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Intrinsic signaling function of APP as a novel target of three V642 mutations linked to familial Alzheimer's disease

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APP_{695} is a transmembrane precursor of Aβ amyloid. In familial Alzheimer's disease (FAD), three mutations V642I/F/G were discovered in APP_{695}, which has been suggested by multiple studies to be a cell surface signaling receptor. We previously reported that normal APP_{695} encodes a potential G_o-linked receptor with ligand-regulated function and that expression of the three FAD mutants (FAD-APPs), not normal APP, induces cellular outputs by G_o-dependent mechanisms. This suggests that FAD-APPs are constitutively active G_o-linked receptors. Here, we provide direct evidence for this notion. Reconstitution of either recombinant FAD-APP with G_o into vesicles induced activation of G_o, which was inhibitable by pertussis toxin, sensitive to Mg^{2+} and proportional in quantity to the reconstituted amounts of FAD-APP. Consistent with the dominant inheritance of this type of FAD, this function was dominant over normal APP, because little activation was observed in APP_{695}–G_o vesicles. Experiments with antibody competition and sequence deletion indicated that His657–Lys676 of FAD-APP, which has been specified as the ligand-dependent G_o-coupling domain of normal APP, was responsible for this constitutive activation, confirming that the three FAD-APPs are mutationally activated APP_{695}. This study identifies the intrinsic signaling function of APP to be a novel target of hereditary Alzheimer's disease mutations, providing an *in vitro* system for the screening of potential FAD inhibitors.

Keywords: amyloid precursor protein/familial Alzheimer V642 mutations/G protein coupling/mutationally activated molecule

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease, and is characterized pathologically by the presence of extracellular Aβ amyloid plaques. Amyloid precursor protein (APP) is the transmembrane precursor of Aβ amyloid (Kang *et al.*, 1987). There are at least 10 isoforms of APP resulting from alternative splicing of a single gene. APP_{695}, consisting of 695 amino acids, is expressed predominantly in neuronal tissues (Neve *et al.*, 1988). Evidence that abnormality of APP causes AD has been provided by missense mutations found in early-onset familial AD (FAD). In some patients with FAD, three mutations have been discovered at V642 in the transmembrane domain of APP_{695} (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Naruse *et al.*, 1991; Murrell *et al.*, 1991; Yoshioka *et al.*, 1991): mutations to Ile, Phe or Gly (the corresponding APP_{695} mutant is referred to as Ile-, Phe- or Gly-APP). Genetic study has clarified that these mutations co-segregate with the AD phenotype (Karlinsky *et al.*, 1992). Furthermore, Games *et al.* (1995) have reported that overexpression of Phe-APP mimics the neuropathology of AD in transgenic mice. Therefore, these types of APP mutants are the first established cause of AD. However, it remains unclear how they cause AD and what abnormality these mutations induce in APP. Only an increase in secretion of Aβ1–42, a longer version of Aβ peptides which is the possible cause of senile plaque formation in AD, has been reported with these mutants (Suzuki *et al.*, 1994). However, most importantly, the role of amyloid plaque formation in the pathophysiology of AD remains totally unknown.

APP has an architecture (Kang *et al.*, 1987), cellular orientation and localization (Weidemann *et al.*, 1989; Schubert *et al.*, 1991) all similar to those of cell surface receptors. Fiore *et al.* (1995) have shown that Fe65 protein, which is partially homologous to the oncogenic signal transducer Shc, binds to the intracellular domain of APP, suggesting that APP has not only the structure but also the function of a cell surface signaling receptor. Earlier, we (Nishimoto *et al.*, 1993) found that the His657–Lys676 peptide of APP selectively activates G_o *in vitro* and that APP_{695} forms a complex with G_o through this cytoplasmic domain in a receptor-mimetic manner. We and another group (Lang *et al.*, 1995) confirmed that the His657–Lys676 peptide of APP activates G_o *in vivo*. Since G_o is a member of the heteromeric G protein family that serves as a signal transducer of cell surface receptors, these findings strongly suggest that APP_{695} is a G_o-linked receptor. This notion is consistent with the fact that APP and G_o as well as GAP-43 co-localize in growth cones and presynapses of neurons (Strittmatter *et al.*, 1990; Ferreira *et al.*, 1993). In further support, it has been shown independently that APP (Breen *et al.*, 1991; LeBlanc *et al.*, 1992; Milward *et al.*, 1992; Jin *et al.*, 1994; Small *et al.*, 1994; Allinquant *et al.*, 1995; Zheng *et al.*, 1995) and G_o (Schuch *et al.*, 1989; Doherty *et al.*, 1991; Sebok *et al.*, 1993; Strittmatter *et al.*, 1994) are involved in virtually

