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Conversion of Forskolin-Insensitive to Forskolin-Sensitive (Mouse-Type IX) Adenylyl Cyclase

SHUI-ZHONG YAN, ZHI-HUI HUANG, REBECCA K. ANDREWS, and WEI-JEN TANG

Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

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ABSTRACT

Forskolin potently activates all cloned mammalian adenylyl cyclases except type IX by interacting with two homologous cytoplasmic domains (C_1 and C_2) that form the catalytic core. A mutational analysis of the IIC_2 protein (C_2 domain from type II adenylyl cyclase) and forskolin analogs suggests that Ser942 interacts with the 7-acetyl group of forskolin. The C_1/C_2 complex has only one forskolin, one ATP, and one binding site for the α subunit of the G protein that stimulates adenylyl cyclase ($G_{s\alpha}$) and its structure may be modeled using the three-dimensional structure of $(IIC_2/forskolin)_2$. The Ser942 mutation defines which forskolin in the $(IIC_2/forskolin)_2$ structure exists in C_1/C_2 complex. Thus, the forskolin-binding site is close to the $G_{s\alpha}$-binding site but distal (15–20Å) from the catalytic site. Mutation from Leu912 of IIC_2 protein to tyrosine or alanine severely reduces $G_{s\alpha}$ activation and completely prevents forskolin activation. The corresponding residue of Leu912 is Tyr1082 at type IX isoform of adenylyl cyclase. Similar to recombinant type IX enzyme, soluble adenylyl cyclase derived from mouse-type IX adenylyl cyclase is sensitive to $G_{s\alpha}$ activation but not to forskolin. Changing Tyr1082 to leucine makes soluble type IX adenylyl cyclase forskolin-responsive.

The diterpene, forskolin, is a cardiac-enhancing drug isolated from the Indian plant *Coleus forskolhii* and is a potent activator of nearly all mammalian adenylyl cyclases (Seamon and Daly, 1986; Laurenza *et al.*, 1989). Forskolin has been immobilized for the affinity purification of the detergent-solubilized adenylyl cyclase, leading to success in cloning the genes that encode mammalian adenylyl cyclases (Pfeuffer *et al.*, 1985; Krupinski *et al.*, 1989). The analysis of nine types of recombinant mammalian adenylyl cyclases reveals that all adenylyl cyclases except type IX can be potently activated by forskolin (Premont *et al.*, 1996; Tang *et al.*, 1997).[1] Mammalian adenylyl cyclases consist of two homologous cytoplasmic domains (C_1 and C_2), each following one transmembrane domain (M_1 and M_2) (Tang *et al.*, 1997; Taussig and Gilman, 1995; Sunahara *et al.*, 1996). The two cytoplasmic domains form the catalytic core; forskolin binds and activates these core domains directly (Tang and Gilman, 1995; Yan *et al.*, 1996; Whisnant *et al.*, 1996; Sunahara *et al.*, 1997; Scholich *et al.*, 1997).

The three dimensional structure of the IIC_2/forskolin dimer, which resembles that of the C_1/C_2 complex, has been solved recently (Zhang *et al.*, 1997; Yan *et al.*, 1997a) (Fig. 1). The IIC_2 structure consists of a $\beta\alpha\beta\beta\alpha\beta$ substructure that is similar to the palm domain of prokaryotic DNA polymerases including *Escherichia coli* DNA polymerase I and *Thermus aquaticus* (*Taq*) polymerase (Artymiuk *et al.*, 1997). The C_1/C_2 complex binds only one $G_{s\alpha}$, one ATP, and one forskolin molecule based on equilibrium dialysis of the C_1 and C_2 domains of type V and type II adenylyl cyclases, respectively (Dessauer *et al.*, 1997). The $G_{s\alpha}$-binding site of adenylyl cyclase has been mapped to a region formed by the $\alpha 2$ and

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[1] Type IX adenylyl cyclase was insensitive to forskolin when expressed in insect Sf9 cells, whereas a 2-fold stimulation was observed by forskolin when type IX enzyme was transiently expressed in human embryonic kidney 293 cells (Premont *et al.*, 1996). The discrepancy remains unresolved; the possible hetero-oligomer formed between isoforms of mammalian adenylyl cyclases may provide the explanation (Tang *et al.*, 1995b).

