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Interaction of the two cytosolic domains of mammalian adenylyl cyclase

(G proteins/forskolin)

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ABSTRACT **Adenylyl cyclase activity can be reconstituted by simple mixture of the two cytosolic domains of the enzyme after their independent synthesis in *Escherichia coli*. We have synthesized and purified the C_{1a} domain of type I adenylyl cyclase and the C_2 domain of the type II enzyme to assess their interactions with each other and with the activators $G_{s\alpha}$ and forskolin. In the absence of an activator, the fragments associate with low affinity and display low catalytic activity. This basal activity can be stimulated more than 100-fold by either forskolin or activated $G_{s\alpha}$. Further, the addition of these activators increases the apparent affinity of the fragments for each other. Stimulation of catalysis by $G_{s\alpha}$ and forskolin is synergistic. These data suggest a model wherein either $G_{s\alpha}$ or forskolin enhances association of the other activator with adenylyl cyclase, as well as facilitating the interaction between the C_1 and C_2 domains of the enzyme.**

All of the mammalian adenylyl cyclases identified to date share the capacity to be activated by both the guanosine triphosphate (GTP)-bound form of the α subunit of the G protein that activates adenylyl cyclase ($G_{s\alpha}$) and the diterpene forskolin (FSK); in several cases, these two activators interact synergistically. These enzymes also share a characteristic structure, consisting of a short and variable amino terminus, followed by two repeats of a module composed of six transmembrane spans (M_1 and M_2) and a roughly 40-kDa cytoplasmic domain (C_1 and C_2). The most highly conserved sequences among the adenylyl cyclases are within the amino-terminal halves of each cytosolic domain (C_{1a} and C_{2a}). These domains are also roughly 50% similar to each other (within a single enzyme) and to the catalytic domains of the guanylyl cyclases (1, 2).

A soluble, chimeric adenylyl cyclase, consisting of the covalently linked C_{1a} and C_2 domains of the type I and type II enzymes, respectively, has been synthesized in *Escherichia coli*, purified, and characterized. This protein displays FSK- and $G_{s\alpha}$-stimulated enzymatic activity and has a turnover number characteristic of the membrane-bound adenylyl cyclases (3, 4). Coexpression of noncovalently linked halves of membrane-bound adenylyl cyclases also permits detection of regulated adenylyl cyclase activity (5), but it was not possible to synthesize these molecules separately and to mix them productively after solubilization. Our goal has been to synthesize the cytosolic domains of adenylyl cyclase separately, in a manner that would permit their mixture and thus the capacity to analyze their interaction.

MATERIALS AND METHODS

DNA Constructs, Antibodies, and G Protein Subunits. To create the DNA for expression of the C_{1a} domain of type I adenylyl cyclase (IC_1), the construct pTrc(271)IC_1IIC_2L3 (3) was digested with *Bsr*BI and ligated with the phosphorylated oligonucleotides 5′-GATCTAGCTAGCTA and 5′-TAGCTAGCTA. The DNA was then digested with *Bsp*HI and *Bgl*II and ligated into pTrcH6 (4) that had been digested with *Nco*I and *Bgl*II. This resulted in a construct with an amino terminal hexa-histidine tag linked to residues 271–484 of type I adenylyl cyclase.

To create the DNA for expression of the C_2 domain of type II adenylyl cyclase (IIC_2), a polymerase chain reaction was performed on pTrc(271)IC_1IIC_2L3 using the primers 5′-ATGAGATCTGGATGCCAAGTTGCTCTGAG and 5′-TGGAGTCATGACACAGAGTGAAT; this created an amino terminal *Bsp*HI restriction site and a carboxy terminal *Bgl*II restriction site. After excision with *Bsp*HI and *Bgl*II, this fragment was ligated into pQE60 (Qiagen, Chatsworth, CA) that had been digested with *Nco*I and *Bgl*II. This created a construct encoding residues 821-1090 of type II adenylyl cyclase with a hexa-histidine tag at the carboxy terminus.

The antibodies utilized in this work have been described (4). $G_{s\alpha}$ was purified and activated with guanosine 5′-[γ-thio]triphosphate (GTP[γS]) as described (4).

