Identification of a G\(_{\text{ia}}\) Binding Site on Type V Adenylyl Cyclase*

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The stimulatory G protein \(\alpha\) subunit \(G_{\alpha}\) binds within a cleft in adenylyl cyclase formed by the \(\alpha_1-\alpha_2\) and \(\alpha_3-\beta_4\) loops of the \(C_2\) domain. The pseudosymmetry of the \(C_1\) and \(C_2\) domains of adenylyl cyclase suggests that the homologous inhibitory \(\alpha\) subunit \(G_{\alpha}\) could bind to the analogous cleft within \(C_1\). We demonstrate that myristoylated guanosine 5'-3'-O-(thio)triphosphate-\(G_{\alpha1}\) forms a stable complex with the \(C_1\) (but not the \(C_2\)) domain of type \(V\) adenylyl cyclase. Mutagenesis of the membrane-bound enzyme identified residues whose alteration either increased or substantially decreased the IC\(_{50}\) for inhibition by \(G_{\alpha1}\). These mutations suggest binding of \(G_{\alpha1}\) within the cleft formed by the \(\alpha_2\) and \(\alpha_3\) helices of \(C_1\), analogous to the \(G_{\alpha1}\) binding site in \(C_2\). Adenylyl cyclase activity reconstituted by mixture of the \(C_1\) and \(C_2\) domains of type \(V\) adenylyl cyclase was also inhibited by \(G_{\alpha1}\). The \(C_1\) domain of the type \(V\) enzyme contributed to affinity for \(G_{\alpha1}\), but the source of \(C_2\) had little effect. Mutations in this soluble system faithfully reflected the phenotypes observed with the membrane-bound enzyme. The pseudosymmetrical structure of adenylyl cyclase permits bidirectional regulation of activity by homologous G protein \(\alpha\) subunits.

The hormone-sensitive adenylyl cyclase system is a well-studied paradigm of G protein-mediated signal transduction. All isoforms of mammalian adenylyl cyclase are stimulated by the heterotrimeric G protein \(G_i\). Appropriate agonist-bound, heptahedral receptors activate \(G_i\) by catalyzing the exchange of GDP for GTP. The GTP-bound \(\alpha\) subunit of \(G_i\), in turn, activates adenylyl cyclase, increasing the rate of synthesis of cyclic AMP from ATP (1, 2). Many other regulatory influences are also brought about on various adenylyl cyclases (nine isoforms are known to date), and these enzymes thus serve critical roles as integrators of these diverse inputs. Other physiological regulators of adenylyl cyclases include \(G_i\), \(G_o\), and \(G_{\alpha}\), which are inhibitors; G protein \(\beta\gamma\) subunits, which can activate or inhibit; free Ca\(^{2+}\) and Ca\(^{2+}\)-bound to calmodulin or calcineurin; and various kinases (3–5). The sites and mechanisms of interaction of these regulators with adenylyl cyclases are being defined by a variety of techniques, particularly including x-ray crystallography and mutagenesis (6–15).

Mammalian adenylyl cyclase is pseudosymmetrical, membrane-bound enzymes. The bulk of the protein consists of two repeats of a unit that includes a hydrophobic membrane-associ-

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were identified by sequencing. Type V adenylyl cyclase was subcloned from the pVL1392 vector (16) into the pAlter-1 mutagenesis vector (Promega) using the restriction sites for BamHI and HindIII. Single-stranded DNA was generated using R408 Helper phage. Regions surrounding the mutations were subcloned back into type V adenylyl cyclase in pVL1392 or VC(670)H4 for expression in SF9 cells or E. coli, respectively.

**SF9 Cell Culture and Recombinant Baculoviruses—** Procedures for the culture of SF9 cells and the production, cloning, and amplification of recombinant baculoviruses have been described by Summers and Smith (32). The plasmids containing mutations in type V adenylyl cyclase were cotransfected into SF9 cells with baculoviral DNA (BacVector-3000, Novagen), and the recombinant viruses were screened by assay of adenylyl cyclase activity in SF9 cell membranes.

