Lett.* **364**, 229 (1995).
16. T. Nakajima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2207 (1993).
17. G. I. Evan, G. K. Lewis, G. Ramsay, J. M. Bishop, *Mol. Cell. Biol.* **5**, 3610 (1985).
18. Electrophoresis was at pH 8.9, and thin-layer chromatography was in isobutyric acid buffer, as described [W. Boyle, P. van der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991)].
19. Abbreviations for the amino acid residues are as follows: 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.
20. I. Sánchez *et al.*, *Nature* **372**, 794 (1994).
21. J.-H. Her *et al.*, *Biochem. J.* **296**, 25 (1993).
22. Wild-type and mutant CHOP were expressed in *Escherichia coli* as His^6-tagged fusion proteins and purified by affinity chromatography on a Ni^{2+} nitrilotriacetic acid–agarose column (Qiagen).
23. M. Hibi, A. Lin, T. Smeal, A. Minden, M. Karin, *Genes Dev.* **7**, 2135 (1993).
24. The DNA-binding domain of Gal4 (amino acids 3 through 147) was amplified by polymerase chain reaction and ligated in frame with wild-type or mutant murine CHOP complementary DNA (cDNA) at the unique Nhe I site of the mutant cDNA, thus deleting the CHOP dimerization domain. The chimeric cDNA was transferred to the pCDNA-Amp mammalian expression plasmid.
25. A. J. Muller *et al.*, *Mol. Cell. Biol.* **11**, 1785 (1991).
26. H. Green and O. Kehinde, *Cell* **1**, 113 (1974).
27. Lipid content was estimated by measuring the integrated intensity of the red signal in scanned images of the plates and is presented in arbitrary units (Fig. 3D). Shown is a typical result of an experiment performed in duplicate and reproduced three times. Equal expression of the wild-type and mutant proteins was observed by immunocytochemistry and immunoblotting (X.-Z. Wang, unpublished data).

28. Supported by U. S. Public Health Service award DK 47119 and by a grant from the Arthritis Foundation. D.R. is a Pew Scholar in the Biomedical Sciences. We thank E. Skolnik and M. Mohammadi (New York University) for advice and the following colleagues for reagents: J. C. Lee (Smithkline Beecham Pharmaceuticals), SB203580; R. J. Ulevitch (Scripps Clinic) and R. J. Davis (U. Mass, Worcester), p38 expression plasmid; L. I. Zon (Children's Hospital, Boston), SAPK-1β expression plasmid; D. J. Templeton (Case Western), activated MEK expression plasmid; and M. Karin (UCSD) and Y. Ben Neriah (Haddash, Jerusalem), GST-Jun expression plasmid.

28 December 1995; accepted 11 March 1996

G Protein–Mediated Neuronal DNA Fragmentation Induced by Familial Alzheimer's Disease–Associated Mutants of APP

Tomoki Yamatsuji,* Takashi Matsui, Takashi Okamoto,† Katsumi Komatsuzaki, Shizu Takeda,‡ Hiroaki Fukumoto, Takeshi Iwatsubo, Nobuhiro Suzuki, Asano Asami-Odaka, Scott Ireland, T. Bernard Kinane, Ugo Giambarella, Ikuo Nishimoto§

Missense mutations in the 695–amino acid form of the amyloid precursor protein (APP_{695}) cosegregate with disease phenotype in families with dominantly inherited Alzheimer's disease. These mutations convert valine at position 642 to isoleucine, phenylalanine, or glycine. Expression of these mutant proteins, but not of normal APP_{695}, was shown to induce nucleosomal DNA fragmentation in neuronal cells. Induction of DNA fragmentation required the cytoplasmic domain of the mutants and appeared to be mediated by heterotrimeric guanosine triphosphate–binding proteins (G proteins).

Alzheimer's disease (AD) is characterized pathologically by the presence of senile plaques and neurofibrillary tangles as well as extensive neuronal loss in the brain (*1*). Senile plaques are extracellular deposits whose major constituent, β-amyloid (Aβ), is cleaved from the transmembrane precursor APP (*2*). Alternative splicing of transcripts from a single gene results in at least 10 isoforms of APP (*3*), of which APP_{695} is preferentially expressed in neuronal tissues. V642I, V642F, and V642G—three missense mutations in which Val^{642} in the transmembrane domain of APP_{695} is replaced by Ile, Phe, or Gly, respectively—have been associated with dominantly inherited familial AD (FAD) (*4*). These mutations cosegregate with the AD phenotype (*5*) and account for most, if not all, of the evidence for linkage of AD to chromosome 21 (*4*). Overexpression of such APP mutants mimics the neuropathology of AD in transgenic mice (*6*).

