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# Negative transactivation of cAMP response element by familial Alzheimer's mutants of APP

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**In familial Alzheimer's disease (FAD), missense point mutations V642I/F/G, which co-segregate with the disease phenotype, have been discovered in amyloid precursor $APP_{695}$. Here, we report that three FAD mutants (FAD-APPs) negatively regulated the transcriptional activity of cAMP response element (CRE) by a $G_o$-dependent mechanism, but expression of wild-type $APP_{695}$ had no effect on CRE. Experiments with various $G\alpha_s$ chimeras demonstrated that Phe-APP coupled selectively to the C-terminus of $G\alpha_o$. Again, wild-type $APP_{695}$ had no effect on its C-terminus. These data indicate that FAD-APPs are gain-of-function mutants of $APP_{695}$ that negatively regulate the CRE activity through $G_o$. This negative transactivation of CRE is the first biochemically analyzed signal evoked by the three FAD-APPs, but not by wild-type $APP_{695}$, in a whole-cell system. We discuss the significance of constitutive CRE suppression by FAD-APPs, which is potentially relevant to synaptic malplasticity or memory disorders.**

*Keywords*: amyloid precursor protein/cAMP response element/familial Alzheimer's disease/$G_o$-dependent mechanism/memory formation

## Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease, characterized pathologically by senile plaques, neurofibrillary tangles and extensive neuronal loss (Katzman, 1986). The senile plaque consists of Aβ amyloid, which derives from amyloid precursor protein (APP). Alternative splicing of a single gene results in at least 10 isoforms of APP (Sandbrink *et al.*, 1994). $APP_{695}$, consisting of 695 residues, and $APP_{770/751}$, consisting of 770/751 residues, are preferentially expressed in neuronal and non-neuronal tissues, respectively. In early-onset familial AD (FAD), missense mutations V642I/F/G have been identified in the transmembrane domain of $APP_{695}$ (Goate *et al.*, 1991; reviewed in Hardy, 1992). These mutations ($APP_{V642X}$ is referred to as X-APP) cosegregate with AD phenotype (Karlinsky *et al.*, 1992), which proves that they are at least one established cause of AD. Recently, Games *et al.* (1995) have reported that overexpression of Phe-APP mimics the neuropathology of AD in transgenic mice. Nevertheless, little has been known about either the molecular function of $APP_{695}$ or its abnormality, which is shared by the three FAD-APPs.

APP has a cell surface receptor-like architecture with a single transmembrane domain (Kang *et al.*, 1987) as well as receptor-like orientation (Dyrks *et al.*, 1988) and cellular localization (Schubert *et al.*, 1991). Accordingly, APP has been implicated in signaling of adhesion (Ueda *et al.*, 1989; Mönning *et al.*, 1992), neurite outgrowth (Milward *et al.*, 1992; Small *et al.*, 1994), synaptic contact (Schubert *et al.*, 1991) and locomotion (Zheng *et al.*, 1995). By using multiple approaches (Nishimoto *et al.*, 1993; Okamoto *et al.*, 1995), we have so far defined a receptor function in $APP_{695}$, which is to couple to the heteromeric G protein $G_o$ through the cytoplasmic domain H657-K676. The APP signaling via $G_o$ is in excellent agreement with a number of reports showing that $G_o$ mediates neuronal signals for adhesion (Schuch *et al.*, 1989; Doherty *et al.*, 1991), neurite outgrowth (Strittmatter *et al.*, 1994) and locomotion (Sebok *et al.*, 1993), all similar to the functions mediated by APP. Thus, wild-type $APP_{695}$ probably encodes a normal receptor which can regulate the intracellular signals through $G_o$.

Although the physiological role of $G_o$ has not been fully understood, this signal-transducing molecule is one of the major proteins abundant in the brain and may be essential for the functioning of neurons, including neural network formation (Schuch *et al.*, 1989; Doherty *et al.*, 1991; Strittmatter *et al.*, 1994), memory formation (Goh and Pennefather, 1989; Guillén *et al.*, 1990) and behavior (Mendel *et al.*, 1995; Ségalat *et al.*, 1995). Also, the dominant inheritance of FAD (Karlinsky *et al.*, 1992) suggests that the FAD mutations evoke dominant abnormality in the actions of $APP_{695}$, suggesting that cellular effects of FAD-APP can be detected by overexpressing FAD-APP cDNA in mammalian cells. Since (i) cAMP response element (CRE) activity is an established mediator of long-term memory formation (Frank and Greenberg, 1994) and (ii) $G_o$ negatively regulates CRE activity in certain cells (Migeon *et al.*, 1994), in this study we examined the potential regulation of CRE by cellular expression of FAD-APP. The results show that each FAD-APP, not $APP_{695}$, negatively regulates the transcriptional activity of CRE by a $G_o$-dependent mechanism. We also show that FAD-APP executes selective coupling to the five C-terminal residues of $G\alpha_o$, providing direct evidence that FAD-APP couples selectively to $G_o$ in a whole-cell environment. The significance of this novel signaling abnormality is discussed.

