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Activating Mutation of Adenylyl Cyclase Reverses its Inhibition by G Proteins

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ABSTRACT

We have implemented a yeast genetic selection developed previously by our laboratory to identify mutant mammalian type V adenylyl cyclases insensitive to inhibition by $G_{i\alpha}$. One mutation isolated was localized to the first cytoplasmic domain at a Phe residue (position 400), which is conserved in all nine isoforms of membrane-bound mammalian adenylyl cyclase. Biochemical characterization of the F400Y mutant revealed a dramatic conversion of the $G_{i\alpha}$ response from inhibitory to stimulatory. This mutation results in additional activating effects. The mutant exhibits an enhanced sensitivity toward activation by either $G_{s\alpha}$ or forskolin. Synergism between $G_{s\alpha}$ and forskolin is not observed for the F400Y mutant, presumably because the mutant already is in the sensitized state. Additionally, an enhancement of the basal unstimulated activity was observed. This mutation, which is the first demonstration of an activating point in a mammalian adenylyl cyclase, mimics a sensitized conformation of the wild-type enzyme that underlies the synergism between stimulatory inputs, and additionally, removes the inhibitory regulatory input provided by $G_{i\alpha}$. Because sensitizing adenylyl cyclase toward its stimulators can have profound biological implications, this raises the possibility that naturally occurring mutations resembling those at the Phe400 residue may be associated with human disease states.

The hormonal regulation of the integral membrane adenylyl cyclase enzymes is primarily responsible for modulating intracellular levels of cAMP. Nine isoforms of membrane-bound mammalian adenylyl cyclase have been identified by molecular cloning techniques, and the activity of these enzymes is regulated by a variety of G protein and non-G protein inputs in an isoform-specific manner (Sunahara et al., 1996; Cooper, 1998; Taussig and Zimmermann, 1998). All membrane-bound adenylyl cyclases consist of a short cytoplasmic amino terminus and six transmembrane segments (called M1) that are followed by a large cytoplasmic domain of ~200 amino acids (called C1); this motif is repeated in the second half of the molecule, which contains a second membrane-spanning (M2) and a cytoplasmic (C2) domain.

The structural motifs of adenylyl cyclase responsible for recognizing and discriminating regulatory molecules and for catalytic activity are presently being elucidated. Mutagenic and crystallographic analyses of adenylyl cyclase have uncovered the binding site for forskolin and $G_{s\alpha}$, the two common stimulators of this enzyme (Tesmer et al., 1997; Yan et al., 1997; Zhang et al., 1997; Zimmermann et al., 1998a). A region in the C2 domain of type II adenylyl cyclase (residues 956 to 982) has been shown to bind the G protein $\beta\gamma$ subunits (Chen et al., 1995), whereas another region of the C1 domain of the type I enzyme (residues 495 to 522) appears to be involved in regulation by calmodulin (Vorherr et al., 1993; Wu et al., 1993). More recently, the mutagenic analysis of adenylyl cyclase has indicated that $G_{i\alpha}$ binds to a region of the C1 domain that is structurally related to the $G_{s\alpha}$-binding site on the homologous C2 domain (Dessauer et al., 1998). In addition, the catalytic mechanism of adenylyl cyclase has been elucidated and appears to involve two Mg^{2+} ions, in manners analogous to the catalytic mechanisms of DNA polymerase (Mitterauer et al., 1998; Zimmermann et al., 1998b).

The characterization of regulatory domains on adenylyl cyclase has also addressed the molecular mechanisms by which stimulators of this enzyme promote catalytic activity. The two cytoplasmic domains of adenylyl cyclase can be expressed independently and reconstitute catalytic activity when mixed in vitro (Whisnant et al., 1996; Yan et al., 1996). The addition of cyclase activators to these domains enhances their affinity for each other and stimulates enzymatic activity, although it is unclear how much of a role this plays in activating the full-length protein in which the two cytoplasmic domains are linked. Because the structure of adenylyl cyclase in the absence of bound stimulators has not been determined, the precise conformational changes associated with activation remain unknown. However, certain features of $G_{s\alpha}$ stimulation have been modeled, based on the available crystal structures of adenylyl cyclase (Tesmer et al., 1997;

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ABBREVIATIONS: GTPγS, guanosine 5′-*O*-thiotriphosphate; WT, wild type.

Zhang et al., 1997). These models predict that the binding of $G_{s\alpha}$ induces a rotation of the two cytoplasmic domains relative to each other and alters the positioning of key catalytic residues at the active site (Skiba and Hamm, 1998; Tesmer and Sprang, 1998).

An additional level of regulatory complexity is established by the ability of various adenylyl cyclase isoforms to integrate coincident regulatory inputs. This regulatory property can be observed when the addition of one activator to adenylyl cyclase enhances the enzyme's responsiveness toward additional stimulators. Types II and IV adenylyl cyclase, for example, are only weakly stimulated by G protein $\beta\gamma$ subunits, but they show a dramatic increase in activity when the $\beta\gamma$ heterodimer is added in the presence of $G_{s\alpha}$ (Gao and Gilman, 1991; Tang and Gilman, 1991); these stimulatory inputs and the interplay among them are regulated further by the phosphorylation state of these cyclases (Zimmermann and Taussig, 1996). In addition, $G_{s\alpha}$ is able to enhance the stimulatory effects of Ca^{2+}-calmodulin by promoting a synergistic activation of the types I and VIII isoforms (Cali et al., 1994; Wayman et al., 1994). $G_{s\alpha}$ and forskolin also display synergism in the activation of several adenylyl cyclase isoforms (Feinstein et al., 1991; Gao and Gilman, 1991; Taussig et al., 1994b), but they do not superactivate type I adenylyl cyclase, in spite of the ability of this isoform to respond to either stimulator individually (Tang et al., 1991). The responsiveness of specific isoforms of adenylyl cyclase toward regulators therefore is highly sensitive to the activation state of the enzyme.