Recent studies of mice in which the APP gene has been disrupted (*7*) have suggested an essential role for normal APP in neuronal development and function. To elucidate the function of APP_{695} and its mutants, we used F11 cells (*8*), hybrids of a primary rat dorsal root ganglion neuron and a mouse

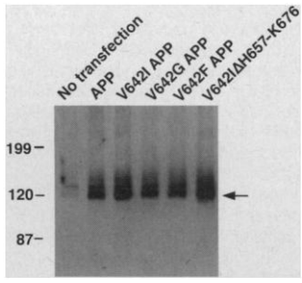

Fig. 1. Immunoblot analysis of the expression of APP_{695}, the three FAD APP mutants, and V642IΔH657–K676 in F11 neuronal cells. Thirty-six hours after transfection with the APP cDNAs, cells were lysed, and the lysate (20 μg of protein per lane) was subjected to SDS–polyacrylamide gel electrophoresis on a 7.5% gel and immunoblot analysis with antibody 22C11 to APP. Similar data were obtained when blots were probed with antibody AC-1 to the COOH-terminus of APP. The arrow indicates the expressed APP proteins. Molecular size standards are indicated in kilodaltons.

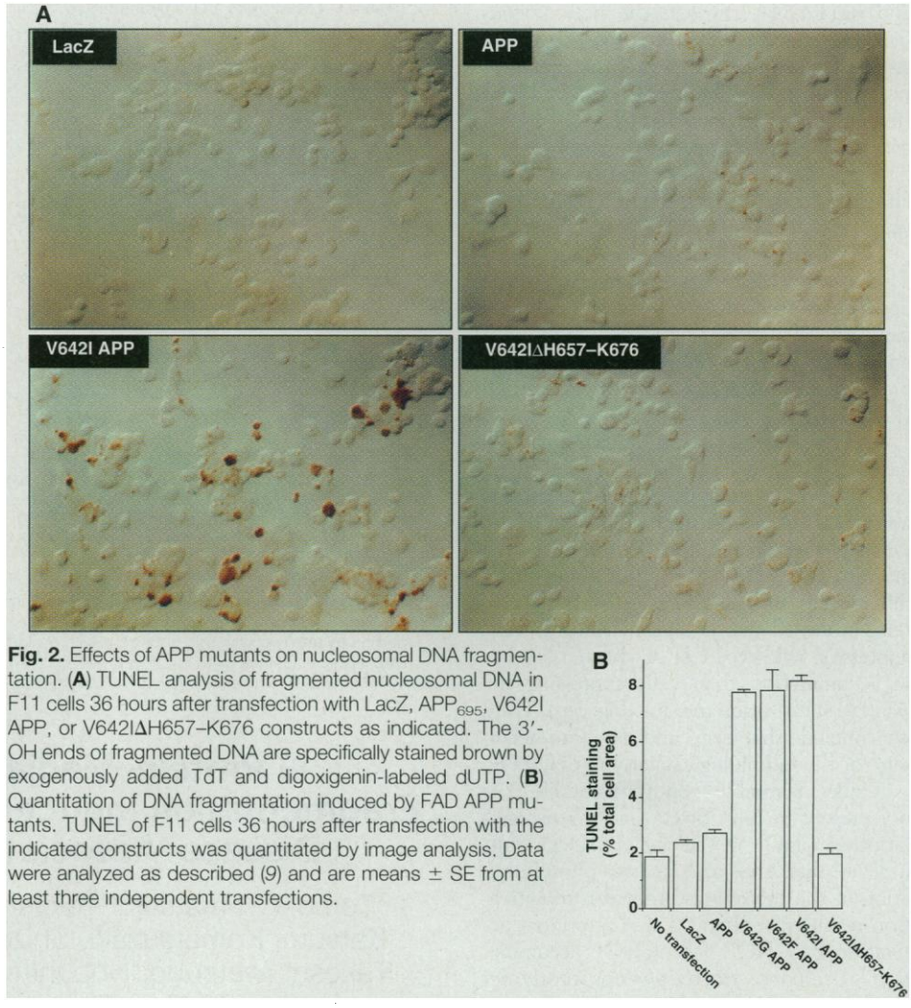

Fig. 2. Effects of APP mutants on nucleosomal DNA fragmentation. (**A**) TUNEL analysis of fragmented nucleosomal DNA in F11 cells 36 hours after transfection with LacZ, APP_{695}, V642I APP, or V642IΔH657–K676 constructs as indicated. The 3′-OH ends of fragmented DNA are specifically stained brown by exogenously added TdT and digoxigenin-labeled dUTP. (**B**) Quantitation of DNA fragmentation induced by FAD APP mutants. TUNEL of F11 cells 36 hours after transfection with the indicated constructs was quantitated by image analysis. Data were analyzed as described (*9*) and are means ± SE from at least three independent transfections.