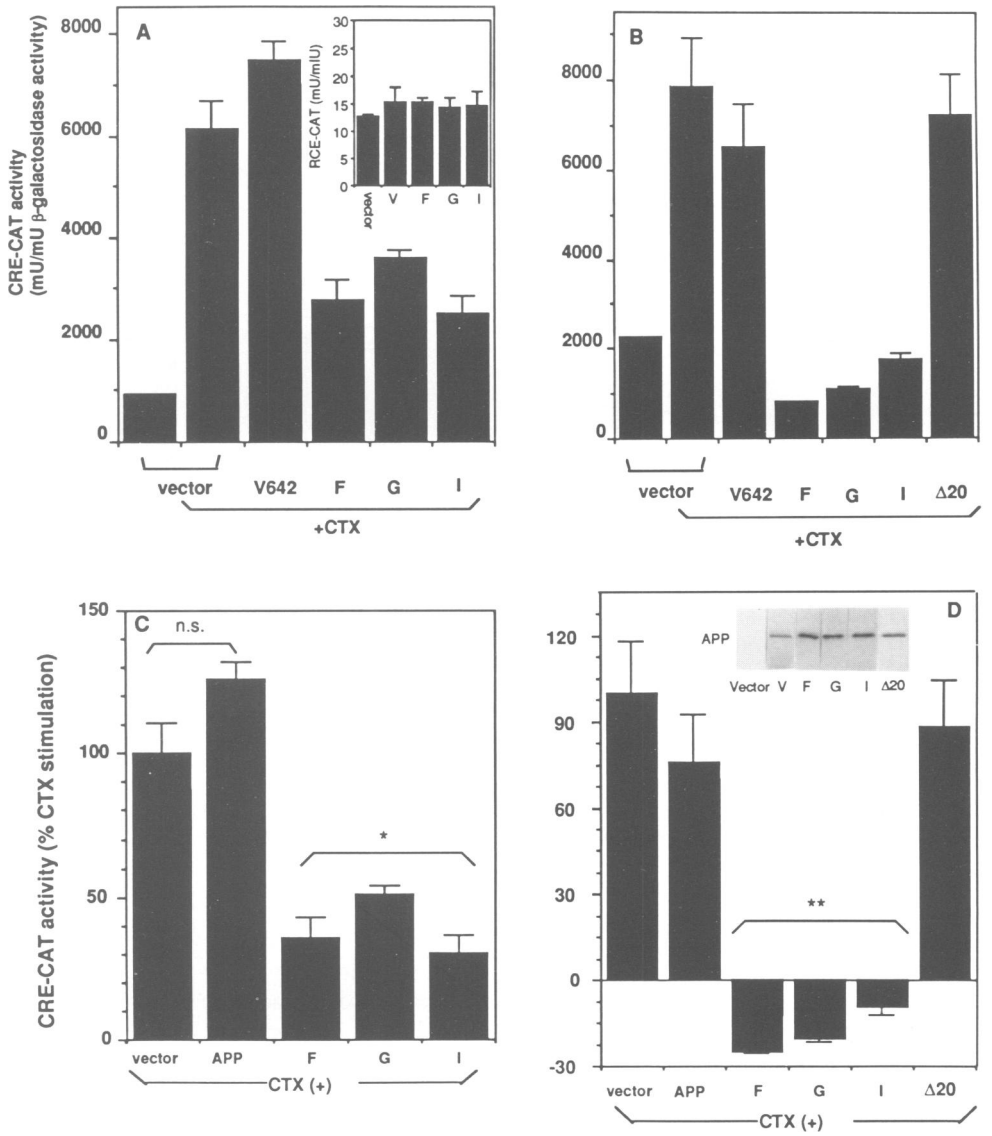


**Fig. 1.** Suppression of CRE activity by FAD-APPs. (A and B) Effect of FAD-APP cDNA transfection on CTX-stimulated CRE-CAT activities in (**A**) NK1 cells and (**B**) NK1 cells overexpressing $G\alpha_o$ ($G_o$-NK1 cells). 1 μg of either $APP_{695}$, FAD-APPs ($APP_{V642X}$, X = F, G and I), Ile-APPΔ20 (Δ20), or vector was transfected into these cells with 0.3 μg CRE-CAT reporter and 0.2 μg pactβgal cDNA, which were also used in Figures 2 and 3. Cells were then treated with 1 μg/ml CTX, and CAT activity was measured. Transfection efficiency was assessed by co-transfection of a control plasmid pactβgal that expresses β-galactosidase activity and used for standardization. All values in the figures of this study represent the means ± SE of three independent experiments with different transfections. (A, inset) Effect of FAD-APP cDNA transfection on CRE-CAT activity. NK1 cells were transfected with 1 μg of either vector, wild-type, $APP_{695}$, or three $APP_{V642X}$ with 0.3 μg CRE-CAT and 0.2 μg pactβgal cDNA; CAT activity was measured in the same protocol as used for CRE-CAT. These insets show representative results of three independent experiments. (**C** and D) Replots of the data presented in (A) and (B). These figures indicate the percentage of the CRE-CAT activity stimulated by CTX, when cells were transfected with vector, wild-type $APP_{695}$, and three FAD-APPs. (**D**, inset) The same samples of $G_o$-NK1 cell homogenates as used for CAT assay were probed with anti-APP antibody 22C11; APP immunoreactivity is shown. $*P<0.05$, $**P<0.01$ versus vector + CTX. n.s., not significant.