We have described previously the development of a genetic selection system for the identification of mutant adenylyl cyclases defective in their regulatory properties (Zimmermann et al., 1998a). This system relies on the expression of mammalian type V adenylyl cyclase in the budding yeast *Saccharomyces cerevisiae*, and has been used to elucidate the $G_{s\alpha}$-binding site on adenylyl cyclase and the role of metal ions in the catalytic mechanism of this enzyme (Zimmermann et al., 1998a,b). In this report, we have used the yeast selection system to identify a mutation in adenylyl cyclase (F400Y) that induces an activated state of the enzyme and results in an enhanced sensitivity toward cyclase stimulators. We predict that this mutation mimics an activated conformation of the wild-type (WT) enzyme that underlies the synergism between stimulatory inputs. The mutation also results in additional activating effects, eliminating the inhibition of the enzyme by $G_{i\alpha}$, leading instead to a $G_{i\alpha}$-dependent stimulation of adenylyl cyclase.

Experimental Procedures

Yeast Strains, Plasmid Construction, and Mutant Selection. The cyclase-deficient yeast strain TC41–1 (Casperson et al., 1985) (*MAT a*, *leu2–3*, *leu2–112*, *ura3–52*, *his3*, *his4*, *cam1–3*, *cyr1* Δ*::URA3*) was a generous gift of Warren Heideman (University of Wisconsin, Madison, WI). The construction of an isogenic derivative of this strain (12229) expressing the rat $G_{s\alpha}$ and the construction of the plasmid pADHprACVLeu encoding the dog type V adenylyl cyclase (Ishikawa et al., 1992) were described previously (Zimmermann et al., 1998a). Plasmid pVT100U-$G_{i\alpha1}$ (Q227L) was constructed by ligating an *Nco*I-*Hin*DIII fragment encoding the coding region of the constitutively active $G_{i\alpha1}$ mutant with the pVT100U vector digested with *Xho*I; before the ligation, these DNA fragments were blunt-ended by incubation with Klenow DNA polymerase and deoxy nucleotide triphosphates. This plasmid was introduced into the 12229 strain to produce the $G_{s\alpha}$- and $G_{i\alpha}$-expressing yeast strain DZ2002–2B. Randomly mutated libraries of plasmid pADHprACV-Leu were generated by error-generating polymerase chain reaction mutagenic techniques and have been described previously (Zimmermann et al., 1998a). Procedures for the selection of type V adenylyl cyclase mutants, characterization of these mutants via DNA sequencing, and the retesting of these mutants in yeast were published previously (Zimmermann et al., 1998a).

Sf9 Cell Culture and Preparation of Cell Membranes. Procedure for the culture of Sf9 cells and generation and amplification of recombinant baculovirus encoding both the WT and the mutant-type V adenylyl cyclase were performed as described previously (Zimmermann et al., 1998a). Sf9 membranes containing individual adenylyl cyclase isoforms were prepared according to methods published previously (Taussig et al., 1994a).

Purification of G Protein Subunits. $G_{s\alpha}$ and myristoylated $G_{i\alpha1}$ were synthesized in bacteria and purified as described by Lee et al. (1994). Protein concentrations were estimated by staining with amido black (Schaffner and Weissmann, 1973). The G protein α subunits were activated by incubation with 50 mM Na-HEPES (pH 8.0), 5 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, and 400 μM guanosine 5′-O-thiotriphosphate (GTPγS) at 30°C for 30 ($G_{s\alpha}$) or 120 ($G_{i\alpha}$) min. Unbound nucleotides were removed by gel filtration in HMED buffer containing: 20 mM Na-HEPES (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, and 3 mM dithiothreitol, as described previously (Graziano et al., 1989).

Adenylyl Cyclase Assay. Adenylyl cyclase activity was measured using the procedure by Smigel (1986). All assays were performed for 10 min at 30°C in a final volume of 100 μl containing: 50 mM Na-HEPES (pH 8.0), 500 μM ATP, 0.6 mM EDTA, 3 mM K_2 phosphoenolpyruvate, 10 mM $MgCl_2$, 500 μM 3-isobutly-l-methylxanthine, 0.1 μg/μl BSA, 1 μg/μl pyruvate kinase, and 20 μg of membrane protein. Activated G protein α subunits were diluted into HMED buffer and mixed with membranes before the start of the assay.

Adenylyl Cyclase Purification and Immunoblotting. WT and mutant type V adenylyl cyclase proteins were purified from Sf9 membranes using published procedures (Taussig et al., 1993). Samples were resolved by SDS-polyacrylamide electrophoresis and immunoblotted as described (Taussig et al., 1994a), using a primary rabbit antibody specific for type V/VI adenylyl cyclase (Gao et al., 1997) as described previously (Zimmermann et al., 1998a).

Data Analysis. Data were analyzed using the GraphPad Prism (GraphPad Software Inc., San Diego, CA) program to determine effective concentration (EC_{50}) values of the dose-response curves.