Membranes were prepared from SF9 cells expressing the mutant proteins (33). Briefly, cells (2 × 10⁹/ml) were infected with the desired baculovirus (1 plaque-forming unit/cell), harvested after 48 h, and lysed by nitrogen cavitation. After removal of nuclei, membranes were pelleted, washed, and resuspended in 20 mM NaHepes (pH 8.0), 2 mM dithiothreitol, and 200 mM sucrose.

**G Protein Subunits—** All G protein subunits were synthesized in *E. coli* as described by Lee et al. (34). Gₐₐₐ was coexpressed with yeast protein N-myristoyltransferase (20) for synthesis of myristoylated protein. Purification of recombinant α subunits was achieved by modification of the methods of Linder et al. (35), as described by Lee et al. (34). Purified α subunits were activated by incubation with 50 mM NaHepes (pH 8.0), 5 mM MgSO₄, 1 mM EDTA, 2 mM dithiothreitol, and 400 mM [³⁵S]GTP[S] at 30 °C for 30 min for Gₛₐₐ or 2 h for Gₛₐ. Free GTP[S] was removed by gel filtration. All G proteins were so activated unless stated otherwise. Gₛₐₐ bound with GDP[S]ₐₐₐ was prepared by incubation of the protein with 400 μM GDP[S] instead of GTP[S] for 2 h on ice, followed by gel filtration.

**Expression and Purification of Adenylyl Cyclase in *E. coli*—** The C₂ domain of type II adenylyl cyclase (IIc₂) and the Cₐₐₐ domain of type V adenylyl cyclase (VCₐₐₐ(591)) were expressed in *E. coli* and purified as described previously (23, 29). VCₐₐₐ and VCₐₐₐ(670) were expressed and purified as described for VCₐₐₐ (591). Briefly, these plasmids were cotransfected into SF9 cells with baculoviral DNA (BacVector-3000, Novagen), and the recombinant viruses were screened by assay of adenylyl cyclase activity in SF9 cell membranes.

Membranes were prepared from SF9 cells infected with a baculovirus encoding type V adenylyl cyclase were assayed for 8 min at 30 °C with the indicated concentrations of Gₛ[S] or Gₛₐₐₐ, in the presence of 100 mM Gₛₐₐₐ or 100 mM Gₛₐₐₐ plus 50 μM forskolin (●). All determinations were performed in duplicate and are representative of three experiments.

**RESULTS**

**Inhibition of Type V Adenylyl Cyclase by Gₛₐₐₐ—** The crystal structure of the catalytically active cytoplasmic domains of adenylyl cyclase associated with Gₛₐₐₐ demonstrated that the switch II region of the G protein α subunits inserts into a groove formed by the α2 helix and the α3'-β4' loop of the C₂ₐ domain of adenylyl cyclase; the G protein also makes an additional contact with the extreme N terminus of C₁. The pseudo-symmetrical structure formed by C₁ and C₂ contains a comparable cleft in the C₁ₐₐₐ domain. Because Gₛₐₐₐ and Gₛₐₐₐ are not competitive regulators of adenylyl cyclase (17), this cleft is an attractive candidate for interaction with Gₛₐₐₐ proteins (15). However, examination of the structure of adenylyl cyclase associated with both forskolin and Gₛₐₐₐ indicated that the cleft in C₁ was not large enough to accommodate the binding of an α subunit in a manner comparable with that observed with the C₂ domain. We were surprised to note that we (and others) have characterized inhibition of Gₛₐₐₐ or forskolin-stimulated adenylyl cyclase activity by Gₛₐₐₐ proteins in the past but have not examined the inhibitory effect of Gₛₐₐₐ in the presence of both Gₛₐₐₐ and forskolin (16–18, 37–39). We now note that Gₛₐₐₐ is a poor inhibitor of Gₛₐₐₐ and forskolin-stimulated type V adenylyl cyclase activity, coupled with effects seen in the presence of Gₛₐₐₐ or forskolin alone (Fig. 1). These results suggest that the crystal structure of the fully activated enzyme may not be appropriate to model sites for interaction with Gₛₐₐₐ.

