Interaction of G\textsubscript{sa} with the Cytosolic Domains of Mammalian Adenyyl Cyclase*

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Forskolin- and G\textsubscript{sa}-stimulated adenylyl cyclase activity is observed after mixture of two independently-synthesized 25-kDa cytosolic fragments derived from mammalian adenylyl cyclases (native $M_r$ 120,000). The C\textsubscript{sa} domain from type V adenylyl cyclase (VC\textsubscript{1}) and the C\textsubscript{bg} domain from type II adenylyl cyclase (IIC\textsubscript{2}) can both be expressed in large quantities and purified to homogeneity. When mixed, their maximally stimulated specific activity, 150 $\mu$mol/min/mg protein, substantially exceeds values observed previously with the intact enzyme. A soluble, high-affinity complex containing one molecule each of VC\textsubscript{1}, IIC\textsubscript{2}, and guanosine 5'-O-(3-thiotriphosphate) (GTP\textsubscript{S})-G\textsubscript{sa} is responsible for the observed enzymatic activity and can be isolated. In addition, GTP\textsubscript{S}-G\textsubscript{sa} interacts with homodimers of IIC\textsubscript{2} to form a heterodimeric complex (one molecule each of G\textsubscript{sa} and IIC\textsubscript{2}) but not detectably with homodimers of VC\textsubscript{1}. Nevertheless, G\textsubscript{sa} can be cross-linked to VC\textsubscript{1} in the activated heterotrimeric complex of VC\textsubscript{1}, IIC\textsubscript{2}, and G\textsubscript{sa}, indicating its proximity to both components of the enzyme that are required for efficient catalysis. These results and those in the accompanying report (Dessauer, C. W., Scully, T. T., and Gilman, A. G. (1997) J. Biol. Chem. 272, 22272–22277) suggest that activators of adenylyl cyclase facilitate formation of a single, high-activity catalytic site at the interface between C\textsubscript{1} and C\textsubscript{2}.

Eleven distinct isoforms of mammalian adenylyl cyclase have been identified to date, and the regulatory properties of several of these proteins have been characterized extensively (1, 2). Although there is remarkable variation in the responsiveness of these enzymes to inhibitory effects of the Gi\textsubscript{a} proteins and to such agents as G protein $\beta\gamma$ subunits and Ca\textsuperscript{2+}, the catalytic activity of all of the known isoforms is stimulated by the $\alpha$ subunit of Gi and, presumably nonphysiologically, by the diterpene forskolin. The adenylyl cyclases share a unique structure for an enzyme, resembling transporters such as the P-glycoproteins topographically. They are intrinsic membrane proteins by virtue of their two large hydrophobic domains, each of which is hypothesized to contain six membrane-spanning helices. The first of these hydrophobic regions follows a short amino-terminal sequence and precedes a roughly 40-kDa cytoplasmic domain (C\textsubscript{1}). The second hydrophobic region separates C\textsubscript{1} from a second cytosolic domain (C\textsubscript{2}) of comparable size. Each of the two cytosolic domains includes a sequence of 200–250 amino acid residues that is typically 50% similar to its counterpart, 50–90% similar to the corresponding domains of other adenylyl cyclase isoforms, and 20–25% similar to the catalytic domains of membrane-bound and cytosolic guanylyl cyclases.

