Truncation and Alanine-Scanning Mutants of Type I Adenylyl Cyclase†

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ABSTRACT: A variety of truncated constructs of type I and type II adenylyl cyclase have been synthesized in Sf9 cells using recombinant baculoviruses, as have a number of type I adenylyl cyclases with point mutations. Truncations indicate that the nonconserved C_{1b} and C_{2b} domains of adenylyl cyclase are not necessary for regulation of enzymatic activity by $G_{s\alpha}$ and forskolin. Point mutations demonstrate the requirement for both of the conserved (and homologous) domains of adenylyl cyclase (C_{1a} and C_{2a}) and the nonequivalence of these domains. Linkage of certain effects of mutations on the K_m for substrate with alterations of the characteristics of P-site inhibition suggest that ATP and P-site inhibitors may bind to different conformations of the same site. However, other mutations affected only P-site inhibition. Although the mutations studied have not permitted assignment of unique functions to the two homologous domains, they have revealed novel phenotypes that appear to reflect the regulatory complexity of mammalian membrane-bound adenylyl cyclases, including the possibility of oligomerization of the enzymes.

Complex systems of interacting proteins have evolved to regulate intracellular concentrations of second messengers in response to environmental signals. In the case of cyclic AMP, most of this regulation is directed at the level of synthesis of the nucleotide from ATP by adenylyl cyclase. There are three primary components in the most prevalent pathways for regulation of cyclic AMP synthesis: heptahelical receptors for hormones, neurotransmitters, odorants, and other regulatory ligands that act at the cell surface; heterotrimeric G proteins[1] (both stimulatory and inhibitory) that are activated by the receptors; and a growing list of adenylyl cyclases that are distinguished by their responses to interactions with G protein subunits and other regulators, particularly calmodulin.

cDNAs encoding eight distinct mammalian adenylyl cyclases have been cloned during the past five years (Bakalyar & Reed, 1990; Feinstein et al., 1991; Gao & Gilman, 1991; Ishikawa et al., 1992; Katsushika et al., 1992; Krupinski et al., 1989, 1992; Premont et al., 1992; Yoshimura & Cooper, 1993; Cali et al., 1994; Watson et al., 1994). Each of these isoforms differs in its tissue distribution, abundance, and regulatory properties (Tang & Gilman, 1992; Iyengar, 1993; Taussig & Gilman, 1994). Each adenylyl cyclase can be activated by the G protein α subunit designated $G_{s\alpha}$ and the diterpene forskolin, and each can be inhibited by certain analogues of adenosine (so-called P-site inhibitors). However, these are the only common major regulatory features. There are also several subtype-specific regulators, including the G protein $\beta\gamma$ subunit complex, Ca^{2+}−calmodulin, Ca^{2+}, and the α subunits of G_i, G_o, and G_z. Certain of these regulators (*e.g.*, $\beta\gamma$) can be either stimulatory or inhibitory, depending on the adenylyl cyclase. When acting concurrently, the effects of regulators can be independent or interdependent (antagonistic or synergistic).

The eight mammalian adenylyl cyclases and the products of the *rutabaga* (*Drosophila*; Levin et al., 1992) and *ACA* (*Dictyostelium*; Pitt et al., 1992) genes have a similar structure (designated $NM_1C_1M_2C_2$). This includes a short amino-terminal region (N) and two roughly 40-kDa cytoplasmic domains (C_1 and C_2), punctuated by two intensely hydrophobic stretches (M_1 and M_2); each of the latter is hypothesized to contain six transmembrane helixes. C_1 and C_2 each contain regions (C_{1a} and C_{2a}) that are homologous to each other, to the catalytic domains of various guanylyl cyclases, and, to a lesser extent, to the catalytic domains of simpler adenylyl cyclases from lower organisms.

Comprehension of the structural correlates of the regulatory complexity of adenylyl cyclases will be challenging. To date, we have shown that concurrent expression of both cytoplasmic domains of type I and type II adenylyl cyclase is necessary for significant enzymatic activity. We have now truncated these domains and replaced some of the most conserved residues within C_{1a} and C_{2a} with alanine to begin to assess the importance of each cytosolic domain for regulated catalytic activity. We also present some observations relevant to the quaternary structure of adenylyl cyclase.

MATERIALS AND METHODS

Materials. The recombinant baculovirus Bac-Pac6 was purchased from Clontech (Palo Alto, CA). *Drosophila* calmodulin was synthesized in *Escherichia coli* and purified as described (Maune et al., 1992). cDNAs encoding $G_{s\alpha}$

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[1] Abbreviations: G protein, guanine nucleotide-binding regulatory protein; $G_{s\alpha}$, the α subunit of the G protein that stimulates adenylyl cyclase; $rG_{s\alpha-s}$, the short form of $G_{s\alpha}$ expressed in *E. coli*; CaM, calmodulin; Fsk, forskolin; GTPγS, guanosine-5′-[γ-thio]triphosphate; GDPβS, guanosine-5′-[β-thio]diphosphate; 2′d-3′AMP, 2′-deoxyadenosine 3′-monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s); EC_{50}, the concentration of activator required for half-maximal stimulation of enzymatic activity; IC_{50}, the concentration of inhibitor causing 50% inhibition. Mutants $G_{s\alpha}$(FT) and $G_{s\alpha}$(CFT) are the same as $G_{s\alpha}$ mutants LR268,269FT and WLR263,268,269CFT, respectively (Itoh & Gilman, 1991).

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mutants with reduced affinities for adenylyl cyclase (Itoh & Gilman, 1991) were excised from plasmids NpT7-5/$G_{s\alpha}$(FT) and NpT7-5/$G_{s\alpha}$(CFT) with *Nco* I and *Hind* III and transferred to the same sites of bacterial expression plasmid pQE6 (Lee et al., 1994). The resulting plasmids were transformed into *E. coli* BL21-DE3 cells to synthesize the mutant $G_{s\alpha}$ proteins. Wild-type and mutant $G_{s\alpha}$ proteins were purified as described (Lee et al., 1994). ATPαS, App(NH)p, forskolin, and Pansorbin were purchased from Calbiochem (LaJolla, CA). 2′-Deoxy-3′-AMP was purchased from Sigma (St. Louis, MO). GTPγS and GDPβS were purchased from Pharmacia (Piscataway, NJ) and purified by FPLC mono-Q column chromatography.

Construction of Phages, Plasmids, and Recombinant Baculoviruses. To produce point mutants, the portions of the cDNA for type I adenylyl cyclase containing the coding region for the C_1 or C_2 domains were ligated into M13 phages, designated M13-mp18-C_1 and M13-mp19-C_2, respectively. The 1.3-kb *Bam* H1−*Sma* I fragment was excised from pSKAClΔ58-13 (Tang et al., 1991) and cloned into the same sites of M13-mp18 to create M13-mp18-C_1. The 1.5-kb *Sma* I−*Pvu* II fragment was excised from pSKAClΔ58-13 and cloned into M13-mp19 (digested with *Sma* I). An M13 clone from which the desired fragment could be excised with *Sma* I and *Xba* I was selected (M13-mp19-C_2). Oligonucleotides (23mers) used for mutagenesis contained 10 complementary nucleotides flanking each side of the target codon, which was replaced by the alanine codon GCC (for alanine scanning mutagenesis, except for mutant R979A, where GCT was used), by the appropriate codon (for mutants V430R, I351T, and R1011K), or by the TGA terminator codon (for $IM_1C_1M_2$[2] and $I\Delta C_{2b}$ at sequences encoding residues 854 and 1071, respectively). Site-directed mutagenesis was performed as described by Kunkel *et al.* (1987); the desired clones were identified by sequencing. The mutant designated $IM_1C_1M_2C_{1/2}$ was constructed by sticky-foot-directed mutagenesis (Clackson & Winter, 1989) using 45mers with the desired 30-base sequence from the C_2 domain and the desired 15-base sequence from the C_1 domain of type 1 adenylyl cyclase. The resulting cDNA encoded a protein that had the C_{2a} domain (residues 838−1020) of type I adenylyl cyclase replaced by the corresponding region of the C_{1a} domain (residues 267−439).

