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G protein βγ complex-mediated apoptosis by familial Alzheimer's disease mutant of APP

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In familial Alzheimer's disease (FAD), three missense mutations, V642I, V642F and V642G, that co-segregate with the disease phenotype have been discovered in the 695 amino acid form of the amyloid precursor protein APP. Expression of these mutants causes a COS cell NK1 clone to undergo pertussis toxin-sensitive apoptosis in an FAD trait-linked manner by activating the G protein G_o, which consists of $G\alpha_o$ and Gβγ subunits. We investigated which subunit was responsible for the induction of apoptosis by V642I APP in NK1 cells. In the same system, expression of mutationally activated $G\alpha_o$ or $G\alpha_i$ induced little apoptosis. Apoptosis by V642I APP was antagonized by the overexpression of the carboxy-terminal amino acids 495–689 of the β-adrenergic receptor kinase-1, which blocks the specific functions of Gβγ. Co-transfection of Gβ2γ2 cDNAs, but not that of other Gβxγz (x = 1–3; z = 2, 3), induced DNA fragmentation in a manner sensitive to bcl-2. These data implicate Gβγ as a cell death mediator for the FAD-associated mutant of APP.
Keywords: amyloid precursor protein/apoptosis/ βγ complex/familial Alzheimer's disease/G protein

Introduction

Alzheimer's disease (AD) is characterized pathologically by extensive neuronal loss, intracellular tangles and extracellular senile plaques, whose major constituent, Aβ amyloid, is cleaved off from the transmembrane amyloid precursor protein (APP) (Kang *et al.*, 1987). Among at least 10 spliced isoforms from a single gene, the 695 amino acid form APP_{695} is preferentially expressed in neurons. In patients with early onset familial AD (FAD), Ile, Phe and Gly mutations have been discovered at V642 in APP_{695} (Hardy, 1992). These mutations co-segregate with the AD phenotype (Karlinsky *et al.*, 1992), demonstrating that V642 mutations in APP are established causes of AD.

Nonetheless, little has been known about what type of abnormality, if any, is induced by APP in these mutations. Suzuki *et al.* (1994) have found that secretion of Aβ1–42, a longer version of Aβ, is a common target of this type of mutation. However, multiple pieces of evidence contradict the notion that Aβ deposition is the cause of AD, although it is the earliest abnormality constantly observed in the AD brain. Despite the neurotoxicity of Aβ *in vitro* (Loo *et al.*, 1993), mice overproducing Aβ1–42 extracellularly showed virtually no neuronal loss (LaFerla *et al.*, 1995). In transgenic mice, overexpression of the V642F type of the APP mutant led to Aβ deposition and senile plaque formation approximating those found in AD patients, but resulted in little neurodegeneration or AD-like signs and symptoms (Games *et al.*, 1995). Conversely, in transgenic mice overexpressing APP mutated in the α-secretase cleavage sites, neurodegeneration and AD-mimetic signs and symptoms occurred without significant deposition of Aβ (Moechars *et al.*, 1996). Furthermore, it has been found that the FAD-associated V642 mutants of APP cause cytotoxicity in cultured cells without Aβ mediation (Yamatsuji *et al.*, 1996a,b). Thus, the significance, as well as the role, of Aβ deposition for AD development remains unclear.

In its structure, orientation and localization, APP is similar to cell surface receptors (Kang *et al.*, 1987; Dyrks *et al.*, 1988; Weidemann *et al.*, 1989; Schubert *et al.*, 1991; Ferreira *et al.*, 1993). The cytoplasmic domain of APP binds Fe65 protein, which has a phosphotyrosine-binding domain related to an oncogenic signal transducer, Shc (Fiore *et al.*, 1995). It also binds APP-BP1, a gene product similar to AXR1 in *Arabidopsis*; AXR1 is required for normal response to the plant growth hormone auxin (Chow *et al.*, 1996). These observations suggest that APP has not only the structure but also the function of a cell surface receptor. Our own earlier study (Nishimoto *et al.*, 1993) found that APP_{695} has an intrinsic G_o-stimulating domain at His657–Lys676 and forms a complex with G_o through this cytoplasmic domain. It has been confirmed that the synthetic His657–Lys676 peptide activates G_o *in vivo* (Lang *et al.*, 1995). We subsequently indicated that intact APP_{695} causes activation of G_o through His657–Lys676 in response to anti-APP monoclonal antibody in reconstituted vesicles (Okamoto *et al.*, 1995). Therefore, APP_{695} has a molecular function as a G_o-coupled receptor. G_o is a heterotrimeric G protein that serves as a signal

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transducer *in vivo*; thus, APP_{695} may play a role as a signaling receptor, even in intact cells. Murayama *et al.* (1996) have reinforced this hypothesis, using APP_{695}-overexpressing gliomas. APP, G_o and growth-associated protein (GAP)-43 co-localize in growth cones in pre-synapses of neurons (Strittmatter *et al.*, 1990; Ferreira *et al.*, 1993). GAP-43 is a specific potentiator for G_o-coupled receptors (Strittmatter *et al.*, 1993), so one can assume that their co-localization may add to the theory of APP being a functional receptor. In further support, APP and G_o have been implicated in virtually identical functions of neurons, such as neurite outgrowth, synaptic contact and cell–cell adhesion (reviewed in Nishimoto *et al.*, 1997).

It has been found that the three FAD-associated V642 mutants of APP, V642I, V642F and V642G, all induce apoptotic cell death when they are expressed in NK1 cells, which are neuron-like transformants of COS cells that endogenously express $G\alpha_o$ (Yamatsuji *et al.*, 1996a). In these cells, the highest incidence of apoptosis was caused by the three FAD mutants; all the other possible mutants at V642, as well as wild-type APP, caused less or no apoptosis. This observation demonstrates that apoptosis by V642 mutants in NK1 cells is phenotypically linked to the AD trait and reflects a key pathological process of FAD.

Significantly, G_o has been implicated in apoptosis after transfection of NK1 cells with the three FAD mutants (Yamatsuji *et al.*, 1996a). First, when the same cells were treated with pertussis toxin (PTX) or transfected with an inactivating $G\alpha_o$ mutant, this apoptosis was blocked. PTX is a known inhibitor specific for $G\alpha_i$ and $G\alpha_o$. Second, all three FAD mutants of APP constitutively activate G_o directly in reconstituted vesicles (Okamoto *et al.*, 1996). Third, in the cytoplasmic domain His657–Lys676 of APP, the FAD mutant V642I APP binds and initiates a cascade message for the induction of apoptosis (Yamatsuji *et al.*, 1996a,b). The only known function of this APP domain has been to activate $G\alpha_o$ selectively among various G proteins (Nishimoto *et al.*, 1993), and this domain is functional for V642I APP to constitutively activate G_o (Okamoto *et al.*, 1996). Fourth, when V642F APP, but not normal APP, was co-expressed in NK1 cells with each of various $G\alpha_s$ chimeras whose C-terminal five residues (the receptor contact site of Gα) were from those of other Gα genes, cAMP response element (CRE) activity was constitutively promoted in $G\alpha_s$–$G\alpha_o$ chimera-transfected cells (Ikezu *et al.*, 1996). It has been established, using the $G\alpha_s$–$G\alpha_x$ chimera, that the signal of the receptor linked to $G\alpha_x$ is converted specifically to the stimulation of adenylyl cyclase and its downstream pathway (Komatsuzaki *et al.*, 1997). The observation mentioned above thus indicates that V642F APP can constitutively and selectively recognize the C-terminal five residues of $G\alpha_o$, i.e. the receptor contact site of $G\alpha_o$, and activate the whole chimeric G protein in NK1 cells. Therefore, V642F APP should constitutively and selectively activate G_o as well through the recognition of the receptor contact site of $G\alpha_o$ *in vivo*. Finally, the three FAD mutants of APP at V642 suppressed the transcriptional activity of CRE when they were expressed in NK1 cells. The suppression of CRE was also reproduced by the expression of constitutively active $G\alpha_o$ mutants in the same cells.