Expression of Proteins in *E. coli*. The IC_1 construct was transformed into *E. coli* strain BL21(DE3), and the cells were grown in the presence of ampicillin (50 μg/ml). The IIC_2 construct was transformed into BL21(DE3) cells also harboring the pREP4 plasmid; cells were grown in ampicillin (50 μg/ml) and kanamycin (50 μg/ml). Cultures were grown to OD_{600} = 0.4 at 30°C; isopropyl β-D-thiogalactoside (30 μM) was then added and cells were grown at room temperature for 15 h before harvesting and freezing in liquid nitrogen. Cell pellets were resuspended with a Polytron homogenizer in 1/15th the culture volume of lysis buffer (50 mM Tris·HCl, pH 8.0/10 mM 2-mercaptoethanol/50 mM NaCl) containing mixed protease inhibitors (4). Cells were lysed by addition of 0.2 mg/ml of lysozyme. After incubation for 30 min at 4°C, DNase was added (0.02 mg/ml plus 5 mM $MgCl_2$). This suspension was centrifuged at 100,000 × *g* for 30 min, and the clarified lysate was collected.

Protein Purification. Clarified lysate from a 10-liter culture was supplemented with NaCl (250 mM final concentration) and loaded onto a 5-ml nickel-nitrilotriacetic acid (Ni^{2+}-NTA) column (Qiagen) that had been equilibrated with lysis buffer. The column was washed with 15 volumes of lysis buffer supplemented with 2 mM $MgCl_2$, 400 mM NaCl (final concentration), and 5 mM imidazole; 12 volumes of 50 mM Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 15 mM imidazole; and 8 volumes of 50 mM Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10 mM NaCl, and 15 mM imidazole. The column was then eluted with 8 volumes of 50 mM Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10 mM NaCl, 2 mM $MgCl_2$, and 150 mM imidazole.

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Abbreviations: $G_{s\alpha}$, the α subunit of the G protein that activates adenylyl cyclase; GTP, guanosine triphosphate; GTP[γS], guanosine 5′-[γthio]triphosphate; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FSK, forskolin.

The Ni^{2+}-NTA column eluate containing IC_1 was adjusted to 400 mM ammonium sulfate and loaded directly onto a 2-ml phenyl Sepharose column that had been equilibrated in buffer A (50 mM Na-Hepes, pH 8.0/2 mM $MgCl_2$/1 mM EDTA/2 mM dithiothreitol) plus 400 mM $(NH_4)_2SO_4$. The column was washed with 10 ml of equilibration buffer and 10 ml of buffer A, followed by elution with a linear gradient (20 ml) of 0 to 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in buffer A and 20 ml of buffer A containing 10 mM CHAPS. The pooled peak was exchanged into buffer A and concentrated to 0.4 ml (≈1 mg/ml).

The Ni^{2+}-NTA column eluate containing IIC_2 was loaded directly onto a 8-ml Mono-Q 10/10 column (Pharmacia), equilibrated in buffer A, and washed with 5 volumes of the same buffer. Protein was eluted with a 120-ml linear gradient of NaCl (0–300 mM) in buffer A, followed by a steep gradient to 1 M NaCl. The IIC_2 protein represents >90% of the total loaded onto the column and elutes at ≈150 mM NaCl. This pooled material was exchanged into buffer A containing 50 mM NaCl and concentrated to 10 mg/ml.

Gel filtration was carried out on a Superdex 200 column (Pharmacia) in 50 mM Na-Hepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, and 150 mM NaCl. All samples were loaded onto the column in less than 0.5 ml.

Adenylyl Cyclase Assays. Adenylyl cyclase activity was quantified as described by Smigel (6). All assays contained 10 mM $MgCl_2$ in a 50-μl final volume. Incubations were for 10 min at 30°C unless otherwise indicated.

RESULTS

The simple mixture of two crude bacterial lysates, each containing either the C_{1a} or the C_2 domains of type I and type II adenylyl cyclase, respectively, permits observation of GTP[γS]-$G_{s\alpha}$- and FSK-stimulated adenylyl cyclase activity (Fig. 1). There was no detectable activity when either IC_1 or IIC_2 was assayed alone in the presence or absence of activators. (The same result was obtained after purification of these fragments.) Based on this result, an assay was defined to facilitate purification of each fragment (Fig. 2). Lysates containing the proteins were purified by Ni^{2+}-NTA column chromatography as described under *Materials and Methods*. A fixed amount of this partially purified material was used as a reagent to assay increasing amounts of the complementary fragment in the presence of 50 μM FSK. Addition of IC_1 to a fixed amount of IIC_2 resulted in a roughly linear increase in enzymatic activity (Fig. 2*A*). Given the smaller amounts of IC_1 in these preparations, saturation was observed when increasing amounts of IIC_2 were added to the partially purified prepa-