neuroblastoma cell line N18TG2 that exhibit neuronal traits. Nucleosomal DNA fragmentation in these cells was directly assessed by the terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay (*9*). The extent of end-labeling was standardized by cell population and quantitated by sampling randomly chosen fields in an entire well with an image analysis system; data were collected from multiple independent transfections (*9*). Normal and mutant APP_{695} cDNAs (*10*) were transiently transfected into F11 cells (*11*), and expression was detected 12 to 48 hours after transfection (Fig. 1). The amount of expression was similar for normal APP_{695} and the FAD mutants. Transfection of F11 cells with *lacZ* cDNA induced little DNA fragmentation after 36 hours (Fig. 2). Under the same conditions, transfection with V642I, V642F, or V642G APP cDNA resulted in marked nucleosomal DNA fragmentation. Transfection with normal APP_{695} cDNA had virtually no effect on DNA fragmentation. Similarly, expression of V642IΔH657–K676, a V642I APP mutant lacking the 20 cytoplasmic residues (His^{657} to Lys^{676}), did not induce DNA fragmentation.

Although Aβ(1–42) is the major and earliest constituent of senile plaques in AD (*12*), fragmentation of DNA was not induced in F11 cells by 50 μM synthetic Aβ(1–42) (Fig. 3) or by 50 μM synthetic Aβ(1–40) (*13*), which has been shown to induce apoptosis in other cultured cells (*14*). Moreover, DNA fragmentation in untransfected F11 cells was not induced by the medium (CM/F11–V642I) conditioned by transfected F11 cells expressing V642I APP, which were themselves undergoing substantial DNA fragmentation. We also excluded the possibility that F11 cells expressing V642I APP secrete a factor that enhances the effect of Aβ(1–42), because the combination of 50 μM Aβ(1–42) and CM/F11–V642I also did not induce DNA fragmentation in untransfected F11 cells.

We next measured the concentrations of Aβ(1–40) and Aβ(1–42) in the conditioned medium of F11 cells transfected with each FAD APP mutant (Table 1) (*15*). As has been described (*16*, *17*), secretion of Aβ(1–40) from cells expressing the three FAD APP mutants was reduced relative to that from cells expressing normal APP_{695}, whereas secretion of Aβ(1–42) was increased. However, the concentrations of both Aβ peptides were in the picomolar range in the medium of F11 cells transfected with the mutant cDNAs. Given that 50 μM Aβ(1–42) failed to induce DNA fragmentation in the presence or absence of CM/F11–V642I, these data indicate that secreted Aβ(1–42) was not responsible for the DNA fragmentation induced by the FAD APP mutants. This conclusion is supported by our results with V642IΔ41/42 (*10*), a mutant V642I APP that lacks the 41st and 42nd residues of the Aβ region and hence encodes Aβ(1–40) but not Aβ(1–42). V642IΔ41/42 was expressed as efficiently as V642I APP in F11 cells (*13*) and also induced DNA fragmentation to a similar extent (Fig. 3).

T. Yamatsuji, T. Matsui, T. Okamoto, K. Komatsuzaki, S. Takeda, U. Giambarella, I. Nishimoto, Cardiovascular Research Center, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Charlestown, MA 02129, USA.
H. Fukumoto and T. Iwatsubo, Department of Neuropathology and Neuroscience, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.
N. Suzuki and A. Asami-Odaka, Takeda Chemical Industries Ltd., Tsukuba, Ibaraki 300-42, Japan.
S. Ireland, Oncor, Gaithersburg, MD 20877, USA.
T. B. Kinane, Renal Unit, Massachusetts General Hospital, Department of Pediatrics, Harvard Medical School, Charlestown, MA 02129, USA.

*Present address: First Department of Surgery, Okayama University School of Medicine, Shikata-cho, Okayama, Okayama 700, Japan.
†Present address: Shriners Hospital for Crippled Children, Department of Anesthesia, Massachusetts General Hospital, Boston, MA 02114, USA.
‡Present address: Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021, USA.
§To whom correspondence should be addressed.