Based upon these multiple lines of evidence, we have concluded that the three FAD-linked mutants of APP activate G_o and thereby induce apoptosis in these cells. Consistent with this notion, recent reports from other laboratories (Carracedo *et al.*, 1995; Yan *et al.*, 1995) have described PTX-sensitive apoptosis in cerebellar neurons and natural killer cells, in both of which endogenous expression of G_o has been documented (Nishida *et al.*, 1991; Sebok *et al.*, 1993). Most recently, Wolozin *et al.* (1997) have described PTX-sensitive apoptosis in PC12 cells induced by overexpression of APP and presenilin-2, another FAD gene product located at human chromosome 1, supporting our theory.

G_o belongs to the oligomeric G protein family, which consists of two functional subunits, Gα and Gβγ. The activity of Gα is strictly regulated by bound guanine nucleotides. Gα stays inactive when it binds GDP. Upon receptor stimulation, Gα undergoes GDP/GTP exchange and become an active GTP-bound form. Through the intrinsic GTP hydrolysis activity built into Gα, the active form returns to the inactive form. Thus, the active form of Gα is the GTP-bound form, and the inactive one is the GDP-bound form. To express the function of the Gα subunit, we usually need to express the GTPase-attenuated Gα mutant, but not the wild-type Gα (Wong *et al.*, 1991), suggesting that most of the expressed wild-type G proteins are in an inactive conformation, which means a GDP-bound form. This is reasonable, because wild-type G proteins have intrinsic GTPase activity, and they are not activated without upstream stimulation. In clear contrast, the active form of Gβγ is the Gα-unbound form, and the inactive one is the Gα-bound form. Therefore, just by overexpressing Gβγ complexes to the extent that they exceed Gα in quantity, we can express the function of the Gβγ. In fact, it has been established that overexpression of wild-type Gβ and wild-type Gγ cDNAs results in stimulation of polyphosphoinositide turnover (Camps *et al.*, 1992; Katz *et al.*, 1992) and MAP kinase activation (Crespo *et al.*, 1994).

The inactive form of Gα is in an oligomeric conformation associated with Gβγ and, in response to upstream receptor activation, the G protein dissociates into the two subunits. Upon stimulation, inactive G_o thus generates two active moieties, $G\alpha_o$ and Gβγ, both of which are capable of activating downstream effectors. Therefore, it is essential to know which subunit of PTX-sensitive G protein G_o is responsible for the induction of apoptosis triggered by the V642 mutants of APP in NK1 cells. This study was conducted to specify the G protein subunit implicated in this apoptosis.

Our strategy was to examine (i) whether mutationally activated Gα mutants of G_o and G_i can induce apoptosis in NK1 cells; (ii) whether wild-type $G\alpha_o$ enhances or impairs apoptosis induced by V642I APP in NK1 cells; (iii) whether the isolated Gβγ-binding domain of βARK1, an established Gβγ inhibitor, attenuates this apoptosis; (iv) whether certain combinations of co-transfected Gβ and Gγ cDNAs cause NK1 cells to undergo apoptosis; and if so, (v) whether the same cells express the subtypes of Gβ and Gγ capable of triggering cellular apoptosis. The results implicate the Gβγ subunit of G_o as a novel effector of the FAD-linked mutant of APP that mediates apoptosis, providing an entirely new insight into the