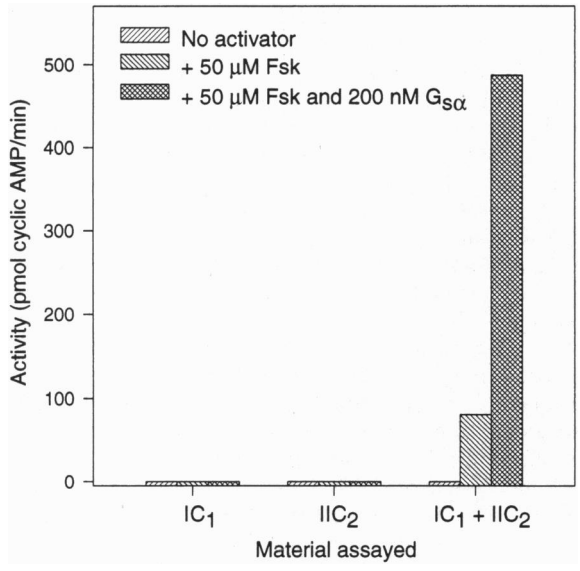

FIG. 1. Simple mixture of the C_{1a} and C_2 domains of adenylyl cyclase reconstitutes adenylyl cyclase activity. Bacterial lysates containing either the IC_1 or the IIC_2 fragments of adenylyl cyclase were assayed as described by themselves (20 μg) or after mixture (10 μg of each) with either no activator, 50 μM FSK, or 50 μM FSK plus 200 nM GTP[γS]-$G_{s\alpha}$. Activities shown as 0 represent less than 1 pmol/min, the limit of detection.

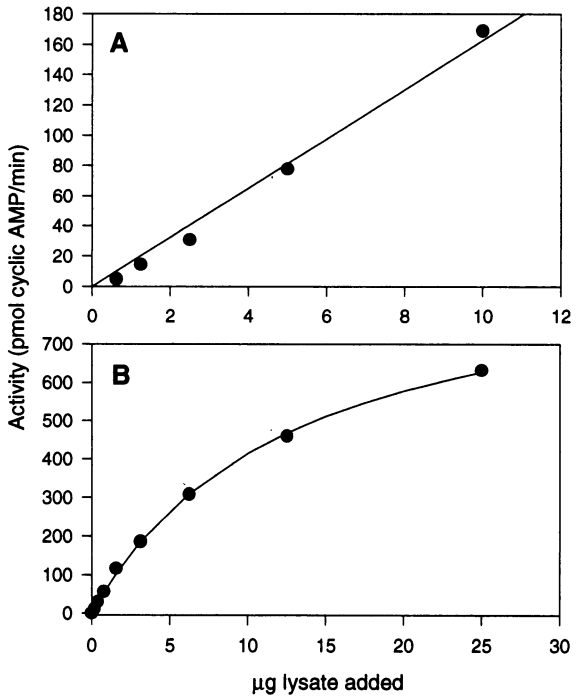

FIG. 2. Definition of a reconstitutive assay for purification of IC_1 and IIC_2. (*A*) Increasing amounts of lysate containing IC_1 were mixed with 1.5 μg of the Ni^{2+}-NTA column eluate containing IIC_2 and assayed with 50 μM FSK. (*B*) Increasing amounts of a lysate containing IIC_2 were mixed with 1 μg of the Ni^{2+}-NTA column eluate containing IC_1 and assayed with 50 μM FSK.

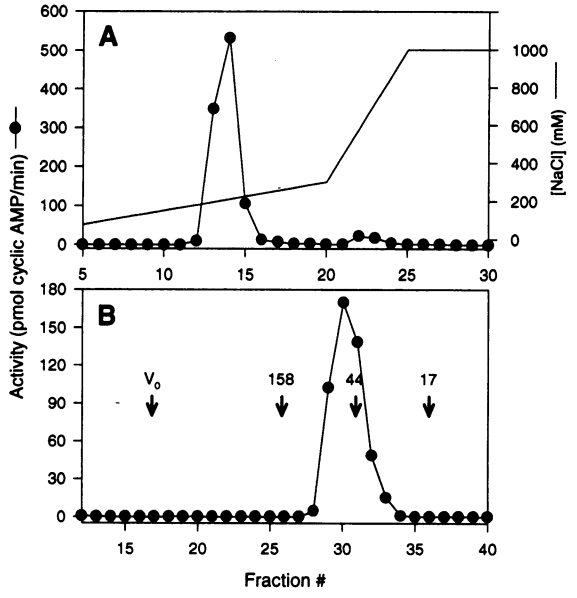

FIG. 3. Purification of IIC_2. (*A*) Mono Q column chromatography. Fractions were 6 ml. (*B*) Gel filtration over Superdex 200. Fractions were 0.5 ml. The positions of molecular weight markers are shown.