Results

Yeast (*Saccharomyces cerevisiae*) require cAMP for growth, and strains lacking a functional adenylyl cyclase (encoded by the *CYR1* locus) are nonviable unless cAMP is added to the medium (Ishikawa et al., 1988). We have described previously the development of a genetic selection system for the identification of mutant adenylyl cyclases with defects in their regulatory properties that uses a *cyr1*-deleted strain of yeast (Zimmermann et al., 1998a). We reasoned that we may be able to use this yeast selection system to identify adenylyl cyclase mutants with defects in their inhibition by $G_{i\alpha}$. As indicated in Fig. 1, expression of mammalian type V adenylyl cyclase in the *cyr1*(−) strain allows the yeast to grow in the presence of cyclase activators (forskolin or $G_{s\alpha}$). The yeast are unable to grow in the absence of activators or when the inhibitory G protein ($G_{i\alpha1}$) is coexpressed, presumably because of the low catalytic activity of the cyclase under these conditions.

By selecting for transformants that grow in the presence of both $G_{s\alpha}$ and $G_{i\alpha}$, mutant cyclases isolated are likely to encode proteins that are insensitive to inhibition by $G_{i\alpha}$. Alternatively, we may isolate mutations that result in elevated catalytic activities and, thus, satisfy the yeast's requirement for cAMP, even when the cyclase activity is reduced by $G_{i\alpha}$. By screening a library of randomly mutated type V adenylyl cyclase (complexity = 4×10^5), we obtained 1.02×10^6 transformants of which 25 were able to grow in the presence of $G_{s\alpha}$ and $G_{i\alpha1}$ expression. Figure 1B depicts the growth phenotype of the F400Y mutant, which was isolated twice by this selection. By contrast to the WT enzyme, this mutant cyclase allowed the yeast to grow in the presence of $G_{i\alpha}$ and, surprisingly, in the absence of cyclase activators as well.

To determine the biochemical basis for both the basal and $G_{i\alpha}$-insensitive growth phenotypes, we overexpressed the F400Y mutant in Sf9 cells and used membrane preparations of these cells to characterize further the effects of this mutation. As indicated in Fig. 2, the mutant cyclase, like the WT, is activated by both forskolin and $G_{s\alpha}$ and, under forskolin-activating conditions, has a similar K_m for substrate MgATP (75 and 95 μM for WT and mutant, respectively). The F400Y mutant, however, displayed a significantly elevated basal activity (13-fold higher than WT) paralleling the yeast growth phenotype, although this activity was very low relative to the forskolin- or $G_{s\alpha}$-stimulated activities. In addition, we found that the F400Y mutant displayed no inhibition in response to $G_{i\alpha}$, suggesting that the growth phenotype of yeast expressing $G_{i\alpha}$ is not simply the result of an elevated basal activity, but actually attributable to the $G_{i\alpha}$-insensitive nature of the mutant.

The lack of $G_{i\alpha}$ inhibition observed for the F400Y mutant was characterized further by measuring the effect of varying concentrations of $G_{i\alpha1}$ on the $G_{s\alpha}$-stimulated activity of this enzyme (Fig. 3). By contrast to the strong inhibition seen for the WT enzyme, increasing the concentration of $G_{i\alpha}$ surprisingly stimulated the activity of the mutant enzyme. This stimulatory effect was observed at slightly higher concentrations of $G_{i\alpha}$ than those required to inhibit the WT enzyme and also was observed for the forskolin-stimulated enzyme (data not shown). Boiling the $G_{i\alpha}$ protein for 10 min eliminated both stimulatory and inhibitory effects, indicating that neither was attributable to buffer components in the $G_{i\alpha}$ preparation (data not shown). It was shown previously that high concentrations of $G_{i\alpha}$ can have modest stimulatory effects on other isoforms of adenylyl cyclase and that this stimulation was proposed to be attributable to the binding of $G_{i\alpha}$ to the stimulatory $G_{s\alpha}$ site on adenylyl cyclase (Taussig et al., 1994b). Thus, it seemed possible that the F400Y mutation alters the $G_{s\alpha}$-binding site in a manner that allows $G_{i\alpha}$ to bind to this site better and thereby activate the cyclase. Such a model appeared unlikely because the Phe400 residue is not located near the $G_{s\alpha}$-binding site (see below), although it remained possible that the mutation may exert its effects on the $G_{s\alpha}$ site via more global conformational changes.

If the stimulation of the F400Y mutant by $G_{i\alpha}$ is attributable to the binding of $G_{i\alpha}$ to an altered $G_{s\alpha}$ site, saturating concentrations of $G_{s\alpha}$ should eliminate the stimulation of the

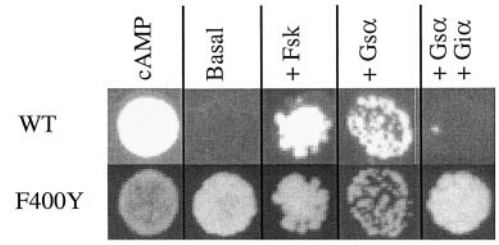

Fig. 1. Growth phenotypes of yeast expressing WT and mutant adenylyl cyclase. Growth characteristics of wild type (WT) or mutant (F400Y) type V adenylyl cyclase constructs were evaluated under the following conditions: addition of 2 mM cAMP to the medium (cAMP), no regulator (Basal), addition of 100 μM forskolin to the medium (+Fsk), coexpression of $G_{s\alpha}$ (+$G_{s\alpha}$), coexpression of $G_{s\alpha}$, and $G_{i\alpha1}$ (+$G_{s\alpha}$ +$G_{i\alpha}$). Growth was assessed in yeast strain TC41–1 (for basal- or forskolin-stimulated conditions), isogenic yeast strains 12229 ($G_{s\alpha}$-stimulated condition), or DZ2002–2B ($G_{i\alpha}$ inhibition of $G_{s\alpha}$-stimulated condition); growth in the presence of cAMP was observed in all three strains (only TC41–1 is shown).