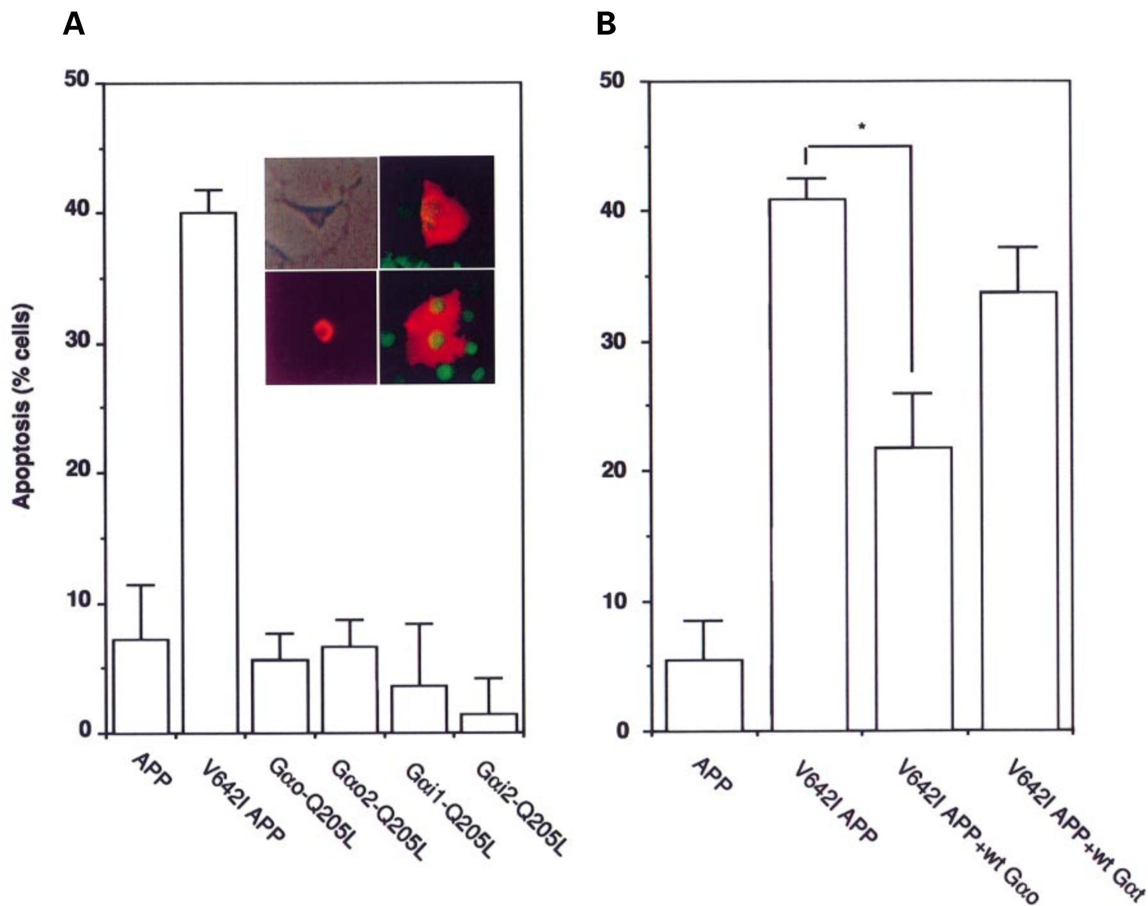

Fig. 1. Lack of effects of mutationally activated $G\alpha_o$ on apoptosis and inhibition of V642I APP-induced apoptosis by wild-type $G\alpha_o$. (**A**) NK1 cells were transfected with wild-type APP_{695} (APP), V642I APP or GTPase-deficient Gα mutant cDNA and, 48 h after transfection, cells were stained red with anti-APP or anti-Gα antibody and their nuclei were stained green with acridine orange. Apoptosis was assessed for the nuclear changes defining apoptosis, which are condensation, fragmentation and compaction. We counted the red cells throughout the well (= a) and the number of red cells having an apoptotic nucleus (= b). The gross incidence of apoptosis by a transfectant (APP, V642I APP or GTPase-deficient Gα mutant) was b/a. In each transfection, background incidence (c) of apoptosis was estimated by counting the frequency of non-transfectant-expressing cells that have apoptotic nuclei in the same sample. The subtraction, b/a–c, represents the specific apoptosis induced by the expression of the transfectant, which is shown here. The data are the means ± SE of three independent transfections. Inset: immunohistochemical staining of $G\alpha_o$-Q205L (right upper) and $G\alpha_{o2}$-Q205L (right lower). Samples were stained red by anti-$G\alpha_o$ antibody 72 h after transfection of the cognate cDNAs under the same conditions used in measuring apoptosis. The photographs were superimposed with the nuclear staining by acridine orange. All the Q205L-transfected cells exhibited widely spread shapes. The left upper panel shows the phase contrast image of untransfected NK1 cells which have typical neurite-like processes. The left lower panel shows the staining of the cells expressing the V642 mutant of APP 72 h after transfection. All the V642I APP-transfected cells became small and round, as described previously (Yamatsuji *et al.*, 1996a). These panels are at the same magnification. (**B**) Cells were transfected with APP_{695}, V642I APP or V642I APP plus either $G\alpha_o$ or $G\alpha_t$ cDNA (0.5 μg for each construct), and the specific incidence of apoptosis was measured as in the left panel. $^*P < 0.05$.

mechanism underlying the cytotoxicity linked to FAD and apoptosis itself.

Results

To specify the responsible subunit, we began by examining whether expression of mutationally activated $G\alpha_o$ causes NK1 cells to undergo apoptosis. Both $G\alpha_o$-Q205L and $G\alpha_{o2}$-Q205L are GTPase-deficient mutants of $G\alpha_o$ and its splicing variant $G\alpha_{o2}$, respectively, which stay in active conformations. The use of this type of mutationally activated Gα has been established in clarifying whether one particular output in cells is triggered by Gα (Wong *et al.*, 1991). Transfection of either mutant did not increase the incidence of nuclear apoptotic changes, i.e. condensation, fragmentation and impaction of nuclei, 48 h after transfection (Figure 1A). In addition, 72 h after transfection, no cells expressing either $G\alpha_o$ mutant showed condensed cytoplasm (Figure 1A, inset, right). Conversely, $G\alpha_o$ mutant-expressing cells had widely spread shapes. In clear contrast, cytoplasmic condensation was observed in all cells expressing V642I APP at 72 h after transfection (Figure 1A, inset, left; and see also Yamatsuji *et al.*, 1996a). These observations indicate that expression of mutationally activated $G\alpha_o$, with certain effects on cytoskeletal machinery, caused no apoptosis. This was also the case with either GTPase-deficient $G\alpha_i$, $G\alpha_{i1}$-Q205L or $G\alpha_{i2}$-Q205L (data not shown). It has been demonstrated that these Q205L constructs of $G\alpha_o$ and $G\alpha_i$ are functional in NK1 cells under similar conditions. The $G\alpha_i$ mutants inhibit adenylyl cyclase activity (Ikezu *et al.*, 1995) and the $G\alpha_o$ mutants suppress CRE (Ikezu *et al.*, 1996), suggesting that their expression levels were above the range allowing functions. As PTX blocks V642I APP-induced apoptosis in NK1 cells (Yamatsuji *et al.* 1996a), the lack of NK1 apoptosis by mutationally activated PTX-

sensitive $G\alpha$ suggests that the $G\beta\gamma$ subunits could be responsible for the induction of this apoptosis.

We next examined how overexpression of wild-type $G\alpha_o$ affects V642I APP-induced apoptosis. If $G\alpha_o$ is utilized as the effector subunit of G_o to induce this apoptosis, the induction of apoptosis is expected to be enhanced or unaltered (if the signal is saturated) by the co-overexpression of wild-type $G\alpha_o$. If $G\beta\gamma$ is the subunit triggering V642I APP-induced apoptosis, overexpression of wild-type $G\alpha_o$ would attenuate this apoptosis, because most of the transfected and expressed wild-type $G\alpha$ is in an inactive GDP-bound conformation, which sequesters free $G\beta\gamma$. We co-transfected wild-type $G\alpha_o$ cDNA with V642I APP cDNA and evaluated the incidence of NK1 apoptosis. Apoptosis induced by V642I APP was significantly diminished by co-transfection of wild-type $G\alpha_o$ (Figure 1B). This inhibitory effect on V642I APP was not non-specific, because similar overexpression of wild-type $G\alpha_o$ potentiates the effect of V642I APP on CRE activity in the same cells (Ikezu *et al.*, 1996). Given the fact that the $G\alpha$ subunit of G_o is involved in the FAD-linked APP-induced suppression of CRE (Ikezu *et al.*, 1996), the present finding offers additional support for the notion that the $G\beta\gamma$ subunit of G_o is involved in the FAD-linked APP-induced apoptosis. Incomplete inhibition of apoptosis by co-transfected wild-type $G\alpha_o$ probably occurred because some of the newly formed trimeric G_o could be activated by V642I APP and join the positive signal for apoptosis. In support of this, inhibition of V642I APP-induced apoptosis by an inactivating $G\alpha_o$ mutant was nearly complete (Yamatsuji *et al.*, 1996a).

As compared with wild-type $G\alpha_o$, transfection of wild-type $G\alpha_t$ resulted in lesser inhibition. $G\alpha_t$ is the phototransducing α subunit of the retina-specific G protein transducin, which frequently has been used to sequester free $G\beta\gamma$ and inhibit $G\beta\gamma$-induced cellular outputs (Federman *et al.*, 1992; Lustig *et al.*, 1993). In these experiments, the expression of V642I APP per cell was not altered by co-transfection of $G\alpha_o$ or $G\alpha_t$ (data not shown). However, for technical reasons, we were not able to compare the expression level of $G\alpha_o$ with that of $G\alpha_t$. The greater inhibition by $G\alpha_o$ than by $G\alpha_t$ may suggest that the $G\beta\gamma$ implicated in V642I APP-induced apoptosis has higher affinity for $G\alpha_o$ than for $G\alpha_t$, implying that the $G\beta\gamma$ complex is not $G\beta1\gamma1$, the transducin $G\alpha_t$-specific $\beta\gamma$ subunit. Alternatively, this finding may suggest that $G\alpha_o$, but not $G\alpha_t$, can switch on an inhibitory pathway for this apoptosis other than through sequestration of $G\beta\gamma$, as is the case with the pheromone-induced mating system of *Saccharomyces cerevisiae*. In this yeast system, the $G\beta\gamma$ stimulates mating and the $G\alpha$ acts as an inhibitor not only by binding to $G\beta\gamma$ but also by turning on a negative signal for this $G\beta\gamma$ output (Doi *et al.*, 1994).

In an effort to confirm the intermediary role of $G\beta\gamma$, we next investigated the effect of the expressed βARK1 C-terminus (βARK1-CT). The βARK1-CT, corresponding to amino acids 495–689 of βARK1, contains an established $G\beta\gamma$-binding site (Koch *et al.*, 1993) and has been employed in multiple studies to inhibit specific actions of $G\beta\gamma$ (Koch *et al.*, 1994a,b; Guo *et al.*, 1995; Hawes *et al.*, 1995; Luttrell *et al.*, 1995a,b). When βARK1-CT cDNA was transiently co-transfected with V642I APP cDNA, V642I APP-induced apoptosis was partially inhibited (Figure 2A), whereas it was not inhibited by the empty plasmid pRK5. The antagonizing effect of βARK1-CT was also observed when V642I APP-induced apoptosis was examined in NK1 cells stably transfected with this construct (Figure 2B). In these cells, DNA fragmentation induced by V642I APP expression was significantly impaired, as compared with that in NK1/Puro cells transfected with a control plasmid or in NK1/βARK1-NT cells transfected with βARK1-NT cDNA, which corresponds to the N-terminus (amino acids 1–494) of βARK1. Although NK1/Puro cells seem to have a certain resistance to V642I APP in causing DNA fragmentation, as compared with parent NK1 cells (Yamatsuji *et al.*, 1996a), these data indicate that V642I APP-induced apoptosis requires the activity of $G\beta\gamma$.