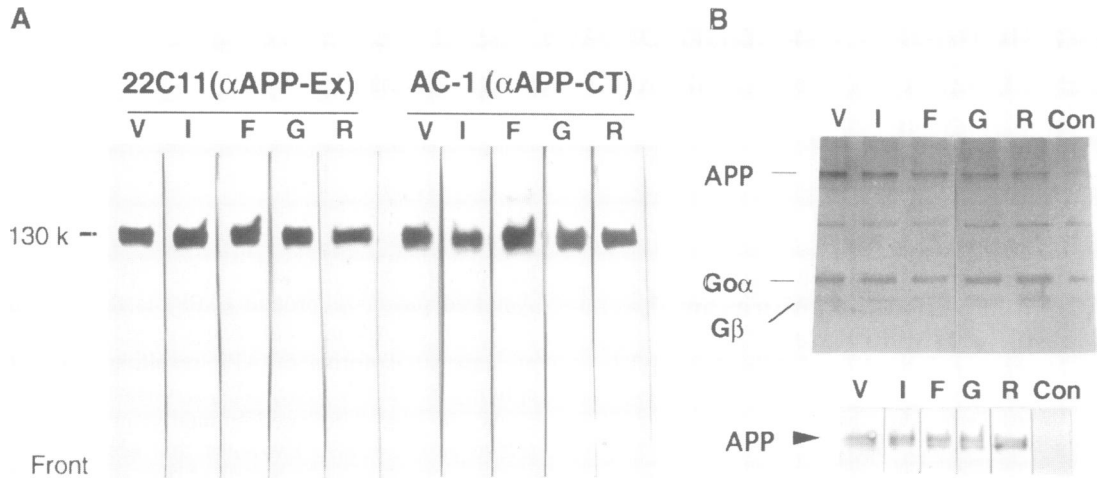

Fig. 1. Vesicles of FAD-linked mutants of APP_{695}. (**A**) Immunoblot analysis of baculovirus-made recombinant APPs. Purified normal APP_{695} (V), FAD-APPs (I, F and G), and Arg-APP (R) were each analyzed by immunoblotting with 22C11 (monoclonal APP ectodomain antibody, 1.0 μg/ml) or AC-1 (polyclonal C-terminus antibody, 1/1000). (**B**) Upper panel: silver staining of reconstituted vesicles. Wild-type APP_{695} (V), three FAD-APPs (I, F and G) or Arg-APP (R), shown in (A), were each reconstituted (each 30 pmol) with trimeric G_o (10 pmol) into phospholipid vesicles. The 130 kDa protein corresponds to recombinant APPs and the 39 kDa protein was $G\alpha_o$, whereas Gβ was weakly stained, which is usually the case with silver staining. Different exposures to the staining ensured that similar amounts of Gβ were reconstituted. As another control, a preparation obtained from mock-infected insect cells (Con) was reconstituted similarly with G_o. Except for APPs and G_o, an 80 kDa protein was the only reproducible band in these vesicles. Lower panel: immunoblot analysis of reconstituted vesicles. Vesicles reconstituted with G_o and either APP_{695} (V), three FAD-APPs (I, F and G), Arg-APP (R) or the mock-infected control preparation (Con) were probed with 1.0 μg/ml 22C11.

identical functions of neurons, such as neurite outgrowth, synaptic contact and cell–cell adhesion. As Growth-Associated Protein (GAP)-43 acts as a G_o-coupled receptor-specific potentiator (Strittmatter *et al.*, 1993), co-localization of GAP-43 further strengthens this theory. Also, Ferreira *et al.* (1993) indicated the presence of APP in clathrin-coated vesicles in hippocampal neurons, lending additional credence to the notion that APP is a functional receptor. Recently, we have provided direct evidence that APP_{695} encodes a G_o-coupled receptor by demonstrating that this APP activates purified G_o in response to anti-APP monoclonal antibody in reconstituted vesicles (Okamoto *et al.*, 1995). Thus, APP_{695} is a potential G_o-coupled receptor with ligand-regulated function, although it is currently unknown what physiological role is played by this function.

Then, what happens to this signaling function of APP_{695} due to the three FAD mutations? Since the trait of FAD linked to these mutations is dominantly transmitted (Karlinsky *et al.*, 1992), the FAD-linked V642 APP mutants (FAD-APPs) would behave as constitutively active or dominantly negative receptors. In strong support of the former possibility, we have two different and independent lines of *in vivo* evidence. Firstly, we found that all three FAD-APPs, but not normal APP, induce apoptotic cell death when they are expressed in a G_o-expressing COS cell NK1 clone (Yamatsuji *et al.*, 1996). This action is most likely mediated by G_o, because Ile-APP-induced apoptosis is inhibited by pertussis toxin (PTX), by dominant-negative $G\alpha_o$, and by deletion of the G_o-activating domain His657–Lys676 from Ile-APP, and also because it did not occur in normal G_o-lacking COS cells. Secondly, we have demonstrated that cellular expression of three FAD-APPs, but not normal APP, results in tonic suppression of transcriptional activity of the cAMP response element (CRE) in a PTX-sensitive, $G\alpha_o$-dependent manner (Ikezu *et al.*, 1996). In the same study, we have also shown, by using $G\alpha_s$ chimeras, that Phe-APP recognizes the C-terminal five residues of $G\alpha_o$ selectively among the $G\alpha_i$ family in a constitutive manner. These findings provide *in vivo* evidence that FAD-APPs are mutationally activated APPs that accelerate G_o activity even in the absence of the ligand. However, there has been no *in vitro* study that examines this hypothesis directly. For this purpose, the present study was conducted, and we have shown that the FAD mutations turn on the G-coupling function intrinsic in normal APP, providing the first evidence that the signaling function of APP is a direct molecular target of the three FAD mutations. We discuss the implications of this finding with respect to the pathophysiology of AD.

Results

We constructed baculoviruses encoding normal APP_{695} and FAD-APPs, expressed each of them in Sf21 cells by infecting the cognate viruses and purified the cognate recombinant proteins by the method described previously (Okamoto *et al.*, 1995). Since soluble forms of APP were possible contaminants, particulate fractions of the infected cells were used as starting materials to ensure purification of full-length APPs. It has been reported that Sf cells infected with baculovirus encoding a full-length APP secrete truncated APP, which lacks its C-terminus (Knops *et al.*, 1991). However, the purified 130 kDa APP_{695} and FAD-linked mutants were all immunoreactive with the C-terminus antibody AC-1 and the ectodomain antibody 22C11 (Figure 1A), confirming that the purified APPs were full-length. The preparations of normal APP_{695} or FAD-APPs as well as a control preparation, obtained from mock-infected insect cells by similar protocols, were each reconstituted with trimeric G_o into phospholipid vesicles. The upper panel of Figure 1B shows the silver staining of these vesicles. A major contaminant in these vesicles was an 80 kDa protein: it was immunoreactive with neither