Table 1. Purification of the IC_1 and IIC_2 fragments of mammalian adenylyl cyclase

Preparation	Fraction	Protein, mg	Total activity, μmol/min	Specific activity, μmol/min·mg
IC_1	Lysate	1300	7.8	0.006
	Ni^{2+}-NTA	15	2.0	0.13
	Phenyl Sepharose	0.40	0.10	0.25
	Gel Filtration	0.22	0.28	1.3
IIC_2	Lysate	1400	220	0.16
	Ni^{2+}-NTA	63	100	1.6
	Mono-Q	57	120	2.1

Each preparation represents a 10-liter culture. Under the assay conditions defined in Fig. 2, activities were linear with respect to time and protein concentration. However, IC_1 and IIC_2 were not used at saturating concentrations, as defined in Fig. 6*B*. Thus, the specific activities for the two preparations shown in this table cannot be compared with each other or to the specific activities for IC_1 shown in Figs. 6 and 7.

ration of IC_1 (Fig. 2*B*). Assays were carried out in the linear ranges of these titrations.

Large amounts (6 mg/liter of culture) of the IIC_2 fragment were readily purified to homogeneity by Ni^{2+}-NTA and Mono Q column chromatography (Fig. 3 and Table 1). The material appeared to be homogeneous after SDS/PAGE (see Fig. 5). Amino acid sequencing revealed that the amino terminus of the protein began at Met-847 of type II adenylyl cyclase, 27 residues downstream from the presumed initiator methionine in the construct. Electron spray mass spectroscopy (not shown) revealed a single species with a M_r of 28,258, consistent with a protein containing Met-847–Ser-1090 (calculated M_r = 28,256). Gel filtration of this material showed a single peak of activity with an apparent M_r of ≈50,000 (Fig. 3). This behavior may represent dimerization of the fragment.

The IC_1 fragment accumulated to much lower levels during bacterial culture. It can be purified by a combination of Ni^{2+}-NTA, phenyl-Sepharose, and gel filtration chromatography (Figs. 4 and 5 and Table 1). Two distinct peaks of activity

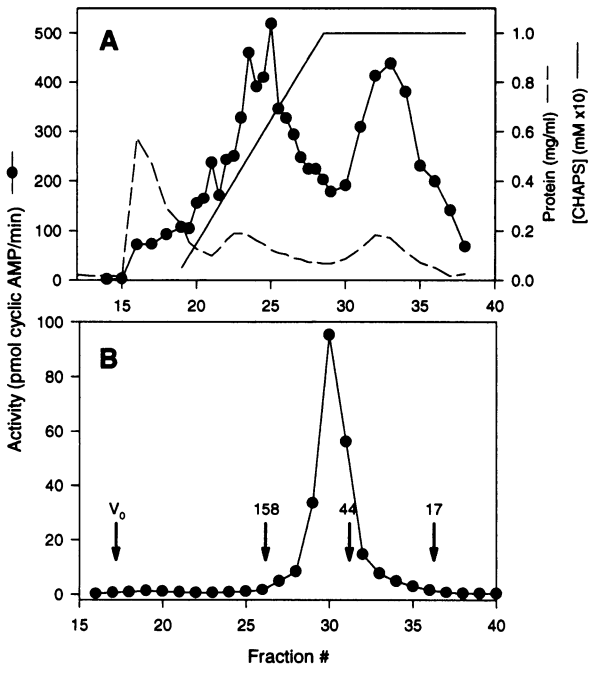

FIG. 4. Purification of IC_1. (*A*) Phenyl-Sepharose column chromatography. Fractions were 2 ml. The first peak of activity was pooled for further purification. (*B*) Gel filtration over Superdex 200. Fractions were 0.5 ml. The positions of molecular weight markers are shown.