**Identification of a Gₛₐₐₐ Binding Site**

![Graph showing inhibition of Gₛₐₐₐ and Gₛ[S]/forskolin-stimulated adenylyl cyclase activity by myristoylated Gₛₐₐₐ.](Image)

**Expression and Purification of Adenylyl Cyclase in *E. coli*—** The C₂ and Cₐₐₐ domains of type V adenylyl cyclase (VCₐₐₐ(591)) were expressed in *E. coli* and purified as described previously (23, 29). VCₐₐₐ and VCₐₐₐ(670) were expressed and purified as described for VCₐₐₐ (591). Briefly, these plasmids were cotransfected into SF9 cells with baculoviral DNA (BacVector-3000, Novagen), and the recombinant viruses were screened by assay of adenylyl cyclase activity in SF9 cell membranes.

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**RESULTS**

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**Identification of a Gₛₐₐₐ Binding Site**

![Graph showing inhibition of Gₛₐₐₐ and Gₛ[S]/forskolin-stimulated adenylyl cyclase activity by myristoylated Gₛₐₐₐ.](Image)
and VC\textsubscript{1}(670), which contain C\textsubscript{1a} and the entire C\textsubscript{1} domain, respectively. These proteins were purified to homogeneity (Fig. 2). We have examined the interactions of the C\textsubscript{1} and C\textsubscript{2} domains with activated G\textsubscript{i1} by gel filtration and sedimentation equilibrium. We have also used both myristoylated G\textsubscript{i1} and the nonmyristoylated protein as a control, as inhibition of adenylyl cyclase activity by G\textsubscript{i1} requires this covalent lipid modification. Under the conditions used, the myristoylated protein migrates more slowly during gel filtration than do both nonmyristoylated G\textsubscript{i1} and a 40-kDa standard, presumably due to the interaction of myristate with the gel filtration matrix (Fig. 3). This gel filtration profile is unaltered in the presence of VC\textsubscript{2}. However, when VC\textsubscript{1}(591) and myristoylated G\textsubscript{i1} are mixed, the migration of both proteins is altered, consistent with formation of a 1:1 complex (compare the lower panels showing migration of VC\textsubscript{1} in the presence of myristoylated and nonmyristoylated G\textsubscript{i1} and the chromatogram of myristoylated G\textsubscript{i1} in the presence and absence of VC\textsubscript{1}). Nonmyristoylated G\textsubscript{i1} does not interact with either VC\textsubscript{1}(591) or VC\textsubscript{2} (Fig. 3B).

Sedimentation equilibrium demonstrates the same pattern of interactions as shown by gel filtration (Fig. 4). Myristoylated G\textsubscript{i1} displays a molecular mass of 37 kDa (actual, 37.7 kDa) in the presence or absence of VC\textsubscript{2}. Addition of VC\textsubscript{1}(591) shifts the molecular mass to 72 kDa but has no effect on the nonmyristoylated protein. The predicted mass for a 1:1 complex of myristoylated G\textsubscript{i1} and VC\textsubscript{1}(591) is 66 kDa. The slightly curved plot of $\ln[G_{i1}/total]$ versus $r^2$ for the complex may indicate an equilibrium of several different multimeric species.

**Mutagenesis of Full-length Type V Adenylyl Cyclase**—Based on the clear interactions between VC\textsubscript{1} and myristoylated G\textsubscript{i1}, we created a series of mutations focused on the $\alpha_2$ helix and the $\alpha_3$-$\beta_4$ loop within the C\textsubscript{1} domain of type V adenylyl cyclase in an attempt to identify residues necessary for G\textsubscript{i1}-mediated inhibition (Fig. 5A). We also mutated additional residues that are conserved between type V and VI adenylyl cyclase but differ in other isoforms of the enzyme that are not inhibited by G\textsubscript{i1}. Baculoviruses encoding native type V adenylyl cyclase containing these point mutations were constructed, the proteins were expressed in Sf9 cells, and membrane preparations were analyzed for inhibition of adenylyl cyclase activity.
were assayed for adenylyl cyclase activity. All of the mutant proteins were active, and they displayed varying sensitivities to myristoylated Gia (Fig. 6, Table I).