## Results

We examined whether FAD-APP expression affects the transcriptional activity of CRE in a whole-cell system, using the CRE-CAT reporter. For this purpose, we used NK1 cells, a naturally occurring transformant of COS cells, which express endogenous $G\alpha_o$. In NK1 cells, the effect of 1 μg/ml cholera toxin (CTX) on CRE activity was 4- to 6-fold over the basal activity. Figure 1A and C shows that the CTX-elevated CRE activity was significantly (50–70%) blocked by transfection of 1 μg of each FAD-APP cDNA. This inhibition did not appear to be an artifact, because similar expression of wild-type $APP_{695}$ had no effect on CRE activity. The action specificity was also supported by the observation that the SV40 promoter activity, as assessed with the reporter plasmid described previously (Ikezu *et al.*, 1994), was not inhibited by transfection of Phe-APP under the same condition (data not shown). Transfection of each FAD-APP cDNA (1 μg) suppressed the basal CRE activity by 20–30% without CTX stimulation (data not shown). We again confirmed the element specificity of the FAD-APP action by using a CRE-CAT plasmid (Ikezu *et al.*, 1994), which contains the retinoblastoma control element. When this plasmid

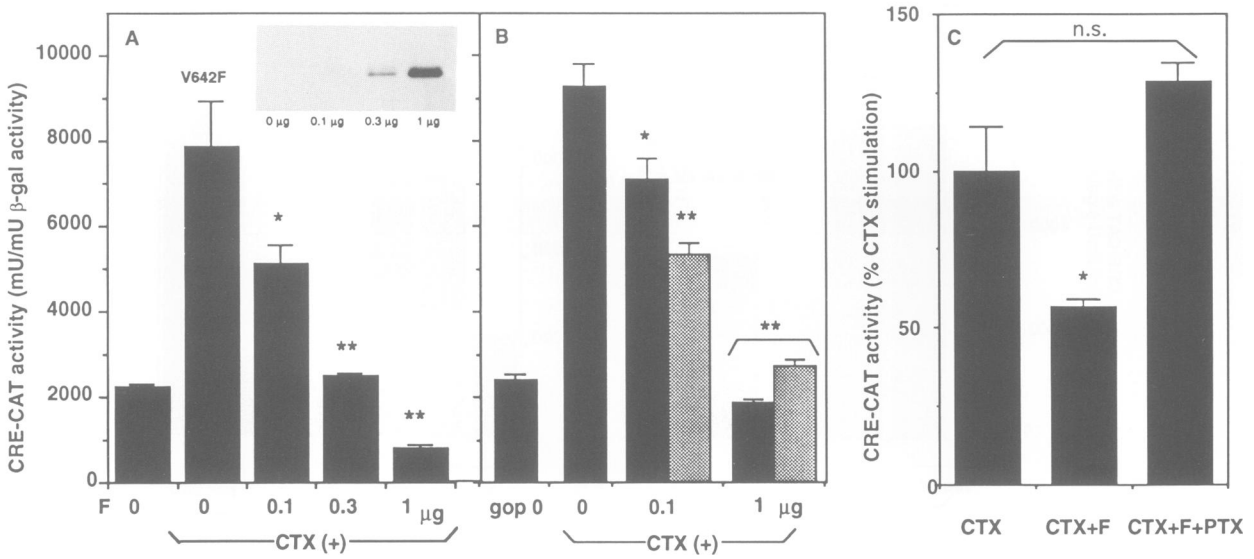


**Fig. 2.** Characterization of Phe-APP inhibition of CRE activity. (**A**) Dose-responsive inhibition by Phe-APP of CTX-stimulated CRE activity in $G_o$-NK1 cells. These cells were transfected with increasing amounts of Phe-APP cDNA in the presence or absence of 1 μg/ml CTX. In each transfection, the total amount of plasmids was adjusted to 1 μg with vector. $*P<0.05$, $**P<0.01$ versus vector + CTX. Inset: the samples of homogenates used for CAT assay were probed with AC-1, and APP immunoreactivity is shown. Although it was hardly visible in 0.1 μg cDNA transfection in this exposure, the band corresponding to Phe-APP was clearly visible in a longer exposure. This inset shows a representative result of three independent experiments. (**B**) Inhibition of CTX-stimulated CRE activity by constitutively activated $G\alpha_o$ mutants in $G_o$-NK1 cells. These cells were transfected with increasing amounts of $G\alpha_oQ205L$ cDNA (solid columns) or $G\alpha_{o2}Q205L$ cDNA (shaded columns) in the presence or absence of 1 μg/ml CTX. In each transfection, the total amount of plasmids was adjusted to 1 μg with vector. $*P<0.05$, $**P<0.01$ versus vector + CTX. (**C**) Effect of PTX on CRE inhibition by Phe-APP in NK1 cells. CRE activity was measured after transfection of 0.1 μg Phe-APP cDNA and subsequent treatment of CTX in the presence or absence of 10 ng/ml PTX. The stimulation of CRE-CAT activity over the basal activity is indicated as percent CTX stimulation. Both 0 and 100% were similar to the values in Figure 1A. $*P<0.05$ versus vector + CTX. There was no significant difference between vector + CTX and Phe-APP + CTX + PTX.

was co-transfected, neither transfection of $APP_{695}$ nor of any of three FAD-APPs resulted in any alteration in CAT reporter activity (Figure 1A, inset).