Detailed biochemical characterization of adenylyl cyclase is impaired by the insolubility, instability, and sparsity of the native enzyme, as well as our incapacity to express necessary amounts of the protein in heterologous systems. To overcome these hurdles we have synthesized (in Escherichia coli) portions of the two cytosolic domains of adenylyl cyclase in the absence of the remainder of the protein, first as a 55-kDa chimeric fusion protein containing the C\textsubscript{1}a domain of type I adenylyl cyclase linked to the C\textsubscript{2} domain of the type II enzyme (3, 4). The specific activity of this engineered enzyme is remarkably high, it is soluble in the absence of detergent, and it is adequately stable. Importantly, it is activated synergistically by Gi\textsubscript{a} and forskolin and inhibited by so-called P-site inhibitors and the G protein $\beta\gamma$ subunit complex, providing ample justification to pursue investigation of this and similar artificial entities. To overcome the remaining hurdle of a relatively low level of accumulation of the chimera in bacterial cytosol, we and others prepared the two cytosolic domains of adenylyl cyclase as distinct entities and found that enzymatic activity with similar regulatory properties can be reconstituted by simple mixture of the two roughly 25-kDa proteins (5, 6). Large amounts (50–100 mg or more) of the C\textsubscript{2} domain of type II adenylyl cyclase can be prepared readily, but similar results are difficult to achieve with the C\textsubscript{1}a domain of the type I enzyme. We have now extended this approach by utilizing a fragment of the C\textsubscript{1} domain of type V adenylyl cyclase, which can be expressed in reasonable (but not exuberant) quantities, and we have characterized the interactions of the two cyclase fragments with each other and with Gi\textsubscript{a}.

**MATERIALS AND METHODS**

Plasmid Construction—DNA containing nucleotides 961–2010 (amino acid residues 321–670) of canine type V adenylyl cyclase (7) was generated with the polymerase chain reaction using oligonucleotides A: 5'-CCATGGCTGAGGTCTCCCAG-3' and B: 5'-CCGGTGAGGCTCCCTGAGG-3', as primers. Note that oligonucleotides A is shorter by 9 than the unique Ncol site in the cDNA for canine type V adenylyl cyclase and includes the codon for Met\textsuperscript{311}. Further truncations of this fragment were generated by taking advantage of unique restriction sites for AvrII (at nucleotide 1815), BstBI (at nucleotide 1858), and AvaI (at nucleotide 1900) to create VC\textsubscript{1} (364–606), VC\textsubscript{2} (364–620), and VC\textsubscript{1} (364–635), respectively. VC\textsubscript{1} (364–567), VC\textsubscript{2} (364–591), and VC\textsubscript{1} (364–591)Flag were generated with the polymerase chain reaction.
using the following pairs of oligonucleotide primers: A and 5′-TTCCTC- CGATACCAAGCTTGCAGCAGACA-3′, A and 5′-GATTCGTTGCTGCTG- CGTCTTGTGATCCGGCAGGTTG-3′. The last construct contains a carboxy-terminal Flag epitope (Kodak), DYKDDDDK, as well as a cleavage site for enterokinase. All of the VC1 constructs were ligated into pQE60-H6 (8) with NcoI and HindIII. Thus, each of the encoded proteins contains a hexa-histidine tag at its amino terminus, followed by the sequence corresponding to Met<sup>644</sup> of canine type V adenylyl cyclase, and terminates at the stop codon contained in pQE60-H6. We will subsequently designate these proteins by reference to their carboxy-terminal residue.

**Expression and Purification of Proteins—** E. coli strain BL21 was co-transformed with pREP4 and pQE60-H6-VC1 (various) and grown overnight at 30 °C in 200 ml of LB medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml). This culture was used to inoculate 10 liters of T7 medium (8), which was incubated at 30 °C until OD<sub>600</sub> reached 1.3. Synthesis of VC1 was then induced with 30 μ M isoprpyl β-thiogalactoside, and incubation was continued for 4 h at room temperature. Cells were harvested by centrifugation, frozen in liquid N<sub>2</sub>, and stored at −70 °C. Frozen cells were pulverized and suspended in 750 ml of buffer A (50 mM Tris-HCl, pH 8, 120 mM NaCl, 1 mM β-mercaptoethanol, and a mixture of protease inhibitors (4)) prior to incubation with 0.25 mg/ml lysozyme in buffer A for 30 min at 4 °C and subsequently 8 mg/ml DNase and 1 mg/ml MgCl<sub>2</sub> in buffer A for an additional 30 min. Cellular debris was removed by centrifugation at 100,000 × g for 40 min at 4 °C.