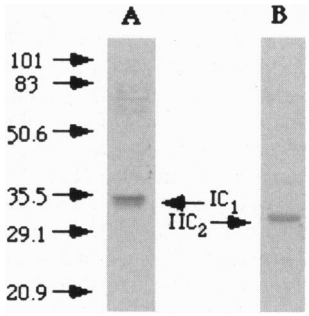

FIG. 5. SDS/PAGE of the purified fragments. IC_1 (1.6 μg) (*A*) and IIC_2 (1.5 μg) (*B*) were resolved on 11% polyacrylamide gels and stained with Coomassie blue. The positions of molecular weight markers and immunoreactive bands are shown with arrows.

were eluted from the phenyl-Sepharose column. The first of these was further purified by gel filtration, where it too displayed an apparent M_r of 50,000. The second phenyl-Sepharose peak displayed a similar gel filtration profile but contained more contaminants; this peak was not investigated further.

We first examined adenylyl cyclase activity obtained by mixture of IC_1 and IIC_2 in the absence of any activator (Fig. 6*A*). Increasing amounts of IIC_2 were added to 0.1 μM IC_1. The highest activity observed (expressed per quantity of IC_1) was 100-fold lower than that obtained in the presence of 50 μM FSK and 500-fold lower than that observed with FSK and activated $G_{s\alpha}$ (Fig. 6*B*). It was not possible to maximize activity by increasing the concentration of IIC_2 in the absence of activators (Fig. 6*A*). However, activity was maximized with apparent EC_{50} values for IIC_2 of 190 nM and 15 nM when

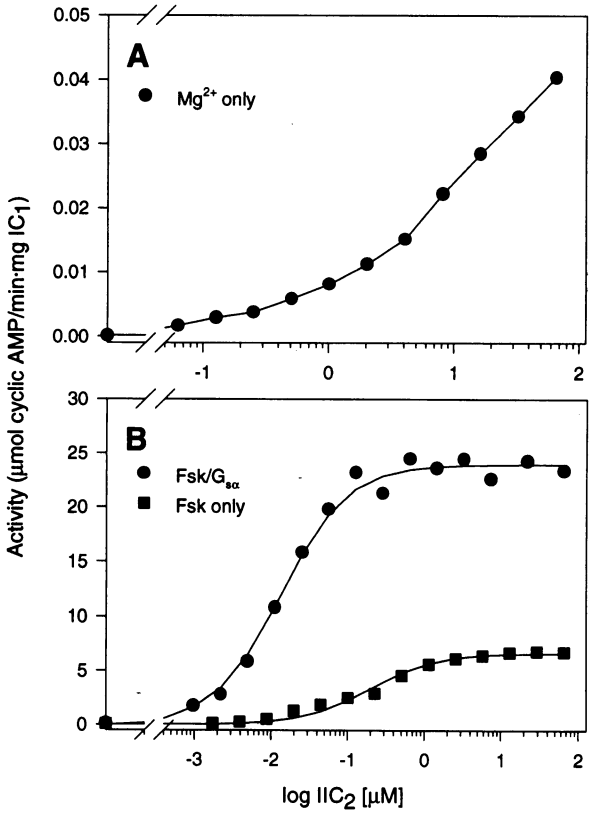

FIG. 6. Interactions of IC_1 with IIC_2. (*A*) The indicated concentrations of IIC_2 were assayed in the presence of 0.1 μM IC_1 for 30 min in the absence of activators. (*B*) The indicated concentrations of IIC_2 were assayed in the presence of 8 nM IC_1 for 10 min with either 50 μM FSK (■) or 50 μM FSK plus 10 μM GTP[γS]-$G_{s\alpha}$ (●). Activities are expressed per mg of IC_1.

stimulated by FSK or FSK plus activated $G_{s\alpha}$, respectively (Fig. 6*B*). Thus, the apparent affinity of IIC_2 for IC_1 was increased substantially by addition of FSK; an even greater shift was observed when activated $G_{s\alpha}$ was also present.

We also examined the capacity of FSK and $G_{s\alpha}$ to stimulate the adenylyl cyclase activity of the mixed fragments as a function of activator concentration (Fig. 7). In these experiments 8 nM IC_1 was mixed with 6.6 μM IIC_2. This is a saturating concentration of IIC_2 in the presence of either FSK or $G_{s\alpha}$ plus FSK. When increasing concentrations of FSK were tested in the presence or absence of 0.5 μM activated $G_{s\alpha}$, maximal activity was increased by $G_{s\alpha}$ and the EC_{50} for FSK was lowered by a factor of 100 (Fig. 7*A*). When increasing concentrations of activated $G_{s\alpha}$ were examined in the presence or absence of 50 μM FSK, maximal activities were similar under the two conditions. However, the inclusion of FSK shifted the EC_{50} for activated $G_{s\alpha}$ by more than 100-fold.