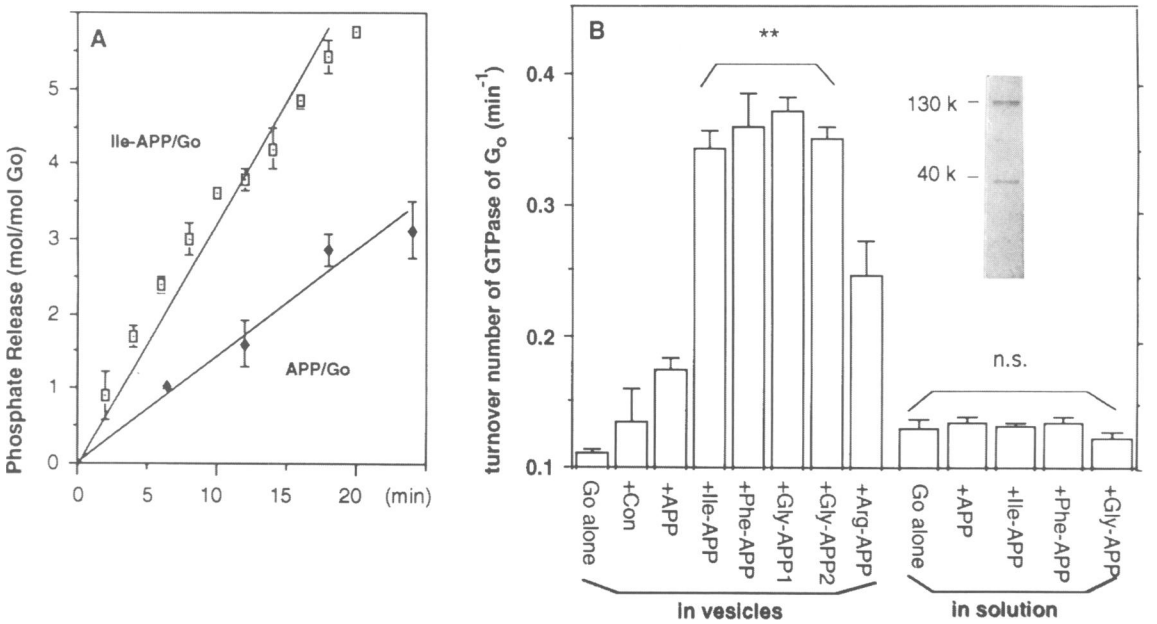

Fig. 2. Constitutive activation of G_o by reconstituted FAD-APP. (**A**) Time course of GTPase activity of G_o in Ile-APP–G_o vesicles. Ile-APP–G_o vesicles were incubated with [γ-^{32}P]GTP in the presence of 20 μM Mg^{2+} at 37°C for the indicated periods, and the radioactivity of phosphate released was measured. As a control, APP_{695}–G_o vesicles were also examined. All values are the means ± SE of at least three independent experiments. (**B**) In the eight columns on the left, the turnover numbers of GTPase activity of G_o promoted by FAD-APPs in various reconstituted vesicles are shown. As in the legend to Figure 1B, recombinant APPs and G_o were reconstituted in vesicles. Each vesicle was incubated with [γ-^{32}P]GTP and the turnover number of GTPase activity of G_o was measured. In parallel experiments, similar amounts of reconstituted APPs were ensured by immunoblot analysis of each vesicle with 22C11. In this analysis, G_o in each lane was set to the same concentration, as assessed by maximal GTPγS binding. In the lane labeled +Gly-APP2, Gly-APP–G_o vesicles which contained little of the 80 kDa protein, whose silver staining is shown in the inset, were used. The data are the means ± SE of at least three assays for each vesicle. $^{**}P<0.05$ versus APP. Inset: silver staining of Gly-APP2 vesicles. Different exposures ensured the presence of Gβ. In the five columns on the right, without reconstitution into vesicles, purified APP_{695} or each APP mutant (each 10 nM) was mixed with trimeric G_o (3.3 nM) in 20 mM HEPES/NaOH (pH 7.4), 20 μM $MgCl_2$, and 100 nM [γ-^{32}P]GTP, and the turnover number of GTPase activity was measured. These concentrations of APPs and G_o were the same as those used for the reconstitution of FAD-APP–G_o vesicles in the left eight columns. Since approximately half of the APP was incorporated into vesicles, the concentrations of FAD-APPs used in the right five columns were estimated to be ~2-fold higher than those in the left eight columns. n.s., not significant versus G_o alone.

AC-1 nor 22C11, and was thought to be derived from insect cells because it was present in the control preparation obtained from mock-infected cells (Figure 1B, Con), but not in vesicles reconstituted with purified G_o alone (G_o-alone vesicles) (data not shown). Because of its relative abundance in insect cells and somewhat similar properties to APP, we were not able to isolate FAD-APPs completely. Its identity was not investigated further. However, multiple lines of evidence outlined below demonstrate that this 80 kDa contaminant was without effect on G_o (also see Discussion). The control vesicles reconstituted with G_o and a preparation obtained from mock-infected insect cells also contained a trace amount of a 130 kDa protein; but we could not identify it. It was not immunoreactive with either 22C11 or AC-1 (Figure 1B, lower panel). For this reason, we assessed the amount of FAD-APP by immunoblot assay.

In the reconstituted APP_{695}–G_o vesicles, the turnover number of GTPase activity of G_o was assessed to be 0.17 ± 0.01/min (mean ± SE, n = 3, the same conditions are used in the following), as we reported previously (Okamoto *et al.*, 1995). The turnover number of the G_o-alone vesicles was 0.11 ± 0.01/min, which represents the basal activity of G_o, consistent with the values reported previously. The turnover number in the control vesicles (reconstituted with G_o and the mock-infected control preparation) was 0.13 ± 0.03/min. The slight but significant increase in basal G_o activity with APP_{695} reconstitution is in a good agreement with the notions that APP_{695} encodes a G_o-coupled receptor (Okamoto *et al.*, 1995) and that there is a basally active fraction in G-coupled receptors (Samama *et al.*, 1993). Here, it was clear that control vesicles and APP_{695}–G_o vesicles showed no and minor activation of G_o, respectively.