ABBREVIATIONS: $G_{s\alpha}$, the α subunit of G protein that stimulates adenylyl cyclase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

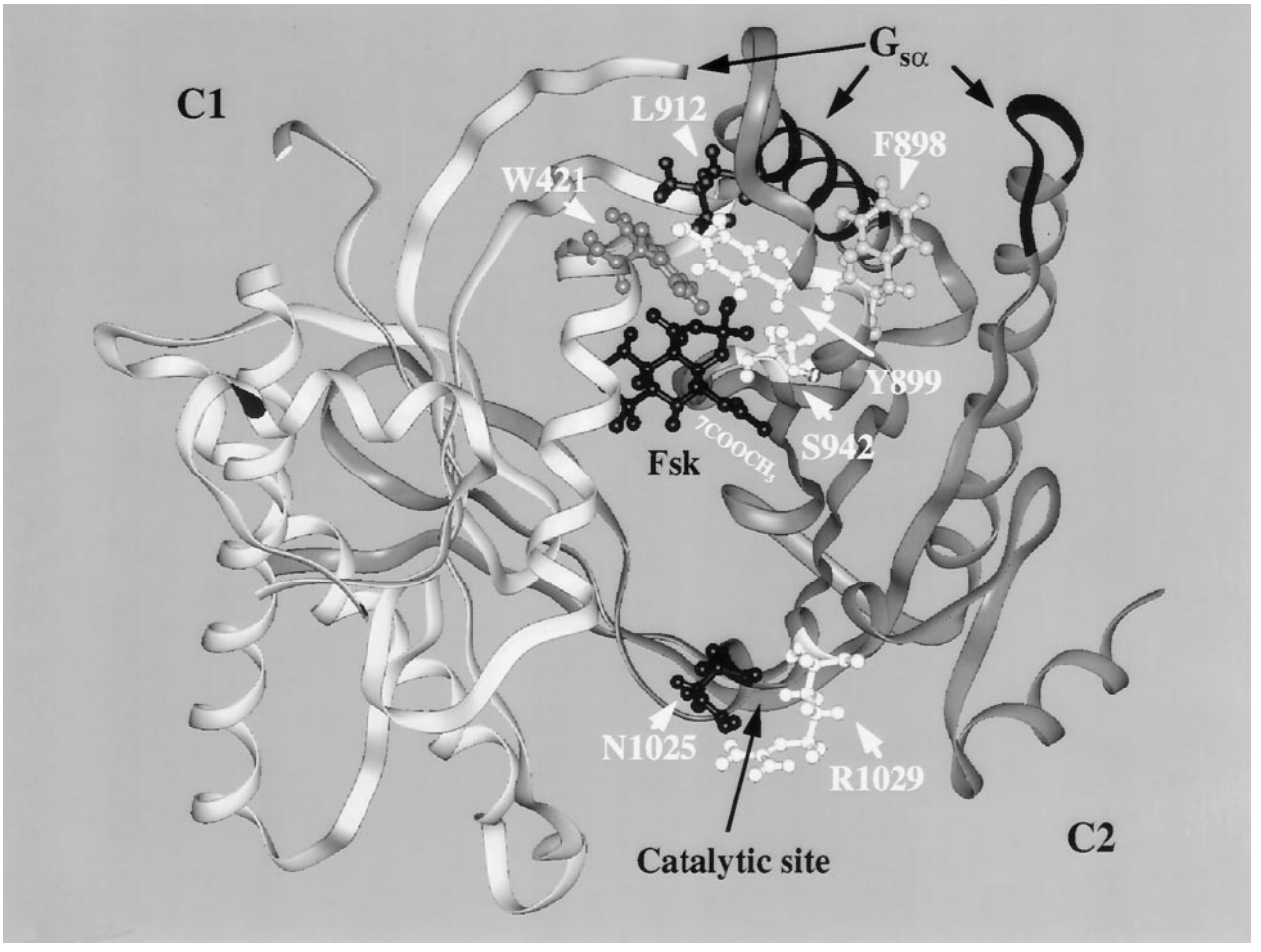

Fig. 1. The interaction of IC_2/IIC_2 complex with forskolin. The IC_1/IIC_2/forskolin structure is based on the three dimensional structure of $(IIC_2/forskolin)_2$ (Zhang *et al.*, 1997). The catalytic and $G_{s\alpha}$-binding sites are based on mutational analysis (Yan *et al.*, 1997a, 1997b).