The largest difference between the C2 domain, to which Gia binds, and the C1 domain is the length of helix 1 and the loop between the a1 and a2 helices, which includes an extra 4 amino acid residues not found in the C1 domain (Fig. 5A). This is one feature that makes the cleft in the C1 domain significantly smaller than its counterpart in C2. Mutation of two residues within the a1 helix had no significant effect on the IC50 for inhibition of adenylyl cyclase by Gia (Table I, Fig. 6A). The lack of a substantial effect of the Q406E or Q406A mutations was surprising, as this residue is exposed to solvent and extends into the cleft formed by the a2 and a3 helices. In addition, this residue is conserved in only those isoforms of adenylyl cyclase that are inhibited by Gia (Fig. 5A).

Mutations within the a2 helix did cause large changes in the response of adenylyl cyclase to Gia (Table I, Fig. 6A). The double mutation of Met-414 and Thr-415 to Ala and the point mutant E411A increased the IC50 for inhibition by Gia by 25- and 75-fold, respectively. In contrast, mutation of Glu-418 to Ala enhanced inhibition, decreasing the IC50 for Gi by 3-fold. Although conserved in type V and VI adenylyl cyclase, mutation of Asn-480 to Asp caused little change. Surprisingly, the relatively small change of Val-479 to an Ile decreased the IC50 for Gia by 4-fold. Except for E398V, none of the residues that were mutated had any effect on the apparent affinity of adenylyl cyclase for Gia, the synergy between Gia and forskolin, or the Km for ATP (Table I). E398V may have a more global effect on adenylyl cyclase, as its Kt for ATP is 2-fold higher than the wild-type value and the EC50 for activation by Gia is increased by 3–5-fold.

Alteration of other residues that are conserved throughout C1 and the N-terminal region of C2 had no effect on inhibition by Gia (Table I). Sequence-alignment strategies have not provided many candidates for possible interactions between Gia and the N terminus of C2 (analogous to the interaction between C1 and Gia). In fact, many of the residues that have the most dramatic effect on inhibition by Gia are conserved in a number of isoforms that do not respond to Gia. Therefore, we cannot rule out additional contacts with other regions of adenylyl cyclase, in particular the N terminus of C2. However, it is clear that the majority of the binding is contributed by the C2 domain and is clustered around the cleft formed by the a2 and a3 helices, as originally predicted. The structural location of residues whose mutation caused more than a 4-fold effect on inhibition by Gia is shown in Fig. 5B.

Inhibition of Reconstituted Adenylyl Cyclase Activity by Gia—Adenylyl cyclase activity can be reconstituted by mixture of VC1(591) or VC2(670) with C2 domains from either type V or type II adenylyl cyclase. The properties of these mixed proteins resemble those described previously with VC1(591) and IIC2 (Ref. 23 and data not shown). Gia inhibits adenylyl cyclase activity obtained by mixture of VC2(670) with VC1 (Fig. 7A). Myristoylated GTPγS-Gia has an IC50 of 0.3 ± 0.1 μM. The IC50 for the GDPγS-ligated protein is increased 5-fold, whereas nonmyristoylated GTPγS-Gia is more than 30-fold less potent than the myristoylated protein. The GTPγS- and GDP-bound forms of both Gia and Gia (23) display relatively small differences in their apparent affinity for adenylyl cyclase. As previously discussed (23), the primary effect of GTP (compared with GDP) is to cause conformational changes that promote dissociation of GTP-α from βγ rather than major changes in the affinity of α for its effectors.

The source of the C2 domain makes little difference; inhibition is nearly equally effective with either IIC2 or VC2, again consistent with primary interaction of Gia with the C2 domain (Fig. 7B). Although the C2 domain may contact Gia, the residues involved do not seem to impart to individual isoforms of adenylyl cyclase the capacity to be inhibited by the G protein α subunit. In the case of Gia, its interaction with the N terminus of C1 is critical for function, and the residues are conserved as expected because activation by Gia is conserved among all isoforms of adenylyl cyclase.

