Isolation and Characterization of Constitutively Active Mutants of Mammalian Adenylyl Cyclase*

A genetic screen in Saccharomyces cerevisiae identified mutations in mammalian adenylyl cyclase that activate the enzyme in the absence of Gsα. Thirteen of these mutant proteins were characterized biochemically in an assay system that depends on a mixture of the two cytosolic domains (C1 and C2) of mammalian adenylyl cyclases. Three mutations, I1010M, K1014N, and P1015Q located in the β4–β5 loop of the C2 domain of type II adenylyl cyclase, increase enzymatic activity in the absence of activators. The K1014N mutation displays both increased maximal activity and apparent affinity for the C1 domain of type V adenylyl cyclase in the absence of activators of the enzyme. The increased affinity of the mutant C2 domain of adenylyl cyclase for the wild type C1 domain was exploited to isolate a complex containing VC1, IIC2, and Gαs-guanosine 5′-3′-(thio)triphosphate (GTPγS) in the absence of forskolin and a complex of VC1, IIC2, forskolin, and P-site inhibitor in the absence of Gαs-GTPγS. The isolation of these complexes should facilitate solution of crystal structures of low activity states of adenylyl cyclase and thus determination of the mechanism of activation of the enzyme by forskolin and Gαs.

Mammalian adenylyl cyclases are membrane-bound enzymes that catalyze the synthesis of the intracellular second messenger cyclic AMP from ATP. Nine isoforms of the enzyme have been detected, and they display characteristic regulatory properties and patterns of cellular distribution (1, 2). Cellular rates of cyclic AMP synthesis are controlled by a variety of extracellular ligands that interact with heptahelical receptors in the plasma membrane. Relevant receptors can either stimulate cyclic AMP synthesis, usually via the intermediary of a G protein1 (Gα) that activates adenylyl cyclase, or inhibit cyclic AMP synthesis, often by interaction with an inhibitory G protein, Gαi. Mammalian adenylyl cyclases can also be activated by the diterpene forskolin (3) and inhibited by certain adenosine analogs and adenine nucleotides called P-site inhibitors (4). Certain adenylyl cyclases are also regulated by Ca2+,-calmodulin, and phosphorylation (5).

Mammalian adenylyl cyclases are integral membrane proteins that appear to contain two sets of six membrane-spanning helices that are separated by a large (~40 kDa) cytoplasmic loop and followed by a similarly sized carboxyl-terminal cytosolic domain (6). The cytosolic domains, termed C1 and C2, have been extensively studied; they are responsible for catalytic activity and most of the regulatory properties of the enzymes (7). The first 200–250 amino acids of each cytosolic domain, designated C1a and C2a, are the most highly conserved regions among adenylyl cyclases. Strikingly, the C1a and C2a domains are approximately 50% similar and 25% identical to each other within a single isoform of adenylyl cyclase, and they are 20–25% similar to the catalytic domains of membrane-bound and cytosolic guanylyl cyclases.

The C1a and C2a domains of adenylyl cyclases can be expressed separately and purified as recombinant proteins. When mixed together, they display the characteristics of membrane-bound adenylyl cyclase with respect to regulation by Gαs, Gai, forskolin, and P-site inhibitors (8–10). The crystal structures of the soluble catalytic core of adenylyl cyclase bound to Gαs and forskolin (11) and of this complex bound with the competitive substrates β1,2,3,3′-ddATP and ATPoS (12) have provided detailed insights into mechanisms of catalysis of cyclic AMP synthesis and regulation of the activity of the enzyme.

Less information is available about the biochemical and structural properties of low activity states of adenylyl cyclase. Crystallization of the C1 and C2 domains in the absence of Gαs and/or forskolin would permit structural comparison of high and low activity states of the enzyme. We have approached this goal with a combination of genetic and biochemical techniques. Full-length mammalian adenylyl cyclase was introduced into a mutant strain of Saccharomyces cerevisiae that does not express the endogenous enzyme. Co-expression of the mammalian protein together with Gαs relieves dependence of cyclic AMP for growth at non-permissive temperatures. We have utilized a genetic screen to isolate constitutively active mutants of adenylyl cyclase within this strain. We describe the isolation of several partially active mutants, as well as the biochemical consequences of these mutations in the context of the soluble, recombinant adenylyl cyclase system.