$\alpha 3/\beta 4$ region of C_2 domain and the amino terminus of the C_1 domain. The $G_{s\alpha}$-binding site is distal (20–30Å) to the catalytic (also ATP-binding) site, which is defined by our mutational analysis (Yan *et al.*, 1997b; Artymiuk *et al.*, 1997). The $(IIC_2/forskolin)_2$ structure has two forskolin molecules, which lie in the hydrophobic pocket in the ventral cleft of the IIC_2 dimer interface. Nine of 13 residues in the C_2 domain involved in the binding of forskolin to the IIC_2 dimer are conserved in the C_1 domain. It remains to be determined which of two forskolin molecules in $(IIC_2/forskolin)_2$ binds at the C_1/C_2 complex and whether the interaction between forskolin and the IIC_2 dimer can serve as a model to study how forskolin binds to the C_1/C_2 complex. In this article, we use mutational analysis to address both questions.

Experimental Procedures

Materials. Forskolin and its analogs were from Calbiochem (La Jolla, CA); restriction enzymes and Vent DNA polymerase were from New England Biolabs (Beverly, MA); Bradford reagent was from Bio-Rad (Hercules, CA); the enhanced chemiluminescence system was from Amersham (Arlington Heights, IL); and Ni-NTA resin was from Qiagen (Chatsworth, CA).

Plasmids. The plasmids used to express mutant forms of IIC_2 were constructed as described except for those used to express IIC_2-L912A and IIC_2-L912Y, which were done using Quickchange (Stratagene, La Jolla, CA) (Yan *et al.*, 1997a). A plasmid used to express IXC_1 protein was constructed by performing polymerase chain reaction using the primers ATTACCATGGGGCAAAGATCTGGAAGTAGAG and TGGGAAGCTTGAATTAATAATCTTTCATCAGGCTGTC, pSK-AC9 as the template, and Vent DNA polymerase. The 1.3-kilobase polymerase chain reaction product was isolated from agarose gel, digested with *Eco*RI and *Hin*dIII, and cloned into pProEx-HAH6 that had been digested with the same enzymes, resulting in the construct pProExHAH6-IXC_1. For the expression of IXC_2, the 1.4 kb *Nco*I/*Xho*I fragment was cut out of pSK-AC9 and ligated to pProEx-HAH6 that was digested with the same enzyme. Because the IXC_2 coding sequences in the resulting plasmid were out of frame compared with those that encoded the hexo-histidine tag, the resulting plasmid and the primer CCGGATTACGCCGGAGATGTGGAGGCCGAC were used to do site-directed mutagenesis for the construction of the plasmid that could be used to express IXC_2, resulting in pProExHAH6-IXC_2 (Kunkel, 1985). Kunkel's method was used to construct IXC_2 mutants from pProExHAH6-IXC_2 as the template; oligonucleotides used for mutagenesis contained 10–12 complementary nucleotides flanking each side of the target codon(s), which were replaced with the appropriate codon (Kunkel, 1985). Mutations were confirmed by dideoxyl nucleotide sequencing of phagemid DNA.