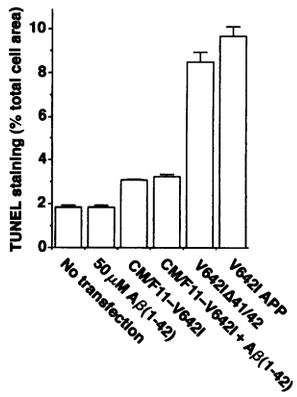

Fig. 3. Effects of Aβ(1–42), CM/F11–V642I, and V642IΔ41/42 on DNA fragmentation. The effects of 50 μM Aβ(1–42), CM/F11–V642I, or both on nucleosomal DNA fragmentation were examined after incubation with untransfected F11 cells for 48 hours (incubation for 36 hours yielded similar results). CM/F11–V642I represents medium conditioned for 48 hours by F11 cells transfected with V642I APP cDNA (medium conditioned for 36 hours yielded similar results). F11 cells expressing V642I APP and V642IΔ41/42 were also analyzed by TUNEL 36 hours after transfection. Data are means ± SE of at least three independent experiments.

Table 1. Concentration of Aβ(1–40) and Aβ(1–42) in the conditioned media of F11 cells transfected with various APP constructs. F11 cells (1×10^6) were seeded into 100-mm dishes 24 hours before transfection with 10 μg of cDNA and 20 μl of Lipofectamine, as described (*11*). Forty-eight hours after transfection, the concentrations of the two Aβ peptides in the medium were determined (*15*). Data are means ± SE of three independent transfections.

Transfectant	Aβ(1–40) (pM)	Aβ(1–42) (pM)
APP_{695}	63.2 ± 3.1	5.3 ± 1.0
V642I APP	47.4 ± 3.1	9.4 ± 0.4
V642G APP	43.2 ± 3.2	10.5 ± 0.6
V642F APP	47.2 ± 3.6	7.1 ± 0.7
V642I-ΔH657–K676	56.1 ± 3.2	9.2 ± 0.7

Our results suggest that residues His^{657} to Lys^{676} are critical for the induction of DNA fragmentation by the FAD APP mutants. It has previously been shown that (i) the His^{657}–Lys^{676} peptide is a specific activator of the heterotrimeric GTP-binding protein G_o (*18*, *19*); (ii) without this region, APP_{695} cannot bind G_o (*18*); (iii) recombinant APP_{695} couples to G_o but not to G_i in reconstituted vesicles (*20*); and (iv) a monoclonal antibody to His^{657}–Lys^{676} blocks this coupling of APP_{695} to G_o (*20*). We therefore examined the role of G proteins in DNA fragmentation induced by V642I APP. Pertussis toxin (PTX) (100 ng/ml), an inhibitor of G_i and G_o, prevented apoptosis induced by V642I APP (Fig. 4A) (*21*); DNA fragmentation was inhibited only slightly by PTX at 10 ng/ml. PTX at 100 ng/ml did not attenuate the expression of V642I APP in these cells or affect secretion of Aβ(1–40) or Aβ(1–42) (*22*). This effective concentration of PTX was typical for its effect on G_o in neuronal cells (*23*).

Cotransfection of F11 cells with cDNA encoding $G\alpha_o$(G204A), a specific inhibitor of the α subunit of G_o in which Gly^{204} is replaced by Ala (*24*), antagonized DNA fragmentation induced by V642I APP (Fig. 4B). In contrast, cotransfection of cells with cDNA encoding $G\alpha_{i2}$(G204A) (*24*), a $G\alpha_{i2}$ homolog of $G\alpha_o$(G204A), had virtually no effect on V642I APP–induced DNA fragmentation under the same conditions. Neither of these cotransfections affected the expression of V642I APP (*13*). Transfection of these two G204A constructs resulted in an increase in cholera toxin–stimulated adenylyl cyclase activity [58 ± 15% and 21 ± 8% of the cholera toxin (0.25 μg/ml)–stimulated activity for the $G\alpha_i$ and $G\alpha_o$ constructs (0.5 μg each), respectively]. Both G proteins achieved similar concentrations in transfected F11 cells (Fig. 4B, lower panel) (*25*). Therefore, functional inactivation of $G\alpha_o$, but not of $G\alpha_i$, resulted in inhibition of V642I APP–induced DNA fragmentation.

Our observation that expression of the three FAD APP mutants induces nucleosomal DNA fragmentation in neuronal cells is consistent with a pathological study by Lassmann *et al.* (*26*). These researchers evaluated, through the use of TUNEL, cell death in the brains of individuals with AD and showed that the number of cells undergoing DNA fragmentation in AD brains was ~30 times that in the brains of age-matched controls. Other studies (*27*) have also provided in situ evidence for extensive DNA fragmentation in AD brains. Although F11 cells are derived from rodents and AD is a human disease, the observation that overexpression of V642F APP causes in transgenic mice neuropathology similar to that of human AD supports the relevance of our observations (*28*).