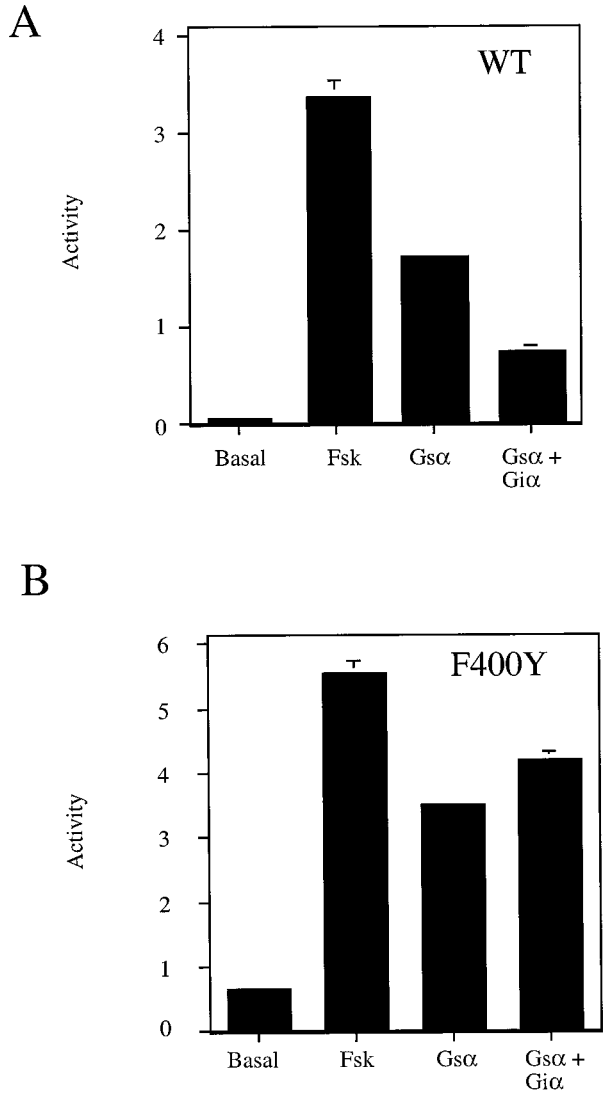

Fig. 2. Regulatory properties of WT and mutant type V adenylyl cyclase. Membranes from Sf9 cells expressing the WT (A) or the F400Y mutant (B) were assayed for adenylyl cyclase activity in the presence of the indicated regulators. No activator (Basal), 100 μM forskolin (Fsk), 100 nM $G_{s\alpha}$ ($G_{s\alpha}$), 100 nM $G_{s\alpha}$, and 1 μM $G_{i\alpha1}$ ($G_{s\alpha}$+$G_{i\alpha}$). Activities are expressed as nmol $min^{-1} \cdot mg^{-1}$ and have been normalized to reflect differences in mutant expression level reported in Table 1. Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments.

enzyme by $G_{i\alpha}$. As shown in Fig. 4, the stimulation of the F400Y mutant by $G_{i\alpha}$ does occur at saturating concentrations of $G_{s\alpha}$, indicating that this stimulatory effect is not attributable to the binding of $G_{i\alpha}$ to the $G_{s\alpha}$-binding site of the enzyme. Consistent with this is our observation that $G_{i\alpha}$ fails to stimulate the F400Y mutant in the presence of both $G_{s\alpha}$ and forskolin (data not shown), conditions in which $G_{i\alpha}$ inhibition is also not seen for the WT cyclase (Dessauer et al., 1998). The data also demonstrates that $G_{i\alpha}$ is unable to stimulate the mutant enzyme in the absence of $G_{s\alpha}$. Stimulating effects by $G_{i\alpha}$ on the mutant also were observed when forskolin was used as the activator, indicating that it is the enzyme activation, and not $G_{s\alpha}$ binding per se, that is necessary for the mutant enzyme to respond to $G_{i\alpha}$. Therefore, it seems likely that $G_{i\alpha}$ is still binding to the $G_{i\alpha}$-binding site of the mutant protein, but that this binding results in an activation of the enzyme instead of the inhibition observed for the WT enzyme.

In the course of performing the experiments described above, we noted that the F400Y mutation also appeared to shift the $G_{s\alpha}$ dose-response curve leftward. This effect is further illustrated in Fig. 5 and indicates that the mutation sensitizes the cyclase to $G_{s\alpha}$ stimulation. It was shown previously that the type V enzyme becomes sensitized to $G_{s\alpha}$ in the presence of forskolin and, thus, it is possible that the mutant cyclase mimics the forskolin-bound conformation of adenylyl cyclase. As shown in Fig. 6, the F400Y mutant displays significant stimulation at much lower concentrations of forskolin than those required to stimulate the WT enzyme, indicating that the conformation induced by the mutation is not equivalent to either the forskolin-bound or the $G_{s\alpha}$-bound states of the WT enzyme. Rather, the mutation appears to induce an activated state of the adenylyl cyclase that sensitizes the enzyme toward both activators and enhances its basal activity.