The 3.5-kb *Hind* III-partial *Pvu* II fragment from pSKAClΔ58-13 was cloned into pBluescript SK^- (digested with *Hind* III and *Sma* I); this plasmid is designated pSKACl-3S. pSKACl-3S has less than 0.2 kb of noncoding sequence and has unique *Sma* I and *Stu* I sites for ease of shuffling the mutated fragments back to the full-length type I adenylyl cyclase cDNA. To transfer the DNA with the desired mutation in the C_1 domain, the fragments (digested with *Stu* I and *Sma* I) were excised from M13 RF and cloned into 5.5-kb pSKACl-3S (digested with *Stu* I and *Sma* I). Clones with the correct orientation were selected. The same strategy was used to transfer cDNAs with the desired mutation in C_2 using the enzymes *Sma* I and *Xba* I. Mutations were again confirmed by double-stranded DNA sequencing. Mutants D338A/D908A and R398A/K923A, which have mutations in both the C_1 and C_2 domains, were constructed by transferring the *Sma* I−*Xba* I fragment encoding the mutated C_2 domain to the desired cDNA containing the mutation in the C_1 domain.

The 3.5-kb cDNAs with the desired mutations (digested with *Hind* III and *Xba* I) were then transferred to pVL1393 (digested with *Sma* I and *Xba* I) (O'Reiley et al., 1992). The resulting plasmids were cotransfected into Sf9 cells with baculoviral DNA (AcRP23-LacZ or Bac-Pak6, digested with *Bsu* 36I), and the recombinant viruses were isolated by plaque purification (O'Reiley et al., 1992). Viruses suitable for expression of the desired mutant proteins were identified by immunoblotting of Sf9 cell membrane extracts or by visualizing the [^{35}S]methionine-labeled products. Recombinant viruses encoding constructs designated INM_1C_1, IM_2C_2, and IIM_2C_2 have been described previously (Tang & Gilman, 1991; Tang et al., 1991). For the construction of mutants $INM_1C_{1(1-484)}$ and $INM_1C_{1(1-527)}$, the termination codon (TAG) was placed into M13-mp18-C_1, and the cDNA fragments encoding the C_1 domain were excised with *Stu* I and *Eco* RI and transferred to pVL1393-ACl. The resulting plasmids were used to construct viruses as described.

Antibodies and Immunoblotting. Antibodies C1-1115 and C2-1077 were raised against peptides corresponding to the carboxyl-termini of type I and type II adenylyl cyclases (C1-1115, CGLAPGPPGQHLPPGASGKEA; C2-1077, TEMSRSLSQSNLAS) and purified as described (Tang et al., 1991). Membranes from Sf9 cells were heated to 80 °C in the presence of 2% sodium dodecylsulfate and 0.2 mM dithiothreitol for 5 min; they were then treated with 50 mM *N*-ethylmaleimide for 10 min at room temperature prior to electrophoresis, transfer to nitrocellulose (1 h at 30 V and 14 h at 80 V), and immunoblotting using the ECL system (Amersham, Arlington Heights, IL).

Expression of Adenylyl Cyclases in Sf9 Cells. Membranes were prepared from Sf9 cells expressing wild-type and mutant type I adenylyl cyclases (Tang et al., 1991). Briefly, cells (10^6/mL) were infected with the desired baculovirus (1 plaque-forming unit/cell), harvested after 48−54 h, and lysed by nitrogen cavitation. After removal of nuclei by centrifugation, membranes were collected, washed, and resuspended. Expression of all constructs utilized in this work was confirmed by immunoblotting or, when necessary, by biosynthetic labeling with [^{35}S]methionine (data not shown). Protein concentrations were determined by the method of Bradford (1976). Adenylyl cyclase activity was measured in the presence of 10 mM $MgCl_2$ as described (Smigel, 1986). GTPγS-bound $G_{s\alpha}$ was prepared as described (Tang et al., 1991). Modulation of adenylyl cyclase activity by GTPγS-$G_{s\alpha}$, Ca^{2+}-calmodulin, forskolin, and the G protein βγ subunit complex was assayed as described (Tang et al., 1991). Adenylyl cyclase activity was calculated by subtracting enzyme activity using Sf9 cell membranes prepared from cells expressing β-galactosidase (about 10 or 100 pmol/min/mg protein for assays performed in the absence or presence of forskolin, respectively) from the value observed with membranes from Sf9 cells expressing type I or mutant adenylyl cyclase. Unless otherwise stated, data are representative of at least two experiments, and standard errors of reported values are less than 10%.

Binding of $G_{s\alpha}$ to Sf9 Cell Membranes Containing Adenylyl Cyclase. [^{35}S]GTPγS-$G_{s\alpha}$ was prepared by incubating 0.6 μM $G_{s\alpha-s}$ (synthesized in *E. coli*) with 1.2 μM [^{35}S]GTPγS for 1 h at 30 °C in 20 mM NaHepes (pH 8.0), 5 mM $MgSO_4$,

[2] Truncation mutants are designated by the domain(s) included (or Δ = deleted) in the constructs, preceded by a roman numeral (I = type I adenylyl cyclase, II = type II adenylyl cyclase).

1 mM dithiothreitol, and 1 mg/mL bovine serum albumin. The reaction mixture was then filtered through Sephadex G25 to remove free nucleotide. The $G_{s\alpha}$ binding assay was performed by mixing Sf9 cell membranes containing wild-type or mutant adenylyl cyclase, variable amounts of [^{35}S]-GTPγS-$G_{s\alpha}$, and variable amounts of nonradiolabeled GTPγS-$G_{s\alpha}$ (or other competitor) in 20 μL of buffer containing 20 mM NaHepes (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM GDPβS. The reaction mixtures were incubated at 30 °C for 7−10 min. [^{35}S]GTPγS-$G_{s\alpha}$ that was not associated with membranes was removed by filtration through 0.22 μm Millipore Duropore membranes, followed by washing with 6 mL of 20 mM NaHepes (pH 8.0), 5 mM $MgCl_2$, and 1 mM dithiothreitol. The amount of labeled GTPγS-$G_{s\alpha}$ retained on the filter was determined by scintillation counting. Specific binding was calculated by subtracting binding to Sf9 cell membranes prepared from cells expressing β-galactosidase from that observed to membranes from Sf9 cells expressing adenylyl cyclase.

Immunoprecipitations. Sf9 cell membranes (500 μg) were solubilized with 0.8% dodecyl maltoside in 125 μL of IP buffer [20 mM NaHepes (pH 8.0), 1 mM EDTA, 400 mM NaCl, 2 mM dithiothreitol, and 20% glycerol]. Soluble proteins were collected after centrifugation at 100 000*g* for 30 min. Fixed *Staphylococcus aureus* bacteria (10 μL of 20% Pansorbin) were added, incubated for 10 min on ice, and removed by brief centrifugation (12 000 rpm for 2 min). Affinity-purified antiserum C1−1115 (5 μL) and 10 μL of 20% Pansorbin were then added to the supernatants for a 1-h incubation at 4 °C. After centrifugation, the pellets were resuspended in 100 μL of IP buffer with 0.05% dodecyl maltoside for enzymatic assay. Adenylyl cyclase activity was measured in the presence of 10 mM $MgCl_2$, 5 mM $MnCl_2$, 100 μM forskolin, and 0.05% dodecyl maltoside.

RESULTS

$G_{s\alpha}$ Binding Assay. A binding assay was developed to detect interaction of activated $G_{s\alpha}$ with membranes from Sf9 cells expressing adenylyl cyclase. Binding of [^{35}S]GTPγS-$G_{s\alpha}$ to membranes from cells expressing type I or type II adenylyl cyclase reached equilibrium in 2−3 min and was about 5-fold higher than values achieved using membranes from cells infected with a baculovirus encoding β-galactosidase (Figure 1A). There was no detectable specific binding of [^{35}S]GTPγS-$G_{i\alpha\text{-}1}$ (nonmyristoylated; synthesized in *E. coli*) to membranes from cells expressing either adenylyl cyclase or β-galactosidase (data not shown). The K_d for binding of [^{35}S]GTPγS-$G_{s\alpha}$ to membranes containing either type I or type II adenylyl cyclase was approximately 10−20 nM (Figure 1B). These values are similar to the EC_{50}'s for activation of these enzymes by GTPγS-$G_{s\alpha}$ (about 25 and 35 nM, respectively; Figure 1E). Forskolin did not alter the binding of [^{35}S]GTPγS-$G_{s\alpha}$ to membranes containing type I adenylyl cyclase and only slightly enhanced binding to membranes containing type II adenylyl cyclase (Figure 1B). The maximal binding of [^{35}S]GTPγS-$G_{s\alpha}$ to 20 μg of Sf9 cell membranes containing type I or type II adenylyl cyclase was 90 or 100 fmol, respectively (Figure 1B). This is in reasonable agreement with concentrations of adenylyl cyclase (about 170 fmol/20 μg) estimated from the enzymatic activities of these membranes and the specific activity of the purified enzymes (Tang et al., 1991; Taussig et al., 1993).