Expression and purification of recombinant C_1 and C_2 protein from *E. coli*. The expression of wild-type and mutant forms of hexo-histidine-tagged IC_1 and IIC_2 has been described previously (Yan *et al.*, 1996). The conditions for expressing hexo-histidine-tagged IXC_1 and IXC_2 wild-type and mutant proteins in *E. coli* BL21(DE3) cells were the same for expressing IC_1 and IIC_2 (Yan *et*

al., 1996). Both IXC_1 and IXC_2 proteins were purified using the Ni-NTA column and Q-sepharose column and IXC_1 was further purified by Superdex 200 column. The conditions and buffers used in purification of IXC_1 and IXC_2 were similar to the purification of IC_1 and IIC_2 (Yan *et al.*,1996). The $G_{s\alpha}$-stimulated activity was used to determine the protein peak in the fractions from Q-sepharose and Superdex 200 columns (Pharmacia, Piscataway, NJ). The concentration of proteins was determined using Bradford reagent and bovine serum albumin as standard (Bradford, 1976).

Adenylyl cyclase assay. The purification of hexo-histidine-tagged $G_{s\alpha}$ was performed as described previously (Lee *et al.*, 1994). $G_{s\alpha}$ was activated by 50 μM $AlCl_3$ and 10 mM NaF, and adenylyl cyclase assays were performed at 30° for 20 min (Yan *et al.*, 1996; Salomon *et al.,* 1976).

Results

Ser942 of IIC_2 protein is important in interacting with forskolin. We have constructed forskolin- and $G_{s\alpha}$-sensitive soluble adenylyl cyclase from the C_1 domain of type I enzyme and the C_2 domain of type II enzyme; such a system is used in analyzing the forskolin-binding site (Tang and Gilman, 1995a; Yan *et al.*, 1996). All adenylyl cyclases except type IX are potently activated by forskolin (Premont *et al.*, 1996).[1] Sequence analysis reveals that eight amino acids in the C_2 domain are absolutely conserved among forskolin-sensitive type I to VIII enzyme, and rutabaga adenylyl cyclases, but differ in forskolin-insensitive mouse-type IX enzyme (Yan *et al.*, 1997a). By mutating six of these eight residues to alanine in the C_2 domain of type II enzyme (IIC_2), we found that *E. coli* lysates containing mutant IIC_2-S942A had relatively normal stimulation by $G_{s\alpha}$, but had moderately reduced activation by forskolin[2] (not shown). This difference is not caused by low expression because immunoblot analysis indicated that lysate containing IIC_2-S942A had similar amounts of IIC_2 compared with wild-type IIC_2 (not shown). Mutant IIC_2-S942A was purified to homogeneity (Fig. 2A). The purified protein was tested for its $G_{s\alpha}$ and forskolin activation when the purified IC_1 protein was added. IIC_2-S942A had nearly normal $G_{s\alpha}$ activation but about a 6-fold reduction in forskolin activation (Fig. 3A, B). In the presence of submaximal $G_{s\alpha}$, the maximal forskolin-stimulated activity of IIC_2-S942A was similar to that of wild-type IIC_2 (Fig. 3C). The 6-fold reduction in forskolin activation of IIC_2-S942A may be related to the apparent reduced affinity indicated by the 6-fold increase in EC_{50} value (0.3 and 1.7 μM for wild type IIC_2 and IIC_2-S942A, respectively; Fig. 3C). $G_{s\alpha}$ activation of the IC_1/IIC_2 complex can be greatly enhanced by forskolin; thus, the reduction in forskolin activation should result in reduced $G_{s\alpha}$ activation. As expected, IIC_2-S942A did have reduced $G_{s\alpha}$ activation when submaximal forskolin (2 μM) was present (Fig. 3D).

The hydroxyl groups at the 1α- and 9α-positions and the acetoxyl group at the 7β-position of forskolin are crucial for forskolin activation of adenylyl cyclase (Sutkowski *et al.*, 1994). The forskolin analog 1-deoxyforskolin did not activate IC_1+IIC_2 complexes; 7-deacetyl- and 9-deoxyforskolin had reduced potency (Fig. 3, E and F; not shown for 1-deoxyforskolin). These results are similar to those observed with