We established an NK1 cell line ($G_o$-NK1) stably overexpressing $G\alpha_o$, by transfecting NK1 cells with $G\alpha_o$ cDNA. In these cells, which express $G\alpha_o$ by 3-fold over parental NK1 cells (data not shown), CTX-promoted CRE activity was totally inhibited and decreased below basal activity by the transfection of each FAD-APP under the same condition as for NK1 cells (Figure 1B and D). CTX stimulation was –25.3 ± 0.1% for Phe-APP; –20.8 ± 0.9% for Gly-APP; –9.6 ± 2.5% for Ile-APP (mean ± SE of three independent transfections), as compared with the basal activity. Again, transfection of $APP_{695}$ cDNA showed no significant inhibition under the same condition. Expression levels of each APP mutant and $APP_{695}$ were similar (Figure 1B, inset). In control NK1 cells stably overexpressing $G\alpha_{i2}$, the suppression by Phe-APP of CTX-stimulated CRE activity was 60.0 ± 8.4% (mean ± SE of three independent transfections), which was similar to that observed in the parental NK1 cells. These findings suggest that $G\alpha_o$ overexpression specifically enhances the action of FAD-APPs on CRE suppression.

The extent of CRE inhibition was proportional to the expression level of Phe-APP in $G_o$-NK1 cells (Figure 2A). It was also noted that transfection of constitutively active $G\alpha_o$ mutant $G\alpha_oQ205L$ cDNA dose-dependently inhibited CRE activity in NK1 cells (Figure 2B, solid columns). Transfection with $G\alpha_{o2}Q205L$ cDNA also resulted in inhibition of CTX-stimulated CRE activity (Figure 2B, shaded columns). In contrast, no inhibition was observed by transfection of wild-type $G\alpha_o$ under the same condition (data not shown).

We also examined the action of Ile-APPΔ20, mutant Ile-APP lacking cytoplasmic H657-K676. In $G_o$-NK1 cells, this mutant was similarly expressed as much as Ile-APP (Figure 1B, inset). Nevertheless, Ile-APPΔ20 failed totally to suppress CRE activity (Figure 1B). This suggests that a specific cytoplasmic mechanism underlies the negative control of CRE by Ile-APP. This also provides direct evidence that FAD-APP suppresses CRE activity by virtue of the cytoplasmic domain, which points to $G_o$ mediation of this action of Ile-APP, because the H657-K676 region has been demonstrated to act as the selective $G_o$-coupling domain of $APP_{695}$ (Nishimoto *et al.*, 1993; Okamoto *et al.*, 1995).

Figure 2C shows the effect of pertussis toxin (PTX), an established inhibitor of $G_i$ and $G_o$. PTX treatment of NK1 cells totally abolished CRE suppression by Phe-APP. Nevertheless, PTX did not alter the expression level of Phe-APP (data not shown). This indicates that FAD-APP negatively regulates CRE activity mainly through PTX-sensitive G proteins.

To assess directly Gα that mediates CRE inhibition by FAD-APP, we next performed a different series of experiments. First, a cDNA specifying a chimeric molecule designated as $\alpha_s/\alpha_o$ was constructed (Figure 3A). In this chimera, the five C-terminal residues were from $G\alpha_o$ and the remaining amino acids were from $G\alpha_s$. We also constructed comparable $G\alpha_s$ chimeras whose five C-terminal residues are from $G\alpha_{i3}$ ($\alpha_s/\alpha_{i3}$) or from $G\alpha_z$ ($\alpha_s/\alpha_z$). All $G\alpha_s$ chimeras were similarly expressed in each