We pursued further evidence for $G\beta\gamma$-induced apoptosis. We co-transfected $G\beta$ and $G\gamma$ cDNAs and examined apoptosis in the same system. Figure 3A shows that co-transfection of $G\beta2\gamma2$ cDNAs induced nucleosomal DNA fragmentation at 48 h after transfection. A single transfection of either $G\beta2$ or $G\gamma2$ cDNA failed to induce chromatin fragmentation (data not shown). At 72 h after transfection, considerable fractions of transfected cells detached from plates. These features are characteristic of apoptosis. In contrast, other co-transfected $G\beta x\gamma z$ ($x = 1, 2, 3$; $z = 2, 3$) caused little DNA fragmentation at 48 h after transfection (Figure 3A) or scarce detachment at 72 h post-transfection (data not shown). Lack of apoptosis by co-transfection of $G\beta3\gamma2$ or $G\beta2\gamma1$ cDNAs (Figure 3A for $G\beta3\gamma2$; not shown for $G\beta2\gamma1$) was consistent with the fact that these combinations do not generate functional complexes (Pronin and Gautam, 1992; Schmidt *et al.*, 1992). Functional dimers of $G\beta1\gamma2$, $G\beta1\gamma3$, $G\beta2\gamma2$ and $G\beta2\gamma3$ have been demonstrated (Graber *et al.*, 1992; Iniguez-Lluhi *et al.*, 1992; Pronin and Gautam, 1992; Robishaw *et al.*, 1992; Schmidt *et al.*, 1992; Boyer *et al.*, 1994). $G\beta2\gamma2$-induced DNA fragmentation was found at least three times by independent transfections by TUNEL (Figure 3B).

To be sure that the cytotoxicity by $G\beta2\gamma2$ was not an artifact, we examined whether it might be regulated by an established apoptosis blocker, bcl-2. To do so, we used NK1 cells (NK1/bcl-2) stably transfected with bcl-2 (Yamatsuji *et al.*, 1996a). Figure 3C indicates that co-expression of $G\beta2\gamma2$ failed to cause DNA fragmentation in NK1/bcl-2 cells, suggesting that the observed DNA fragmentation by $G\beta2\gamma2$ is sensitive to bcl-2 and is associated with the typical feature of apoptosis. Because apoptosis by V642I APP is also sensitive to bcl-2 (Yamatsuji *et al.*, 1996a), these data lend additional credence to the notion that $G\beta\gamma$ is a cell death mediator of FAD-associated APP.

The expression of $G\beta1$, $G\beta2$, $G\gamma2$ and $G\gamma3$ associated with transfection of cognate cDNAs was verified (Figure 3D). Immunoblot analysis with anti-$G\beta$ common antibody revealed that the major $G\beta$ in mock-transfected NK1 cells was 36 kDa $G\beta$. In response to $G\beta1$ or $G\beta2$ cDNA transfection, corresponding protein expression at 36 or 35 kDa was observed. The anti-$G\beta2$-specific antibody selectively detected the 35 kDa protein, confirming that the 36 and 35 kDa bands correspond to $G\beta1$ and $G\beta2$, respectively. NK1 cells endogenously expressed 10 kDa $G\gamma2$ protein, which was recognized by anti-$G\gamma2$-specific antibody, and overexpressed it when transfected with

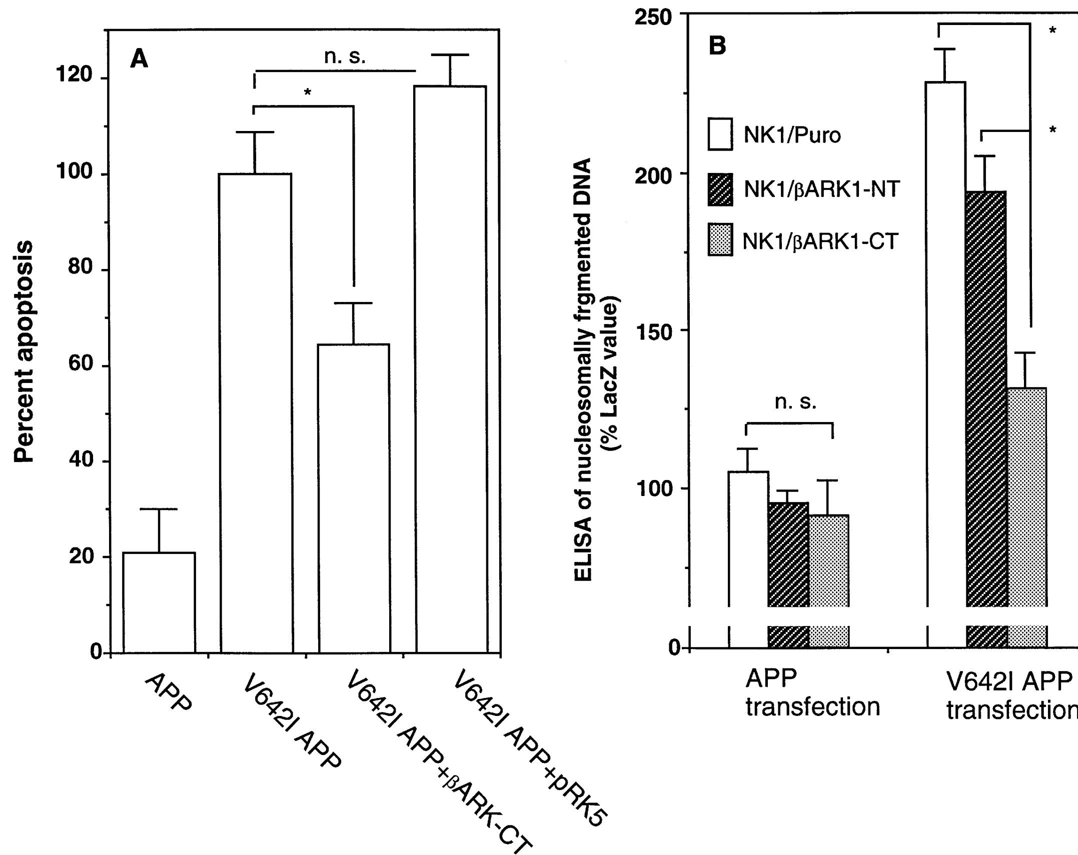

Fig. 2. Inhibition of V642I APP-induced apoptosis by the C-terminus of βARK1. (**A**) Cells were transfected with V642I APP cDNA (0.5 μg), with βARK1-CT cDNA or pRK5 (each 0.5 μg), and apoptosis was examined in cells expressing V642I APP; 100% indicates the specific incidence of apoptosis induced by V642I APP, which was similar to that in Figure 1. The data are the means ± SE of three independent transfections. $^*P < 0.05$. n.s., not significant. (**B**) After either LacZ, APP or V642I APP cDNA (each 0.5 μg) was transfected into NK1 stable cell lines, histone-associated oligonucleosomally fragmented DNA was measured by ELISA. NK1/βARK1-CT, NK1/βARK1-NT or NK1/Puro were the cells stably transfected with βARK1-CT cDNA, βARK1-NT cDNA or pBabe/puro (an empty plasmid for puromycin resistance), respectively. The data shown here as the percentage of LacZ-induced DNA fragmentation are the means ± SE of three independent transfections. $^*P < 0.05$. n.s., not significant.