DISCUSSION

We have expressed the IC_1 and IIC_2 domains of mammalian adenylyl cyclase separately and reconstituted $G_{s\alpha}$- and FSK-stimulated adenylyl cyclase activity by their mixture. Neither protein has detectable adenylyl cyclase activity by itself (with or without activators), and the mixture has a very low basal activity in the absence of an activator. However, the activity observed in the presence of FSK and/or activated $G_{s\alpha}$ provides compelling evidence that adenylyl cyclase activity is dependent on the association of the C_1 and C_2 domains of the protein. The stimulated level of activity observed is comparable to that seen with the native, membrane-bound enzyme. Furthermore, the activation produced by FSK and $G_{s\alpha}$ is synergistic. We thus believe that this system provides a valuable tool for definition

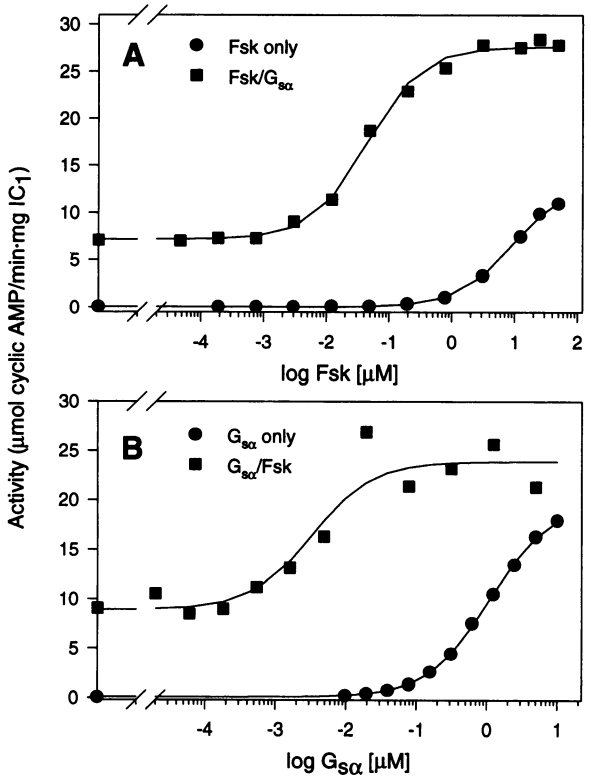

FIG. 7. Interactions between FSK and GTP[γS]-$G_{s\alpha}$. (*A*) Fragments IIC_2 (6.6 μM) and IC_1 (8 nM) were mixed and assayed in the presence of the indicated concentrations of FSK, with (■) or without (●) 0.5 μM GTP[γS]-$G_{s\alpha}$ (*B*) Fragments IIC_2 (6.6 μM) and IC_1 (8 nM) were mixed and assayed in the presence of the indicated concentrations of GTP[γS]-$G_{s\alpha}$, with (■) or without (●) 50 μM FSK.

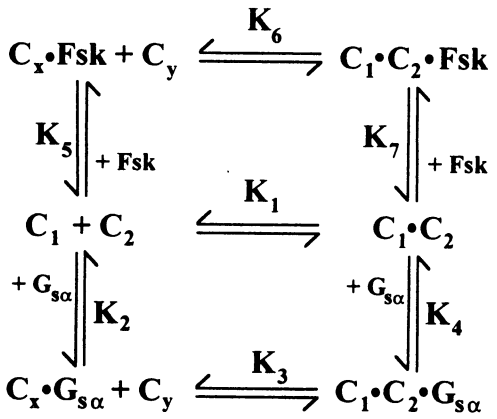

FIG. 8. A simple model for the interactions of IC_1, IIC_2, $G_{s\alpha}$, and FSK. The two domains of adenylyl cyclase are designated C_x and C_y where their identities are unknown; they are designated C_1 and C_2 when associated. See text for further discussion.

of mechanisms of regulation of adenylyl cyclase activity. As a first step toward this goal, we provide the simple model shown in Fig. 8 for analysis of the data presented above.