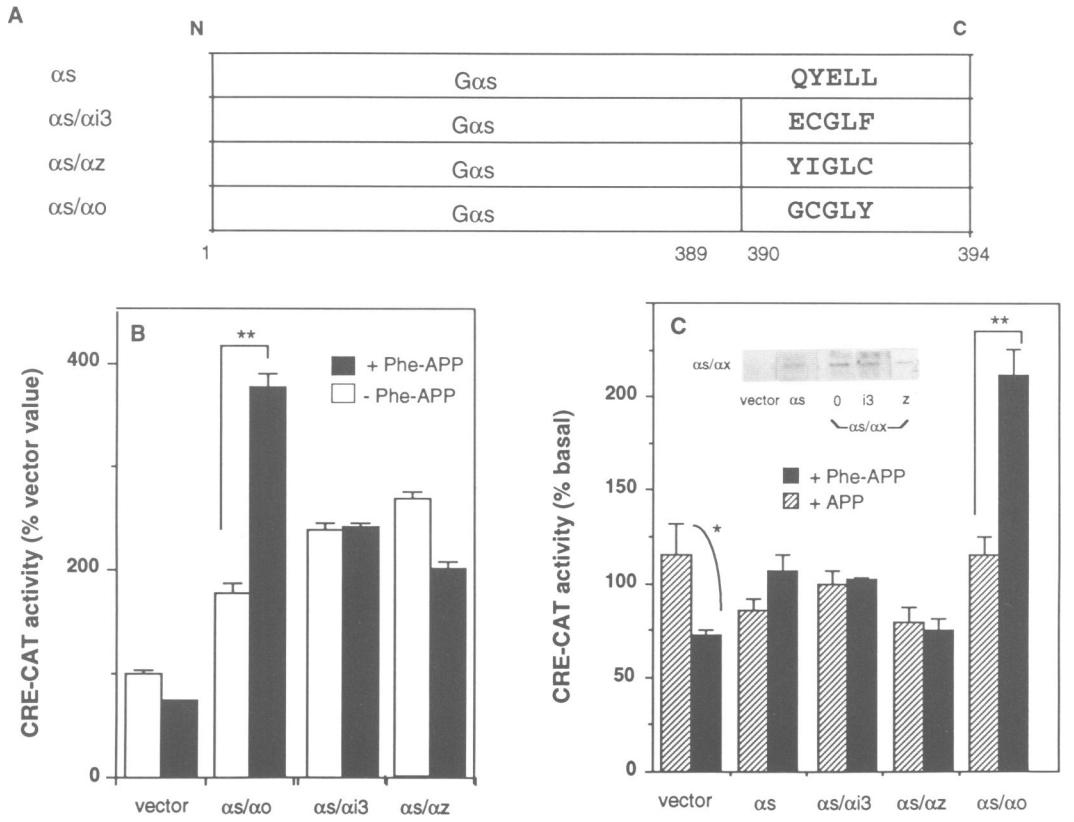


**Fig. 3.** Selective recognition of $G\alpha_o$ by Phe-APP. (**A**) Schematic illustration of $G\alpha_s/\alpha_x$ chimeras. (B and C) Effect of co-transfected $G\alpha_s$ ($\alpha_s$) or $G\alpha_s$ chimeras ($\alpha_s/\alpha_o$, $\alpha_s/\alpha_{i3}$, $\alpha_s/\alpha_z$) on the Phe-APP-regulated CRE activity in NK1 cells. After transfection of 0.3 μg of each $G\alpha_s$ chimera cDNA with or without 0.7 μg Phe-APP or $APP_{695}$ (plus 0.2 μg pactβgal and 0.3 μg CRE-CAT), and subsequent CTX treatment, CRE activity was measured. (**B**) Absolute values of CRE activity when each $G\alpha_s$ chimera was transfected with or without Phe-APP. The basal CRE activity in transfecting vector (instead of chimeras) with another vector (instead of Phe-APP) was 1549 ± 46 mU/mIU β-galactosidase activity, which corresponds to 100% in this figure. This figure also shows the effect of each $G\alpha_s$ chimera on the basal activity. These effects were less than maximal stimulation because CTX-stimulated CRE activity was 8503 ± 558 mU/mIU, corresponding to 550%, under the same conditions. $^{**}P<0.01$ versus vector + $\alpha_s/\alpha_o$. (**C**) The effects of Phe-APP or $APP_{695}$ on each $G\alpha_s$ chimera. The effect of Phe-APP was assessed by dividing CRE activity when a $G\alpha_s$ chimera was transfected with Phe-APP by the basal CRE activity when the same $G\alpha_s$ chimera was transfected with vector (instead of Phe-APP), and is indicated in solid columns as a percentage of the basal activity (100% indicates no action of Phe-APP). As another control, wild-type $APP_{695}$ cDNA was used instead of Phe-APP cDNA (open columns). To make figures more understandable, we used the same data as for Phe-APP effect in Figures 3B and C, whereas experiments were repeated three times with similar results. $^{*}P<0.05$ versus $APP_{695}$ + vector; $^{**}P<0.01$ versus $APP_{695}$ + $\alpha_s/\alpha_o$. Inset: each transfected sample was probed with common Gα antibody (1/1000 dilution, NEN) and the 52 kDa immunoreactivity is shown (52 kDa is the expected molecular weight of these $G\alpha_s$ chimeras). The inset shows a representative result of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus wild-type $APP_{695}$ effect.

transfection (Figure 3, inset). With co-transfection of each construct, we compared CRE activity in the presence or absence of Phe-APP transfection.

In NK1 cells, single transfection of either $G\alpha_s$ chimera resulted in significant increase in CRE activity (Figure 3B, open columns). However, these activities were 170–280% of the vector value (1549 ± 46 mU/mIU β-galactosidase activity) and sufficiently less than maximal stimulation (CTX stimulation was 549 ± 36% of the vector value under the same condition). Co-transfection of Phe-APP with $\alpha_s/\alpha_o$ resulted in a further increase in CRE activity (Figure 3B), which was more than 200% of the CRE activity observed in single transfection of $\alpha_s/\alpha_o$ and 370% of the vector value (Figure 3C, left panel). This Phe-APP-stimulated activity was quantitatively significant, as it corresponds to ~70% of the maximal stimulation by CTX under the same condition, and is qualitatively significant relative to the activity induced by similar transfection of normal $APP_{695}$ (Figure 3C, right panel). In clear contrast, co-transfection of vector, $\alpha_s$, $\alpha_s/\alpha_{i3}$ or $\alpha_s/\alpha_z$ with Phe-APP resulted in no further stimulation of CRE activity relative to the basal activity observed in their single transfection without Phe-APP (Figure 3B and C). Without activating $G\alpha_s$ chimeras, transfection of FAD-APP should result in significant inhibition of CRE activity, because transfection of FAD-APP by 20–30% inhibited the basal CRE activity in NK1 cells (see earlier). Consistent with this idea, Phe-APP transfection with a vector resulted in ~75% value of the basal activity (Figure 3, vector). These data indicate that Phe-APP recognizes and couples selectively to the five C-terminal residues of $G\alpha_o$ in whole cells.

As another control, we transfected each $G\alpha_s$ chimera with wild-type $APP_{695}$ cDNA into NK1 cells under the same condition and similarly compared the CRE activities with and without $APP_{695}$ transfection (Figure 3). The results revealed that co-transfection of $APP_{695}$ with either construct resulted in virtually 100% CRE activity of the basal activity (the activity observed in the vector transfection with each $G\alpha_s$ construct). This indicates that

wild-type APP has no activating effect on either $G\alpha_s$ construct including $\alpha_s/\alpha_o$. Figure 3C shows that a significant difference was observed between the actions of Phe-APP and wild-type APP only when vector or $\alpha_s/\alpha_o$ was co-transfected. These indicate that: (i) Phe-APP, not wild-type APP, inhibits CRE activity through an endogenous mechanism; and (ii) Phe-APP, not wild-type APP, activates CRE activity selectively through $\alpha_s/\alpha_o$.