All purification steps were performed at 4 °C. The supernatant (750 ml) was applied to a 5-ml metal chelate column (Talon, CLONTECH) equilibrated in buffer A, and the column was washed with 20 volumes of buffer A containing 500 mM NaCl and 10 volumes of buffer B (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM β-mercaptoethanol, and protease inhibitors). H6-VC1 was eluted from the column in 2-ml fractions with buffer B containing 100 mM imidazole. Peak fractions were pooled, concentrated to 3 mg/ml unless noted otherwise.

**Results**

**Expression and Purification of Proteins Containing the VC<sub>1a</sub> Domain—** The C<sub>1</sub> domain of type V adenylyl cyclase was truncated at its carboxy terminus in attempts to obtain a pure, stable protein with high specific enzymatic activity when reconstituted with IIC<sub>2</sub>. Bacterial lysates containing constructs as large as VC<sub>1</sub>(670), for example, supported high forskolin-stimulated adenylyl cyclase activity (>50 nmol/min/mg in the lysate), but immunoblot analysis of SDS-PAGE gels indicated contamination with several proteolytic products (data not shown). Extracts containing truncations VC<sub>1</sub>(635), VC<sub>1</sub>(621), or VC<sub>1</sub>(606) displayed progressively less such contamination, but susceptibility to degradation was again high with VC<sub>1</sub>(676); the latter protein is analogous to the C<sub>1</sub> domain of type I adenylyl cyclase described previously (5). However, extracts of bacteria expressing VC<sub>1</sub>(591) or its carboxy-terminally Flag-tagged counterpart, VC<sub>1</sub>(591)Flag, contained only a single significant proteolytic product (molecular mass ~25 kDa; mass of VC<sub>1</sub>(591) ~28 kDa), and its accumulation was minimized by shortening the length of time for expression to 4 h. The specific activities of such lysates for reconstitution of adenylyl cyclase activity with IIC<sub>2</sub> were typically 50–100 nmol/min/mg. These values are roughly 1% of those observed with purified native adenylyl cyclases (12–14).

**Metal chelate column chromatography (Talon resin) of extracts containing VC<sub>1</sub>(591)Flag resulted in >100-fold or more purification of the protein with loss of 60% of the total activity (Table I). The remaining activity was not found in the flowthrough or column washes. Subsequent Mono Q column chromatography resulted in 2–3-fold purification and removal of the remaining major contaminants. This final product appeared essentially homogeneous when analyzed electrophoretically (Fig. 1) and had a reconstitutive specific activity of 30 μmol/min/mg when assayed with maximally effective concentra-

**TABLE I**

| Purification step | Volume | Protein | Specific activity | Total activity | Recovery | Purification |
|------------------|--------|---------|-------------------|----------------|----------|-------------|
| lysates           | 1000   | 7100    | 0.05              | 355            | 100      |             |
| Talon pool        | 10     | 12      | 12                | 144            | 41       | 240         |
| Mono Q pool       | 4.5    | 4       | 30                | 120            | 34       | 600         |

**Adenylyl Cyclase Assays—** Adenylyl cyclase activity was measured as described by Snigiel (12). All assays were performed for 10 min at 30 °C in a volume of 100 μl. In reconstitutive assays the final concentration of IIC<sub>2</sub> was at least 1–5 μg to maintain linearity with variable concentrations of VC<sub>1</sub>. The final concentration of ATP was 1 mM unless stated otherwise, and an ATP regenerating system was used only when crude preparations of enzyme were assayed.

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trations of purified IIC2 and 50 μM forskolin as the sole activator. This purified protein will subsequently be designated VC1.

Adenylyl Cyclase Activity Reconstituted from VC1 and IIC2—
The adenylyl cyclase activity observed in mixtures of VC1 and IIC2 resembles that seen previously with mixtures of IC1 and IIC2 (5, 6); many of the regulatory features that characterize native membrane-bound adenylyl cyclases are retained. Activities shown in Fig. 2, A, B, and D, were measured with low concentrations of IIC2 and either near saturating (2 μM) or 400 nM GTP and IC2 (1 nM) were activated with increasing concentrations of either GDP-lysate; lane B, 1 μg of Talon column eluate; lane C, 1 μg of Mono Q pool.