It is not known if the individual protein fragments studied here exist as dimers, as suggested by their gel filtration profiles. If true, their affinity for homooligomerization is very high, since the gel filtration pattern is unaltered at very low protein concentrations. We also do not know if membrane-bound adenylyl cyclase or the soluble IC_1IIC_2 construct (7–9) is multimeric. However, the specific activity of IC_1 used in these studies is constant over a broad range of concentrations (200 pM to 100 nM) when assayed in the presence of saturating concentrations of IIC_2 and 50 μM FSK, and the covalent IC_1IIC_2 construct similarly has a constant specific activity over a similar range of concentrations when activated with either FSK or FSK plus $G_{s\alpha}$ (data not shown). If the catalytic entity is dependent on the formation of homooligomers, we suggest that such structures are dominant at the protein concentrations used in this study. This justifies consideration of the data presented here in the context of the model shown in Fig. 8.

The data of Fig. 6*A* provide an estimate of the lower limit for the affinity of IIC_2 for IC_1 in the absence of any activator: $K_1 \geq 10$ μM (Fig. 8). In the presence of FSK (Fig. 6*B*), both a higher specific activity and a lower EC_{50} for IIC_2 are apparent. The expression for fractional activation of IC_1 in the presence of FSK is as follows:

$$Act_{fr} = \frac{[IIc_2]}{\frac{K_1K_7}{[Fsk]} + [IIC_2]\left(\frac{K_7}{[Fsk]} + 1\right)}. \quad [1]$$

This neglects the activity due to unregulated complexes of IC_1 and IIC_2, which is insignificant. This derivation also relies on the assumption that K_5 is relatively large—i.e., that most of the IIC_2 in the assay is free and not bound to FSK. At FSK concentrations that are high relative to K_7, Eq. **1** approximates a normal binding isotherm, where the EC_{50} is equal to $K_1K_7/[FSK]$. From the EC_{50} of 190 nM (Fig. 6*B*), $K_7 = 1$ μM. A similar analysis of the FSK activation curve (Fig. 7*A*) also provides an estimate for K_7:

$$Act_{fr} = \frac{[Fsk]}{K_7\left(\frac{K_1}{[IIC_2]} + 1\right) + [Fsk]}. \quad [2]$$

Eq. **2** yields a value for K_7 of 3.7 μM, in reasonable agreement with the value obtained from Eq. **1** (1 μM).

An identical analysis can be done for stimulation of activity by $G_{s\alpha}$ by using Eq. **3** and the EC_{50} for activated $G_{s\alpha}$ from Fig. 7*A*.

$$Act_{fr} = \frac{[G_{s\alpha}^*]}{K_4\left(\frac{K_1}{[IIC_2]} + 1\right) + [G_{s\alpha}^*]}. \quad [3]$$

This analysis yields a value for K_4 of 0.4 μM. Similarly, the value of K_2 must be sufficiently high to permit this analysis to be meaningful. Attempts are underway to isolate the interactions of the individual fragments with these activators to determine the values of K_2 and K_5.

Qualitatively, it is clear that there is positive cooperativity among the four molecules involved in the formation of active complexes. The inclusion of FSK when titrating IIC_2 shifts the EC_{50} for IIC_2 from a value of $\geq$10 μM to 190 nM. Thus, this activator facilitates association of the two adenylyl cyclase fragments (K_6 is less than K_1). The inclusion of activated $G_{s\alpha}$ shifts this EC_{50} to an even lower value, indicating that $G_{s\alpha}$ and FSK both shift the equilibrium toward association of IC_1 and IIC_2. The binding of these two activators is also positively cooperative with respect to each other. Thus, the presence of activated $G_{s\alpha}$ during the FSK titration lowers the EC_{50} for FSK dramatically (Fig. 7*A*), just as the presence of FSK lowers the EC_{50} for $G_{s\alpha}$ (Fig. 7*B*). These results are similar to those obtained with the covalently linked IC_1IIC_2 construct (4) and the type II enzyme (10). We conclude that the presence of either activator in the active complex acts to enhance association of the other activator, as well as facilitating the interaction of the C_1 and C_2 domains of adenylyl cyclase.

This model can be confirmed and described explicitly for a single activator if the affinity of the activator for the individual protein domains (K_2 and K_5) can be determined. It is not known which of the two domains, if either, binds FSK or $G_{s\alpha}$ to any significant extent. However, the existence of this system will permit analysis of such interactions.

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