Gγ2 cDNA but not with Gγ3 cDNA. Anti-Gγ3 antibody detected Gγ3 protein of a slightly larger size in NK1 cells transfected with Gγ3 cDNA. Parental cells expressed little of this protein. This anti-Gγ3 antibody less potently detected the 10 kDa Gγ2 overexpressed in Gγ2-transfected cells, while it could not detect the smaller amount of endogenous Gγ2. These data are also consistent with multiple earlier studies showing that the Gβ2γ2 complex is a minor member of Gβγ (Woolkalis and Manning, 1987; Asano *et al.*, 1993; Yan *et al.*, 1996).

To ensure that co-transfected Gβ1γ2 and Gβ2γ3, like Gβ2γ2, form functional complexes, we checked the effects of co-transfection of Gβ1γ2, Gβ2γ2 and Gβ2γ3 cDNAs on the promoter activity of the stromelysin gene. As shown in the left panel of Figure 3E, co-transfection of Gβ1γ2, Gβ2γ2 and Gβ2γ3 each resulted in similar stimulation of the stromelysin promoter activity located at –1303 to –754. As controls, each of these Gβγ complexes was tested with the stromelysin promoter at –1218 to –1202, and none of them stimulated this promoter activity (Figure 3E, right panel). The known nuclear transcriptional element located at –1303 to –754 outside –1218 to –1202 of the stromelysin gene is the Ras-responsive element (Sanz *et al.*, 1994), and Ras activation is the known effect of Gβγ complexes (Crespo *et al.*, 1994). It is therefore likely that these Gβγ complexes activate the stromelysin promoter through Ras activation. These findings demonstrate that co-transfection of either Gβ1γ2, Gβ2γ2 or Gβ2γ3 cDNAs leads to the expression of the cognate functional complexes which generate nuclear signals to similar extents, but that only Gβ2γ2 can turn on the pathway for apoptosis.

We examined the native expression of Gβ2 and Gγ2 in NK1 cells. As shown above, the immunoblot analysis detected endogenous expression of Gγ2 but not of Gβ2. However, it has been established that Gβ2 is ubiquitously expressed as a minor message (Woolkalis and Manning, 1987). To confirm expression in NK1 cells, mRNA was purified from these cells and reversely transcribed into cDNA, and a fragment of each subunit was amplified by using subtype-specific primers in the PCR (Figure 4). Three Gβ (β1, β2 and β3) and three Gγ (γ2, γ4 and γ5) subunits were found to be expressed (the β3 band was only weakly visible). Negative detection of β4, γ1, γ3 or γ7 was confirmed by using a second set of PCR primers. To ensure that the PCR bands shown here represent the segments of Gβ2 and Gγ2, we performed Southern blot analysis of the PCR products from Gβ2, Gβ3, Gβ4, Gγ2, Gγ3, Gγ4 and Gγ5 from the NK1 cells, using as probes the labeled Gβ2 and Gγ2 oligonucleotides. As shown in Figure 4B, the Gβ2 probe specifically detected the 160 bp PCR band of Gβ2 and the Gγ2 probe detected the 110 bp