The two activators of type V adenylyl cyclase have been shown to stimulate the enzyme in a synergistic manner. Thus, the addition of one cyclase activator enhances the sensitivity of the enzyme toward the second stimulator. The activated state of the F400Y mutant thus may mimic a sensitized conformation that can be induced in the WT enzyme by the binding of either cyclase stimulator. Alternatively, the F400Y mutant might be sensitized further by the binding of an activator. Therefore, we tested whether the mutant exhibits synergism between forskolin and $G_{s\alpha}$. Whereas the addition of forskolin to the WT enzyme results in a leftward shift in the $G_{s\alpha}$ dose-response curve (Fig. 7A and Table 1), as well as an increase in the maximum enzyme activity, the effects of forskolin and $G_{s\alpha}$ appear to be merely additive for

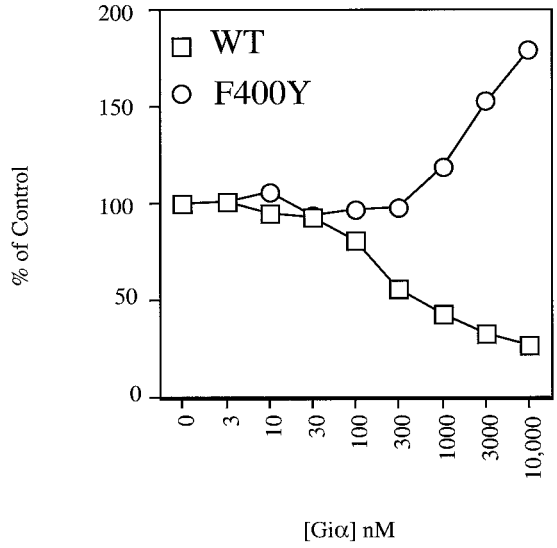

Fig. 3. Regulation of WT and F400Y adenylyl cyclase by $G_{i\alpha}$. Sf9 membranes containing the WT (□) or mutant (○) adenylyl cyclase constructs were assayed for adenylyl cyclase activity in the presence of 100 nM $G_{s\alpha}$ and indicated amounts of $G_{i\alpha 1}$. Activities are expressed as percent control (measured in the absence of added $G_{i\alpha 1}$). Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments.

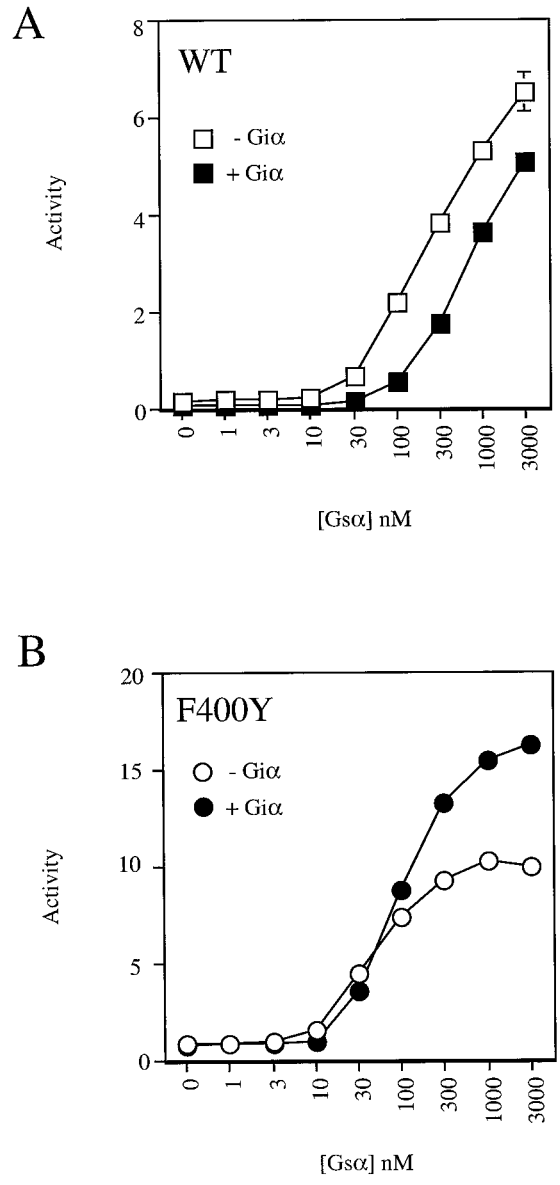

Fig. 4. $G_{i\alpha}$ stimulates the F400Y mutant by binding to the $G_{i\alpha}$ site on the cyclase. Membranes from Sf9 cells expressing the WT (A) or the F400Y mutant (B) were assayed for adenylyl cyclase activity in the presence of indicated amounts of $G_{s\alpha}$, either in the absence (open symbols) or presence (solid symbols) of 1 μM $G_{i\alpha 1}$. Activities are expressed as nmol $min^{-1} \cdot mg^{-1}$ and have been normalized to reflect differences in mutant expression level reported in Table 1. Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments.

the F400Y mutants (Fig. 7B and Table 1). The same behavior was observed when the synergism between stimulators was tested by evaluating the effect of $G_{s\alpha}$ on the forskolin dose-response curve (data not shown). The EC_{50} values presented in Table 1 indicate that the F400Y mutant displays the same sensitivity toward $G_{s\alpha}$ (43 nM) as does the forskolin-stimulated WT enzyme (42 nM), indicating that the partially activating conformation induced by the F400Y mutation is very similar to the sensitized state of the WT enzyme in the presence of cyclase activators. However, the F400Y mutation has the unique property of sensitizing adenylyl cyclase without inducing substantial levels of basal enzymatic activity.