## Discussion

We have herein shown that expressed FAD-APPs, not wild-type APP, negatively regulate the transcriptional activity of cAMP response element CRE in whole cells. We have recently reported that NK1 cells cause apoptosis by expression of FAD-APPs (Yamatsuji *et al.*, 1995). However, multiple lines of independent evidence indicate that this transcriptional control by FAD-APPs is not due to apoptotic changes. First, at the time point we measured CRE activity in this study, most of the FAD-APP-expressing NK1 cells have no apoptotic cytoplasm (Yamatsuji *et al.*, 1995). Secondly, in each assay, β-actin promoter activity was used as a reference, which allowed for the measurement of specific changes in CRE activity. In fact, the FAD-APP-induced decrease in transcriptional activity was actually specific for CRE in that similarly transfected plasmids carrying the SV40 promoter or the CRE promoter showed no reduced activities in FAD-APP-transfected NK1 cells. Thirdly, in these cells, the FAD-APP suppression of basal CRE activity was 20–30% in quantity, which was not proportional to the FAD-APP suppression of CTX-stimulated activity. Fourthly, when $\alpha_s/\alpha_o$ was co-expressed, expression of FAD-APP positively regulated CRE activity in NK1 cells. Fifthly, we observed considerable potentiation of CRE suppression by FAD-APPs in $G_o$-NK1 cells. As opposed to this enhancement, apoptosis by FAD-APPs was significantly inhibited in these cells (T.Yamatsuji and I.Nishimoto, unpublished observation). Finally, FAD-APPs, not wild-type APP, similarly inhibited CRE activity in NK1 cells overexpressing bcl-2. In these cells, CTX stimulation was 4.5 ± 0.9% for Phe-APP, 25.2 ± 2.0% for Gly-APP, 11.5 ± 3.4% for Ile-APP and 82.8 ± 7.1% for $APP_{695}$ (mean ± SE of three independent transfections) of the CTX stimulation when vector was transfected. Bcl-2 is an anti-apoptotic gene, and in NK1 cells overexpressing bcl-2, no apoptosis is induced by FAD-APPs under the same condition used in the present study (Yamatsuji *et al.*, 1995). This provides compelling evidence that FAD-APP-induced suppression of CRE is not the result of apoptosis.

Thus, all three FAD-APPs, as the specific target, share the nuclear signal that negatively controls the transcriptional activity of CRE. CRE is an established nuclear target of multiple important signaling pathways involving cAMP-dependent kinase (Gonzalez and Montminy, 1989) and $Ca^{2+}$/calmodulin-dependent kinase (Sun *et al.*, 1994). Hence, FAD-APPs may potentially regulate these pathways. As CRE is typified by the consensus palindromic sequence TGACGTCA and is present in the promoters of many genes, it is conceivable that the expression of FAD-APPs deteriorates the expression of many genes via this transcriptional control. The intracellular abnormality induced by the expression of three FAD-APPs has not yet been analyzed biochemically. Negative transactivation of CRE is thus the first biochemically analyzed intracellular signal that has been evoked by all three FAD-APPs, but not by wild-type APP, in a whole-cell system.

Multiple lines of evidence indicate that FAD-APP-induced negative control of CRE is mediated by a $G_o$-dependent mechanism. This action of FAD-APP was potentiated by overexpression of $G\alpha_o$, not $G\alpha_i$, inhibited by PTX, and reproduced by constitutively activated $G\alpha_o$ mutant. Furthermore, lack of the action by Ile-APPΔ20 was notable. Despite the fact that it was similarly expressed as Ile-APP, this mutant caused no suppression on CRE transcriptional activity. Ile-APPΔ20 specifically lacks the cytoplasmic H657-K676 domain. This stretch of sequence has been shown to constitute the only known functional domain in the cytoplasmic region of APP, which directly couples $APP_{695}$ selectively to $G_o$ (Nishimoto *et al.*, 1993; Okamoto *et al.*, 1995). Moreover, this domain is identical among all three FAD-APPs. Therefore, FAD-APPs should negatively regulate CRE by virtue of this cytoplasmic function and relevant activation of $G_o$.

This study also provides the direct evidence that FAD-APP selectively recognizes and couples to the C terminus of $G\alpha_o$ in the same whole-cell system. Here, we have constructed $\alpha_s/\alpha_x$ chimeras having the five C-terminal residues of $G\alpha_x$ with the remainder being $G\alpha_s$. Studies by Bourne and co-workers (Conklin *et al.*, 1993; Voyno-Yasenetskaya *et al.*, 1994) have specified that the four or five C-terminal residues of Gα are the major determinant of receptor specificity and thereby constitute a principal receptor contact domain. Given the fact that cAMP stimulates CRE activity, their theory suggests that the signal of $G\alpha_x$-coupled receptors can stimulate CRE activity by the expression of $\alpha_s/\alpha_x$. Conversely, if we observe receptor-dependent CRE stimulation only with the transfection of $\alpha_s/\alpha_x$, we can assign the $G\alpha_x$-coupling function to the receptor. In the case of FAD-APP, co-expression of $\alpha_s/\alpha_o$ chimera was expected to change the effect of FAD-APP on CRE from inhibition to stimulation, because FAD-APP inhibits basal CRE activity as mentioned above.