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† The abbreviations used are: G protein, heterotrimeric guanine nucleotide-binding protein; Gα, the α subunit of the G protein that stimulates adenylyl cyclase; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3′-(thio)triphosphate; ATPoS, adenosine 5′-(α-thio)-triphosphate; ACII, type II adenylyl cyclase; kb, kilobase pair; PCR, polymerase chain reaction.
Experimental Procedures

Genetic Screen—To achieve high level constitutive expression of type II adenyl cyclase (ACII) in yeast, a 0.94-kb fragment containing the S. cerevisiae PGK1 promoter and a 3.3-kb fragment encoding rat ACII (PCR-amplified from a template plasmid provided by Randall Reed, The Johns Hopkins University) were subcloned into pRS425 (13). The resulting expression plasmid, Cp1512 (genotype LEU2 PGK1-ACII 2α-ori REP2 1) was used as the template for error-prone PCR amplification of two separate fragments as follows: nucleotides 5514–6384, which encode the Cα domain, and nucleotides 7033–8264, which encode the Cα domain (primer sequence available upon request). The mutated fragments encoding the Cα domain were then used to transform yeast strain TC41F2-1 (genotype MATA cyr1::ura3 can1 can2 cam1 leu2-3 leup-2 12 his3 523 his4 ura3; provided by Warren Heideman, University of Wisconsin) along with a “gapped” vector prepared as follows. Cp1512 was digested with ApoI (which cuts at nucleotide 5682 of ACII) and DraIII (which cuts at nucleotide 6254), and the 10.1-kb fragment, which lacks the Cα coding sequence, was isolated. Similarly, TC41F2-1 was co-transformed with mutagenized fragments encompassing the Cα domain and a gapped vector prepared as follows. Cp1512 was digested with PvuII (which cuts at nucleotide 7111) and BamHI (which cuts at nucleotide 8168), and the 9.7-kb fragment, which lacks the Cα coding region, was isolated. For both transformations, cells were plated on SC-Leu (14) without supplemental cyclic AMP and incubated at room temperature. Colonies from these plates were replica-plated to SC-Leu plates and incubated at 34 °C. Colonies that grew at 34 °C were expected to harbor stably replicating episomes that result from in situ recombination between the gapped vector and a PCR-amplified fragment and that, as a result of this recombination, express an adenyl cyclase that has constitutive (i.e. Gα-independent) activity.

In all cases, the plasmid dependence of growth under selective conditions (on SC-Leu at 34 °C) was further tested by allowing spontaneous loss of plasmid under non-selective conditions (on YEPD with supplemental cyclic AMP at 30 °C), followed by re-testing under selective conditions. Plasmids were rescued from those colonies whose growth under selective conditions was dependent on plasmid; the plasmids were then reintroduced into naïve host strain TC41F2-1, and the transformants were tested for growth on SC-Leu at 34 and at 37 °C. All plasmids reported here confer plasmid-dependent growth at 34 °C when introduced into TC41F2-1.

Mutagenesis of Adenyl Cyclase Domains—The mutants that appeared to confer the highest level of constitutive activity in yeast were isolated using the soluble mammalian adenyl cyclase system. The vectors pQE60-H6-VC1(591)FLAG and pQE60-IIcH6 were co-transformed with mutagenized fragments encompassing the Cα domain of rat type II adenyl cyclase corresponding to the mutations in the rat type II adenyl cyclase using the vector pQE60-IIcH6. The vector pQE60-ArgC-IIC2 (11) served as templates for site-directed mutagenesis (QuikChangeTM, Stratagene). The mutations in the Cα domain were made in the conserved residues in the Cα domain from canine type V adenyl cyclase corresponding to the mutations in the rat type II cyclase domain obtained from the yeast screen. The mutations in the Cα domain from the yeast screen were made in the Cα domain of rat type II adenyl cyclase using the vector pQE60-IIcH6. The vector pQE60-ArgC-IIC2 was used to generate a non-tagged IIC2-K1014N. Sequences of synthetic mutagenic sense and antisense primers are available upon request.

Expression and Purification of Proteins—Wild type and mutant H6-VC1(591)FLAG and IIcH6 were expressed in Escherichia coli and purified as described previously (8, 10). Non-tagged ArgC-IIcH6 wild type and mutant K1014N were expressed and purified as described (11). Bovine Gα (short form) was purified and activated by GTP[S] as described (15).