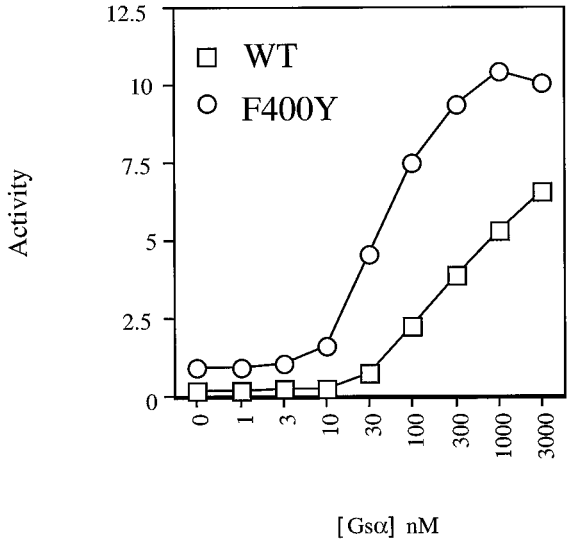

Fig. 5. F400Y mutant adenylyl cyclase is sensitized to $G_{s\alpha}$ stimulation. Membranes from Sf9 cells expressing either the WT (□) recombinant type V adenylyl cyclase or the F400Y mutant (○) were assayed for adenylyl cyclase activity in the presence of indicated amounts of $G_{s\alpha}$. Activities are expressed as nmol $min^{-1} \cdot mg^{-1}$ and have been normalized to reflect differences in mutant expression level reported in Table 1. Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments.

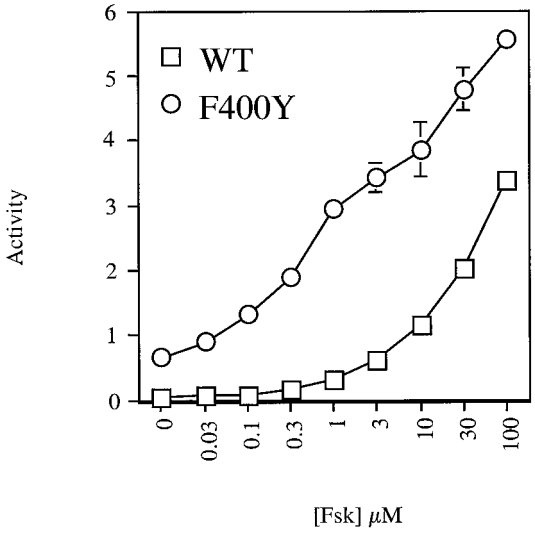

Fig. 6. F400Y mutant adenylyl cyclase is sensitized to forskolin stimulation. Sf9 membranes containing the WT (□) or F400Y mutant (○) adenylyl cyclase constructs were assayed for adenylyl cyclase activity in the presence of indicated concentrations of forskolin. Activities are expressed as nmol $min^{-1} \cdot mg^{-1}$ and have been normalized to reflect differences in mutant expression level reported in Table 1. Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments.

Discussion

Our studies demonstrate that the F400Y substitution promotes an activated state that alters the responsiveness of type V adenylyl cyclase toward both stimulatory and inhibitory regulators. The Phe400 residue is conserved across the nine isoforms of mammalian adenylyl cyclase, and inspection of the structure of the adenylyl cyclase catalytic core reveals that this residue does not form part of the binding sites for $G_{s\alpha}$, $G_{i\alpha}$, or forskolin (Fig. 8A). Closer inspection reveals that this residue is part of the α1 helix in the C1 domain and is in close proximity to critical catalytic residues at the active site

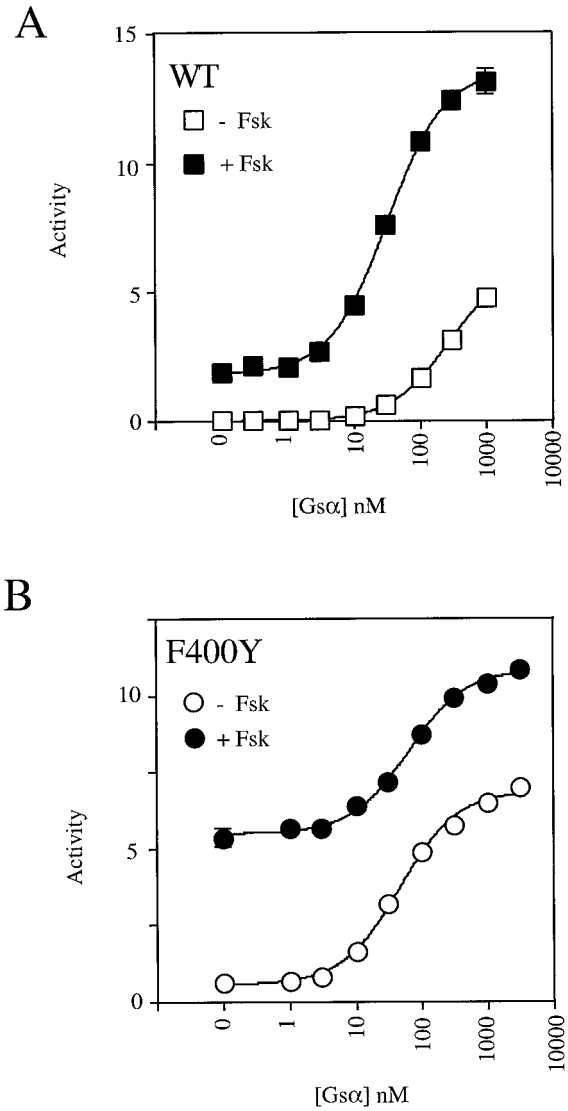

Fig. 7. F400Y mutant lacks synergistic activation by forskolin and $G_{s\alpha}$. Sf9 membranes containing the WT (A) or F400Y mutant (B) adenylyl cyclase constructs were assayed for adenylyl cyclase activity in the presence of varying amounts of $G_{s\alpha}$ either in the absence (open symbols) or presence (solid symbols) of 50 μM forskolin. Activities are expressed as nmol $min^{-1} \cdot mg^{-1}$ and have been normalized to reflect differences in mutant expression level reported in Table 1. Assays were performed in duplicate (bars, S.D.), and results are representative of at least three experiments. The indicated curves were fit to a sigmoidal dose-response using GraphPad Prism.