We conclude that G proteins, specifically G_o, mediate V642 APP mutant–induced DNA fragmentation and that the cytoplasmic domain (His^{657} to Lys^{676}) of the mutant proteins is critical for this neurotoxicity. Constitutively activated mutants of rhodopsin, a G protein–coupled receptor, cause the retina to develop features of retinitis pigmentosa, which at the level of retinal neuronal cells is characterized by DNA fragmentation (*29*). Further research will be necessary to determine whether G protein–mediated DNA fragmentation is implicated in other subtypes of AD.

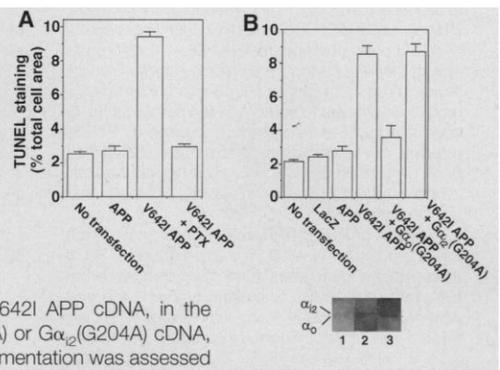

Fig. 4. Role of G proteins in DNA fragmentation induced by V642I APP. (**A**) Inhibition of V642I APP–induced DNA fragmentation by PTX. Twenty-four hours after transfection of F11 cells with V642I APP cDNA, the culture medium was changed to Ham's F12 medium with or without PTX (100 ng/ml) and the cells were cultured for an additional 12 hours. Cells were then subjected to TUNEL analysis. (**B**) Upper panel: Inhibition of V642I APP–induced DNA fragmentation by $G\alpha_o$(G204A) but not by $G\alpha_{i2}$(G204A). F11 cells were transfected with 0.5 μg of V642I APP cDNA, in the absence or presence of 0.5 μg of $G\alpha_o$(G204A) or $G\alpha_{i2}$(G204A) cDNA, with the use of 1 μl of Lipofectamine. DNA fragmentation was assessed by TUNEL 36 hours after transfection. Nontransfected cells and cells transfected with LacZ or APP_{695} constructs are shown as controls. Lower panel: Expression of $G\alpha_o$(G204A) or $G\alpha_{i2}$(G204A) by cDNA transfection in F11 cells. Cells were transfected with V642I APP cDNA and either $G\alpha_{i2}$(G204A) cDNA (lane 2) or $G\alpha_o$(G204A) cDNA (lane 3); after 36 hours, cell lysates were prepared and subjected to immunoblot analysis with antibodies to $G\alpha_o$ and to $G\alpha_i$. Lane 1 indicates analysis of untransfected F11 cells. Data in (A) and (B) are means ± SE from at least three independent transfections.

REFERENCES AND NOTES

1. R. Katzman, *N. Engl. J. Med.* **314**, 964 (1986).
2. J. Kang *et al.*, *Nature* **325**, 733 (1987).
3. R. Sandbrink, C. L. Masters, K. Beyreuther, *J. Biol. Chem.* **269**, 1510 (1994).
4. A. Goate *et al.*, *Nature* **349**, 704 (1991); S. Naruse *et al.*, *Lancet* **337**, 978 (1991); K. Yoshioka, T. Miki, T. Katsuya, T. Ogihara, Y. Sakaki, *Biochem. Biophys. Res. Commun.* **178**, 1141 (1991); J. Murrell, M. Farlow, B. Ghetti, M. D. Benson, *Science* **254**, 97 (1991); M.-C. Chartier-Harlin *et al.*, *Nature* **353**, 844 (1991); J. Hardy, *Nature Genet.* **1**, 233 (1992).
5. H. Karlinsky *et al.*, *Neurology* **42**, 1445 (1992).
6. D. Games *et al.*, *Nature* **373**, 523 (1995).
7. U. Müller *et al.*, *Cell* **79**, 755 (1994); H. Zheng *et al.*, *ibid.* **81**, 525 (1995).
8. D. Platika, M. H. Boulos, L. Baizer, M. C. Fishman, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3499 (1985). F11 cells are a mixture of at least four different clones—small and large cells with and without neurite-like processes—although all exhibit neuronal traits. Cells were grown in Ham's F12 medium supplemented with 18% fetal bovine serum (FBS), penicillin, and streptomycin.
9. Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, *J. Cell Biol.* **119**, 493 (1992). The TUNEL assay was performed with the Apoptag kit (Oncor). This method