Adenyl Cyclase Assays—Adenyl cyclase activity was measured as described by Smigel (16). All assays were performed in a volume of 100 μl for 10 min at 30 °C. The final concentration of MgCl2 was 10 mM. The concentration of ATP was 1 mM unless otherwise stated. Kinetic constants were determined by varying MgATP from 20 μM to 5 mM with a fixed excess of Mg2+2. Unless otherwise stated, assays contained a limiting concentration of VC1 and an excess of IIcH6 for both wild type and mutant assays. All specific activities reported are with respect to the concentration of the limiting domain. Each experiment was repeated two to three times.

Gel Filtration—Proteins (Gα and adenyl cyclase domains) were separated by fast protein liquid chromatography with tandemly arranged Superdex 75 and 200 (HR10/30) columns. Proteins were eluted in a buffer containing 20 mM NaHepes, pH 8.0, 2 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, and 50 mM NaCl. Flow rates were 0.2 ml/min, and 300-μl fractions were collected. An aliquot of each fraction (15 μl) was analyzed by SDS-PAGE on a 15% acrylamide gel and stained with Coomassie Blue.

Talon Column Binding Studies—H6-VC1(591)FLAG (15 μM) and ArgC-IIcH6 wild type or K1014N (75 μM) were incubated on ice for 15 min in 150 μl of buffer A (20 mM NaHepes, pH 8.0, 2 mM MgCl2, 1 mM EDTA, 50 mM NaCl) in the presence or absence of 100 μM forskolin, 100 μM 2',3'-AMP, and 100 μM PP. The mixture was applied to a 25-μl metal chelate column (TalonTM, CLONTECH) equilibrated with buffer A. The column was washed twice with 100 μl of buffer B (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM MgCl2, 100 μM forskolin, 100 μM 2',3'-AMP, and 100 μM PP). The column was then eluted with 100 μl of buffer B containing 100 μM imidazole. An aliquot (15 μl) of the eluate was resolved by SDS-PAGE on a 15% acrylamide gel and stained with Coomassie Blue.

Results

Genetic Screen—We isolated 24 plasmids that conferred cyclic AMP-independent growth at 34 °C when introduced into strain TC41F2-1; this phenotype is presumed to reflect the expression of Gα-independent adenyl cyclase activity. The mutagenized regions of these plasmids were sequenced, and their mutations are listed in Table I. Cp4465, which encodes...
The mutations were made in the C1a domain of canine type V adenylyl cyclase system to facilitate biochemical characterization. A yeast screen identified variant colonies that were grew to a given size 4 times faster than a colony with wild type growth of 0.5. TC41F2–1 transformed with CP1512, which encodes wild type ACII, was used to generate the two plasmids encoding ACII 2-K1014N and ACII2-K1014N. It is possible that plasmid-dependent variations in copy number could account for the apparent affinity or V.max values for the mutant proteins in the presence of forskolin, however, the low level of expression of this protein precluded detailed characterization. The mutations I1010M, K1014N, and P1015Q, all located in the β4−β5 loop of IIC2, caused an increase in V.max in both the basal and the forskolin-stimulated conditions.

### Table II

| Plasmid | Amino acid substitutions | Corresponding residue in Vc1 | Growth at 34°C | Growth at 37°C |
|---------|--------------------------|-------------------------------|---------------|---------------|
| Cp4416  | Y496C                    | Asn-592                       | 2             | 0.5           |
| Cp4466  | N315S                    | Asn-417                       | 2             | 1             |
| Cp4467  | F295Y                    | Phe-400                       | 2             | 0.25          |
| Cp4468  | T494P                    | Gly-589                       | 2             | 1             |
| Cp4474  | P1015Q                   | —                             | 2             | 0.5           |
| Cp4476  | L877P                    | —                             | 2             | 0             |
| Cp4521  | V377I                    | —                             | 2             | 1.5           |
| Cp4525  | K334R                    | Lys-436                       | 2             | 1.5           |
| Cp4526  | E309K                    | Glu-411                       | 2             | 1             |
| Cp4527  | V429A                    | Ala-531                       | 2             | 1             |
| Cp4533  | I1010M                   | —                             | 2             | 1.5           |
| Cp4534  | K896E                    | —                             | 2             | 1.5           |
| Cp4563  | Y402C                    | Phe-504                       | 2             | 1.5           |
| Cp4702  | C305R                    | Cys-407                       | 2             | 1             |

* Growth was determined by visual inspection of streaked colonies.
* These mutants are in ACII2.