of the enzyme (Fig. 8B). The mutagenic analysis of adenylyl cyclase has targeted other residues on this short α1 helix with no apparent effect on the stimulation by $G_{s\alpha}$ or inhibition by $G_{i\alpha}$ (Dessauer et al., 1998), indicating further that this helix is not involved directly in the binding of $G_{s\alpha}$ or $G_{i\alpha}$.

Phe400 forms a hydrophobic pocket that includes Ile397, Leu403, Leu412, and Leu416. The substitution of Tyr for the Phe at position 400 does not disrupt this hydrophobic pocket; rather, the hydroxyl group of the Tyr residue is predicted to hydrogen-bond with the carbonyl group of Gly439 (type V numbering) and the N7 amine of Asn1025 (type II numbering). These residues occur on the α4 helix (C2 region) and the $\beta2\beta3$ hairpin loop (C1 region) that position the essential catalytic residues Arg1029 and Asp440 (Fig. 8B). We believe that the net result of these new interactions is the narrowing of the catalytic cleft by altering the positioning of the α4 helix and the $\beta2\beta3$ hairpin loop, and reducing the distance between the pair of C1 aspartates (positions 396 and 440) and Arg1029, therefore optimizing the positioning of these key catalytic residues relative to the substrate.

The F400Y mutant is characterized by an enhanced level of basal activity and a higher sensitivity toward both $G_{s\alpha}$ and forskolin. It is likely that the narrowing of the catalytic cleft underlies all of these properties, because it resembles the proposed mechanism by which $G_{s\alpha}$ activates adenylyl cyclase (Skiba and Hamm, 1998; Tesmer and Sprang, 1998). Because a sensitized conformation of the enzyme in the presence of either activator promotes the synergistic stimulation of adenylyl cyclase by the other activator, the F400Y mutant may assume a conformation similar to the stimulator-bound cyclase. Synergism between $G_{s\alpha}$ and forskolin is not seen for the F400Y mutant, presumably because the mutant already is in the sensitized state before the binding of the first activator.

At this time, it is unclear exactly how these added interactions might reverse the inhibitory effect of $G_{i\alpha}$. However, it is tempting to speculate that the hydrogen bonds will restrict the proposed movement of the α1 helix in response to $G_{i\alpha}$ binding (Dessauer et al., 1998), and almost certainly alter the $G_{i\alpha}$-bound conformation of the active site by promoting a more favorable positioning of the key catalytic moieties.

The type V F400Y mutant has the striking property of sensitizing adenylyl cyclase without inducing substantial levels of basal enzymatic activity. It is now clear that sensitized conformations of adenylyl cyclase can be induced under various conditions in an isoform-specific manner. For exam-

TABLE 1

Properties of F400Y mutant expressed in Sf9 cells

Expression levels of active type V adenylyl cyclase mutants in Sf9 membrane preparations were determined by Western blotting and are reported as the percent of WT expression level. As described previously (Zimmermann et al., 1998a), relative expression levels were calculated by determining the activity of the membrane preparations and dividing this value by the specific activity of the WT and mutant enzymes. First, purified WT or mutant adenylyl cyclase protein (equivalent activities) were quantified by Western blot analysis to calculate the specific activity of the mutant relative to the WT enzyme. Relative specific activity = (catalytic activity loaded on Western blot)/(optical density of cyclase band as % of WT). Next, expression levels were calculated by dividing the activity of Sf9 membrane preparations by the specific activity of the WT and mutant enzymes. Relative expression level = (catalytic activity of Sf9 membranes)/(relative specific activity of mutant cyclases). Data represent the average of four experiments. The EC_{50} values of adenylyl cyclase $G_{s\alpha}$ and $G_{s\alpha}$ (+Fsk) stimulation were derived from experiments represented in Fig. 7.

	Expression	$G_{s\alpha}$ EC_{50}	$G_{s\alpha}$ (+Fsk) EC_{50}
	% of WT	*nM*	*nM*
WT	100	260 (±6)	42 (±5)
F400Y	87 (±19)	43 (±15)	52 (±16)