In the reconstituted Ile-APP–G_o vesicles, a 2- to 2.5-fold higher activity in the turnover number of G_o was observed over the basal G_o activity in the APP_{695}–G_o vesicles (Figure 2A). This stimulation level of G_o in the Ile-APP–G_o vesicles was equivalent to >3-fold of the G_o activity observed in the G_o-alone vesicles. GTPase activity was negligible in vesicles reconstituted with Ile-APP alone or APP_{695} alone (data not shown), indicating that no possible contaminants in these vesicles were G proteins and that the G protein activity was attributable to the reconstituted G_o.

Pre-treatment of the Ile-APP–G_o vesicles with PTX significantly reduced the promoted activity of G_o. The turnover number of G_o activity in PTX-treated Ile-APP–G_o vesicles was 0.15 ± 0.01, whereas that in untreated Ile-APP–G_o vesicles was 0.34 ± 0.01 under the same condition (mean ± SE, n = 3). Note that the former value was almost identical to basal G_o activity in APP_{695}–G_o vesicles, implying that the effect of PTX was complete. These data provide an additional, independent line of

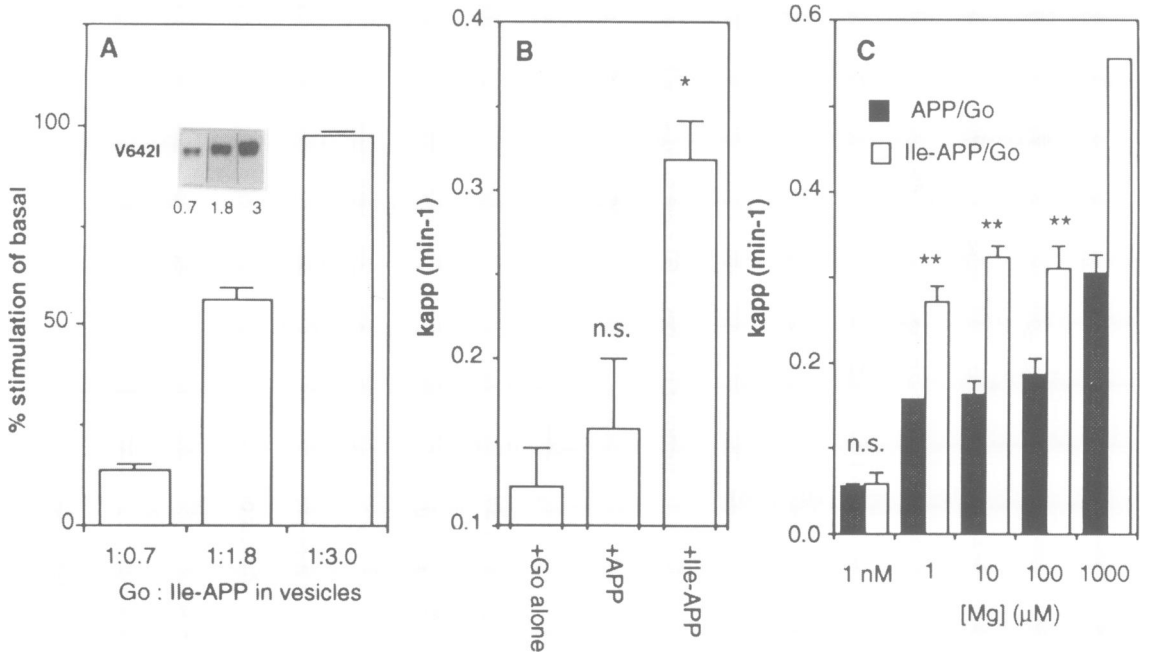

Fig. 3. Characterization of Ile-APP-induced G_o activation. (**A**) Dose effect of reconstituted Ile-APP for activation of G_o. Ile-APP was reconstituted into vesicles with G_o at various final APP:G_o ratios. When the concentration of G_o in each vesicle was set to 1.5 nM, that of Ile-APP was 1, 2.7 or 4.5 nM at 1:0.7, 1:1.8 and 1:3.0 vesicles, respectively. These contents were assessed after reconstitution. Stimulation of GTPase activity of G_o is indicated as percentage stimulation of the basal G_o activity in G_o-alone vesicles, which was similar to that presented in Figure 2B. The data in these figures are the means ± SE of three assays for each vesicle. Inset: the reconstituted Ile-APP in each vesicle is shown by immunoblot analysis with AC-1. (**B**) The GTPγS binding to G_o promoted by Ile-APP. Vesicles (left, G_o-alone vesicles; middle, APP–G_o vesicles; right, Ile-APP–G_o vesicles) were incubated with [γ-^{35}S]GTPγS in the presence of 20 μM Mg^{2+} at 37°C for 2 min and the rate constant k_{app} of GTPγS binding to G_o was measured. *P<0.05 versus G_o alone. (**C**) Mg^{2+} dependence of GTPγS binding to G_o in Ile-APP–G_o vesicles, as compared with that in APP_{695}–G_o vesicles. Vesicles were incubated with [γ-^{35}S]GTPγS for 2 min at various Mg^{2+} concentrations, and the rate constant was measured. The SE of the rate constant for Ile-APP–G_o vesicles in the presence of 1 mM Mg^{2+} was 0.1. **P<0.05 versus APP–G_o vesicle.

evidence that G_o was constitutively activated in Ile-APP–G_o vesicles.

Reconstitution of either Phe-APP or Gly-APP with G_o into vesicles also resulted in stimulation of G_o activity comparable with that with Ile-APP (Figure 2B, left), despite the fact that these vesicles contained various amounts of the 80 kDa contaminant. Reconstitution of Arg-APP also significantly stimulated the reconstituted G_o, but the effect was apparently less than that of FAD-APPs, suggesting that the residue at position 642 plays a critical role in quantitatively regulating an active conformation of APP. The intermediate activation of G_o by Arg-APP agrees well with the observation that expression of Arg-APP induces apoptosis with intermediate efficacy (three FAD-APPs, the highest; wild-type APP, one of the lowest) in NK1 cells, where apoptosis by FAD-APP is mediated by G_o activation (Yamatsuji *et al.*, 1996). Again, GTPase activity was negligible in vesicles reconstituted with each APP mutant alone (data not shown). Similar amounts of APP mutants were in each vesicle when the G_o concentration was set equal (Figure 1B, lower). Figure 2B also indicates the result obtained from Gly-APP–G_o vesicles which contained an apparently smaller amount of the 80 kDa contaminant (Figure 2B, inset), which revealed that G_o activation was induced as effectively in these vesicles as in the Gly-APP–G_o vesicles shown in Figure 1. This provides an additional line of evidence that G_o activation is not due to the contaminant but due to reconstituted FAD-APPs. No detectable degradation was observed in APP_{695} or each APP mutant during 30 min incubation of the reconstituted vesicles at 37°C, as assessed with silver staining and immunoblotting (data not shown), indicating that it was intact FAD-APP that activated G_o.