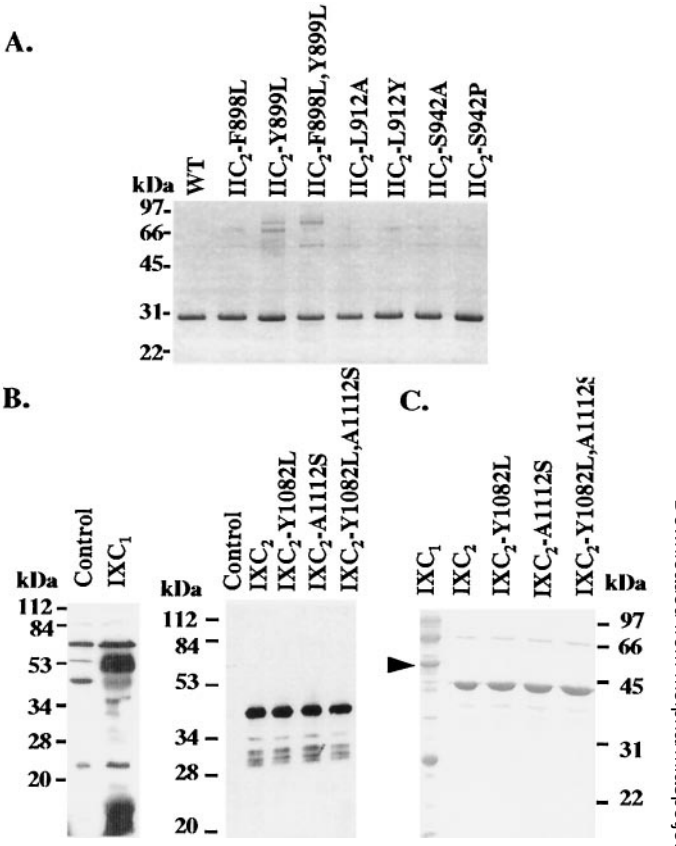

Fig. 2. Analysis of IIC_2, IXC_1, and IXC_2 wild-type and mutant proteins. A, Purified IIC_2 mutant proteins (2.5 μg) were electrophoresed on 13% SDS-PAGE and stained with Coomassie Blue. B, High-speed supernatants of lysate from *E. coli* BL21(DE3) cells (15 μg of that containing IXC_1 and 0.2 μg of those containing IXC_2 and IXC_2 mutant proteins) were prepared 3 hr after induction by isopropyl-1-thio-galactopyranoside, electrophoresed on 13% SDS-PAGE and immunoblotted with a monoclonal antibody, 12CA5. C, Purified IXC_1 (5 μg), IXC_2, and its mutant proteins (2.5 μg) were electrophoresed on 13% SDS-PAGE and stained with Coomassie Blue.

membrane-bound adenylyl cyclase (Sutkowski *et al.*, 1994). When mixed with IC_1, activation of IIC_2-S942A by 9-deoxyforskolin was reduced about 3–7-fold compared with that of wild-type IIC_2, similar to the observed reduction in activation by forskolin. In contrast, the activation of IIC_2-S942A by 7-deacetylforskolin was only about 2–3-fold less than that of wild-type IIC_2. This corroborates the hydrogen bonding between the 7β-acetyl group of forskolin and the hydroxyl group of Ser942 observed in the IIC_2/forskolin crystal structure (Zhang *et al.*, 1997).[3]

The $(IIC_2/forskolin)_2$ structure indicates that the 7-acetyl group of forskolin forms hydrogen bonds with both the main and side chain of Ser942 (Zhang *et al.*, 1997) (Fig. 1). The model predicts that a Ser942-to-proline mutation would disrupt hydrogen bonding in both the main and the side chains to the 7-acetyl group of forskolin. Thus, such a mutation should have a profound reduction in forskolin activation without a reduction in $G_{s\alpha}$ activation. IIC_2-S942P had 25–30% reduction in $G_{s\alpha}$ activation when either lysate containing IIC_2-S942P or purified mutant protein was used (Fig. 3B). In contrast to the moderate reduction in $G_{s\alpha}$ stimulation,

[2] The six mutated residues are Q880, S881, L912, S942, S990, and N992. We did not test A1012 and S1032 of IIC_2 (both are threonine in the type IX enzyme).

[3] The observation suggesting the interaction between Ser942 of the type II enzyme and the 7-acetyl group of forskolin was obtained without prior knowledge of the molecular structure of IIC_2/forskolin dimer.