As expected, co-transfection of Phe-APP with $\alpha_s/\alpha_o$ resulted in significant increase in CRE activity as compared with the activity observed in control transfection of $\alpha_s/\alpha_o$ without Phe-APP, indicating that Phe-APP activated $\alpha_s/\alpha_o$. Among $\alpha_s$, $\alpha_s/\alpha_{i3}$, $\alpha s/\alpha_z$ or $\alpha_s/\alpha_o$, this increase by Phe-APP was only observed when $\alpha_s/\alpha_o$ was co-transfected, demonstrating the selective linkage of Phe-APP to $\alpha_s/\alpha_o$. This was surprising, because these four chimeras are identical to $G\alpha_s$ except for their five C-terminal residues, yielding ~99% identity; and nevertheless, only $\alpha_s/\alpha_o$ assisted the stimulation of CRE by Phe-APP. Particularly, it should be noted that $\alpha_s/\alpha_o$ and $\alpha_s/\alpha_{i3}$ have only two amino acids different among 394 residues. One might consider that Phe-APP activates endogenous $G_i$, and the released $G\beta\gamma$ in turn promotes CRE activity. However, this seems unlikely, because: (i) $G\beta\gamma$ requires the basal $G\alpha_s$ activity to stimulate CRE; (ii) $\alpha_s/\alpha_o$ exhibited the lowest basal $G\alpha_s$ activity among these chimeras (Figure 3B, open columns); and (iii) co-expression of other $G\alpha_s$ chimeras did not assist Phe-APP stimulation of CRE, despite the fact that they showed considerably high basal $G\alpha_s$ activity. The $\alpha_s$ chimera data therefore provide direct evidence that Phe-APP recognizes the C-terminus of $G\alpha_o$

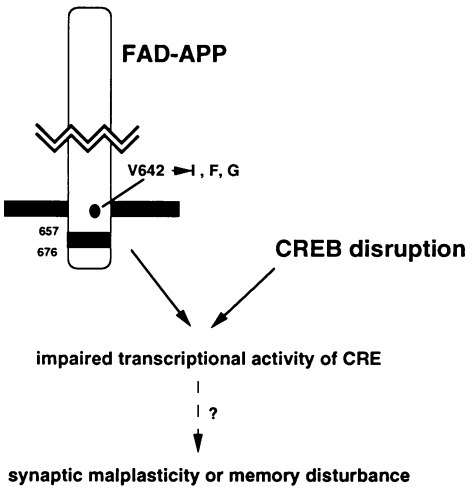


**Fig. 4.** Schematic model of the regulation abnormality of CRE by FAD-APPs. This figure proposes a model for the signal transduction abnormality induced by FAD-APPs and how it could contribute potentially to the pathogenesis of FAD. This study indicates that FAD-APPs suppress the transcriptional activity of CRE in whole cells. Since the transcriptional activity of CRE is essential for long-term memory formation, sustained inhibition of CRE activity by FAD-APP may deteriorate memory-related systems, as does CREB deficiency, potentially contributing to synaptic malplasticity or memory loss in FAD.

highly selectively and executes *in vivo* coupling to this G protein.

In contrast to the dominant actions of FAD-APPs, normal $APP_{695}$ produced neither CRE inhibition nor $\alpha_s$/$\alpha_o$ stimulation in the same systems. We previously reported that wild-type $APP_{695}$ behaves like a normal $G_o$-coupled receptor in phospholipid vesicles (Okamoto *et al.*, 1995). Since normal receptors generate no signal in non-liganded conformations, these results are consistent with the theory that APP is a normal receptor. Therefore, the dominant function of the three FAD-APPs shown here further supports the idea that they encode gain-of-function mutants that constitutively activate $G_o$ in whole cells. This idea closely agrees with another line of the present results indicating that $G_o$ mediates the action of FAD-APPs.

Because all three FAD-APPs have a cytoplasmic domain entirely identical to that of wild-type $APP_{695}$, it is highly likely that transmembrane FAD mutations cause the conformational change of $APP_{695}$ and turn on its cytoplasmic signaling function. In fact, we have demonstrated that three FAD-APPs behave like constitutively activated $G_o$-coupled receptors in reconstituted vesicles (T.Okamoto *et al.*, manuscript submitted). Frameworks which are similar in mechanism and are turned on by transmembrane mutations have been described for receptor tyrosine kinases such as neu/c-erbB2 (Bargmann *et al.*, 1986) and IGF-I receptor (Takahashi *et al.*, 1995). It should also be emphasized that this action predominance of FAD-APPs over wild-type $APP_{695}$ fits well with the dominant inheritance of this type of FAD (Karlinsky *et al.*, 1992), which suggests the presence of action in FAD-APPs.