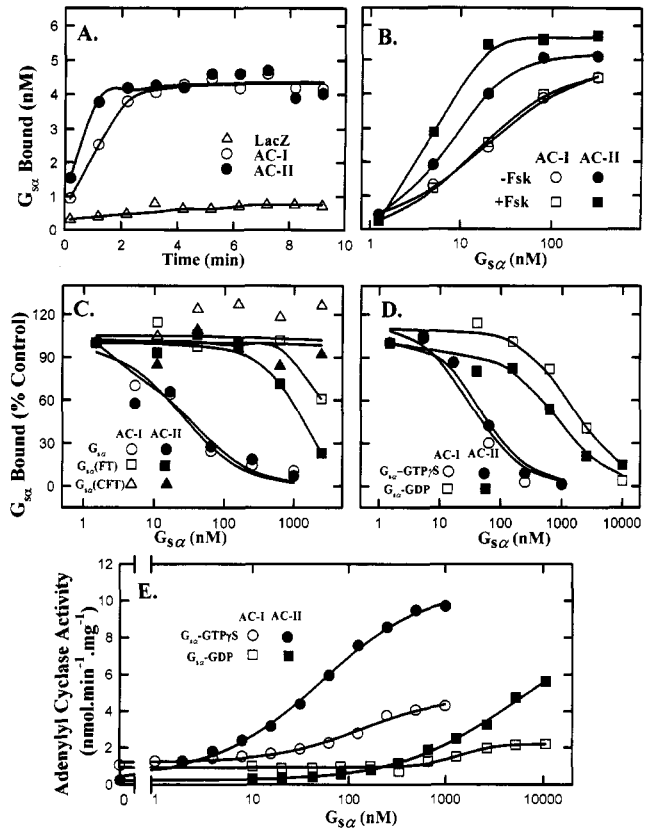
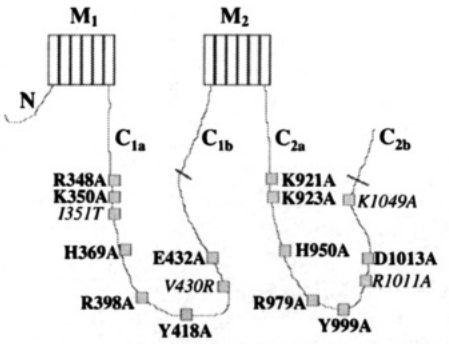


FIGURE 1: Binding of [^{35}S]GTPγS-$G_{s\alpha}$ to Sf9 cell membranes containing adenylyl cyclases. (A) Time course of binding to membranes (30 μg) containing type I (○) or type II (●) adenylyl cyclase or β-galactosidase (△). The concentration of [^{35}]SGTPγS-$G_{s\alpha}$ was 6 nM. (B) Specific binding of GTPγS-$G_{s\alpha}$ to membranes (20 μg) of Sf9 cells containing type I (○,□) or type II (●,■) adenylyl cyclase. When present (●, ●), the forskolin concentration was 100 μM during the incubation and 10 μM during the washes. (C) Competition for [^{35}S]GTPγS-$G_{s\alpha}$ binding to Sf9 cell membranes containing type I (open symbols) or type II (closed symbols) adenylyl cyclase by GTPγS-$G_{s\alpha}$ (○, ●), GTPγS-$G_{s\alpha}$(FT) (□, ■), or GTPγS-$G_{s\alpha}$(CFT) (△, ▲). The concentration of [^{35}S]GTPγS-$G_{s\alpha}$ was 1.5 nM. The amount of labeled $G_{s\alpha}$ bound (to 20 μg of Sf9 cell membranes) in the absence of competitors was 5.1 and 7.7 fmol for type I and type II adenylyl cyclase, respectively. (D) Competition for [^{35}S]GTPγS-$G_{s\alpha}$ binding to Sf9 cell membranes containing type I (open symbols) or type II (closed symbols) adenylyl cyclase by GTPγS-$G_{s\alpha}$ or GDP-$G_{s\alpha}$. The amount of labeled $G_{s\alpha}$ bound (to 20 μg of Sf9 cell membranes) in the absence of competitors was 5.5 and 6.1 fmol for type I and type II adenylyl cyclase, respectively. (E) Activation of type I (open symbols) and type II (closed symbols) adenylyl cyclase by GTPγS-$G_{s\alpha}$ or GDP-$G_{s\alpha}$. The adenylyl cyclase activity of membranes (10 μg) was measured after 5 min with 1 mM ATP in the absence of an ATP regenerating system. Enzyme activity was linear for 6−7 min. GDPβS (50 μM) was included in the assay. Binding of [^{35}S]GTPγS-$G_{s\alpha}$ and adenylyl cyclase activities were measured in duplicate, and the data are representative of at least two experiments.

Two mutants of $G_{s\alpha}$, $G_{s\alpha}$(FT) and $G_{s\alpha}$(CFT), have normal guanine nucleotide binding properties but only 10% and 1%, respectively, of the apparent affinity of wild-type $G_{s\alpha}$ for adenylyl cyclase (measured in activation assays) (Itoh & Gilman, 1991). Competition for [^{35}S]GTPγS-$G_{s\alpha}$ binding to Sf9 cell membranes containing type I or type II adenylyl cyclase (Figure 1C) or direct binding measurements (not shown) also indicate that these mutant proteins have a reduced affinity for their target enzyme. The affinity of [^{35}S]-GTPγS-$G_{s\alpha}$(FT) for adenylyl cyclase is reduced by about

Table 1: Effects of Activators and Inhibitors on the Enzymatic Activity of Point Mutants and Binding of $G_{s\alpha}$[a]



class	protein	Mg^{2+}	Mn^{2+}+Fsk[a]	$G_{s\alpha}$[b] EC_{50}	$G_{s\alpha}$[b] V_{Max}	CaM[b] EC_{50}	CaM[b] V_{Max}	CaM/CaM+$\beta\gamma$[a]	$G_{s\alpha}$ binding[c]
	wild type	0.9	6.8	23 ± 2	3.8	40 ± 3	5.5	5.1/1.7	100%
$C_1 = C_2 =$ GC = AC-s[d]	R398A	0.01	2.1	−	0	−	0.05	−	84 ± 3
	R979A	0	0.3	−	0.2	−	0	−	20 ± 5
$C_1 = C_2 =$ AC-s, not to GC	K350A	0.1	5.4	168 ± 20	3.5	−	0.6	0.6/0.2	90 ± 2
	K923A	0.1	2.5	−	0.4	−	0.3	0.3/0.2	92 ± 4
$C_1 = C_2$ =GC not to AC-s	R348A	1.2	8.3	20 ± 3	4.3	71 ± 5	4	4.4/1.7	113 ± 6
	K921A	0.05	1.3	−	0.4	−	0.3	0.3/0.2	60 ± 5
	H369A	0.6	5.5	13 ± 4	2	33 ± 4	2.6	2.9/1.1	87 ± 8
	H950A	0.01	0.3	−	0.2	−	0.05	−	36 ± 3
	Y418A	0.6	4.4	25 ± 2	1.6	28 ± 4	3.4	3.3/1.7	119 ± 7
	Y999A	0.1	0.3	−	0.2	−	0.2	0.2/0.1	23 ± 2
	E432A	0.1/1.4[e]	0.3/7.2[e]	10 ± 2[f]	0.4/7.4[e]	39 ± 5[f]	0.3/4.3[e]	4.7/2.8[f]	57 ± 4
	D1013A	0.17	4.0	19 ± 3	2.1	63 ± 4	1.4	1.2/0.2	85 ± 3
conserved in C_2	R1011A	0.09	0.5	−	0.2	−	0.5	−	129 ± 5
	R1011K	0.60	15.0	45 ± 4	1.6	32 ± 4	3.6	2.5/0.8	22 ± 6
double mutant	D338A/D908A	0	0.1	−	0.2	−	0	−	0 ± 4
	R398A/K923A	0.02	0.1	−	0.1	−	0	−	51 ± 5

[a] Membranes were assayed for adenylyl cyclase activity (nmol/min/mg) as indicated. Background activity (~0.01−0.10 nmol/min/mg) has been subtracted. Assays were performed using 10 mM $MgCl_2$ (Mg^{2+}, 100 μM forskolin, and 10 mM $MgCl_2$ with 5 mM $MnCl_2$ (Mn^{2+}Fsk), or 300 nM calmodulin and 50 μM $CaCl_2$, without or with 100 nM bovine brain G protein $\beta\gamma$ subunits (CaM/CaM+$\beta\gamma$). The diagram shows the locations of the mutations. Paired mutations are in bold type; nonpaired mutations are in italics. [b] Values for EC_{50} and V_{max} were calculated from least squares fits to a simple rectangular hyperbola. Values of EC50 for those mutants that have very low activity cannot be estimated. Concentrations of GTPγS-$G_{s\alpha}$ and calmodulin were varied to a maximum of 2 μM; the concentration of Ca^{2+} was 50 μM. [c] Assayed with 5 nM [^{35}S]GTPγS-$G_{s\alpha}$. [d] Except that the residue is lysine or threonine in type V adenylyl cyclase or the 70-kDa subunit of cytosolic guanylyl cyclase, respectively. [e] Assayed without or with 5 mM $MnCl_2$ ($-Mn^{2+}/+Mn^{2+}$). [f] Assayed with 5 mM $MnCl_2$.