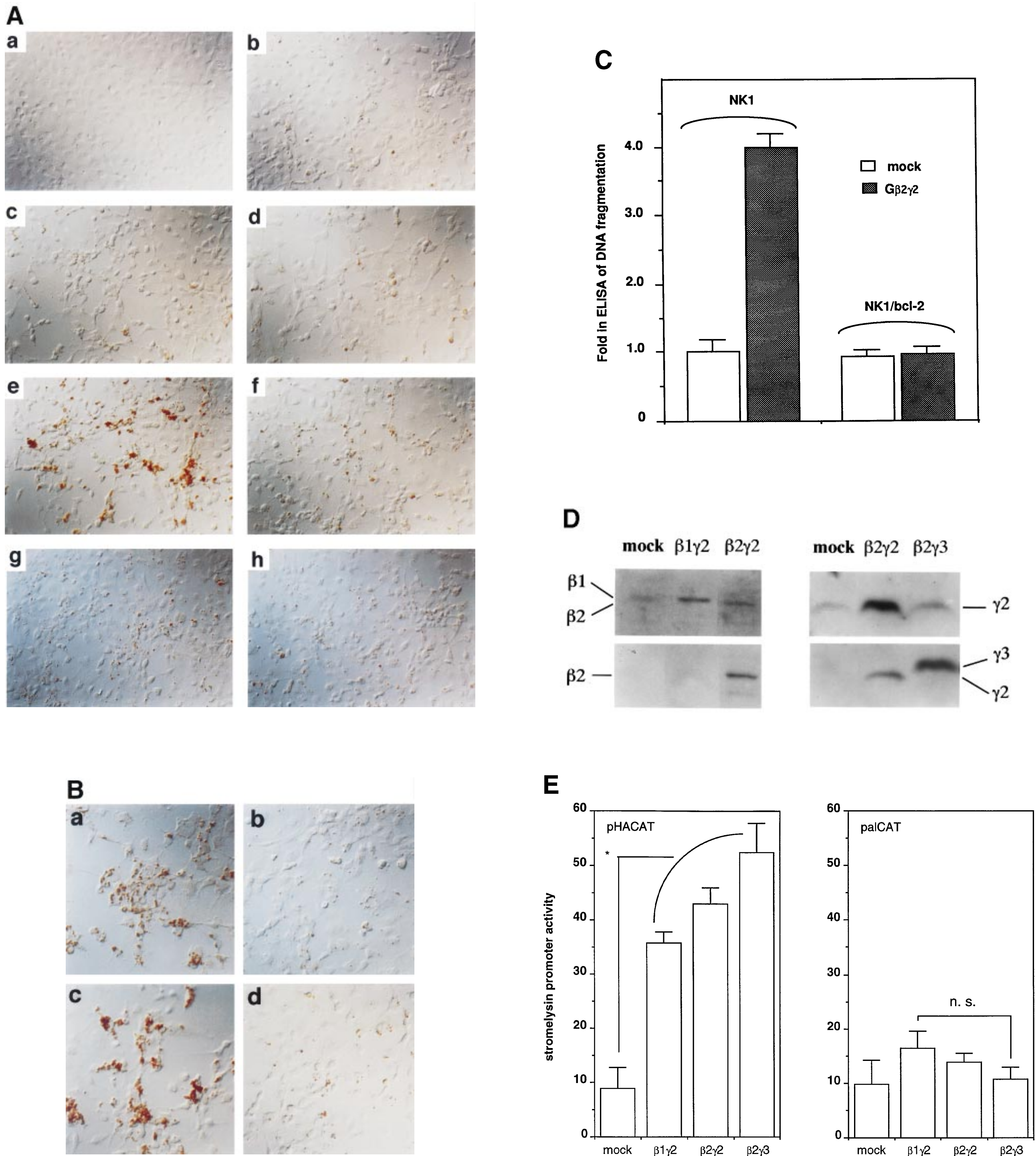

Fig. 3. Co-transfection of Gβγ cDNAs induces apoptosis. (**A**) NK1 cells were co-transfected with Gβ and Gγ cDNAs [0.25 μg each; Gβ1γ2 (c), Gβ1γ3 (d), Gβ2γ2 (e), Gβ2γ3 (f), Gβ3γ2 (g) and Gβ3γ3 (h)]. At 48 h after transfection, fragmented DNA was stained *in situ* by TUNEL. Nucleosomally fragmented DNA is stained brown in these figures. As controls, cells were transfected with 0.5 μg LacZ (b) or no transfection procedure (a). (**B**) Reproducible DNA fragmentation by Gβ2γ2. Cells were transfected with Gβ2γ2 cDNAs (a and c) or Gβ2γ3 cDNAs (b and d). After transfection, nucleosomally fragmented DNA was stained *in situ* by TUNEL. Note that transfected cells started to detach from a plate at 48 h after transfection of Gβ2γ2 (see text). Experiments were done independently of those shown in (A). (**C**) Effects of bcl-2 on Gβ2γ2-induced DNA fragmentation. Parental NK1 cells and NK1 cells stably expressing bcl-2 (NK1/bcl-2) were transfected with or without Gβ2γ2 cDNAs; 48 h after transfection, DNA fragmentation was measured by ELISA and indicated as the fold of DNA fragmentation observed in mock-transfected NK1 cells (100% indicates 0.333 in this figure). The data are the means ± SE of three independent transfections. In these experiments, the expression levels of transfected Gγ2 were similar between NK1 and NK1/bcl-2 cells. (**D**) Expression of Gβ and Gγ in Gβγ co-transfection. Cells were transfected with Gβγ cDNAs (as indicated) under the same conditions as in (A), and cell lysates were immunoblotted with anti-Gβ common antibody (left upper panel), anti-Gβ2-specific antibody (left lower panel), anti-Gγ2-specific antibody (right upper panel) or anti-Gγ3 antibody (right lower panel). The weak cross-reactivity of anti-Gγ3 antibody for Gγ2 was suggested by the fact that the antigen peptide for this antibody is 50–60% identical to that for anti-Gγ2 antibody. (**E**) Effects of Gβγ co-transfection on the promoter activities of stromelysin. Cells were transfected with either Gβ1γ2, Gβ2γ2 or Gβ2γ3 cDNAs with pHACAT (the stromelysin promoter –1303 to –754) or palCAT (the stromelysin promoter –1218 to –1202), and CAT activity was indicated as mU/well. *P <0.01. n.s., not significant.

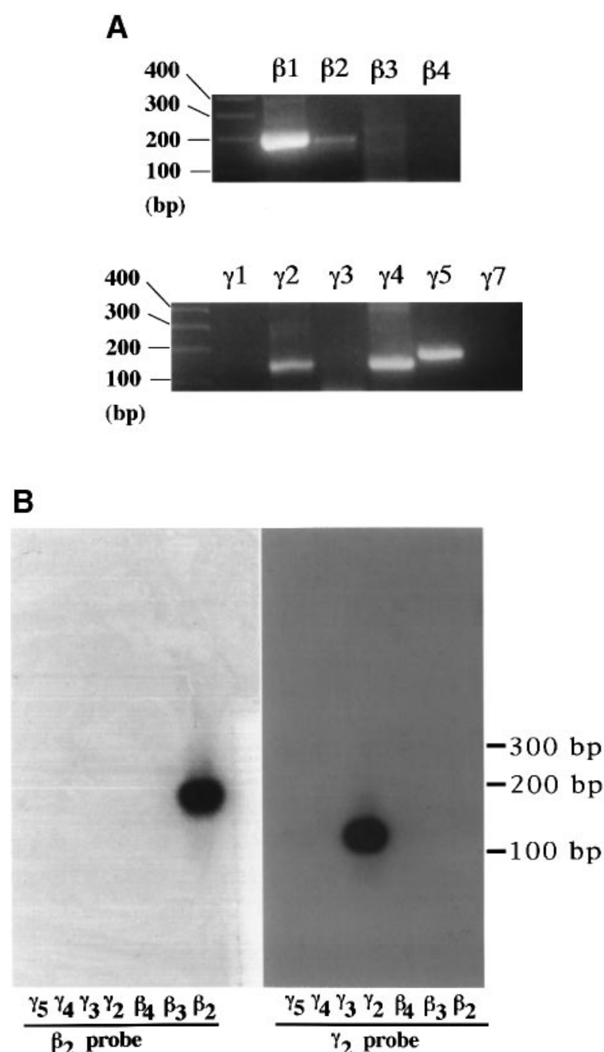

Fig. 4. Amplification of G protein β and γ subunit fragments from NK1 cells. (**A**) Amplified DNA fragments of the cDNA of Gβ subunits (upper panel) and Gγ subunits (lower panel) were separated on an agarose gel and visualized by staining with ethidium bromide. Numbers on the left indicate molecular weight standards in base pairs. The figures are representative of three independent experiments with similar results. (**B**) The amplified PCR fragments were separated on an agarose gel and transferred onto Southern blotting sheets. The sheets were incubated with labeled probes specific for Gβ2 (left panel) and Gγ2 subunits (right panel).

band of Gγ2. These data demonstrate the endogenous expression of Gβ2 and Gγ2 in NK1 cells, although there is a possibility that the negative subunits were present but could not be detected by the primers used (originally designed for rat subunit detection, but selected from well-conserved regions).

As the major purpose of this study was to specify the G_o subunit that executes V642I APP-induced apoptosis in NK1 cells, in which V642 APP-induced apoptosis is phenotypically linked to the FAD trait (Yamatsuji *et al.*, 1996a), it seemed to be beyond our aim to investigate the generality of Gβγ-induced cell apoptosis. However, we screened various cell lines to observe whether they were susceptible to Gβγ-induced apoptosis. The Gβ2 and Gγ2 cDNAs were transfected to HEK293, CHO, Rat-1 and the usual COS-7 cells. Despite considerable co-expression of Gβ2 and Gγ2, virtually no apoptosis occurred under the same conditions as used in the present study (data not shown). Hence, it was likely that these non-neuronal cells lack the downstream machinery for Gβ2γ2-induced apoptosis. However, we could not totally exclude the possibility that, although those cells were susceptible to Gβ2γ2-induced apoptosis, the quantitative duration of exogenous Gβ2γ2 expression was not sufficient or its time profile was not appropriate to cause them to undergo apoptosis. We also transfected the Gβ2 and Gγ2 cDNAs into neuronal cell lines, PC12, F11 and Ntera-2. However, none of these cells allowed for co-expression of Gβ and Gγ subunits under our experimental conditions set for NK1 cells (data not shown). We were not able, therefore, to assess whether the Gβ2γ2 complex can induce apoptosis in neuronal cells. It has been reported recently that the V642 type of FAD mutants of APP cause F11 cells to undergo apoptotic death (Yamatsuji *et al.*, 1996b). Therefore, the investigation of whether co-expressed Gβ2 and Gγ2 can kill F11 cells would be especially important.

Discussion

Expression of the FAD-associated V642I APP causes NK1 cells to undergo apoptosis in a PTX-sensitive manner (Yamatsuji *et al.*, 1996a). Wild-type APP activates G_o but not G_i in a ligand-dependent manner (Okamoto *et al.*, 1995). All three V642 mutants, V642I, V642F and V642G, of APP have the molecular function of constitutively activating G_o through the region involved in the ligand-dependent activity of APP (Ikezu *et al.*, 1996; Okamoto *et al.*, 1996). These observations indicate that V642 mutants of APP activate G_o and induce PTX-sensitive apoptosis in NK1 cells. Upon stimulation, G proteins dissociate into two functional moieties, Gα and Gβγ. Here we indicate that (i) expression of mutationally activated $G\alpha_o$ or $G\alpha_i$ induced no apoptosis in NK1 cells; (ii) multiple strategies designed to block the functions of Gβγ antagonized NK1 apoptosis by V642I APP; (iii) co-expression of Gβγ cDNAs caused NK1 apoptosis in a subtype-specific manner; and (iv) NK1 cells express endogenous Gβγ subunits that are able to mediate apoptosis. These findings implicate Gβγ complexes as the effector of V642I APP to trigger apoptosis in our system. As V642I APP activates the trimeric form of G_o through His657–Lys676 (Okamoto *et al.*, 1996), V642I APP should activate the trimeric form of G_o and release Gβγ, which then turns on the pathway for apoptosis. In further support of this concept, V642I APP fails to induce apoptosis without His657–Lys676 (Yamatsuji *et al.*, 1996a,b).