The C11 domain contributes to the potency of inhibition by Gia. The IC50 levels for inhibition of the adenylyl cyclase activity of mixtures of C1 and C2 domain proteins containing VC1(670) (both the C1 and C11 domains) are six times lower than those containing VC1(591) protein, which includes only a few residues of C1p. The C11 domains of the various isoforms of adenylyl cyclase differ significantly. It is possible that much of the selectivity for inhibition of adenylyl cyclase activity by Gia proteins is contributed by this region of adenylyl cyclase. The elevated IC50 and changes in the assay conditions explain why inhibition was not detected previously with VC1(591) and IIC2 (see “Materials and Methods”).

Effect of Mutations in VC1(670) on Inhibition by Gia—To determine whether the reconstituted system reflects the inhib-
itory effects of G \(i_o\) observed with full-length type V adenylyl cyclase, we made the mutations described above in VC1(670), expressed the proteins in E. coli, and purified them by Talon metal chelate chromatography. Except for E411A, all of the mutant proteins were synthesized at levels comparable with the wild-type protein (data not shown) and had similar specific activities. Adenylyl cyclase activity was measured by reconstitution of these mutant proteins with VC2 in the presence of activated G\(s_a\) and myristoylated G\(i_a\)1 (Fig. 8). As demonstrated with the full-length protein, mutants E411A, MT414A, L472A, and M476A all showed a clear reduction in G\(i_a\)-mediated inhibition. Although the enhancement of inhibition of mutant M476V is not as pronounced as that shown in the full-length protein, there is a very marked increase in the potency of inhibition of mutant E418A by G\(i_a\)1.

**DISCUSSION**

The inability of myristoylated G\(i_o\) to inhibit effectively G\(s_a\)- and forskolin-stimulated type V adenylyl cyclase activity suggests that the binding site for G\(i_o\) is highly sensitive to the conformational changes imposed on the enzyme by stimulators of catalysis. Hints of this phenomenon were revealed previously by Taussig et al. (17). Thus, the capacity of myristoylated G\(i_o\) to inhibit type V adenylyl cyclase decreased as the concentration of G\(s_a\) used to activate the enzyme increased (although the IC\(_{50}\) seemed largely unchanged). The simplest explanation of these results is that G\(s_a\) and G\(i_o\) can bind simultaneously to adenylyl cyclase. However, given sufficient concentrations of G\(s_a\) or addition of another activator such as forskolin, the enzyme can largely overcome the inhibitory effect of G\(i_o\), despite apparent binding of the inhibitory G protein \(\alpha\) subunit. These studies also indicate that the recently solved three-dimensional structure of the forskolin- and G\(s_a\)-bound VC1-IIC2 complex may not be the optimal state of adenylyl cyclase to model its inhibitory interactions with G\(i_o\).

Previous work by Taussig et al. (17) suggested separate binding sites for G\(i_o\) and G\(s_a\). Considering the pseudosymmetry of adenylyl cyclase, Sunahara et al. (23) and Tesmer et al. (15) suggested that G\(i_o\) may bind to the C1 domain of the enzyme in
Identification of a $G_{i\alpha}$ Binding Site

![Graphs showing inhibition of type V adenylyl cyclase mutants by myristoylated $G_{i\alpha}$](image)

**Fig. 6. Inhibition of type V adenylyl cyclase mutants by myristoylated $G_{i\alpha}$.** Membranes (30 μg) from Sf9 cells expressing either wild-type V adenylyl cyclase or mutant proteins were assayed with 100 nM activated $G_{i\alpha}$ in the presence of 7 nM–7 μM myristoylated GTP-S-$G_{i\alpha}$. Assays were performed in duplicate, and the results are representative of two experiments.