20-fold; there was no detectable competition by GTPγS-$G_{s\alpha}$-(CFT) at the highest concentration tested (2 μM).

We also tested the capacity of GDP-$G_{s\alpha}$ to compete for binding of [^{35}S]GTPγS-$G_{s\alpha}$ to adenylyl cyclase−containing Sf9 cell membranes (Figure 1D). Interestingly, GDP-$G_{s\alpha}$ appeared to be a competitor, although its affinity was about 20 to 40-fold lower than that of GTPγS-$G_{s\alpha}$. Similarly, GDP-$G_{s\alpha}$ was capable of activating type I and type II adenylyl cyclase (Figure 1E). Again, the potency was about 50-fold less than that of GTPγS-$G_{s\alpha}$. Although it is difficult to exclude the possibility that nucleotide exchange might explain these results, this should be minimal under the conditions utilized (i.e., no ATP regenerator, GDPβS in the assay, short incubation times).

Truncation Mutants. Each cytoplasmic domain of the membrane-bound adenylyl cyclases (C_1 and C_2) can be divided further (C_{1a}/C_{1b}; C_{2a}/C_{2b}). C_{1a} and C_{2a} are about 55% similar to each other, and they are homologous to the related domains of the other adenylyl cyclases and to the catalytic domains of the guanylyl cyclases. C_{1b} and C_{2b} are dissimilar to each other and are poorly conserved among the adenylyl cyclases. We have constructed truncation mutants of type I adenylyl cyclase that consist of either the amino-terminal half ($INM_1C_{1(1-570)}$) or the carboxyl-terminal half (IM_2C_2) of the protein. When expressed, each protein alone has no detectable adenylyl cyclase activity (Table 1; see also Tang et al., 1991). Similarly, the carboxyl-terminal half of type II adenylyl cyclase (IIM_2C_2) has no activity itself but contributes to a functional enzyme when coexpressed with INM_1C_1 (Table 1; see also Tang & Gilman, 1991). In the absence of folding problems, this result indicates that both cytoplasmic domains are necessary for catalysis.

To study the functions of the poorly conserved C_{1b} and C_{2b} regions, we have truncated type I adenylyl cyclase and the NM_1C_1 and M_2C_2 constructs at their carboxyl termini. Protein IΔC_{2b} lacks domain C_{2b} (64 amino acids). Membranes containing IΔC_{2b} have about 25−50% of the activity of membranes containing the wild-type enzyme (Figure 2). This could be due to lower expression or impaired folding of the protein. More importantly, the responses of the protein to regulators appear to be intact (including the inhibitory

Protein	Mg^{2+}	$G_{s\alpha}$	Mn^{2+}+Fsk
Wild type	0.6±0.05	2.8±0.2	6.9±0.3
$I\Delta C_{2b}$	0.2±0.01	1.1±0.1	3.2±0.2
$INM_1C_{1(1-570)}$	0	0.06	0.06
$INM_1C_{1(1-484)}$	0.01	0.03	0.06
IM_2C_2	0.01	0.02	0.02
IM_2C_{2a}	0.02	0.01	0.05
IIM_2C_2	0	0.02	0
$INM_1C_{1(1-570)}$+IM_2C_2	0.6±0.05	2.0±0.1	3.4±0.2
$INM_1C_{1a(1-484)}$+IM_2C_2	0.8±0.1	1.8±0.02	1.5±0.2
$INM_1C_{1(1-570)}$+IM_2C_{2a}	0.3±0.02	0.9±0.1	2.3±0.1
$INM_1C_{1a(1-484)}$+IM_2C_{2a}	0.7±0.05	0.7±0.05	1.2±0.2
$INM_1C_{1(1-570)}$+IIM_2C_2	0.02	0.3±0.02	2.1±0.3
$INM_1C_{1(1-484)}$+IIM_2C_2	0.01	1.4±0.2	4.1±0.2

FIGURE 2: Adenylyl cyclase activity of truncation mutants. Membranes of Sf9 cells were assayed for adenylyl cyclase activity (nanomoles of cyclic AMP per minute per milligram of membrane proteins). Mg^{2+} = 10 mM $MgCl_2$; Mn^{2+} = 5 mM $MnCl_2$; $G_{s\alpha}$ = 200 nM GTPγS-bound $G_{s\alpha}$; Fsk = 100 μM forskolin. Standard errors for those values less than 0.1 nmol/min/mg are less than 20%.

effects of 2′-deoxy-3′-AMP and G protein $\beta\gamma$ subunits; not shown in Figure 2).

The two mutants $INM_1C_{1(1-570)}$ and $INM_1C_{1(1-484)}$ consist of N, M_1, and C_1 terminated 86 and 0 residues from the end of C_{1a}, respectively. Mutant IM_2C_{2a} begins just prior to M_2 (the same as IM_2C_2) and terminates at the end of C_{2a}. None of these proteins exhibits significant adenylyl cyclase activity (Figure 2). However, coexpression of the two constructs containing C_{1a} with IM_2C_2, IM_2C_{2a}, or IIM_2C_2 in the six possible combinations results in substantial adenylyl cyclase activity that is elevated in response to most activators (particularly GTPγS-$G_{s\alpha}$ and forskolin; Figure 2). Thus, neither C_{1b} nor C_{2b} is essential for regulated adenylyl cyclase activity.

Paired Mutants. To assess the role of each cytoplasmic domain in catalysis, we have made single amino acid substitutions of residues that are conserved in varying patterns in the C_{1a} and C_{2a} domains of adenylyl cyclases from mammals, *Drosophila*, and *Dictyostelium*; the conserved domains of adenylyl cyclases that contain one such domain (yeast, *Dictyostelium*, *Trypanosoma brucei*, and *Brevibacterium liquefaciens*; AC-s in Table 1); and the conserved domains of membrane-bound and soluble guanylyl cyclases (mammalian and *S. purpuratus*) (Kataoka et al., 1985; Krupinski et al., 1989; Schulz et al., 1989; Thorpe & Garbers, 1989; Yamawaki-Kataoka et al., 1989; Alexandre et al., 1990; Bakalyar & Reed, 1990; Nakane et al., 1990; Chinkers & Garbers, 1991; Feinstein et al., 1991; Gao & Gilman, 1991; Peters et al., 1991; Ishikawa et al., 1992; Levin et al., 1992; Pitt et al., 1992). We have focused on charged residues and have mutated them to alanine to minimize structural alterations (Cunningham & Wells, 1989). Eight pairs of residues were chosen (four basic, three acid, one aromatic). All of the mutant proteins were expressed in Sf9 cells using recombinant baculoviruses (verification of expression by immunoblotting not shown). Membranes were assayed for adenylyl cyclase activity, and their capacities to bind [^{35}S]GTPγS-$G_{s\alpha}$ were also assessed (Table 1).

We have attempted to place the mutant proteins into groups based on the pattern of conservation of the altered residue. In the first group, residues are conserved among all four of the types of sequences listed above (C_{1a}, C_{2a}, AC-s, GC). Two glycine residues meet this criteria but were avoided. There are six other residues that show this pattern (with one or two exceptions in each case among the 24 sequences examined). Only one of these (Arg398/979 in type I adenylyl cyclase) is charged. Mutant R398A had an unaltered capacity to bind [^{35}S]GTPγS-$G_{s\alpha}$, but its catalytic activity was very low unless assayed in the presence of both forskolin and Mn^{2+}. Mutant R979A also had very low enzymatic activity under all conditions, as well as a reduced capacity to bind activated $G_{s\alpha}$.