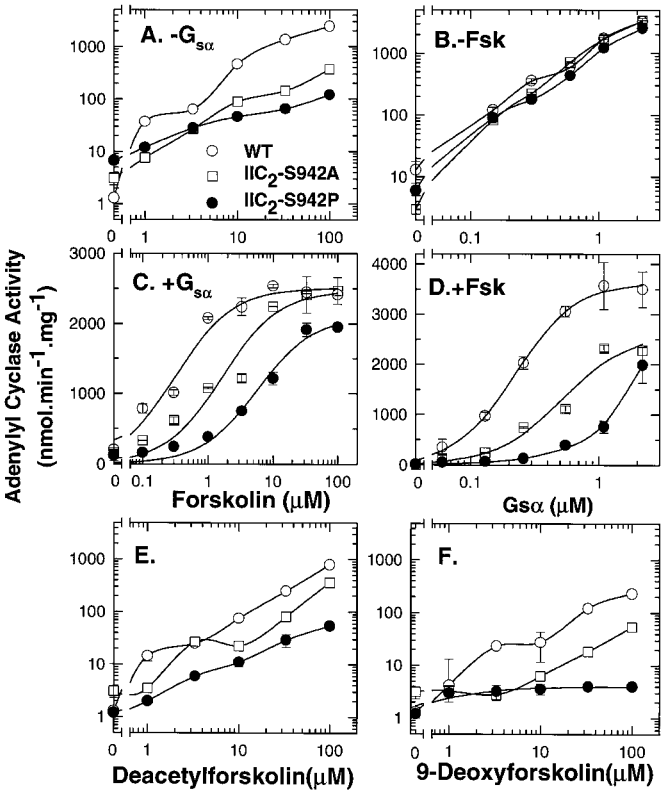

Fig. 3. Characterization of IIC_2-S942A and IIC_2-S942P. Adenylyl cyclase activity of purified wild-type IIC_2 and IIC_2-S942A (0.26 μM) mixed with IC_1 (0.1 μM) and activated by forskolin (A), $G_{s\alpha}$ (B) forskolin and 0.2 μM $G_{s\alpha}$ (C), $G_{s\alpha}$ with 2 μM forskolin (D), deacetylforskolin (E), and 9-deoxyforskolin (F). The data (mean ± standard deviation) are representative of at least two experiments.

IIC_2-S942P had a 20- to 40-fold reduction in forskolin and 9-deoxyforskolin stimulation, substantially more than that of IIC_2-S942A. IIC_2-S942P had about 25% reduction in maximal forskolin stimulation when activated with submaximal $G_{s\alpha}$ (Fig. 3C). The reduction in forskolin activation is obvious in the EC_{50} values, not in the V_{max} values (Fig. 3C; the EC_{50} values for wild-type IIC_2 and IIC_2-S942P were 0.3 and 5.8 μM, respectively, and V_{max} values were 2.5 and 2.1 μmol/min/mg, respectively). In the presence of forskolin (2 μM), IIC_2-S942P had significant reduction in $G_{s\alpha}$ activation, presumably because of the drastically reduced forskolin activation of IIC_2-S942P mutant (Fig. 3D).

Based on equilibrium dialysis of the VC_1/IIC_2 complex, there is only one forskolin in the C_1/C_2 complex (Dessauer *et al.*, 1997). Our data show that S942 of IIC_2 is essential in interacting with the 7-acetyl group of forskolin. Thus, the forskolin molecule binds the site that is close to the $G_{s\alpha}$-binding site but distal to the ATP-binding site (Fig. 1). Based on the $(IIC_2/forskolin)_2$ model, IIC_2-S942A or IIC_2-S942P should not have significantly reduced activation by 7-deacetylforskolin relative to wild-type IIC_2. However, we observed 2- and 10-fold reductions when IIC_2-S942A and IIC_2-S942P were stimulated by 7-deacetylforskolin, respectively. Possible reasons for such reductions include changes in the local conformation at the forskolin-binding site by Ser942 to the alanine or proline mutations and the structural differences at the forskolin-binding site between the C_1/C_2 complex and the IIC_2 dimer. Further experiments are required to resolve this discrepancy.