ACII I259V/Y402C, was used to generate the two plasmids encoding ACII 259V and ACII Y402C; similarly, Cp4522 was used to generate two plasmids encoding ACII M253V and ACII C305R. In this way it was found that ACII C305R and ACII Y402C are constitutively active mutants. In several cases mutations that were observed in genetically selected plasmids were engineered by site-directed mutagenesis into wild type ACII to test their effects independently of coincident mutations. Table II lists the mutations and the growth characteristics of all constitutively active ACII single mutants.

It is possible that plasmid-dependent variations in copy number could account for the apparent affinity or V.max values for the mutant proteins in the presence of forskolin. There was no difference in either the EC50 or the V.max in the presence of both forskolin and Gs-GTPyS, demonstrating that the mutation does not create a hyperactive enzyme. Similar results were observed using limiting concentrations of IIC2 and varying concentrations of VC1 (data not shown). The apparent affinity of Gs-GTPyS for adenylyl cyclase is shown in Fig. 1D. The EC50 for Gs-GTPyS was 0.05 μM for K1014N compared with 0.4 μM for the wild type IIC2. These values were 6 and 25 μM, respectively, in the presence of forskolin.

**Gel Filtration of the VC1, IIC2-K1014N and Gs-GTPyS Complex—**Puriﬁed H6-VC1 and wild type or IIC2-K1014N were combined with Gs-GTPyS and gel-ﬁltered using tandem Superdex 75 and 200 columns. Fractions were analyzed by SDS-PAGE (Fig. 3). In the absence of forskolin, there is no evidence for formation of a complex between VC1, wild type IIC2, and Gs (Fig. 3B). The largest apparent species (78 kDa) is likely a heterodimer consisting of IIC2 and Gsα; similar results have been reported previously (10). In contrast, protein in the mixture of VC1, IIC2-K1014N, and Gsα eluted as two major peaks with the largest species representing a 100-kDa complex. Analysis by SDS-PAGE indicates a complex of VC1, IIC2-K1014N, and Gs-GTPyS.

**Isolation of a Complex of VC1, IIC2, Forskolin2′,3′-AMP-PPi—**Puriﬁed H6-VC1 and non-tagged wild type or IIC2-K1014N were combined and applied to a metal chelate chro-
The apparent affinities and maximal activities were determined for each mutant protein by assaying a limiting concentration of VC1 (30 nM for basal; 2 nM with forskolin) with increasing concentrations of IIC2 in the absence and presence of 100 µM forskolin. The values in parentheses are the values for the wild type protein in each assay.

| Mutant | Basal EC<sub>50</sub> µM | Ratio WT/mutant | Basal V<sub>max</sub> nmol/min/mg | % WT | Forskolin EC<sub>50</sub> µM | Ratio WT/mutant | Forskolin V<sub>max</sub> nmol/min/mg | % WT |
|--------|----------------|----------------|----------------|------|----------------|----------------|----------------|------|
| Y383H<sup>a</sup> | 5 (4.8) | 0.96 | 460 (550) | 84 | 1.7 (1.3) | 0.76 | 85 (130) | 65 |
| F400Y<sup>a</sup> | 18 (8) | 0.44 | 380 (840) | 45 | 3 (1.4) | 0.47 | 89 (130) | 68 |
| N417S<sup>a</sup> | 7 (8) | 1.10 | 260 (410) | 63 | 1.5 (1.4) | 0.93 | 56 (100) | 56 |
| K436E<sup>a</sup> | 12 (5.7) | 0.48 | 310 (570) | 54 | 1.9 (1.4) | 0.74 | 37 (88) | 42 |
| V479I | 8 (7) | 0.88 | 980 (660) | 150 | 0.55 (1.7) | 3.1 | 81 (130) | 62 |
| L877P<sup>a</sup> | 3 (2) | 0.67 | 250 (170) | 150 | 1.1 (1.5) | 1.4 | 48 (44) | 110 |
| K896E<sup>a</sup> | 3 (2.4) | 0.80 | 150 (150) | 100 | 1.3 (1.2) | 0.92 | 34 (55) | 62 |
| C407R | 3 (4) | 1.30 | 680 (650) | 105 | 0.89 (0.83) | 0.93 | 120 (93) | 130 |
| I1010M<sup>b</sup> | 4.5 (2.7) | 0.60 | 410 (190) | 220 | 2.2 (1.3) | 0.59 | 110 (65) | 170 |
| K1014N<sup>b</sup> | 1.6 (2.4) | 1.50 | 1200 (170) | 700 | 0.33 (1.4) | 4.20 | 78 (60) | 130 |
| P1015Q<sup>b</sup> | 4.2 (2.2) | 0.52 | 510 (160) | 320 | 1.4 (1.2) | 0.86 | 100 (56) | 180 |