Two charged residues (K350/923 and D419/1000) are absolutely conserved in C_{1a}, C_{2a}, and the soluble adenylyl cyclases; they differ but are conserved (K as E, D as C) in the guanylyl cyclases. Mutants K350A and K923A have essentially normal capacities and affinities for $G_{s\alpha}$ binding under standard assay conditions (Table 1; Figure 3A). Both of these mutants show substantial alterations in regulated adenylyl cyclase activities. Neither mutant responds to calmodulin (Figure 3F), which is both interesting and mysterious.[3] Mutant K923A has poor adenylyl cyclase activity unless Mn^{2+} is present, in which case forskolin-stimulated activity is roughly half the wild-type value (Figure 3C,D). However, the EC_{50} for forskolin in the presence of Mn^{2+} is elevated about 20-fold compared to wild type (Figure 3C). This mutant is also notable for its poor affinity for P-site inhibitors, its moderately elevated K_m for substrate (see below), and little response to $G_{s\alpha}$. Mutant K350A has low basal activity. Although near wild-type activities can be achieved in the presence of activators, the EC_{50} values for both forskolin and $G_{s\alpha}$ are elevated by 20−30 fold (Figure 3C,D,E). This shift in the apparent affinity of mutant K350A for GTPγS-$G_{s\alpha}$ introduces a substantial discrepancy between the curves for activation of enzymatic activity and competition for binding (compare Figure 3A with 3E). We thus examined binding of GTPγS-$G_{s\alpha}$ under the usual assay conditions and in the presence of reagents normally included in the adenylyl cyclase assay. Of interest, ATP causes a modest reduction (25%) in the binding of subsaturating concentrations of GTPγS-$G_{s\alpha}$ to membranes containing wild-type adenylyl cyclase and a substantial reduction (84%) in binding to membranes containing mutant K350A (Figure 3B). Although we have not yet explored this phenomenon in detail, there appears to be a negative heterotropic binding interaction between $G_{s\alpha}$ and ATP that is accentuated in the mutant and that explains the discrepancy between binding and activation curves.

A double mutant, R398A/K923A, was constructed in an attempt to eliminate catalytic activity with retention of $G_{s\alpha}$ binding activity; this goal was achieved (Table 1). This

[3] The calmodulin-binding domain of type I adenylyl cyclase is believed to reside within residues 495−522 (Wu et al., 1993). Failure of these mutants to be activated by calmodulin is thus presumably not due to direct alteration of this primary site of interaction.

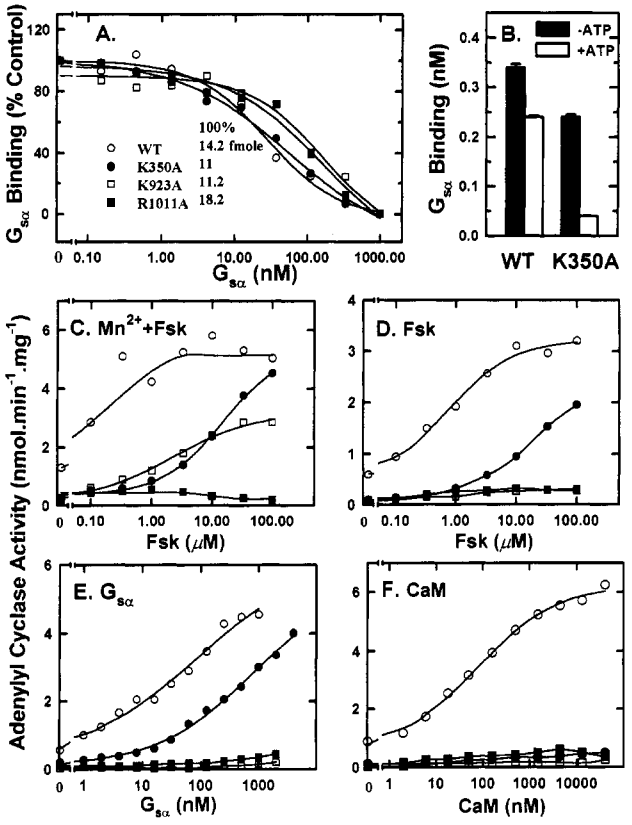


FIGURE 3: Binding of [^{35}S]GTPγS-$G_{s\alpha}$ and enzymatic activities of K350A, K923A, and R1011A adenylyl cyclase. (A and B) Binding of [^{35}S]GTPγS-$G_{s\alpha}$ to Sf9 cell membranes containing wild-type and mutant adenylyl cyclases. The concentration of labeled GTPγS-$G_{s\alpha}$ was 4 nM, and unlabeled GTPγS-$G_{s\alpha}$ was included at the indicated concentrations as competitor. The amount of labeled $G_{s\alpha}$ bound to 20 μg (A) or 10 μg (B) of Sf9 cell membranes in the absence of competitors is indicated. When present (B), the concentration of ATP was 0.5 mM. (C−F) Adenylyl cyclase activities of Sf9 cell membranes (10 μg) containing wild-type and mutant (K350A, K923A, and R1011A) adenylyl cyclases in the presence of forskolin and 5 mM $MnCl_2$ (C), forskolin (D), GTPγS-$G_{s\alpha}$ (E), and calmodulin and 50 μM $CaCl_2$ (F). Binding of [^{35}S]-GTPγS-$G_{s\alpha}$ and adenylyl cyclase activities were assayed in duplicate, and data are representative of at least two experiments.

double mutant resembles the other mutant pair in this group—D419A and D1000A. Both of these proteins have very little catalytic activity, although $G_{s\alpha}$ binding activity is approximately 50% of the wild-type value (data not shown).

There are 17 amino acid residues that are relatively well conserved among the C_{1a} and C_{2a} domains of the adenylyl cyclases and the catalytic domains of the guanylyl cyclases; they differ in the adenylyl cyclases with a single conserved domain. Five of these pairs were mutated, and the resulting proteins display a few different phenotypes. Both membranes of the D338A/D908A pair are essentially inactive and have substantially reduced ability to bind activated $G_{s\alpha}$ (data not shown). Three pairs of mutants are similar (R348A/K921A, H369A/H950A, and Y418A/Y999A). Each member of these pairs with the mutation in C_{1a} is relatively normal. Each member with the mutation in C_{2a} has little to no catalytic activity, as well as reduced binding of GTPγS-$G_{s\alpha}$ (Table 1). Both members of the last pair in this group, E432A and D1013A, have good catalytic activity. Interestingly, however, mutant E432A is exceedingly dependent on Mn^{2+} and has an elevated K_m for substrate (see below).

Table 2: Effects of Nucleotides[a]

protein	activity[a] Mn^{2+} + FSK (nmol/min/mg)	$K_{m,ATP}$ (μM)[b]	$IC_{50,2'd3'\text{-AMP}}$ (μM)[b]
wild type	6.8 ± 0.2	38 ± 8	5 ± 0.7
R348A	8.3 ± 0.3	51 ± 12	66 ± 14
K921A	1.3 ± 0.1	53 ± 11	35 ± 5
K350A	5.4 ± 0.3	53 ± 11	83 ± 17
K923A	2.5 ± 0.2	124 ± 16	1300 ± 300
E432A	5.6 ± 0.1	371 ± 27	25 ± 3
D1013A	5.6 ± 0.2	44 ± 11	4 ± 0.6
H369A	5.5 ± 0.3	40 ± 12	6 ± 1
R398A	2.1 ± 0.2	72 ± 16	120 ± 30
Y418A	4.4 ± 0.3	78 ± 16	13 ± 3
V430R	8 ± 0.2	63 ± 10	6 ± 1
I351T	1 ± 0.1	48 ± 10	9 ± 2
T924A	4.7 ± 0.2	53 ± 9	5 ± 1
R1011K	15 ± 0.5	50 ± 10	9 ± 1
K1049A	5.7 ± 0.1	50 ± 12	41 ± 4

[a] All assays were performed with 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 100 μM forskolin. [b] Values for $K_{m,ATP}$ and $IC_{50,2'd3'\text{-AMP}}$ were calculated from least squares fits of adenylyl cyclase activity to a simple rectangular hyperbola. The $IC_{50,2'd3'\text{-AMP}}$ was determined with 0.5 mM ATP.