**Fig. 1.** Purification of H6-VC1(364–591)Flag. H6-VC1(364–591)Flag was expressed in E. coli as described under “Materials and Methods.” Aliquots from various stages in the purification were resolved by SDSPAGE on a 15% polyacrylamide gel, and proteins were visualized by staining with Coomassie Blue. Lane A, 5 μg of E. coli lysate; lane B, 1 μg of Talon column eluate; lane C, 1 μg of Mono Q pool.

The adenylyl cyclase activity observed in mixtures of VC1 and IIC2 resembles that seen previously with mixtures of IC1 and IIC2, GTP-Gs, could not activate a mixture of VC1 and IIC2 maximally. The effects of forskolin and GTP-Gs-Gsa are synergistic; the presence of one activator increasing the apparent affinity (EC50) of the enzyme for the other by 40-fold or more. In the absence of an activator, the rate of cyclic AMP synthesis is low and the two domains of the enzyme have an apparent affinity for each other of greater than 5 μM (Fig. 2D, inset). This value is lowered in the presence of activators; the apparent affinity of VC1, and IIC2 is 0.66 μM with GTP-Gs-Gsa, 1.2 μM with forskolin, and 150 nM when both activators are present.

Activation of adenylyl cyclase activity by Gsa is dependent on the nature of the bound nucleotide, but not to the extent usually assumed. GDP-Gsa is only 10-fold less potent than GTP-Gs-Gsa and is equally efficacious (in the presence or absence of forskolin (Fig. 2B)). Identical results were obtained with limiting concentrations of either VC1 or IIC2 (data not shown).

**Gel Filtration and Sedimentation Equilibrium Centrifugation**—We have examined the interactions of VC1 and IIC2 with each other and with Gs by gel filtration, sedimentation equilibrium centrifugation, and (see below) chemical cross-linking. Gel filtration of VC1 or IIC2 on tandem columns of Superdex 75 and 200 suggests that each of these proteins exists in solution as roughly 50-kDa homodimers (Fig. 3, A and B); each individual protein has a molecular mass of approximately 28,000. Similarly, sedimentation equilibrium analysis of IIC2 revealed a molecular weight of 46,000 (Fig. 6D). Gel filtration of a mixture of VC1 and IIC2 in the presence (Fig. 3B) or absence (not shown) of forskolin revealed a single peak of protein with an apparent molecular weight of 50,000, while sedimentation...
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**Fig. 3.** Gel filtration of VC1 and IIC2. A. VC1 (500 μg; approximately 100 μM) was gel filtered on a Superdex 200 (HR 10/30) column. B. VC1 (100 μM) was gel filtered on a Superdex 200 (HR 10/30) column by itself, with IIC2 (100 μM), or in the presence of IIC2 and GTPγS-Gsα (100 μM). IIC2 was also gel filtered by itself. Forskolin (50 μM) was present in the samples and the elution buffer. The positions of elution of molecular weight markers are shown.

Equilibrium analysis of the mixture indicates a molecular weight of 52,000 (Fig. 6B). Because of the similarity of the molecular masses of VC1 and IIC2, we do not know if these values represent homodimers, heterodimers, or a mixture of the two.

Mixture of VC1, IIC2, and GTPγS-Gsα (in the presence of forskolin) results in formation of a high-affinity complex. This is detected by gel filtration with the appearance of a new peak of optical density, migrating with an apparent molecular weight of 104–107 kDa complex when either Gsα, VC1, or both, since the cyclase domains have similar molecular weights. This was also complicated by the inability of available IIC2 antibodies to detect IIC2-Gsα complexes by immunoblotting. Cross-linking studies performed at higher protein concentrations (2 μM VC1, IIC2, and Gsα) and analyzed with an anti-Gsα antibody reveal a IIC2-Gsα cross-linked species in the absence of VC1 (Fig. 7E, lane 4). Moreover, a large species (Mᵣ ~ 95,000) appears in the presence of all three proteins and 100 μM forskolin; we presume this to be the VC1-IIC2-Gsα heterotrimer (Fig. 7E, lane 7).