<sup>a</sup> Protein purified by metal affinity column followed by anion exchange chromatography.

<sup>b</sup> Protein purified by metal affinity column.

**FIG. 1.** The apparent affinity of IIC2-K1014N for VC1, and G<sub>α</sub>·GTP·pYs. VC1 was mixed with wild type IIC2 or IIC2-K1014N and assayed for adenylyl cyclase activity in the presence of the indicated activators. A, adenylyl cyclase activity was assayed with VC1 (30 nM) and increasing concentrations of IIC2 (open symbols) or IIC2-K1014N (closed symbols) in the absence of activators. B, adenylyl cyclase was assayed with VC1 (2 nM) and increasing concentrations of IIC2 (open symbols) or IIC2-K1014N (closed symbols) in the presence of 100 µM forskolin. C, adenylyl cyclase was assayed with VC1 (2 nM) and increasing concentrations of IIC2 (open symbols) or IIC2-K1014N (closed symbols) in the presence of 400 nM G<sub>α</sub>·GTP·pY·S with (filled symbols) and without (open symbols) 100 µM forskolin. D, adenylyl cyclase was assayed with 2 nM VC1 and 2 µM IIC2 (open symbols) or IIC2-K1014N (closed symbols) with increasing concentrations of G<sub>α</sub>·GTP·pY·S in the presence (filled symbols) and absence (open symbols) of 100 µM forskolin.

**TABLE III**

| Mutant | Basal EC<sub>50</sub> µM | Basal V<sub>max</sub> nmol/min/mg | Forskolin EC<sub>50</sub> µM | Forskolin V<sub>max</sub> nmol/min/mg |
|--------|----------------|----------------|----------------|----------------|
| Y383H<sup>a</sup> | 5 (4.8) | 460 (550) | 1.7 (1.3) | 85 (130) |
| F400Y<sup>a</sup> | 18 (8) | 380 (840) | 3 (1.4) | 89 (130) |
| N417S<sup>a</sup> | 7 (8) | 260 (410) | 1.5 (1.4) | 56 (100) |
| K436E<sup>a</sup> | 12 (5.7) | 310 (570) | 1.9 (1.4) | 37 (88) |
| V479I | 8 (7) | 980 (660) | 0.55 (1.7) | 81 (130) |
| L877P<sup>a</sup> | 3 (2) | 250 (170) | 1.1 (1.5) | 48 (44) |
| K896E<sup>a</sup> | 3 (2.4) | 150 (150) | 1.3 (1.2) | 34 (55) |
| C407R | 3 (4) | 680 (650) | 0.89 (0.83) | 120 (93) |
| I1010M<sup>b</sup> | 4.5 (2.7) | 410 (190) | 2.2 (1.3) | 110 (65) |
| K1014N<sup>b</sup> | 1.6 (2.4) | 1200 (170) | 0.33 (1.4) | 78 (60) |
| P1015Q<sup>b</sup> | 4.2 (2.2) | 510 (160) | 1.4 (1.2) | 100 (56) |

<sup>a</sup> Protein purified by metal affinity column.

<sup>b</sup> Protein purified by metal affinity column.