Nonpaired Mutants. Two residues (T924 and R1011) are conserved in the C_{2a} domains of the adenylyl cyclases and in the corresponding domains of the guanylyl cyclases and the single-conserved-domain adenylyl cyclases; these residues differ in the C_{1a} domains. Mutant T924A is essentially normal (data not shown). Mutant R1011A binds activated $G_{s\alpha}$ normally but is essentially inactive catalytically (Table 1, Figure 3). Mutant R1011K had excellent catalytic activity (particularly with Mn^{2+} + forskolin) but paradoxically reduced $G_{s\alpha}$ binding activity and response to this protein. One residue (D354) is conserved in the C_{1a} domains as well as the guanylyl cyclases and the soluble adenylyl cyclases. Mutant D354A is completely inactive but retains significant $G_{s\alpha}$ binding activity (not shown).

Effects of Nucleotides. Values of $K_{m,ATP}$ for type I adenylyl cyclase in the absence of activators or in the presence of GTPγS-$G_{s\alpha}$ or Ca^{2+}-calmodulin are between 30 and 60 μM. Surprisingly, the K_m is elevated approximately 10-fold (to 440 μM) when forskolin is the activator. In the presence of Mn^{2+}, all values, including that with forskolin, are in the 30−60 μM range. This phenomenon, which has apparently been overlooked, is also true of type II, type V, and *Drosophila (rutabaga)* adenylyl cyclases.

The $K_{m,ATP}$ for mutants E432A and K923A are elevated about 10-fold and 3-fold, respectively (Table 2). These relative differences in $K_{m,ATP}$ are maintained under other assay conditions. For example, with forskolin or GTPγS-$G_{s\alpha}$ but no Mn^{2+}, the $K_{m,ATP}$ for K923A is 1.8 or 0.5 mM, respectively.

Membrane-bound adenylyl cyclases are inhibited directly by adenosine and certain of its analogues (Johnson et al., 1989; Johnson & Shoshani, 1990). These compounds have been termed P-site inhibitors because of the importance of an intact purine ring; 2′- or 5′-deoxy and 3′-phosphoryl compounds are the most potent. Inhibition is not competitive with respect to Mg^{2+}-ATP, and stimulated forms of adenylyl cyclase are more susceptible to inhibition than is basal activity. A potent P-site inhibitor, 2′-deoxy-3′-AMP, was tested for its inhibitory effect on 14 mutants that are activated by Mn^{2+} and forskolin (Table 2). Based on their sensitivity to 2′-deoxy-3′-AMP, we have grouped the mutants roughly

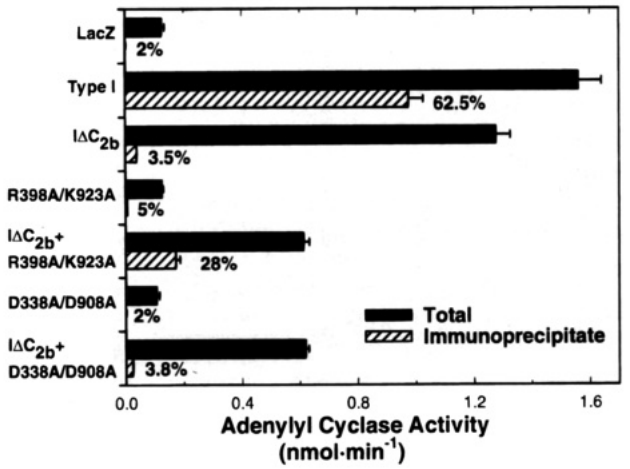


FIGURE 4: Immunoprecipitation of adenylyl cyclase activity from detergent extracts of membranes containing wild-type and mutant enzymes. Membranes (500 μg) from Sf9 cells expressing the indicated proteins were solubilized and immunoprecipitated with type I adenylyl cyclase-specific antiserum C1-1115. The filled bar shows the total adenylyl cyclase activity that was solubilized with dodecyl maltoside. The hatched bar shows activity that was immunoprecipitated. The percent activity that was immunoprecipitated is also indicated. Assays were performed in duplicate, and data are representative of two experiments.

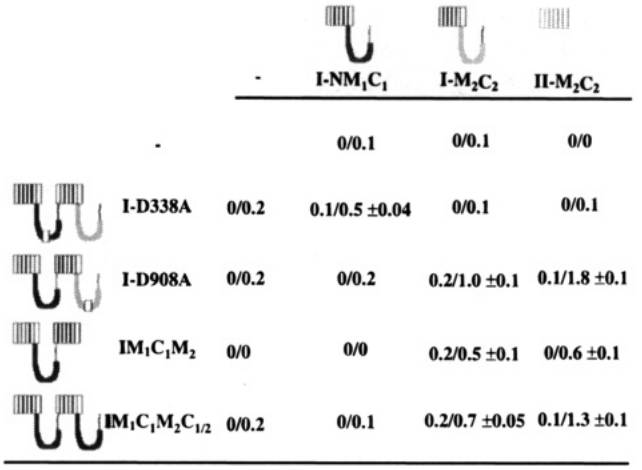


FIGURE 5: Adenylyl cyclase activity of membranes containing different combinations of mutant enzymes. Complementation of mutations in the C_2 domain by coexpression of truncation mutants. Membranes (10 μg) were assayed for adenylyl cyclase activity (nmol/min/mg protein). Values shown are assays with either 10 mM $MnCl_2$ or both 10 mM $MnCl_2$ and 100 μM forskolin. Assays were performed in duplicate, and data are representative of at least two experiments.

into three categories. There is little change in the sensitivity to the inhibitor among the seven mutants in group II (D1013A, H369A, Y418A, V430R, 1351T, T924A, R1011K). In the second group, (six of the mutants described above), sensitivity to the inhibitor is reduced by 5−24 fold (R348A, K921A, K350A, E432A, R398A, K1049A). Mutant K923A, the only member of the third group, is very insensitive to P-site inhibition.

Quaternary Structure. Hydrodynamic analyses of detergent-solubilized, unstimulated adenylyl cyclases from brain and S49 cells have indicated molecular weights of about 200 000, suggestive of dimerization (Haga et al., 1977; Neer et al., 1984; Yeager et al., 1985; Smigel, 1986). We have attempted to use certain of the mutants described above to assess this possibility. Antiserum C1-1115 recognizes the carboxyl-terminus of type I adenylyl cyclase and can be used to immunoprecipitate significant enzymatic activity from detergent extracts of Sf9 cells that express the protein (Figure 4).[4] (The immunoprecipitated activity can be stimulated by $G_{s\alpha}$, calmodulin, or forskolin.) Mutant $I\Delta C_{2b}$ has substantial enzymatic activity (Figure 2) but is not recognized by antiserum C1-1115 (the epitope has been removed); adenylyl cyclase activity is not immunoprecipitated from extracts of cells expressing this mutant (Figure 4). Double mutants D338A/D908A and R398A/K923A are totally inactive, but both retain the epitope for antiserum C1-1115. Mutant R398A/K923A retains $G_{s\alpha}$ binding activity; D338A/D908A does not (Table 1). When mutants $I\Delta C_{2b}$ and R398A/K923A were coexpressed in Sf9 cells, a significant amount (28%) of the enzymatic activity could be immunoprecipitated. This was not true when $I\Delta C_{2b}$ and D338A/D908A were synthesized together. The result indicates interaction of $I\Delta C_{2b}$ and R398A/K923A that is dependent on the structural integrity of the double mutant (as assessed by retention of the capacity to bind $G_{s\alpha}$). (Note: the amount of activity that could be immunoprecipitated following coexpression of $I\Delta C_{2b}$ and R398A/K923A is very reasonable if $I\Delta C_{2b}$ also forms homodimers.)

We have also attempted to detect interactions between truncated and full-length constructs, which could be revealed by complementation between mutant proteins. These experiments utilized some of the point mutants described above, as well as INM_1C_1, IM_2C_2, IIM_2C_2, $IM_1C_1M_2$ (entire C_2 deleted from type I), and $IM_1C_1M_2C_{1/2}$ (C_{2a} replaced by C_{1a}). The truncation mutants and $IM_1C_1M_2C_{1/2}$ have little or no adenylyl cyclase activity (Figure 5). Results of adenylyl cyclase assays performed with membranes from Sf9 cells infected with various combinations of baculoviruses encoding these mutants are shown in Figure 5. Coexpression of proteins that have alterations in C_{2a} (point mutation [D908A], truncation [$IM_1C_1M_2$], or exchange [$IM_1C_1M_2C_{1/2}$]) with proteins representing the carboxyl-terminal half of type I or type II adenylyl cyclase resulted in complementation (3- to 7-fold increase in adenylyl cyclase activity). Appropriately, this was not observed when NM_1C_1 replaced M_2C_2. We were also able to detect weak complementation between point mutant D338A and INM_1C_1 (but not between D338A and M_2C_2 constructs). These results again support the hypothesis that both C_{1a} and C_{2a} are necessary for catalytic activity. They further indicate productive interactions between full-length and truncated constructs of adenylyl cyclase. However, we were not able to observe catalysis as the result of interaction between two full-length constructs with inactivating point mutations, one in C_{1a} and the other in C_{2a} (*i.e.*, coexpression of D338A or R398A with H950A or K923A) (Tang et al., 1992).