The conformational requirement for the action of FAD-APP was shown by the following experiment. When each of the three FAD-APPs was mixed with G_o in solution using the same amounts as used for the reconstitution (10 nM for APP mutants, 3.3 nM for G_o), no stimulation of G_o activity was observed (Figure 2B, right). These findings not only show that activation of G_o in FAD-APP–G_o vesicles is not mediated by soluble APP, but also indicate that an appropriate conformation of APP_{695}, which is ensured by a lipid environment, is required for the FAD mutations to turn on the signaling function of APP. It is less likely that FAD-APP is concentrated locally by reconstitution or that the elevated concentration triggers G_o activation, as normal APP_{695} hardly activated G_o under the same conditions.

Figure 3A shows that the activation of G_o was dependent on the amount of Ile-APP reconstituted into vesicles. When Ile-APP was reconstituted with G_o at a 70% concentration of G_o, only a slight stimulation of G_o was observed. When the amount of reconstituted Ile-APP increased, the turnover number of G_o was augmented proportionally.

The GTPγS binding assay also revealed an ~2-fold higher stimulation of G_o activity in the Ile-APP–G_o vesicles than in the APP_{695}–G_o vesicles and an ~3-fold higher stimulation than that of the G_o-alone vesicles (Figure 3B). The magnitude of stimulation was comparable with that