specifically labels the 3′-OH ends of fragmented nucleosomal DNA in situ in the presence of exogenously added TdT and digoxigenin-labeled dUTP. F11 cells were seeded on a cover glass precoated with poly-D-lysine (Boehringer Mannheim) and cultured for 24 hours in Ham's F12 medium supplemented with 18% FBS and penicillin-streptomycin. After transfection, the cells were cultured for a further 36 hours and then subjected to the TUNEL assay. The extent of DNA fragmentation was quantitated with a Videometric 150 Image Analysis System (Oncor), as described [I. Joffe *et al.*, *Calcif. Tissue Int.* **53**, 45 (1993)]; briefly, it was determined from the ratio of the area of stained cells to the total cell area, the latter of which was obtained after detecting and highlighting the edges of all cells in the field. Cell areas were determined as total number of pixels. The maximal extent of DNA fragmentation was ~10%. For each transfection, data were analyzed to obtain means ± SE of the extent of DNA fragmentation in 20 randomly chosen fields (the SE values were <5% of the mean values in all instances), and the obtained mean values were further analyzed to yield means ± SE for at least three independent transfections. To ensure objective evaluation, different groups of researchers (i) performed transfections as well as stained and numbered the samples; (ii) subjected the anonymous numbered samples to image analysis; and (iii) collected data and collated them with the recorded numbers.

10. APP mutants were constructed as follows: The Sac I–Bam HI fragment of APP_{695} cDNA (*18*) was subcloned in M13mp18. Oligonucleotide-directed mutagenesis was performed as described [T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)]. The Sac I–Xba I fragment of each APP mutant cDNA was inserted into pVL1393-APP (*18*). The oligonucleotides used were 5′-ACCGTGATTATCATCACCC for V642I APP, 5′-ACCGTGATTTTCATCACCC for V642F APP, 5′-CCGTGATTGGCATCACCCT for V642G APP, and 5′-GATGATAATCACGGTGACAACGCCGCCCAC for V642IΔ41/42. All mutations were verified by DNA sequencing. The Bam HI fragment of each APP mutant cDNA was subcloned in pcDNA1 and pECE. V642IΔH657–K676 cDNA was constructed with the following primers: 5′-AAACAGTACACATCCATCATGCAGCAG (internal primer 1), 5′-ATATCCGTTCTGCTGCATGATGGATGT (internal primer 2), 5′-AACGACGCTCTCATGCCT (external primer 1), and 5′-AATGGGGAAGCTGTCTTCCAT (external primer 2). Two fragments of V642I APP cDNA were amplified by the polymerase chain reaction (PCR) with two combinations of the primers (external primer 1 and internal primer 2, and external primer 2 and internal primer 1), mixed, and amplified by PCR with the two external primers to generate a fragment lacking the sequence encoding His^{657} to Lys^{676}. This fragment was subcloned into pVL-APP with the use of Sac I and Xba I restriction sites. The sequence was verified as lacking the His^{657}–Lys^{676} coding sequence and shown not to contain unwanted mutations. The V642IΔH657–K676 cDNA was finally subcloned in pcDNA1 and pECE. The *lacZ* construct contained the *Escherichia coli lacZ* gene under the control of the chicken β-actin gene promoter.

11. F11 cells were seeded at a density of 4×10^4 cells per well on a cover glass in 24-well plates (for DNA fragmentation assay) or at 10^6 cells per 100-mm dish (for immunoblot analysis) and cultured for 24 hours in Ham's F12 medium supplemented with 18% FBS and penicillin-streptomycin. Cells were transfected with cDNA with the use of Lipofectamine (Gibco) in serum-free medium. Unless otherwise specified, 0.5 μg of cDNA and 1 μl of Lipofectamine were used for DNA fragmentation assays, and 10 μg of cDNA and 20 μl of Lipofectamine were used for immunoblot analysis. After incubation for 24 hours in serum-free medium, transfected cells were cultured for a further 12 hours in Ham's F12 medium containing 10 nM nerve growth factor (NGF) and antibiotics (in some experiments, cells were cultured in the absence of NGF or in the presence of 10 nM NGF and 1% FBS; the results were similar, although NGF reduced the error bars in the quantitation of DNA fragmentation). Cells were then fixed or lysed. For experiments in which F11 cells were treated with Aβ(1–42), cells were exposed to 50 μM Aβ(1–42) plus 10 nM NGF for 48 hours to simulate the conditions of APP transfection experiments. Because F11 cells contain abundant endogenous APP, we could not identify the individual cells that express transfected APP mutants by immunocytochemistry with antibodies to APP.