**Fig. 4.** Purification of VC1, associated with IIC2 and GTPγS-Gsα. VC1 (100 μM), IIC2 (300 μM), and GTPγS-Gsα (100 μM) were incubated for 30 min on ice in gel filtration buffer containing 50 μM forskolin. The mixture was applied to a Superdex 75 (HR 10/30) gel filtration column in tandem with a Superdex 200 (HR 10/30) column. The column was eluted with gel filtration buffer containing 50 μM forskolin. Fractions 5–17 (5 μl of each 400-μl fraction) were resolved by SDS-PAGE on a 15% polyacrylamide gel and stained with Coomasie Blue. The positions of elution of two gel filtration standards are indicated.

Chemical Cross-linking—Although direct interactions between VC1 and Gsα were not detected by gel filtration or sedimentation equilibrium, chemical cross-linking studies do suggest that the molecules are at most 11 Å away from each other when tightly complexed with IIC2. Fig. 7A illustrates the forskolin (and IIC2, not shown)-dependent appearance of a 70-kDa species representing covalent coupling of Gsα and VC1 by the 11-Å cross-linker, disuccinimidyl suberate. Analysis of the same fractions with an anti-Gsα antibody also reveals a 70-kDa species that appears in a forskolin-dependent manner. It is unclear in this panel if this species represents Gsα-VC1, Gsα-IIC2, or both, since the cyclase domains have similar molecular weights. This was also complicated by the inability of available IIC2 antibodies to detect IIC2-Gsα complexes by immunoblotting. Cross-linking studies performed at higher protein concentrations (2 μM VC1, IIC2, and Gsα) and analyzed with an anti-Gsα antibody reveal a IIC2-Gsα cross-linked species in the absence of VC1 (Fig. 7E, lane 4). Moreover, a large species (Mᵣ ~ 95,000) appears in the presence of all three proteins and 100 μM forskolin; we presume this to be the VC1-IIC2-Gsα heterotrimer (Fig. 7E, lane 7). Observation of both the cross-linked heterotrimer and the IIC2-Gsα heterodimer is dependent on the presence of GTPγS-activated Gsα (Fig. 7E, lane 3 versus 4 and lane 6 versus 7).

Chemical cross-linking also revealed forskolin-dependent formation of heterodimers between VC1 and IIC2 (Fig. 7, C and D). Although each domain exists as a homodimer under non-activating conditions, formation of heterodimers occurs in a forskolin (and Gsα)-dependent manner.

**DISCUSSION**

We have expressed and purified a fragment of the C1 domain of type V adenylyl cyclase, consisting of amino acid residues
364–591 and including hexa-histidine and Flag tags at the amino and carboxyl termini, respectively. Although this protein itself has no adenylyl cyclase activity, catalysis of cyclic AMP synthesis is restored by simple mixture of VC1 with an appropriate fragment from the second cytosolic domain of the enzyme, such as IIC2. This interaction is similar to that described previously between IC1 and IIC2 (5, 6); the major advantages are the yield of VC1, which exceeds that of IC1 by 20-fold, and the apparent homogeneity of the product. The adenylyl cyclase activity of the VC1–IIC2 mixture is stimulated markedly by either Gs or forskolin, and these two regulators activate the enzyme synergistically when present simultaneously. These are characteristics of native type II and type V adenylyl cyclases.
A notable difference between this reconstituted adenylyl cyclase and the native enzyme is the maximal stimulated activity, which typically exceeds 100 μmol/min/mg in the case of the mixture of VC₁ and IIC₂. Values of 10 μmol/min/mg typify purified preparations of native enzymes (12–14). Although the source of this discrepancy is not known, we suspect that the values observed with the VC₁/IIC₂ mixture may indeed approximate a true V_{max} for mammalian adenylyl cyclase. Several factors may cause underestimation of maximal activity when dealing with a native adenylyl cyclase. Overexpression of these enzymes in Sf9 cells is plagued by production of nonfunctional enzymes; detergents are necessary to maintain solubility of the native proteins but may alter estimates of specific activity; lengthy purification schemes may cause denaturation of these labile entities. Alternatively, it is possible that inhibitory domains have been removed from the soluble constructs described above or that the membrane spanning domains of the enzymes do not permit optimal orientation of the interacting cytosolic segments. Another notable difference between native adenylyl cyclases and the engineered soluble enzymes (whether or not the cytosolic domains are linked covalently) is the relative low (reduced 20–50-fold) apparent affinities of the soluble enzymes for Gₐₐₙ whereas the stimulatory properties of the constructs utilized may contribute to the binding site for Gₐₐₙ. Nevertheless, the essential features of activation of adenylyl cyclase by the G protein α subunit are retained.