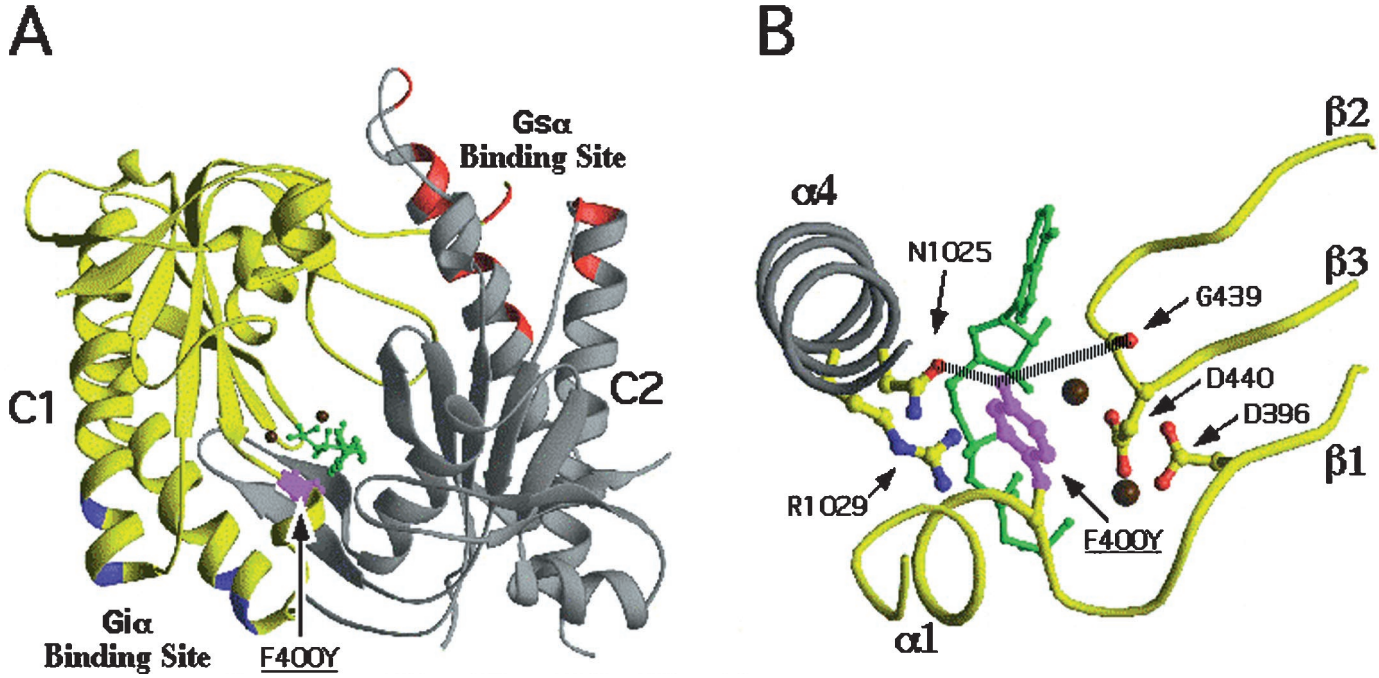

Fig. 8. Location of F400Y mutant in adenylyl cyclase structure. A, a structural representation of the heterodimer of the C1 domain of type V adenylyl cyclase (yellow) and the C2 domain of the type II isoform (gray) are shown. Residues that make up the $G_{s\alpha}$-binding site are depicted in red, whereas those involved in the binding of $G_{i\alpha}$ are shown in blue. ATP (green), the mutant Tyr residue at position 400 (magenta), and two catalytic magnesium ions (black) are depicted in their approximate positions. B, detailed view of mutant Tyr residue at position 400. Color scheme for ATP, magnesium ions, and C1 and C2 domains is same as in A. Atoms of key catalytic residues (D396, D440, R1029) and residues predicted to interact with mutant Tyr residue (G439, N1025) are depicted as small spheres colored yellow (carbon), blue (nitrogen), and red (oxygen). Putative hydrogen bonds are indicated by broken lines. See text for additional discussion.

ple, forskolin and $G_{s\alpha}$ activate synergistically most adenylyl cyclase isoforms; however, their effect on the type I isoform is merely additive. Phosphorylation by protein kinase C will induce a sensitized conformation of the type II enzyme that displays an enhanced sensitivity for $G_{s\alpha}$, but does not alter the basal activity of the cyclase substantially (Zimmermann and Taussig, 1996); type V is also phosphorylated by protein kinase C, but this leads to a direct elevation of basal activity (it was not determined whether the sensitivity toward activators also was affected) (Kawabe et al., 1994). These data indicate that the conformational changes leading to high levels of catalytic activity are distinct from those that sensitize the enzyme to stimulators, and both are critical in determining the regulatory properties of this family of enzymes. Our current study is the first to demonstrate that mutations in mammalian adenylyl cyclases likewise can alter the conformation of the enzyme and thus lead to sensitization toward activators.

Recent reports indicate that sensitized states of adenylyl cyclase may play important roles in a variety of physiological and pathophysiological conditions. Chronic activation of receptors coupled to G proteins of the G_i class can lead to an adenylyl cyclase superactivation on withdrawal of the inhibitory agonist. This effect is thought to play a role in the development of opiate tolerance and dependence as a result of prolonged exposure to opiate drugs (Avidor-Reiss et al., 1995). It seems likely that the cyclase superactivation is the result of a sensitized conformation of adenylyl cyclase, because the basal activity of the enzyme is not altered significantly by the opioid treatment, but the enzyme appears to become more sensitive to activation. Similarly, prolonged exposure to dopamine or muscarinic agonists also results in a higher sensitivity of the cyclase to forskolin and an enhanced responsiveness to $G_{s\alpha}$ (Thomas and Hoffman, 1996; Watts and Neve, 1996). These effects are cyclase isoform-specific, and are likely mediated by a phosphorylation of the cyclase (Watts and Neve, 1996; Avidor-Reiss et al., 1997; Varga et al., 1999). These reports demonstrate that sensitizing adenylyl cyclase toward its stimulators can have profound biological implications and raise the possibility that naturally occurring mutations resembling those at the Phe400 residue may be associated with human disease states.

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