DISCUSSION

Described in this report is a series of truncation mutations designed to test the functions of the nonconserved domains of membrane-bound adenylyl cyclases. Regulated adenylyl cyclase activity is observed in the absence of both C_{1b} and C_{2b}. These findings have guided our successful efforts to synthesize a truncated, soluble adenylyl cyclase by linkage of the C_{1a} domain of type I adenylyl cyclase with the C_{2a}

[4] J. Iñiguez-Lluhi and A. G. Gilman, unpublished observations.

domain of the type II enzyme (Tang & Gilman, 1995). This soluble enzyme has extremely low basal activity and is activated markedly by $G_{s\alpha}$ and forskolin. This system will be used to study G protein-regulated adenylyl cyclases both biochemically and genetically.

A variety of point mutations have been made in the duplicated domains of type I adenylyl cyclase. Of the 21 mutations to alanine that are described, 10 are in C_{1a} and 11 are in C_{2a}. Of these, we classify four as relatively normal (H369, Y418, T924, and D1013) and four as uninterpretably inactive (maximal enzymatic activity 0.3 nmol/min/mg or less, $G_{s\alpha}$ binding $\leq$ 25% of wild type; H402, D908, R979, and Y999). There is no obvious tendency of these mutations to arise from any particular group or to fall in one domain or the other. Thirteen mutants are thus left for analysis. We classify seven of these (three in C_{1a}, four in C_{2a}) as having substantially reduced enzymatic activity in the presence of any activator despite retention of significant $G_{s\alpha}$ binding activity (D338, D354, D419, K921, H950, D1000, and R1011). Two others (one in each domain) have significant defects in P-site inhibition without any other notable alteration (R348 and K1049). Four mutants have more complex phenotypes (K350, R398, E432, and K923). All have impaired P-site inhibition. Two of them have clearly elevated K_m's for substrate (E432 and K923); one is tending in this direction (R398). These three mutants are also characterized as "dependent" on Mn^{2+} for enzymatic activity. The concentration dependence for activation of mutant K923 by forskolin is shifted to the right. The same is true for mutant K350 for both forskolin and $G_{s\alpha}$; this mutant also displays a curious relationship between binding of ATP and GTPγS-$G_{s\alpha}$.

Certain phenotypic characteristics appear to be linked. The four mutants listed above with severely impaired $G_{s\alpha}$ binding activity all have exceedingly low enzymatic activities, despite adequate expression detected by immunoblotting. This appears to justify use of $G_{s\alpha}$ binding activity as a gross measure of the structural integrity of the constructs. The mutant R1011K is an exception, displaying excellent activity with forskolin (and calmodulin) despite poor $G_{s\alpha}$ binding and a relatively poor response to this protein. However, it is unlikely that this residue is crucial for interaction with $G_{s\alpha}$ because its mutation to alanine impaired all enzymatic activities but left $G_{s\alpha}$ binding intact. Stated in another way, we have failed to find mutants with meaningful, isolated deficiencies in $G_{s\alpha}$ binding and activation by the protein in the face of normal responses to other activators. We ascribe this to the fact that the residues that were mutated were chosen because of homologies with non$-G_{s\alpha}$-responsive nucleotide cyclases.

Although the numbers are small, there also appears to be linkage among $K_{m,ATP}$ defects, loss of P-site inhibition, and dependence on Mn^{2+}. (However, the confidence with which one makes this statement is tempered by the apparent ease of obtaining so-called P-site mutants.) At least some of this phenomenon is explicable by the fact that forskolin raises $K_{m,ATP}$ significantly and this effect is reversed by Mn^{2+}.

One of the stronger conclusions that can be drawn from study of these mutants is that both cytosolic domains of membrane-bound adenylyl cyclases are required for characteristic enzymatic activity. Mutations in either cytosolic domain can largely eliminate catalysis with retention of substantial $G_{s\alpha}$ binding activity (*e.g.*, D354, D419, D1000, R1011). Furthermore, the two cytosolic domains are not equivalent. The individual effects of paired mutations differ (*e.g.*, Y418/999, H369/950). Replacement of domain C_{2a} with C_{1a} is ineffective. Despite the inequivalence of the domains, we cannot ascribe unique functions to them. For example, there is little or no evidence to support the notion that one putative nucleotide-binding domain constitutes the P site, while the other is responsible for catalysis. "P-site mutants" are found everywhere. Two mutants have altered K_m's for substrate. Although the magnitude of the effects differs, one (E432) is in C_{1a}, the other (K923) in C_{2a}. We note that the effect of mutation on $K_{m,ATP}$ exceeds that on the IC_{50} for 2′deoxy-3′-AMP for mutant E432A, while the reverse is true for mutant K923A; unfortunately little can be made of this observation at the moment.

Given the ability of mutations in either domain to interfere with nucleotide-related phenomena, we suggest that both the C_{1a} and C_{2a} domains bind ATP. Given their inequivalence, we further speculate that one of these domains is predominantly catalytic while the other is predominantly regulatory. The results of synthesis of each cytoplasmic domain in *E. coli* are consistent with this notion (Tang & Gilman, 1995). Expression of C_{2a} results in weak complementation of bacteria that lack endogenous adenylyl cyclase; expression of C_{1a} does not. Mammalian phosphofructokinase and hexokinase appear to provide precedents. These enzymes have two homologous domains, and only one is involved in catalysis (Fothergill-Gilmore & Michels, 1993). The other domain arose by gene duplication, with subsequent mutation of several residues that are critical for catalysis. The ultimate evolutionary outcome has been to increase greatly the regulatory power of these enzymes compared to their single-domain yeast and bacterial counterparts.

Adenylyl cyclase is largely inactive in the absence of stimulators, despite the presumption that it can bind substrate readily. In addition, there is little inhibition of enzymatic activity by product. We thus suggest that the enzyme is regulated by an open/closed transition of the catalytic domain (Figure 6) (Schulz, 1991). Substrate is bound in the open state. The conformational change leading to the closed state brings crucial residues to bear on the α-phosphoryl and 3′-OH groups of ATP, where catalysis must occur. After catalysis, the open state could reform either prior or subsequent to release of product. Return to the open conformation from the nucleotide-free closed state could thus limit the rate of reaction, as could the rate of formation of the closed state prior to catalysis. Either step could be regulated by activators and/or inhibitors. We favor these conformational arguments because of the effects of P-site inhibitors.

P-site inhibitors resemble cyclic AMP, yet product inhibition of adenylyl cyclase is weak. P-site inhibitors resemble substrate, but inhibition is not competitive. Inhibition is activity-dependent (Florio & Ross, 1983; Johnson & Shoshani, 1990). The P site cannot be clearly distinguished from a catalytic site by the mutational analysis presented above. Rather, mutants with altered K_m's for substrate have altered P-site characteristics as well. The converse is not always true; alterations in apparent affinity for the P-site inhibitor do not dictate altered values of K_m. This presumably reflects the mechanism of inhibition and highlights the need to be able to measure nucleotide binding directly. Despite these uncertainties, we hypothesize that P-site inhibitors and

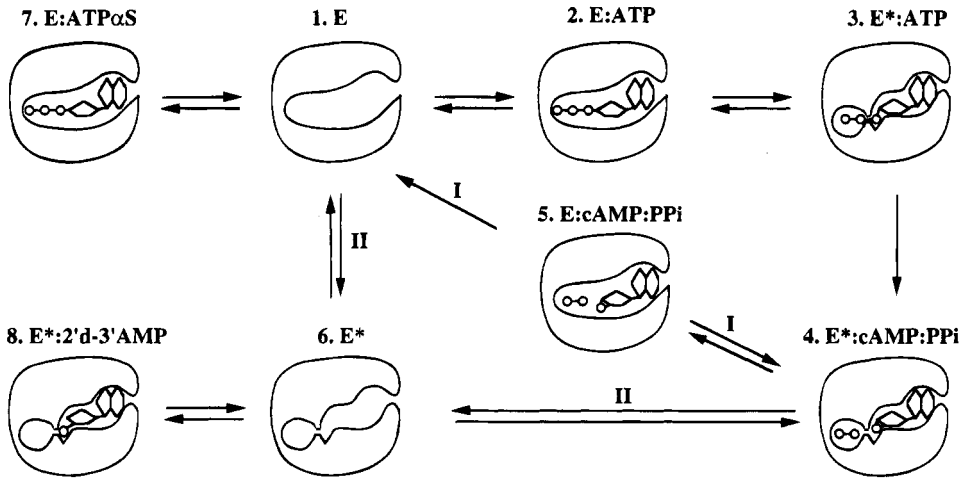


FIGURE 6: Model of conformational changes at the catalytic site of adenylyl cyclase. See text for description.

substrate bind to different conformations of the same site(s). Substrate can bind only to the open conformation. P-site inhibitors have high and preferential affinity for the closed state. The activity dependence of P-site inhibition is explained if stimulators promote formation of the closed state. We further suggest that impairment of enzyme regulation in a variety of ways could upset the normal balance between open and closed conformations, perhaps explaining the frequency with which alterations in P-site inhibition are seen among the mutants described above.