Mutations near the forskolin-binding site affect both $G_{s\alpha}$ and forskolin activation of C_1/C_2 complex. We constructed and analyzed two sets of IIC_2 mutants that had mutations surrounding forskolin (Fig. 1). Tyr899 is conserved among all the mammalian adenylyl cyclases. Based on the IC_1/IIC_2 model using $(IIC_2/forskolin)_2$ structure, Tyr899 is located at the C_1/C_2 interface and is in contact with 13-methylenyl and 13-methyl groups of forskolin and Trp421 of C_1 protein (Fig. 1). Thus, mutation at Tyr899 of IIC_2 is likely to affect both interaction with IC_1 and forskolin. We constructed a IIC_2 mutant with the mutation of Tyr899 to Leu, and IIC_2-Y899L was expressed normally (not shown). As expected, the purified IIC_2-Y899L protein had substantially higher reduction in forskolin stimulation (~40-fold) than in $G_{s\alpha}$ activation (6-fold) compared with wild-type IIC_2 (Fig. 4, A and B). Submaximal $G_{s\alpha}$ partially rescued forskolin activation (Fig. 4, A and C). We also mutated Phe898, which is conserved among all mammalian adenylyl cyclase and seems to be involved in coordinating the $G_{s\alpha}$-binding site. To our surprise, mutation of Phe898 to Leu drastically reduced both $G_{s\alpha}$ and forskolin activation of IIC_2-F898 (Fig. 4, A and B). IIC_2-F898 seemed to have reduced affinity to forskolin depicted by the increased EC_{50} value of forskolin activation in the presence of submaximal $G_{s\alpha}$ (Fig. 4C). The purified IIC_2

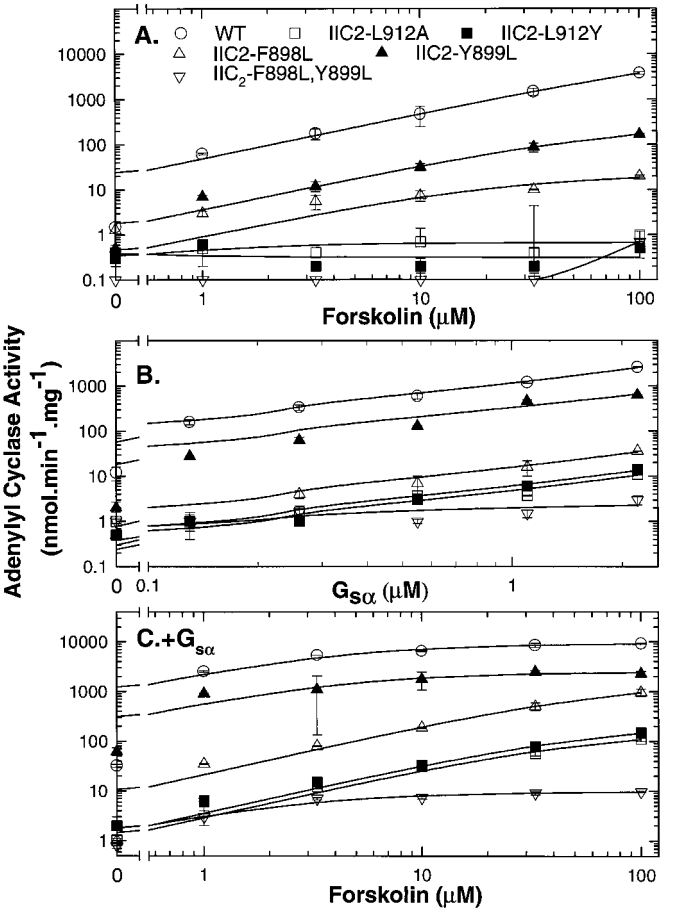

Fig. 4. Characterization of IIC_2-L912A, IIC_2-L912Y, IIC_2-F898L, IIC_2-Y899L, and IIC_2-F898L, Y899L. Adenylyl cyclase activity of purified wild-type IIC_2 and IIC_2 mutants (0.26 μM) mixed with IC_1 (0.1 μM) and activated by forskolin (A), $G_{s\alpha}$ (B), and forskolin and 0.2 μM $G_{s\alpha}$ (C). The data (mean ± standard deviation) are representative of at least two experiments.

protein that had mutated both Tyr899 and Phe898 to Leu had nearly no enzyme activity (Fig. 4).