We demonstrate herein that the C₁ and C₂ domains of adenylyl cyclase interact to form a catalytically active adenylyl cyclase and that the apparent affinity of C₁ for C₂ is enhanced in the presence of Gₐₐₙ and/or forskolin. Gel filtration, equilibrium sedimentation, and cross-linking analyses all demonstrate that Gₐₐₙ interacts with the C₂ domain of adenylyl cyclase in a GTP-enhanced manner and that this interaction is further stabilized by C₁. Direct interactions between VC₁ and Gₐₐₙ were not detected by gel filtration or sedimentation equilibrium. However, these two proteins could be cross-linked by disuccinimidyldiarsenite in a C₂-dependent manner. We further demonstrate that Gₐₐₙ, C₁, and C₂ form a relatively high-affinity complex with a 1:1:1 stoichiometry. Although this is not surprising, the similarities in the primary sequence of the C₁ and C₂ domains of adenylyl cyclase raised the possibility of binding sites for two molecules of Gₐₐₙ. Several adenylyl cyclase isoforms are inhibited by Gₐₐₙ. Although high concentrations of Gₐₐₙ can apparently compete for the Gₐₐₙ-binding site, inhibition of adenylyl cyclase activity by Gₐₐₙ is not dependent on Gₐₐₙ and is not competitive with the stimulatory α subunit (15). We presume that there is a distinct binding site for Gₐₐₙ on certain adenylyl cyclases; Gₐₐₙ may well be found to interact predominantly with C₁.

Homodimerization of C₁ and C₂ is difficult to interpret. The phenomenon may clearly be an artifact of protein engineering and have no significance with regard to the membrane-bound native enzyme. However, we have previously detected oligomers of near native adenylyl cyclases in detergent solution, suggesting the possibility of relevance of homodimerization of the cytosolic domains (16).

The relative capacities of GTP- and GDP-bound Gₐₐₙ proteins to interact with their effectors has been long debated and may be dependent on the system in question. However, no such interaction has probably been examined previously in a system containing such highly purified enzymes. The Gₐₐₙ, VC₁, and IIC₂ proteins utilized herein are all expressed abundantly in bacteria, greatly facilitating their purification to a high degree. Significant levels of contaminating nucleotide kinases are extremely unlikely, obviating such concerns as conversion of GDP to GTP in the presence of ATP. Nevertheless, the apparent affinity of GDP-Gₐₐₙ for adenylyl cyclase is only about 10-fold less than that of GTPγS-Gₐₐₙ. The GTPase activity of a G pro-
tein α subunit thus facilitates deactivation of the system by reducing the affinity of Gα for its effector. However, the affinity of the G protein βγ subunit complex for Gα is more highly dependent on the nature of the bound nucleotide. GTP hydrolysis thus greatly favors association of α with βγ, and it is this interaction that prevents access of either α or βγ to effectors.

More critical and microscopic analyses of the interactions of the two cytosolic domains of adenylyl cyclase with each other and with their regulators are necessary. Although this need has been recognized for a long time, progress has been greatly limited by the availability and purity of reagents. Milligram quantities of G protein-regulated adenylyl cyclase domains may now be prepared readily, as can a high-affinity complex of Gsα with the crucial components of the cyclase. We hope that all relevant tools can now be utilized to understand the complex regulatory features of these interesting enzymes.

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