**Table I**

**Characterization of mutations in type V adenylyl cyclase**

| Structural location | MyrG$_{i\alpha}$ IC$_{50}$ | $K_m$ | V$_{max}$ | G$_{i\alpha}$ EC$_{50}$ | $G_{i\alpha}$ EC$_{50}$ | Fsk EC$_{50}$ |
|--------------------|-----------------|------|---------|----------------|----------------|--------------|
| $\mu M$            | $\mu M$         | nMol/min/mg | nM | nM | nM |
| Type V, wt         | 0.15            | 22     | 2.4    | 49 | 3.0 |
| E398VG              | 0.23            | 40     | 1.7    | 171| 18  |
| S402A               | 0.14            | 20     | 2.1    | 56 | 4.9 |
| Q406A               | 0.42            | 17     | 1.3    | 54 | 3.3 |
| Q406E               | 0.21            | 25     | 2.9    | 22 | 5.0 |
| E411A               | 0.02 >0.15      | 15     | 1.0    | 48 | 3.0 |
| MT414A              | 3.2             | 20     | 1.7    | 54 | 2.0 |
| A41S                | 0.025           | 17     | 1.0    | 74 | 4.3 |
| L472A               | 3.9 >9          | 26     | 1.4    | 58 | 5.5 |
| M476A               | 5 >3            | 23     | 1.5    | 80 | 4.6 |
| M476V               | 0.045           | 24     | 1.5    | 59 | 4.7 |
| V479I               | 0.036           | 20     | 1.5    | 44 | 5.8 |
| N480D               | 0.25            | 22     | 4.1    | 61 | 2.3 |
| A525P               | 0.19            | 27     | 1.8    | 56 | 7.6 |
| Q844K               | 0.12            | 20     | 3.2    | 44 | 7.0 |
| A945F               | 0.3             | 18     | 3.2    | 63 | 6.0 |

*The variation in the V$_{max}$ for each mutant is due in large part to differences in the age of Sf9 cells used for expression rather than the stability of individual proteins. Endogenous adenylyl cyclase activity of Sf9 membranes infected with β-galactosidase is 0.03 nMol/min/mg.

The location of each mutant residue is based on the secondary structure of the C1 and C2 domains of type V adenylyl cyclase.

A location analogous to that for interaction of $G_{i\alpha}$ with the C2 domain. We have expressed and purified fragments of the C1 and C2 domains of type V adenylyl cyclase and demonstrate herein the interaction of the C1 domain of type V adenylyl cyclase with $G_{i\alpha}$ by two independent methods. Both gel filtration and sedimentation equilibrium indicate formation of a 1:1 complex between V1 and G$_{i\alpha}$, as was observed previously with G$_{i\alpha}$ and IIC2 (23). We were unable to detect interactions between V2 and myristoylated G$_{i\alpha}$. We have to date been unable to detect formation of the tetrameric complex containing VC1, VC2, G$_{i\alpha}$, and G$_{i\alpha}$. Nevertheless, we believe that G$_{i\alpha}$ and G$_{i\alpha}$ can bind simultaneously to adenylyl cyclase, as stated above. Binding of G$_{i\alpha}$ to G$_{i\alpha}$-G$_{i\alpha}$-VC1, and VC2 may weaken interactions between the cytosolic domains of the enzymes. Detection of simultaneous binding of G$_{i\alpha}$ and G$_{i\alpha}$ to adenylyl cyclase may thus require covalent linkage of C1 and C2.

A series of mutations was created in the C1 domain of type V adenylyl cyclase that specifically targeted the α1-α2 and α3-β4 cleft, the site analogous to that for binding of G$_{i\alpha}$ to IIC2. Several mutations within the α2 and α3 helices led to either increased or decreased IC$_{50}$ levels for inhibition of adenylyl cyclase activity by G$_{i\alpha}$. Mutations in other regions of the enzyme had no effect on this parameter. The point mutations E411A, L472A, and M476A, and the double mutation M414A,T415A increased the IC$_{50}$ for G$_{i\alpha}$-mediated inhibition of these enzymes by 25–75-fold, whereas the mutations E418A, M476V, and V479I decreased the IC$_{50}$ for G$_{i\alpha}$ inhibition by 4–6-fold. All of the side chains at these positions point into the cavity created by the flanking helices, α2 and α3, except for Leu-472 (Fig. 5B). This side chain might participate in contacts with the α3-β5 loop of G$_{i\alpha}$, as do residues in the C2 domain with G$_{i\alpha}$ or even with the C1b domain of adenylyl cyclase.