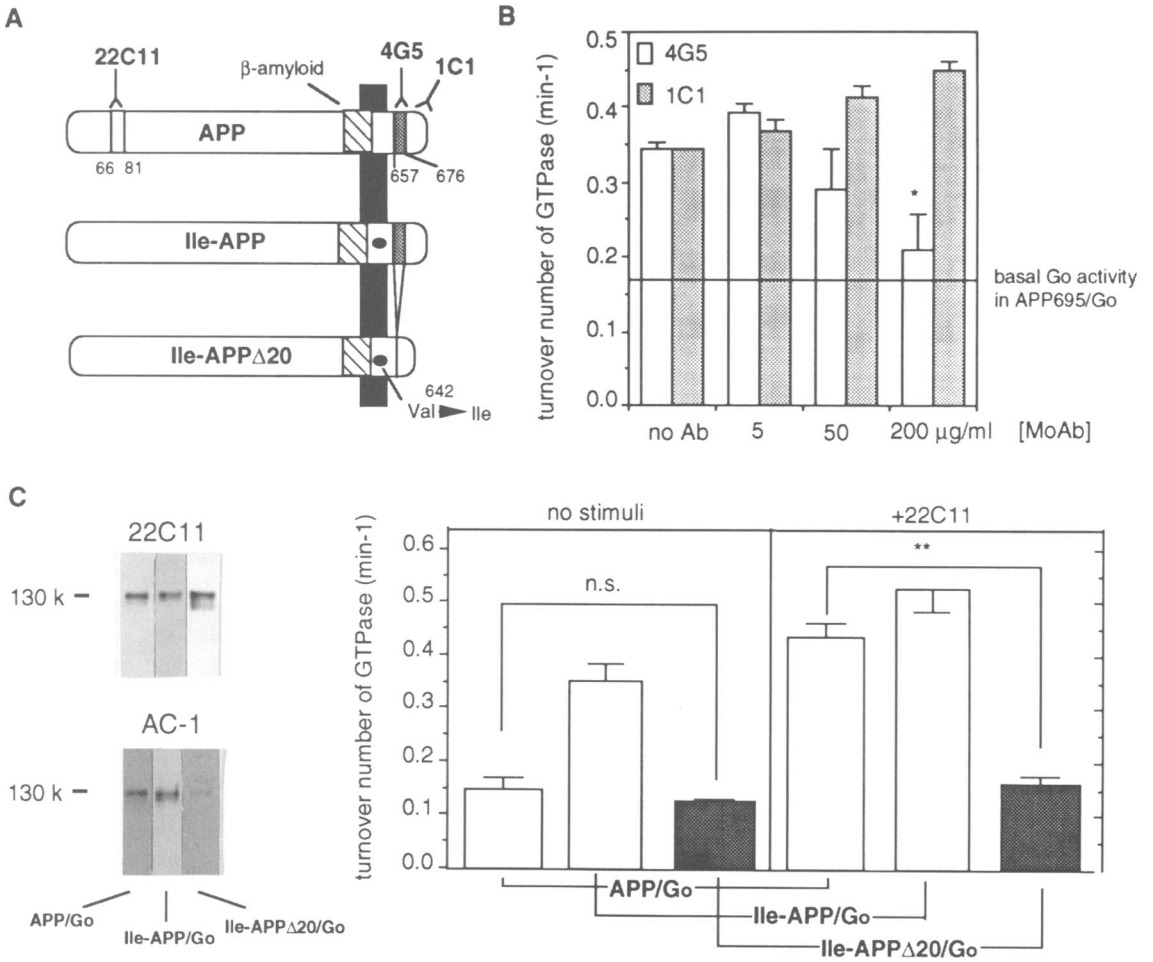

Fig. 4. Activation of G_o by Ile-APP is mediated by its cytoplasmic His657–Lys676 domain. (**A**) Wild-type APP_{695}, Ile-APP and Ile-APPΔ20 are illustrated. This figure also indicates the epitope regions in APP_{695} for 22C11, 4G5 and 1C1. (**B**) Effect of 4G5 on increased G_o activity in Ile-APP–G_o vesicles. After mixing vesicles for 1 h with increasing concentrations of 4G5 or 1C1 with sonication at 4°C, Ile-APP–G_o vesicles were incubated with [γ-^{32}P]GTP for 20 min, and the turnover number of GTPase activity of G_o was measured. The level of the basal G_o activity in APP_{695}–G_o vesicles is shown by a line, which signifies the putative non-stimulated activity of G_o in vesicles reconstituted with Ile-APP. All values represent the means ± SE of three independent experiments. *P<0.05 versus no antibody. (**C**) Left: immunoblot analysis of APP_{695}–G_o, Ile-APP–G_o and Ile-APPΔ20–G_o vesicles with anti-APP antibodies (22C11 and AC-1). In this analysis, the concentration of G_o in each lane was set equal, as assessed with maximal GTPγS binding. These vesicles were used in the experiments shown on the right. Right: reconstituted Ile-APPΔ20 fails to activate G_o. The Ile-APPΔ20–G_o vesicles were incubated with vehicle (left panel) or 5 μg/ml 22C11 (right panel) for 20 min at 37°C. The turnover number of the G_o activity was measured (right lanes). APP_{695}–G_o (left lanes) or Ile-APP–G_o vesicles (middle lanes) were similarly incubated with water or 5 μg/ml 22C11, and the turnover number of G_o was measured. The data show the means ± SE of three independent assays for each vesicle, and were reproduced by at least two different batches of reconstituted vesicles. **P<0.01. n.s., not significant.

observed in the turnover number of G_o activity in FAD-APP–G_o vesicles, suggesting that the increase in the turnover number was due mainly to the increase in GDP–GTP exchange of this G protein. Figure 3C shows that Ile-APP-induced activation of G_o in reconstituted vesicles, which was assessed with the rate constant k_{app}, was critically affected by Mg^{2+} concentrations. These data offer additional support to the notion that FAD-APP triggers G_o activation by a receptor-mimetic mechanism.

We next examined the involvement of His657–Lys676 in the action of FAD-APP. 4G5 is a monoclonal antibody raised against His657–Lys676 of APP_{695} (Nishimoto *et al.*, 1993; and Figure 4A), and has been shown to block ligand-stimulated G_o activation by APP_{695} (Okamoto *et al.*, 1995). In Ile-APP–G_o vesicles, the elevated activity of G_o was dose-dependently attenuated by 4G5 and was lowered by 200 μg/ml 4G5 to a level comparable with basal G_o activity observed in APP_{695}–G_o vesicles (Figure 4B). Two hundred μg/ml of 4G5 is the concentration at which ligand-dependent stimulation of G_o is abolished in APP_{695}–G_o vesicles (Okamoto *et al.*, 1995). In contrast, 1C1 (monoclonal antibody against Met677–Asn695, the 19 residue region adjacent to the 4G5 epitope) failed to inhibit activity. 4G5 and 1C1 had no effect on G_o activity in APP_{695}–G_o vesicles (Okamoto *et al.*, 1995) or in G_o-alone vesicles (data not shown). These data indicate that G_o stimulation by Ile-APP was attributable to its cytoplasmic domain, His657–Lys676. It was unclear why 1C1 stimulated G_o in Ile-APP–G_o vesicles, but this may suggest that the C-terminal Met677–Asn695 region has a tonic suppression activity on the Ile-APP–G_o interaction. Such opposite actions of Met677–Asn695 and His657–Lys676 have also been observed previously (Nishimoto *et al.*, 1993). The His657–Lys676 peptide inhibits the complex formation of APP with G_o, while the Met677–Asn695 peptide enhances it. However, as no enhancement

by 1C1 has been observed for the action of 22C11 in APP_{695}–G_o vesicles, the significance of this region needs further investigation.

To verify the requirement for His657–Lys676, we constructed mutant Ile-APP cDNA specifically devoid of this domain (Ile-APPΔ20) and purified baculovirus-made Ile-APPΔ20, which was reconstituted with trimeric G_o to phospholipid vesicles in the same manner. The reconstituted Ile-APPΔ20–G_o vesicles contained almost the same amounts of Ile-APP–20 and G_o as those in Ile-APP–G_o or APP_{695}–G_o vesicles examined in parallel experiments (Figure 4C, left upper). Both Ile-APP–G_o and Ile-APPΔ20–G_o vesicles contained the 80 kDa protein in similar amounts to that shown in the Ile-APP–G_o vesicles in Figure 1B. The reconstituted APP proteins in Ile-APPΔ20–G_o vesicles were comprised of two 22C11-immunoreactive bands: a major 130 kDa band and a faint 110 kDa band (Figure 4C, left upper). As Sf21 cells do not express 22C11-interactive APP endogenously, these proteins were derived from Ile-APPΔ20 cDNA. The fact that the 130 kDa protein showed immunoreactivity to both 22C11 and AC-1 indicated that this protein was full-length Ile-APPΔ20. We also emphasize that this 130 kDa protein was recognized very weakly by AC-1, despite the fact that APP and Ile-APP were recognized efficiently by this antibody (Figure 4C, left lower). As the polyclonal epitopes of AC-1 are in the C-terminus of APP, including the His657–Lys676 region (which is not present in Ile-APPΔ20), this finding indicates that the 130 kDa protein lacks the complete C-terminus of APP, providing additional evidence that this protein is Ile-APPΔ20. In further support, Ile-APPΔ20 expressed in COS cells has the same size (130 kDa) as that of expressed APP and Ile-APP (Ikezu *et al.*, 1996), and is weakly recognized by AC-1 as compared with the latter APPs (our unpublished observation). The integration of Ile-APPΔ20 into vesicles did not differ from that of Ile-APP (Figure 4C, left upper).