12. A. Tamaoka *et al.*, *J. Biol. Chem.* **269**, 32721 (1994); T. Iwatsubo *et al.*, *Neuron* **13**, 45 (1994).
13. T. Yamatsuji and I. Nishimoto, unpublished data.
14. D. T. Loo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7951 (1993).
15. Sandwich enzyme-linked immunosorbent assay (ELISA) of Aβ peptides was performed as described (*17*), with some modifications. The monoclonal antibody BNT77, generated against synthetic Aβ(11–28), was immobilized on 96-well microtiter plates by incubation of 1 μg of antibody in 0.1 ml of 0.1 M sodium carbonate buffer (pH 9.6) in each well at 4°C overnight. After washing with 0.3 ml of a phosphate-buffered saline (PBS) solution containing 0.1 M sodium phosphate (pH 7.4) and 0.1 M NaCl, 0.3 ml of PBS containing 1% Block Ace (Snow Brand Milk Products, Sapporo, Japan) and 0.05% NaN_3 was added to each well. Standards were prepared by dilution of stock Aβ(1–40) and Aβ(1–42) (Backem) with buffer EC [20 mM sodium phosphate (pH 7.0), 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace, 0.2% bovine serum albumin, 0.05% CHAPS detergent, and 0.05% NaN_3]. The standard solutions and samples were incubated in the BNT77-coated microtiter plate at 4°C overnight. After three washes with PBS, the plate was incubated at room temperature for 6 hours with 0.1 ml per well of horseradish peroxidase–conjugated detector antibody (BA27 or BC05) diluted appropriately with buffer C [20 mM sodium phosphate buffer (pH 7.0), 0.4 M NaCl, 2 mM EDTA, and 1% bovine serum albumin]. The plate was washed four times with PBS, and bound enzyme activity was measured with substrate solution of the TMB microwell peroxidase system (Kirkegaard Perry, Gaithersburg, MD). The enzyme reaction was stopped with 0.1 ml of 1 M phosphoric acid, and absorbance at 450 nm was measured with a microplate reader. The specificities of BA27 and BC05 for the COOH-terminal residues of Aβ(1–40) and Aβ(1–42), respectively, have been demonstrated (*17*). Because the epitope recognized by the capture antibody (BNT77) is located at the midportion of Aβ, this ELISA can detect Aβ with a truncated NH_2-terminus as well as full-length Aβ [A. Asami-Odaka *et al.*, *Biochemistry* **34**, 10272 (1995)].
16. X.-D. Cai, T. E. Golde, S. G. Younkin, *Science* **259**, 514 (1993).
17. N. Suzuki *et al.*, *ibid.* **264**, 1336 (1994).
18. I. Nishimoto *et al.*, *Nature* **362**, 75 (1993).
19. J. Lang *et al.*, *EMBO J.* **14**, 3635 (1995).
20. T. Okamoto, S. Takeda, Y. Murayama, E. Ogata, I. Nishimoto, *J. Biol. Chem.* **270**, 4205 (1995).
21. The effect of PTX was clearly preventive, as virtually no DNA fragmentation was observed at 24 hours after transfection with APP mutant cDNAs. Thus, DNA fragmentation appears to occur between 24 and 36 hours after transfection. Given that expression of the APP mutants was already apparent at 24 hours, these data further suggest that the mutants require ≥12 hours to achieve DNA fragmentation. In contrast, only 2 to 3 hours were required for PTX to mediate ADP (adenosine diphosphate) ribosylation of G proteins in our system, allowing for the total inhibitory effect of this toxin added 24 hours after transfection.
22. Expression of V642I APP in F11 cells treated with PTX at 100 ng/ml was 100.5 ± 3.9% (mean ± SD of three independent transfections) of that apparent with untreated cells. Secretion of Aβ(1–40) and Aβ(1–42) from V642I APP–expressing F11 cells treated with PTX (100 ng/ml) was 98.3 ± 6.3% and 93.9 ± 8.0% (means ± SE of three independent transfections), respectively, of the corresponding values for untreated cells.
23. M. Igarashi, S. M. Strittmatter, T. Vartanian, M. C. Fishman, *Science* **259**, 77 (1993).