**DISCUSSION**

The crystal structure of the cytosolic portions of adenylyl cyclase demonstrates that the C<sub>1</sub> and C<sub>2</sub> domains are arranged as a pseudo-2-fold symmetrical dimer (see inset in Fig. 5) (11). The contributions of several residues within each domain to substrate and Mg<sup>2+</sup> binding, as well as catalysis, have been investigated in previous studies. Adenylyl cyclases, and presumably guanylyl cyclases, contain palm domains. These domains were defined previously in DNA polymerases, enzymes that catalyze very similar reactions (18). Crystal structures of the cytosolic domains of adenylyl cyclase have revealed significant conformational changes upon substrate binding (11). The α1 and α2 helices and the α3 and β4 helix/strand of C<sub>2</sub> collapse around the nucleotide and align the nucleotide and two metal ions for catalysis. Located within the active site is the β2-β3 loop of C<sub>2</sub>, containing aspartate residues 396 and 440 that coordinate two Mg<sup>2+</sup> ions. These divalent cations participate in deprotonation of the 3′ hydroxyl of the ribose moiety (a critical step in the synthesis of cyclic AMP) and stabilize the pentavalent transition state. The conserved aspartate residues are also found among DNA polymerases and...
guanylyl cyclases (11, 12, 19–22).

The β4-β5 loop of C2 supports the β2-β3 loop of C1 (Fig. 5). Perturbations in either the contact regions or the loop-fold could have dramatic effects on C1:C2 structure and hence activity. Several residues in both the β2-β3 loop and the a2 helix of C1 and the β4-β5 loop of C2 have been investigated by site-directed mutagenesis and have various effects on adenylyl cyclase activity. Structural evidence strongly suggests that Asp-424 in the a2 helix and Arg-434 in the β2 sheet of C1 engage in extensive hydrogen bonding with the β4-β5 loop of C2 (Fig. 5). Asp-424 forms a salt bridge with Arg-434 and a hydrogen bond with the backbone nitrogens of Ala-1012 and Gln-1013. The side chain of Arg-434 forms a hydrogen bond with the side chain of Gln-1016; the backbone carbonyl of Arg-434 forms a hydrogen bond with the side chain of Gln-1013. Mutations of either Asp-424 or Arg-434 have previously been shown to have detrimental effects on adenylyl cyclase activity. Mutations of these residues have broad effects on cyclase activity without affecting the affinity of the enzyme for Gsα, as follows: R434A increases the IC50 value for P-site inhibitors (23); R434S increases the Kmax value for MgATP, the Vmax value for ATPαS, and the EC50 value for Mg2+ (24); D424A and D424N decrease forskolin- and Gsα-stimulated enzymatic activity (23, 25).

Mutations of residues in the β4-β5 loop of C2 have also been shown to affect adenylyl cyclase activity. The mutations Y1017A and D1018A (Y999A and D1000A in type I adenylyl cyclase) obliterate activity without eliminating Gsα binding (23). Asp-1018 coordinates substrate binding through the pu-

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**TABLE IV**

| Condition     | Protein         | K<sub>m</sub>ATP (μM) | V<sub>max</sub> (μmol/min/mg) |
|---------------|-----------------|------------------------|-----------------------------|
| Basal         | K1014N          | 210 ± 28               | 0.78 ± 0.23                 |
|               | IIC2            | 1300 ± 310             | 0.20 ± 0.07                 |
| Forskolin     | K1014N          | 380 ± 65               | 86 ± 8                      |
|               | IIC2            | 840 ± 85               | 66 ± 2                      |
| Gsα           | K1014N          | 35 ± 5                 | 26 ± 1                      |
|               | IIC2            | 220 ± 45               | 28 ± 4                      |
| Gsα + Forskolin| K1014N         | 160 ± 27               | 92 ± 18                     |
|               | IIC2            | 210 ± 26               | 106 ± 24                    |

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**Fig. 2. Substrate kinetics with IIC2-K1014N.** The kinetic constants for ATP were determined from the Michaelis-Menten plots generated by measuring adenylyl cyclase activity with a limiting concentration of VC1 and an excess of IIC2 while varying the ATP concentration from 20 μM to 5 mM. A, 30 mM VC1 and 10 μM IIC2 (□) or IIC2-K1014N (○) were assayed in the absence of activators. B–D, 2 mM VC1 and 3 μM IIC2 (□) or IIC2-K1014N (○) were assayed in the presence of 100 μM forskolin (B), 400 nM G<sub>αGTP</sub>S (C), or 100 μM forskolin and 400 nM G<sub>αGTP</sub>S (D).
staining with Coomassie Blue. Resolved by SDS-PAGE on a 15% polyacrylamide gel and visualized by washing and then eluted with imidazole. Aliquots of the eluates were cated. The mixtures were applied to 25- of IIC2. The side chains of the stick models. The b side chain–side chain and side chain–main chain hydrogen bonds. Car-