We examined whether the activity of G_o was elevated in the Ile-APPΔ20–G_o vesicles. Ile-APPΔ20–G_o vesicles exhibited no increase in the turnover number of G_o activity, which clearly contrasted with the results from Ile-APP–G_o vesicles (Figure 4C, right panels). The failure of Ile-APPΔ20 to activate G_o was demonstrated further using anti-APP monoclonal antibody 22C11. In APP_{695}–G_o vesicles, 22C11 increased the G_o activity by >2-fold, as reported previously (Okamoto *et al.*, 1995). Even in Ile-APP–G_o vesicles, this antibody further augmented G_o activity. In Ile-APPΔ20–G_o vesicles, however, this effective concentration of 22C11 failed to augment G_o activity. Therefore, His657–Lys676 was essential for Ile-APP to activate G_o. It is also likely that this domain mediates G_o activation by other FAD-APPs, because it is identical in all of them.

The additional effect of 22C11 in Ile-APP–G_o vesicles suggested that the autoactive function of Ile-APP was submaximal in quantity. The fact that there was no significant difference in the maximal stimulation between APP_{695}–G_o and Ile-APP–G_o vesicles (Figure 4C) suggested that the V642I mutation turned on the function of APP_{695} by a mechanism similar to that of ligand stimulation. Similar observations have been reported with autoactive mutants of thyrotropin and luteinizing hormone receptors (Parma *et al.*, 1993; Shenker *et al.*, 1993), which were found in hyperfunctioning thyroid adenomas and in male precocious puberty, respectively. In cells expressing these mutants, cAMP was elevated submaximally and further stimulated by ligands to the maximal levels attained by ligand-stimulated wild-type receptors. In this regard as well, FAD-APP greatly resembles the constitutively active hormone receptors implicated in human diseases.

Discussion

This study has provided direct evidence that APP_{695} with FAD mutations is able to activate G_o in the absence of ligands, whereas normal APP_{695} does so only ligand-dependently. There are multiple lines of evidence that G_o activation in FAD-APP–G_o vesicles was due to FAD-APP, and not due to an 80 kDa protein, a major contaminant, or others: (i) G_o activation was not observed in the mock-infected cell-derived control vesicle that contained the same amount of 80 kDa protein as in Ile- or Phe-APP–G_o vesicles; (ii) the APP_{695}–G_o vesicle, containing comparable or even higher amounts of the 80 kDa protein than either FAD-APP–G_o vesicle, showed little activation of G_o; (iii) the Gly-APP–G_o vesicle with an apparently lesser amount of the 80 kDa protein yielded stimulation of G_o which was as efficient as the Gly-APP–G_o vesicle with more of this protein; (iv) none of the FAD-APP preparations activated G_o in solution; (v) anti-APP antibody further activated G_o in Ile-APP–G_o vesicles; and (vi) G_o activation observed in Ile-APP–G_o vesicles was inhibited by two different strategies, antibody competition and sequence deletion, both designed to block the function of His657–Lys676 of Ile-APP. Except for APP (and typical G-coupled receptors), GAP-43 is the most potent activator of G_o so far reported, which requires concentrations of from many hundreds of nanomolar to a few micromolar to attain 2- to 2.5-fold activation of G_o (Strittmatter *et al.*, 1991). At concentrations of a few nanomolar, however, liganded APP activates G_o to comparable extents in reconstituted vesicles (Okamoto *et al.*, 1995). Because the concentrations of the major contaminant 80 kDa protein in vesicles were estimated to be below a few nanomolars, it is very unlikely that the observed activation of G_o in these vesicles was due to this and other contaminants. The fact that all FAD-APPs share a peptide region that activates G_o *in vitro* (Nishimoto *et al.*, 1993) and *in vivo* (Lang *et al.*, 1995) offers additional credence to the notion that G_o is activated directly by FAD-APPs. Although we could not totally exclude the possibility that very potent intermediary or co-factor proteins were included in FAD-APP–G_o vesicles, this possibility does not conflict with G_o activation by FAD-APPs. In further support, Phe-APP couples to the C-terminal five residues of $G\alpha_o$ and induces the output of this G protein in a whole cell system (Ikezu *et al.*, 1996).

APP_{695} encodes a potential G_o-coupled receptor with ligand-regulated function (Okamoto *et al.*, 1995). While most G-coupled receptors consist of a seven-transmembrane configuration, there are at least two additional examples of single transmembrane proteins directly linked to G proteins: the insulin-like growth factor II receptor (Murayama *et al.*, 1990; Okamoto *et al.*, 1990; Ikezu *et al.*, 1995) and the sperm surface ZP3 receptor β1,4-galactosyltransferase (Gong *et al.*, 1995), both of which

couple to G_i. We previously showed that ligand-dependent activation of G_o by APP_{695} occurs through a mechanism shared by typical G-coupled receptors (Okamoto *et al.*, 1995). The present data, regarding PTX sensitivity, Mg^{2+} dependency and reconstitution requirement, indicate that constitutive activation of G_o by FAD-APP also shares the mechanism with G-coupled receptors. Furthermore, we have also shown here that the constitutive function of Ile-APP is mediated by its His657–Lys676 region, which acts as a ligand-dependent G_o-coupling domain of non-mutated APP_{695} (Okamoto *et al.*, 1995). These observations confirm that the three FAD-linked mutations switch on the intrinsic signaling function of APP, indicating that FAD-APPs are mutationally activated APP_{695}.

As the degree of G_o activation in reconstituted APP vesicles reflects the proportion of APP associated with an active conformation, our data suggest that at a basal state (i) most of wild-type APP_{695} is in an inactive conformation; and (ii) large fractions of three FAD-APPs and an intermediate one of Arg-APP are in active conformations. Thus, V642 mutations should crucially affect the conformation of APP_{695} and alter the proportion of its active versus inactive conformations, suggesting the importance of the transmembrane residue at position 642 in the conformational regulation of APP_{695}. This notion is consistent with the report of Lefkowitz and his co-workers (Kjelsberg *et al.*, 1992) indicating that all possible substitutions at a single residue in G-coupled receptors create constitutively active receptors with different degrees of G protein activation. They explained this phenomenon by theorizing that mutations cause various equilibria of active versus inactive receptor conformations (Bond *et al.*, 1995), which is consistent with our interpretation.