24. V. Z. Slepak, T. M. Wilkie, M. I. Simon, *J. Biol. Chem.* **268**, 1414 (1993); S. Hermouet, J. J. Merendino Jr., J. S. Gutkind, A. M. Siegel, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10455 (1991); I. Corre and S. Hermouet, *Blood* **86**, 1776 (1995). We constructed $G\alpha_{i2}$(G204A) cDNA with the use of bovine $G\alpha_{i2}$ cDNA in pUC18 as a template. The entire $G\alpha_{i2}$ cDNA was amplified by PCR with two oligonucleotides—5′-AAATTTAAGCTTATGGGCTGCACCGTGAGCG and 5′-AAATTTTCTAGATCAGAAGAGGGCCGCAGTCC—that yield new restriction sites for Hind III and Xba I at the 5′ and 3′ ends of the gene, respectively. The amplified fragment was digested with Hind III and Xba I and was subcloned in pcDNA1 (pcDNA1-$G\alpha_{i2}$). The pcDNA1-$G\alpha_{i2}$ insert was sequenced to verify that it did not contain unwanted mutations. After digestion of pcDNA1-$G\alpha_{i2}$ with Hind III and Bam HI, the ~600–base pair fragment was subcloned into M13mp18 (M13-$G\alpha_{i2}$). Oligonucleotide-directed mutagenesis was performed with 5′-ACGTGGGCGCTCAGCGGTC. After confirming by sequencing that C was present at nucleotide position 611 instead of the original G, the mutant M13-$G\alpha_{i2}$ was digested with Hind III and Bam HI, and the ~600–base pair fragment was inserted into pcDNA1-$G\alpha_{i2}$. Again, the sequence of this plasmid was verified.
25. $G\alpha_o$(G204A) or $G\alpha_{i2}$(G204A) cDNA was cotransfected with V642I APP cDNA, and the 39-kD $G\alpha_o$ and 41-kD $G\alpha_i$ in lysates of transfected F11 cells were detected by immunoblot analysis with antibodies to $G\alpha_o$ (GC/2; 1/100 dilution) and to $G\alpha_i$ (AS/7; 1/100 dilution). Because the reactivities of these antibodies with the cognate Gα are similar, the antigenic bands observed at 39 and 41 kD reflect the relative amounts of the recombinant $G\alpha_o$ and $G\alpha_i$ constructs. Densitometric analysis indicated that transfection with $G\alpha_{i2}$(G204A) and $G\alpha_o$(G204A) cDNAs resulted in 1.8- and 2.1-fold increases (means of three experiments) in the intensity of the 41- and 39-kD bands, respectively, relative to the intensity of the endogenous 39-kD $G\alpha_o$ band, indicating that the two G204A constructs were expressed in similar amounts. Transfection with $G\alpha_o$(G204A) cDNA appeared to enhance slightly the expression of endogenous $G\alpha_i$ at 41 kD, probably because the $G\alpha_i$ gene promoter responded to an increase in the concentration of adenosine 3′,5′-monophosphate induced by the construct, as described [T. B. Kinane *et al.*, *J. Biol. Chem.* **268**, 24669 (1993)].
26. H. Lassmann *et al.*, *Acta Neuropathol.* **89**, 35 (1995).
27. J. H. Su, A. J. Anderson, B. J. Cummings, C. W. Cotman, *Neuroreport* **5**, 2529 (1994); M. Dragunow *et al.*, *ibid.* **6**, 1053 (1995); G. Smale, N. R. Nichols, D. R. Brady, C. E. Finch, W. E. Horton, *Exp. Neurol.* **133**, 225 (1995).
28. We have also examined DNA fragmentation in human postmitotic hNT neurons (Stratagene), differentiated from Ntera2 cells [V. M. Y. Lee and P. W. Andrews, *J. Neurosci.* **6**, 514 (1986)], 36 hours after transfection with various APP cDNAs. Oligonucleosomally fragmented DNA was assessed by ELISA (Boehringer Mannheim) [E. Bonfoco *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7162 (1995)]. The results (expressed as the ratio of absorbance at 405 nm to that at 492 nm, per 4×10^4 cells) were 0.025 ± 0.001 for nontransfected cells, 0.018 ± 0.007 for cells transfected with 0.5 μg of APP_{695} cDNA, and 0.056 ± 0.001 for cells transfected with 0.5 μg of V642I APP cDNA (means ± SE of three independent transfections). These data indicate that V642 mutant–induced DNA fragmentation occurs in human neurons.
29. V. R. Rao, G. B. Cohen, D. D. Oprian, *Nature* **367**, 639 (1994); G.-Q. Chang, Y. Hao, F. Wong, *Neuron* **11**, 595 (1993).

30. Supported in part by grants from Bristol-Myers Squibb. We thank M. C. Fishman for F11 cells, advice, and critical reading of the manuscript; M. Miura and J. Yuan for the *lacZ* construct and advice; S. M. Strittmatter for $G\alpha_o$(G204A) cDNA; A. H. Tashjian, E. Haber, and J. H. Growdon for helpful discussions; E. Neer and B. Yankner for reading a prior version of this manuscript; M. H. Boulos, Y. Tamai, Y. Murayama, and E. Ogata for support of this work; and D. Wylie and L. Duda for help with preparation of the manuscript.

21 July 1995; accepted 6 March 1996