Although the significance of apoptosis in AD has not been finally established, a number of recent studies (Su *et al.*, 1994; Dragunow *et al.*, 1995; Lassmann *et al.*, 1995; Smale *et al.*, 1995) have all agreed that apoptosis associated with DNA fragmentation is the major feature in the sporadic form of AD (see Nishimoto *et al.*, 1997 for the significance of apoptosis in AD). Studies that examine DNA fragmentation in the brain from FAD patients carrying V642 mutations in APP have not been reported. Although little apoptosis has occurred in the brain in V642F APP-overexpressing transgenic mice, in which Aβ amyloidogenesis is the major feature in pathology (Games *et al.*, 1995), these mice have scarcely developed the signs and symptoms of AD. This finding

does not conflict with, but potentially supports, the positive interactions between apoptosis and AD development. The relationship between apoptosis and FAD genes has also been suggested by the studies of D'Adamio and colleagues; Vito *et al.* (1996) reported that the 103 residue portion of presenilin-2, another FAD gene product located at human chromosome 1, inhibits apoptosis in T cells; Wolozin *et al.* (1997) reported that APP and presenilin-2 induce PTX-sensitive apoptosis in PC12 cells. Although a study using neuronal cells comparable with the present research may be required in the future, no neuronal system has allowed examinations comparable with those of the present study, including transient co-expression of Gβγ. Although we recently have established a neuronal system where nucleosomal DNA fragmentation is induced by transient expression of the three FAD-linked mutants of APP (Yamatsuji *et al.*, 1996b), so far we have not been able to co-express Gβ and Gγ cDNAs in that system. We emphasize, however, that the observed apoptosis in NK1 cells is phenotypically linked to the FAD trait, because three FAD-associated APP mutants cause the highest incidence of apoptosis among all of the possible 19 mutants at V642 and wild-type APP (Yamatsuji *et al.*, 1996a). Therefore, the Gβγ action implicated in this apoptosis by FAD-associated APP should be relevant to the mechanism linked to FAD.

This study also provides direct evidence that Gβγ expression triggers apoptosis. Gβ2γ2-induced apoptosis was subtype-specific and regulated by bcl-2. So far, Gβ2γ2-induced apoptosis in COS cells has not been reported. The positive data in NK1 cells could be attributable to cellular differences between NK1 and other COS cells. In strong support of this, expression of the FAD mutants causes NK1 cells but not the usual COS cells to undergo apoptosis (Yamatsuji *et al.*, 1996a), and NK1 cells express tissue-specific proteins such as $G\alpha_o$ and Gγ2 (Yamatsuji *et al.*, 1996a; this study) that other COS cells do not express (Katz *et al.*, 1992). In addition, co-expression of Gβ2 and Gγ2 cDNAs in the usual COS-7 cells caused no DNA fragmentation. It is therefore highly likely that NK1 cells also express the cell-specific downstream target of Gβ2γ2 for apoptosis. Diverse effectors or effector systems of Gβγ have been identified: adenylyl cyclases (Iñiguez-Lluhi *et al.*, 1992), βARK family kinases (Inglese *et al.*, 1992), phospholipase C-β (Camps *et al.*, 1992; Katz *et al.*, 1992), K^+ channels (Reuveny *et al.*, 1994), phosphatidylinositol 3-kinases (Stephens *et al.*, 1994; Thomason *et al.*, 1994), Ras/mitogen-activated kinases (Crespo *et al.*, 1994) and stress-activated protein kinases (Coso *et al.*, 1996). No functional differences between Gβ1γ2 and Gβ2γ2 have so far been specified for activation of these known targets, suggesting that a hitherto unidentified target pathway(s) may be involved in the Gβ2γ2-induced apoptosis.

The present data, particularly the data obtained from the C-terminus of βARK, demonstrate the involvement of Gβγ in apoptosis by V642I APP. However, we emphasize that it remains unknown whether and how greatly Gβ2γ2 is involved in V642I APP-induced apoptosis. The scarcity of endogenous Gβ2 and Gγ2 may suggest that other Gβγ subtypes may mediate this apoptosis. The fact that there was an apparent difference in the expression levels between native and transfected Gβ2γ2 may suggest that the Gβγ implicated in apoptosis by V642I APP is different from Gβ2γ2. However, it is nearly impossible to measure the real concentration of Gβ2γ2 and compare the concentration of transfectionally expressed Gβ2γ2 with that of the native complex, because inside the cell, there are various Gβγ complexes, different from Gβ2γ2, that consist of either Gβ2 or Gγ2. In addition, whereas native Gβγ is post-translationally modified, considerable fractions of transfectionally overexpressed Gβγ may stay unmodified; the modification of Gβγ critically affects its workings (Maltese and Robishaw, 1990; Kisselev *et al.*, 1995). Therefore, we cannot simply compare the functional amounts of Gβγ between native and overexpressed complexes by measuring their concentrations, even if such measurement becomes possible. For the same reason, we cannot exclude the possibility that only a small fraction of transiently expressed Gβ2γ2 is accessible to its putative target for apoptosis. Alternatively, only prolonged activation of the minor Gβ2γ2 by V642I APP may be able to induce apoptosis. This idea fits well with the accompanying result that expression of Gβ1γ2 or Gβ1γ3 did not induce apoptosis. They are the major Gβγ in the brain (Wilcox *et al.*, 1994), which could transiently be released intracellularly from G proteins in response to many neurotransmitter stimulations, which do not induce death of neurons. Apoptosis not by a major Gβγ but by a minor one could allow for specific cell death by the signal that constitutively activates G proteins.

In summary, we conclude that activation of G_o by V642I APP results in the generation of two distinct signals, $G\alpha_o$ and Gβγ (Figure 5). $G\alpha_o$ turns on its proper signaling pathways; it negatively regulates transactivation of CRE (Ikezu *et al.*, 1996), which potentially contributes to long-term memory disturbance and synaptic malplasticity (Frank and Greenberg, 1994). In a parallel manner, specific Gβγ complex released from G_o should transmit the signal for apoptosis, which most likely causes organic degeneration. As V642F and V642G APPs can also activate G_o with similar potencies to that of V642I APP (Okamoto *et al.*, 1996) and their G_o-activating domains are identical, this model is applicable to all three mutants of APP associated with FAD. The signal of the V642 mutants of APP thus diverges at the level of G proteins into at least two distinct messages. These G protein subunits probably activate many other effectors and produce much wider spectra of cellular and tissue responses. Such signaling divergence could contribute to a mechanism generating complicated pathophysiology in FAD.

Materials and methods

All Gα constructs were described previously (Ikezu *et al.*, 1994, 1995; Strittmatter *et al.*, 1994). The cDNAs of βARK1-CT and βARK1, both in pRK5, corresponding to the 495–689 and the entire region of human βARK1, respectively, were kindly provided by Dr R.J.Lefkowitz. The βARK1-CT cDNA was described previously (Koch *et al.*, 1994a). To construct the cDNA encoding βARK1-NT, PCR was employed using βARK1 cDNA as a template with the sense oligonucleotide AAATTTG-AATTCTGAGCATGGCCATGTGAGAAT, and the antisense nucleotide AAATTTTCTAGATTATTTTGTGTCCTCCTCATCAAAG. The sense oligonucleotide was designed to possess an additional *Eco*RI restriction site, whereas the antisense oligonucleotide was given an additional *Xba*I restriction site together with the termination codon. The PCR product was digested with *Eco*RI and *Xba*I, and then subcloned into pcDNA-1. Sequencing confirmed that the PCR-driven part did not contain unwanted