Residues within the G$_{i\alpha}$ binding pocket are conserved among the different isoforms of adenylyl cyclase as expected, as all isoforms respond to this activator. However, only type I, V, and VI adenylyl cyclases are inhibited by G$_{i\alpha}$, and we expected to find the greatest effect of mutation of residues that are conserved among only these isoforms. However, residues Glu-411 and Glu-418 are conserved in nearly all isoforms of adenylyl cyclase, and Val-479 is conserved in about half of these, including several that are not inhibited by G$_{i\alpha}$. Residues Leu-472, Met-476, Met-414, and Thr-415 are not conserved among other isoforms of adenylyl cyclase, but they also differ significantly in the type I enzyme. A residue such as Gln-406, located at the entrance of the G$_{i\alpha}$-G$_{i\alpha}$-mixed inhibition by G$_{i\alpha}$ when mutated to either alanine or glutamate. It is thus unclear how specificity for interaction with G$_{i\alpha}$ is achieved.

The main structural difference between the G$_{i\alpha}$ binding pocket and that proposed for G$_{i\alpha}$ is the size of the cleft, which is largely dictated by the angle between the α2 and α3 helices and the insertion of four residues in C2 in the loop between α1 and α2. This provides a wider entrance to this cleft for contact with switch II of G$_{i\alpha}$. There are significant conformational differences in the G$_{i\alpha}$ binding pocket when one compares the IIC2 homodimeric structure (14), proposed to mimic an inactive ground state, with that of the G$_{i\alpha}$-Fek-VC1-IIC2 complex (15). It is unknown what changes may occur in the C2 domain and the G$_{i\alpha}$ binding pocket as the enzyme is converted from a low to a high activity state. However, this cleft undergoes conformational changes upon P-site inhibitor and pyrophosphate binding.

Inhibition of adenylyl cyclase activity by G$_{i\alpha}$ was also detected using the purified C1 and C2 domains of the type V enzyme. The IC$_{50}$ for inhibition was only 2-fold greater than that observed with full-length, membrane-bound type V adenylyl cyclase (0.3 versus 0.15 μM). This is a small difference.
Identification of a \( G_{ia} \) Binding Site

comparing the 10-fold or more loss of apparent affinity for \( G_{ia} \) when the purified soluble VC1/IIC2 system was compared with the holoprotein. Full inhibition by \( G_{ia} \) required both activation of the protein with GTP-S and myristoylation. Myristoylation was also required for binding of \( G_{ia} \) to VC1 as measured by gel filtration and sedimentation equilibrium. Modeling of \( G_{ia} \) in the binding pocket identified by mutagenesis (Fig. 9) places its N terminus and attached myristate near the presumed location of the plasma membrane. The requirement for myristoylation of \( G_{ia} \) for inhibition of membrane-bound adenylyl cyclases is not understood. However, insertion of myristate in the plasma membrane would increase the local concentration and apparent affinity of \( G_{ia} \) for adenylyl cyclase, and there are also several examples of intermolecular and intramolecular protein-lipid interactions. Recoverin and Arf contain myristate groups that are sensitive to the conformational states of these proteins (40–42). Lipid-mediated protein-protein interactions are prevalent in binding of regulatory molecules to Ras-related G proteins (43). The requirement for myristate in a completely soluble system suggests that myristate does not serve simply to concentrate \( G_{ia} \) at the membrane but rather interacts directly with either the protein moiety of \( G_{ia} \), adenylyl cyclase, or both to increase the affinity of their interaction.

The inhibitory effect of \( G_{ia} \) on reconstituted adenylyl cyclase activity was largely unchanged when the C2 domain of the type II enzyme replaced VC2, suggesting that C2 does not play a significant role in binding \( G_{ia} \) and thereby dictating isofrom-specific recognition of the regulatory protein. However, inhibition by \( G_{ia} \) was enhanced when the full C1 domain (VC1(670)) was included compared with C1a alone (VC1(591)). Isoform specificity may be partially dictated by C1b. This region is highly variable among the different isoforms of adenylyl cyclase. The difference in inhibition by \( G_{ia} \) between VC1a and VC1(670) was not observed when the C1 and C2 domains were linked covalently (30). It is not clear why this should make a difference. However, mutations in VC1(670) do faithfully mimic the phenotypes of those made in the full-length protein.