Augmented cleavage of Aβ1–42 from APP has so far been the only known molecular target of the V642 residue substitutions (Suzuki *et al.*, 1994). On the other hand, we and another group (Nishimoto *et al.*, 1993; Fiore *et al.*, 1995) have reported independently that APP is able to play a normal role as a signaling molecule by interacting with intracellular proteins through its cytoplasmic domain. The present study indicates, for the first time, that such a signaling function of APP is another molecular target of the FAD-associated V642 mutations that causes constitutive activation of signal transducers of APP.

It is thus tempting to clarify what abnormal outputs G_o propagates in intact cells when this G protein is continuously activated by FAD-APPs. Although the physiological role of G_o is not well defined, mammalian G_o has been implicated in neuronal network formation (Schuch *et al.*, 1989; Doherty *et al.*, 1991; Strittmatter *et al.*, 1994) and long-term potentiation of hippocampal synapses (Ito *et al.*, 1988; Goh and Pennefather, 1989). The targeted disruption of G_o in *Caenorhabditis elegans* causes behavioral disorders (Mendel *et al.*, 1995; Ségalat *et al.*, 1995). It should also be noted that the tissue distribution of G_o in human brain coincides with the areas severely afflicted by AD. These findings prompt us to envisage that the aberrant activation of G_o by FAD-APP disturbs pivotal functions of neurons potentially linked to the primary processes of FAD. Consistent with this idea, we have found recently that FAD-APPs, not normal APP, cause apoptotic cell death by a G_o-dependent mechanism (Yamatsuji *et al.*, 1996) and also induce tonic suppression of transcriptional activity of CRE in a $G\alpha_o$-dependent manner (Ikezu *et al.*, 1996). The apoptosis by the V642 mutants observed in the former study is phenotypically linked to the FAD trait (Yamatsuji *et al.*, 1996). The suppression of CRE function is thought to be relevant to synaptic malplasticity and long-term memory disturbance (Frank and Greenberg, 1994), which are the major manifestations of AD. These findings suggest that G_o activation by FAD-APPs, demonstrated here, is linked to AD-associated disorders *in vivo*. Therefore, the present *in vitro* system will prove especially useful for screening of reagents that potentially act as FAD inhibitors.

In summary, it is highly likely that FAD mutations transform APP_{695} to the APP that constitutively activates G_o, implying that the intrinsic signaling function of APP is a novel target of these mutations. This is not surprising, because rapidly expanding information about presenilins, products of a gene family responsible for another type of FAD (Rogaev *et al.*, 1995; Sherrington *et al.*, 1995), shows that their homolog of *C.elegans* functions as a co-subunit of a cell surface signaling receptor Notch (Jarriault *et al.*, 1995; Levitan and Greenwald, 1995), suggesting that the putative signaling function may also be the target of FAD mutations found in human presenilins. The mechanism whereby FAD mutations turn on the function of APP also needs further investigation. Obviously, dimerization is the most likely mechanism, as is the case with antibody-induced cross-linking of normal APP (Okamoto *et al.*, 1995), sperm surface G_i-coupled receptor β1,4-galactosyltransferase (Gong *et al.*, 1995) and the transmembrane mutation in c-erbB2, a tyrosine kinase receptor (Stern *et al.*, 1988).

Materials and methods

Materials

APP_{695} and APP mutant cDNAs were described previously (Yamatsuji *et al.*, 1996). Baculoviruses encoding APP mutants were constructed as described earlier (Nishimoto *et al.*, 1993). Recombinant APP_{695} and its FAD mutants were purified from Sf21 cells infected with each baculovirus encoding the cognate gene, as described in detail previously (Okamoto *et al.*, 1995). Trimeric G_o was purified from bovine brain to homogeneity, as described (Katada *et al.*, 1986); stored in 20 mM HEPES/NaOH (pH 7.4), 0.1 mM EDTA and 0.7% CHAPS, and diluted ≥10-fold for reconstitution. The APPs and the trimeric G_o were reconstituted with 1 mg/ml azolectin by gel filtration with G-50 Sepharose, as described (Okamoto *et al.*, 1995). Unless otherwise specified, they were reconstituted into vesicles in a G_o:APP ratio of 1:3, which has been used for optimal reconstitution of receptors and G proteins (Okamoto *et al.*, 1994). While approximately half of the G_o and APP was incorporated into vesicles, we monitored their amounts in vesicles after each reconstitution by total GTPγS binding for G_o and by immunoblotting for APP. AC-1 (Yoshikawa *et al.*, 1992) was provided by Dr K.Yoshikawa, and 4G5 and 1C1 were as described previously (Nishimoto *et al.*, 1993). PTX was pre-activated in 50 mM Tris–HCl (pH 7.5), 100 mM dithiothreitol (DTT) and 100 μM ATP for 15 min at 30°C. Vesicles were then treated with or without 5 μg/ml pre-activated PTX in a buffer containing 100 mM Tris–HCl (pH 7.5), 100 μM GTP, 1 mM EDTA and 10 μM NAD for 15 min at 30°C. At least three different batches were constructed for each FAD-APP, and similar results were obtained, unless otherwise specified.

Assays

The turnover number of GTPase activity was assayed as described (Okamoto *et al.*, 1995). Unless otherwise specified, the turnover number was measured by incubating vesicles with 100 nM [γ-^{32}P]GTP in the presence of 20 μM Mg^{2+} for 20 min at 37°C. The total amount of G_o in vesicles was measured after reconstitution, as described (Okamoto *et al.*, 1995). The time course of GTPase activity of either APP_{695}– or

APP mutant–G_o vesicles was linear during 20 min incubation. The rate constant k_{app} of GTPγS binding to G_o in vesicles was assessed as described (Okamoto and Nishimoto, 1992). Immunoblot analysis was performed as described previously (Nishimoto *et al.*, 1993). All assays shown here were performed at least three times with similar results. Statistical analysis was carried out with the Student's *t* test.

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