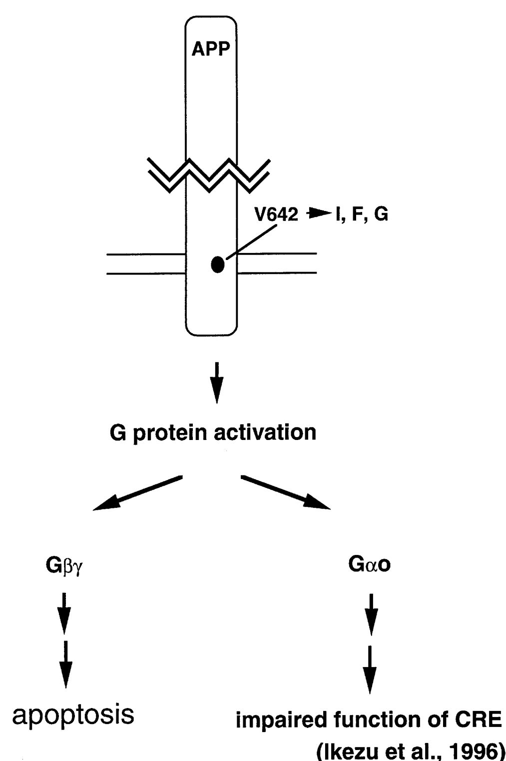

Fig. 5. Diagram of divergence of V642 APP mutant signal at the G protein level. This study suggests that V642I APP-induced apoptosis involves Gβγ subunits. In a different study (Ikezu *et al.*, 1996), we have shown that this FAD mutant of APP induces CRE suppression through $G\alpha_o$. Therefore, the generated signal of V642I APP diverges at the level of G proteins into two distinct moieties having different intracellular roles, suggesting the versatile function of the FAD-associated mutants of APP.

mutations. Gβ1, Gβ2 and Gγ2 cDNAs (Katz *et al.*, 1992), Gβ3 cDNA (Levine *et al.*, 1990) and Gγ3 cDNA (Gautam *et al.*, 1990) were described in the indicated literature. The Gβ1, Gβ2 and Gβ3 cDNAs were inserted in pcDNA-1.

Transient transfection was done with Lipofectamine, as described previously (Yamatsuji *et al.*, 1996a). Briefly, NK1 cells were seeded at 4×10^4/well in a 24-well plate and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and antibiotics. Cells were then exposed to DNA transfection using Lipofectamine in DMEM without serum. Unless otherwise specified, 0.5 μg of cDNA (in total) and 1 μl of Lipofectamine were used for each well. After 24 h serum-free culture, media were changed to DMEM with 1% calf serum. After another 24 h culture, cells were fixed and submitted to the assays. βARK1-CT cDNA was stably transfected with pBabe/puro (puromycin resistance gene) into NK1 cells by calcium phosphate precipitation, as described previously (Yamatsuji *et al.*, 1996a). Cells were selected by puromycin resistance and amplified for further usage. Cells stably transfected with βARK1-NT cDNA or pBabe/puro alone were similarly established. NK1/bcl-2 cells were as described previously (Yamatsuji *et al.*, 1996a).

All assays for apoptosis (immunohistochemical analysis with nuclear staining, TUNEL assay and ELISA of fragmented DNA) were performed using the same protocols as described previously (Yamatsuji *et al.*, 1996a). In brief, for immunohistochemical analysis, NK1 cells were transfected with wild-type APP_{695}, V642I APP or GTPase-deficient Gα mutants and, 48 h after transfection, cells were fixed, incubated with phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA) and 5% calf serum for 1 h, and stained with anti-APP antibody (0.5 μg/ml 22C11) followed by Texas red-labeled anti-mouse IgG (1/100) or anti-Gα antibody [rabbit anti-$G\alpha_o$ antibody at 1/500 and rabbit anti-$G\alpha_i$ antibody at 1/100 (both UBI)] followed by rhodamine-labeled anti-rabbit IgG (1/100) (the first and second antibody for each 1 h). The samples were stained with acridine orange and examined with a fluorescence microscope. Apoptosis was assessed with the nuclear changes defined as apoptosis, nuclear condensation, fragmentation and compaction (Kerr and Harmon, 1991). The incidence of apoptosis in cells expressing the transfectant was then measured and indicated as transfectant-specific by subtracting the incidence of apoptosis in non-transfectant-expressing cells (background apoptosis) in the same sample. In all experiments, the incidence of background apoptosis was ~20%, as described previously (Yamatsuji *et al.*, 1996a), which was induced by transfection procedures and 2 day serum starvation.

To measure the effect of Gβγ co-transfection on the promoter activity of stromelysin, cells were transfected with Gβγ cDNAs with pactβgal and either pHACAT containing the stromelysin promoter –1303 to –754 or palCAT containing the stromelysin promoter –1218 to –1202 (Gβ, Gγ, CAT reporter, pactβgal: 0.5, 0.5, 0.3, 0.2 μg, respectively; this condition allowed expression of Gβ and Gγ comparable with that seen in other experiments without reporter co-transfection). CAT assay was performed, as described (Ikezu *et al.*, 1994). The two stromelysin promoter CAT constructs (Sanz *et al.*, 1994) were kindly provided by Dr J.Moscat. For detection of Gγ immunoreactivity, we used Tris-Tricine gel electrophoresis, as described previously (Schagger and von Jagow, 1987). Cell lysates were immunoblotted with anti-Gβ common antibody (MS/1, 1/500), anti-Gβ2-specific antibody (1/100, Santa Cruz), anti-Gγ2-specific antibody (1/100, Santa Cruz) or anti-Gγ3 antibody (1/100, Santa Cruz).

Reverse transcriptase–PCR of Gβγ subunits in NK1 cells was done as described by Kalkbrenner *et al.* (1995) using the same PCR primers. NK1 cells (~10^6 in a 100 mm dish) were collected in PBS, washed and immediately frozen at –80°C. mRNA was prepared and measured using kits from Invitrogen, and was reverse transcribed into cDNA using a kit from Stratagene. A fragment of each subunit was amplified using subtype-specific primers in the PCR with 40 cycles. *Taq* polymerase was from Perkin Elmer. For Southern blot analysis of the PCR bands, the PCR fragments of Gβ and Gγ subunits amplified from the NK1 cell mRNA were transferred onto Hybond-N (Amersham). The Gβ2 oligonucleotides used for the PCR reaction were in position 223–245 at the 5′ end, and position 396–411 at the 3′ end: CAGATCACA-GCTGGGCTGGA and AGCTGTCCCAGATGATGAGC, respectively. These primers were designed from the sequences within the primers used for the RT–PCR experiment, to ensure the specificity of the Southern blot data. To probe the Gβ2 subunit, we used the PCR product obtained from these oligonucleotides, which gave us a 160 bp fragment of Gβ2. The Gγ2 oligonucleotides were in position 139–158 of the 5′, and 238–219 of the 3′ end: AGCATAGCACAAGCCAGGAA and AGTAGGCCATCAAATCTGCA, which gave us a 110 bp PCR fragment of Gγ2. We amplified these oligonucleotides by incubating them (2 pg of each) with 10 ng of human Gβ2 cDNA (for the Gβ2 probe) or 10 ng of bovine Gγ2 cDNA (for the Gγ2 probe) in the presence of 2 mM $MgCl_2$ and 0.8 nM dNTPs for 40 cycles. The probes were purified from the corresponding bands in a 2% low-melting agarose gel with a resin purification kit (Promega). We then labeled 25 ng of each probe with [α-^{32}P]dCTP and used them in overnight hybridization of each filter, blotted with the PCR products of Gβ and Gγ subunits. The filters were washed extensively under highly stringency conditions and exposed to X-ray films. All other materials used in this study were described previously (Yamatsuji *et al.*, 1996a) or obtained from commercial sources. Statistical analysis was performed by Student's *t*-test.

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