Catalytic core of adenylyl cyclase. Inset, the heterodimeric complex formed by VC, (khaki) and IIC, (mauve) viewed along its pseudo-2-fold axis toward the hypothesized cytoplasmic face. Forskolin (Fsk) and ATP bind between VC and IIC, and are shown as stick models. The β4-β5 loop of IIC containing I1010M, K1014N, and P1015Q is highlighted in green. Bottom right, interactions of β4-β5 loop of IIC. The side chains of the β4-β5 loop (green) of IIC, (mauve) and their interactions with VC, (khaki) are shown. Dashed gray lines show side chain–side chain and side chain–main chain hydrogen bonds. Carbon atoms are gray, nitrogen atoms blue, and oxygen atoms red.

Constitutively Active Adenylyl Cyclase

The β4-β5 loop of the C1 domain has a congrous interaction with the β2-β3 loop of C2 because of the pseudosymmetrical structure of adenylyl cyclase. Of the clones obtained from the genetic screen, only V506I displayed significant enhancement of activity when tested in the soluble adenylyl cyclase system. V506I adds a methyl group that may form a primary contact with forskolin and increase the hydrophobicity of the forskolin-binding pocket. Another possible explanation is that substitution of the isoleucine may enhance C1-C2 interactions by altering van der Waals contacts with neighboring residues in the β4-β5 loop of C1. The structural effect of this minor change at the base of the loop may be amplified along the length of the loop and thus alter interactions with the β2-β3 loop of C2.

It is difficult to determine why other mutations displayed strong phenotypes in yeast but failed to produce substantial changes in the soluble adenylyl cyclase assays. As demonstrated in this study, mutations that increase favorable interactions between the β2-β3 loop of C1 and the β4-β5 loop of C2 likely account for the increased activity that was observed. Alternatively, increased activity may be related to the method of protein expression, since the soluble C1 and C2 domains remain as homodimers when expressed and purified individually. Mutations in the interface region may alter homodimerization. Mutations that impair homodimerization may favor heterodimerization and hence increase adenylyl cyclase activity. More likely, the lack of increased basal activity in the in vitro assays of many of the mutants could be explained by the inherent sensitivity to small changes in cyclic AMP concentrations of the yeast screen. The lack of change in activity in vitro could also be explained by the ablation of the membrane domains and the putative regulatory C1β domain in the soluble constructs. For example, the F400Y mutation has been shown to increase both basal activity and sensitivity to the activators Gα and forskolin and to abrogate inhibition by Gβ (28). However, when assayed in the soluble system, this mutation caused no increase in basal activity or sensitivity to forskolin compared with the wild type enzyme. The contribution of the C1β domain to activity are not known, nor is there any structural information on this domain.

As mentioned previously, all complexes of adenylyl cyclase whose structures have been determined to date contain both Gα and forskolin. Endogenous forskolin-like substances have yet to be discovered, begging the questions of the physiological significance of the C1-C2-forskolin-Gα-GTPyS structure and the degree to which it resembles the structure of C1-C2-Gα-GTPyS. Or rather, what is the mechanism of activation of adenylyl cyclase by forskolin? This is a particularly interesting question, since some forms of adenylyl cyclase (types II, IV, and V–VII) are activated synergistically by forskolin and Gα-GTPyS, whereas others (2) are activated only additively (types I, III, and VIII). The K1014N mutation facilitated the isolation of a C1-C2-Gα-GTPyS complex in the absence of forskolin. Determination of the structure of this complex would further our understanding of activation of adenylyl cyclase by the diterpene.

The crystal structure of a homodimer of the C2 domain bound with two molecules of forskolin has also been determined (29).
The structure also contains a 2-fold symmetrical arrangement of the domains and has been used as a model for the basal, nonactivated form of adenylyl cyclase. However, the presence of two forskolin molecules in a complex without an active site inherently precludes this structure as a precise model of non-activated adenylyl cyclase. It is our hope that the structure of the constitutively active mutants described herein, particularly that of VC1 associated with IIC2-K1014N, may represent a closer approximation of the low activity basal state.

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