FIG. 7. Inhibition of the soluble form of type V adenylyl cyclase by \( G_{ia} \). A, purified VC1(670) (50 nm) was reconstituted with 0.5 \( \mu \)M VC2 and assayed with 400 nM activated \( G_{ia} \) in the presence of 10 nm–10 \( \mu \)M myristoylated GTP-S-Ga mounts (●), myristoylated GDP-S-Ga mount (■), or nonmyristoylated GTP-S-Ga mount (△). B, VC1(670) (●, ■) or VC1(591) (△, ▽) was reconstituted with 0.5 \( \mu \)M VC2 and assayed with 400 nM \( G_{ia} \) in the presence of the indicated concentrations of myristoylated GTP-S-Ga mounts. Activities are expressed as percentages of control values: 3.3, 5.6, 8.7, and 8.5 nmol/min/mg for VC1(670)/VC2, VC1(670)/IIC2, VC1(591)/VC2, and VC1(591)/IIC2, respectively. All values shown are the averages of duplicates, and the results are representative of three experiments.
C2 domains of adenylyl cyclase, are shown as ball-and-stick models. To this helix. The molecule of GTP bound to each function by disrupting the active site, decreasing the affinity of C1 for and rendered using RASTER3D.

The mechanism of activation of adenylyl cyclase by Gs based on the enzyme’s interactions with Gs and Gi found along the A derived from the crystal structure of Gs. A side view of the complex. The N and C termini of all the subunits are side view of the complex. The N and C termini of all the subunits are

FIG. 9. Two views of a theoretical complex between the catalytic domains of adenylyl cyclase and the stimulatory and inhibitory G protein α subunits Gs and Gi. Evidence for such a complex is from kinetic studies of full-length adenylyl cyclase that revealed no competitive inhibition of Gs-stimulated activity by Gi (17). As yet, there is no evidence that such a tetrameric complex exists for the soluble system whose components are portrayed here. Thus, these models are only meant to show the pseudosymmetrical relationship of both α subunits to the active site of adenylyl cyclase. A side view of the complex. The N and C termini of all the subunits are

A new position of Giα was generated with the program BOBSCRIPT and rendered using RASTER3D.

cyclase could interact with this region of Gia, adding additional stability to this complex.

The mechanism of activation of adenylyl cyclase by Gs is not completely understood, and discussion of the mechanism of inhibition by Gi can thus be only speculative. However, the close proximity of the Giα binding site to the active site of the enzyme certainly poises Gia to affect catalysis by a number of mechanisms. A simple model of binding of Giα to adenylyl cyclase based on the enzyme’s interactions with Gia (Fig 9) does not explain all of the effects of the mutations in adenylyl cyclase described above or those in Gsα reported previously (45). However, this model is consistent with the general location of the binding site for Giα, that is indicated by the mutations. Therefore, much in the same way that binding of Giα is proposed to widen the binding cleft in C2, inducing a domain rotation that reorients residues within the active site (15), Giα may widen its binding pocket in C1 and generate a movement of the α1-α2 loop toward the catalytic site to disrupt or block the active site. The addition of forskolin to the Giα-bound enzyme increases the affinity of the two domains for one another and may serve to lock the enzyme in a highly activated state that does not favor Gsα binding to C1. However, we suggest that Gsα and Giα do bind simultaneously to adenylyl cyclase, forming a large complex (200 kDa with the native enzyme) whose pseudosymmetry extends from the duplicated structure of adenylyl cyclase to these two regulatory proteins. The pseudosymmetry of adenylyl cyclase is a clear structural correlate of bidirectional regulation of the enzyme by homologous G protein α subunits.

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Identification of a Gia Binding Site
Identification of a $G_{i\alpha}$ Binding Site

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