Both members of one pair of mutants, K350A and K923A, hint at very interesting and important regulatory phenomena—linkage of binding of activators and nucleotides. The EC_{50}'s for activation of the enzymatic activity of mutant K350A by both forskolin and GTPγS-$G_{s\alpha}$ are shifted to the right. The affinity of GTPγS-$G_{s\alpha}$ for the enzyme is lowered by ATP. Mutant K923A is unresponsive to P-site inhibitors and appears to have an altered affinity for forskolin. This mutant also displays apparent positive cooperativity for inhibition of activity by GTP (not shown). We strongly suspect that understanding of these phenomena will provide great insight into mechanisms of regulation of adenylyl cyclase activity. However, detailed analyses of these effects will require knowledge of nucleotide binding to the proteins. In view of the affinities involved, this will require reasonably large amounts of enzyme—soluble and pure. We place a high priority on design and synthesis of a regulable, soluble adenylyl cyclase that can be expressed and purified in reasonable yields.

We have obtained evidence for interaction between mutant adenylyl cyclases by immunoprecipitation and by complementation following coexpression of selected mutant constructs. We have not been able to observe such complementation by mixing of solubilized components. Interaction may be established only during synthesis, or a competent complex may be necessary for proper protein folding. There is as yet no evidence for oligomerization of adenylyl cyclase in the plasma membrane. Further, our experiments do not speak rigorously to the possibility of dimerization of wild-type adenylyl cyclase (only to mutants) or to a requirement for oligomerization for catalysis. However, there is evidence for oligomerization (even tetramerization) of membrane-bound guanylyl cyclases (Iwata et al. 1991; Chinkers & Wilson, 1992; Lowe, 1992). The existence of oligomers of adenylyl cyclase could presumably increase regulatory complexity greatly.

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REFERENCES

Alexandre, S., Paindavoine, P., Tebabi, P., Pays, A., Halleux, S., Steinert, M., & Pays, E. (1990) *Mol. Microbiol. 43*, 279.
Bakalyar, H. A., & Reed, R. R. (1990) *Science 250*, 1403.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M. F., & Krupinski, J. (1994) *J. Biol. Chem. 269*, 12190.
Chinkers, M., & Garbers, D. L. (1991) *Annu. Rev. Biochem. 60*, 553.
Chinkers, M., & Wilson, E. M. (1992) *J. Biol. Chem. 267*, 18589.
Clackson, T., & Winter, G. (1989) *Nucleic Acids Res. 17*, 10163.
Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081.
Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W.-J., Krupinski, J., Gilman, A. G., & Reed, R. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10173.
Florio, V. A., & Ross, E. M. (1983) *Mol. Pharmacol. 24*, 195.
Fothergill-Gilmore, L. A., & Michels, P. A. M. (1993) *Prog. Biophys. Mol. Biol. 59*, 105.
Gao, B., & Gilman, A. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10178.
Haga, T., Haga, K., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5776.
Ishikawa, Y., Katsushika, S., Chen, L., Halnon, N. J., Kawabe, J., & Homcy, C. J. (1992) *J. Biol. Chem. 267*, 13553.
Itoh, H., & Gilman, A. G. (1991) *J. Biol. Chem. 266*, 16226.
Iwata, T., Uchida-Mizuno, K., Katafuchi, T., Ito, T., Hagiwara, H., & Hirose, S. (1991) *J. Biochem. 110*, 35.
Iyengar, R. (1993) *FASEB J. 7*, 768.
Johnson, R. A., & Shoshani, I. (1990) *J. Biol. Chem. 265*, 11595.
Johnson, R. A., Yeung, S.-M. H., Stübner, D., Bushfield, M., & Shoshani, I. (1989) *Mol. Pharmacol. 35*, 681.
Kataoka, T., Broek, D., & Wigler, M. (1985) *Cell 43*, 493.
Katsushika, S., Chen, L., Kawabe, J. I., Nilakantan, R., Halnon, N. J., Homcy, C. J., & Ishikawa, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8774.
Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., & Gilman, A. G. (1989) *Science 244*, 1558.

Krupinski, J., Lehman, T. C., Frankenfield, C. D., Zwaagstra, J. C., & Watson, P. A. (1992) *J. Biol. Chem. 267*, 24858.
Kunkel, T. A., Roberts, J. D., & Zabour, R. A. (1987) *Methods Enzymol. 154*, 367.
Lee, E., Linder, M. E., & Gilman, A. G. (1994) *Methods Enzymol. 237*, 146.
Levin, L. R., Han, P.-L., Hwang, P. M., Feinstein, P. G., Davis, R. L., & Reed, R. R. (1992) *Cell 68*, 479.
Lowe, D. G. (1992) *Biochemistry 43*, 10421.
Maune, J. F., Klee, C. B., & Beckingham, K. (1992) *J. Biol. Chem. 267*, 5286.
Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., & Murad, F. (1990) *J. Biol. Chem. 265*, 16841.
Neer, E. J., Lok, J. M., & Wolf, L. G. (1984) *J. Biol. Chem. 259*, 14222.
O'Reiley, A., Miller, L. K., & Luckow, N. A. (1992) (Abstract).
Peters, E. P., Wilderspin, A. F., Wood, S. P., Zvelebil, M. J. J. M., Sezer, O., & Danchin, A. (1991) *Mol. Microbiol. 5*, 1175.
Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., & Devreotes, P. N. (1992) *Cell 69*, 305.
Premont, R. T., Chen, J. Q., Ma, H. W., Ponnapalli, M., & Iyengar, R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9809.
Schulz, G. E. (1991) *Curr. Opin. Struct. Biol. 1*, 883.
Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., & Garbers, D. L. (1989) *Cell 58*, 1155.
Smigel, M. D. (1986) *J. Biol. Chem. 261*, 1976.
Tang, W.-J., & Gilman, A. G. (1991) *Science 254*, 1500.
Tang, W.-J., & Gilman, A. G. (1992) *Cell 70*, 869.
Tang, W.-J., & Gilman, A. G. (1995) *Science 268*, 1769.
Tang, W.-J., Krupinski, J., & Gilman, A. G. (1991) *J. Biol. Chem. 266*, 8595.
Tang, W.-J., Iñiguez-Lluhi, J. A., Mumby, S. M., & Gilman, A. G. (1992) in *The Cell Surface—Symposium 57* (Anonymous) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, in press.
Taussig, R., & Gilman, A. G. (1995) *J. Biol. Chem. 270*:1.
Taussig, R., Quarmby, L. M., & Gilman, A. G. (1993) *J. Biol. Chem. 268*, 9.
Thorpe, D. S., & Garbers, D. L. (1989) *J. Biol. Chem. 264*, 6545.
Watson, P. A., Krupinski, J., Kempinski, A. M., & Frankenfield, C. D. (1994) *J. Biol. Chem. 269*, 28893.
Wu, Z., Wong, S. T., & Storm, D. R. (1993) *J. Biol. Chem. 168*, 23766.
Yamawaki-Kataoka, Y., Tamaoki, T., Choe, H.-R., Tahaka, H., & Kataoka, T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5693.
Yeager, R. E., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1985) *Biochemistry 24*, 3776.
Yoshimura, M., & Cooper, D. M. F. (1993) *J. Biol. Chem. 268*, 4604.

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