Accumulated evidence indicates that CRE activity is tightly linked to synaptic plasticity and long-term memory formation (Frank and Greenberg, 1994). In *Aplysia* neurons, cAMP and CREB, the CRE-binding protein, both of which positively regulate CRE, activate the expression of genes required for long-term synaptic plasticity (Dash *et al.*, 1990; Kaang *et al.*, 1993; Alberini *et al.*, 1994). Also, both early and late stages of hippocampal long-term potentiation depend on cAMP activity (Huang *et al.*, 1994). Recent studies (Bourtchuladze *et al.*, 1994; Yin *et al.*, 1994) with transgenic technology have revealed that the functional knock-out of CREB results in a loss of long-term memory in mice and *Drosophila*. These findings confirm a fundamental role of CRE activity for memory formation at both cellular and whole-animal levels. It is therefore conceivable that negative transactivation of CRE by FAD-APPs may afflict synaptic plasticity and memory formation during a long course of action, as has been observed in CREB deficiency (Figure 4). While extensive neuronal loss clearly constitutes one of the major reasons for memory disorder in AD, it is also accepted that memory loss is the earliest manifestation of this disease, observed long before the occurrence of brain atrophy. This study may thus provide a molecular clue to the potential bases for such functional abnormalities in AD pathophysiology which are not revealed by organic changes.

## Materials and methods

$APP_{695}$ cDNA and all mutants used in this study were as described (Yamatsuji *et al.*, 1995). The $G\alpha_s$ chimeras $\alpha_s/\alpha_o$, $\alpha_s/\alpha_{i3}$ and $\alpha_s/\alpha_z$ were constructed as follows. First, PCR was performed to add *Afl*II and *Xba*I sites at the 3′ end of $G\alpha_s$ cDNA using the following two primers: ATCTGGAATAACAGATGGCTGC and AAACTAGTCTAGACTA-GCTCAAATTCTTAAGTGCATGCGCTGGATGATGTCA. The PCR product was digested with *Bgl*II and *Xba*I and subcloned into pcDNA-1-$G\alpha_s$ ($G\alpha_s$-AX), which was predigested with the same enzymes. After confirming by sequencing that the PCR-driven part contains both *Afl*II and *Xba*I sites, $G\alpha_s$-AX was digested with those enzymes and ligated with synthetic oligonucleotides designed to possess *Afl*II and *Xba*I sites at the both ends. The nucleotides were TTAAGAGGTTGCGGCTTGTA-CTAAT and CTA-GATTAGTACAAGCCGCAACCTC (for construction of $\alpha_s/\alpha_o$), TTAAGAGAATGCGGC-TTATTTTAAT and CTAGATTAA-AATAAGCCGCATTCTC (for $\alpha_s/\alpha_{i3}$), TTAAGATA-CATTGGCCT-TTGCTAAT and CTAGATTAGCAAAGGCCAATGTATC (for $\alpha_s/\alpha_z$). It was confirmed by sequencing that the final products encoded the designed sequences. Creation of the *Afl*II site in $G\alpha_s$ cDNA did not affect the original amino acid residues, thus allowing the expression of $\alpha_s/\alpha_x$ chimeras as designed. Bovine $G\alpha_o$ cDNA was kindly provided by Dr T.Nukada. Unless specified, $G\alpha_o$ denotes $G\alpha_{o1}$ in this study. $G\alpha_oQ205L$ cDNA was as described (Ikezu *et al.*, 1994). $G\alpha_{o2}Q205L$ cDNA was described previously (Strittmatter *et al.*, 1994).

NK1 cells were grown in DME plus 10% FCS and antibiotics. This cell line is a naturally occurring transformant of COS-7 cells, which express endogenous $G\alpha_o$ (Yamatsuji *et al.*, 1995). Transient transfection was performed by the lipofection method as described (Ikezu *et al.*, 1994). For stable expression of $G\alpha_o$, NK1 cells were transfected by the calcium phosphate method using 10 μg of $G\alpha_{o2}$ cDNA and 1 μg pBabe-Puro. Cells were then selected with 3 μg/ml puromycin in DME plus 10% CS. After 2–3 weeks, colonies were picked, transferred to a 24-well plate, tested for immunoblot analysis with anti-$G\alpha_o$ antibody (UBI), and amplified for future studies. NK1 cells overexpressing bcl-2 were described previously (Yamatsuji *et al.*, 1995).

CRE is typified by the consensus palindromic sequence TGACGTCA and present in the promoters of many genes. Our CRE-CAT reporter, described previously (Takahashi *et al.*, 1993), has CRE located in the promoter of the somatostatin gene, which is highly responsive to cAMP stimulation. For CAT assay, $10^5$ cells were seeded onto a 6-well plate 24 h before transfection. 1 μg cDNA of interest, 0.2 μg pactβgal, 0.3 μg CRE-CAT reporter were co-transfected with LipofectAMINE (3 μl, Gibco). Media were changed at 24 h after transfection. CTX (1 μg/ml) was added to these media and cells were incubated for another 24 h. For PTX treatment, cells were treated with 10 ng/ml PTX 12 h after transfection, incubated for 12 h, and incubated with PTX and CTX for 24 h. CAT assay was performed as described (Ikezu *et al.*, 1994). CAT

activity was normalized by β-galactosidase activity. CRE-CAT and SV40 promoter-PAP (Ikezu *et al.*, 1994) were similarly used, with their reporter activities being normalized by β-galactosidase activity.

Immunoblot analysis was performed as described (Nishimoto *et al.*, 1993). For detection of transfected APP genes, AC-1 (kindly provided by K.Yoshikawa) and 22C11 (Boehringer-Mannheim) were used at 1/10000 dilution and 50 ng/ml, respectively. HRP-conjugated anti-rabbit IgG was used as a second antibody (1/4000 dilution, Calbiochem), and the antigenic bands were visualized by ECL.

All results presented in this study were repeated at least three times with independent sets of transfection, each of which yielded similar results. Statistical significance was determined with Student's *t* test.

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