Leu912 is one of eight amino acid residues that are conserved among forskolin-sensitive adenylyl cyclases but differ in the forskolin-insensitive type IX enzyme. Based on the $(IIC_2/forskolin)_2$ structure, Leu912 is located at the interface of C_1/C_2 complex and 6Å away from forskolin. Thus, it is involved in coordinating the binding of Tyr899 of IIC_2 and Trp421 of IC_1 to forskolin (Fig. 1). The corresponding residue of Leu912 in type II enzyme is Tyr1082 in the type IX enzyme. Tyrosine substitution of Leu912 in the IIC_2 protein should cause steric conflicts between the substituted residue and Tyr899 and Trp421 and consequently disturb the structure of the forskolin-binding pocket (Fig. 1). We had mutated Leu912 of IIC_2 to alanine or tyrosine and both IIC_2-L912A and IIC_2-L912Y could be expressed and purified similarly to wild-type IIC_2 (Fig. 2A). When mixed with IC_1, both IIC_2-L912A and IIC_2-L912Y had > 100-fold reduction in $G_{s\alpha}$-stimulated activity. Both mutant proteins showed little forskolin stimulation unless $G_{s\alpha}$ (0.2 μM) was present (Fig. 4, B and C).

Tyr1082-to-leucine mutation converts type IX adenylyl cyclase to be forskolin-sensitive. Type IX adenylyl cyclase is forskolin-insensitive. One or more mutations that render type IX enzyme sensitive to forskolin would highlight the residue(s) important for forskolin activation. To find such residue(s), we first constructed the C_1 and C_2 domains from type IX adenylyl cyclase (IXC_1 and IXC_2, respectively) and tested whether they could form the functional enzyme and exhibit the proper biochemical properties.[4] IXC_1 and IXC_2 proteins were both tagged with the influenza hemoagglutinin epitope, and immunoblot analysis showed that IXC_1 and IXC_2 proteins had the expected 50- and 42-kDa size (Fig. 2B). Small molecular-weight proteins were seen in the lysates containing IXC_1 and IXC_2 proteins, presumably proteolytic products. *E. coli* lysates containing either IXC_1 or IXC_2 protein alone had no detectable adenylyl cyclase activity. However, significant $G_{s\alpha}$-stimulated enzyme activity was detected when the two lysates were mixed together (not shown). Lysates containing IXC_1 and IXC_2 protein did not exhibit forskolin sensitivity with or without $G_{s\alpha}$ (not shown). This result is consistent with the observation that type IX adenylyl cyclase is $G_{s\alpha}$-sensitive, but is insensitive to stimulation by forskolin (Premont *et al.*, 1996).[1]

IXC_2 protein was expressed in at least 100-fold greater quantity than IXC_1 protein based on immunoblot analysis. IXC_2 protein could be purified to near homogeneity with the use of a Ni-NTA column and then a Q-sepharose column. The yield of IXC_2 protein was about 6 mg/liter of *E. coli* culture (Fig. 2C). The purification of IXC_1 protein was less effective. After running the lysates through three columns (Ni-NTA, Q-sepharose, and fast performance liquid chromatography Superdex 200), 50 μg of IXC_1 protein, which was only about 20% pure, could be obtained from a liter of *E. coli* culture (Fig. 2C).[5] Thus, the specific activity of the purified IXC_1 and IXC_2 mixed proteins was only about 15-fold higher than that of *E. coli* lysates. Similar to the results obtained from *E. coli* lysates, the purified IXC_1 and IXC_2 proteins could be activated by $G_{s\alpha}$ but not by forskolin (Fig. 5A).

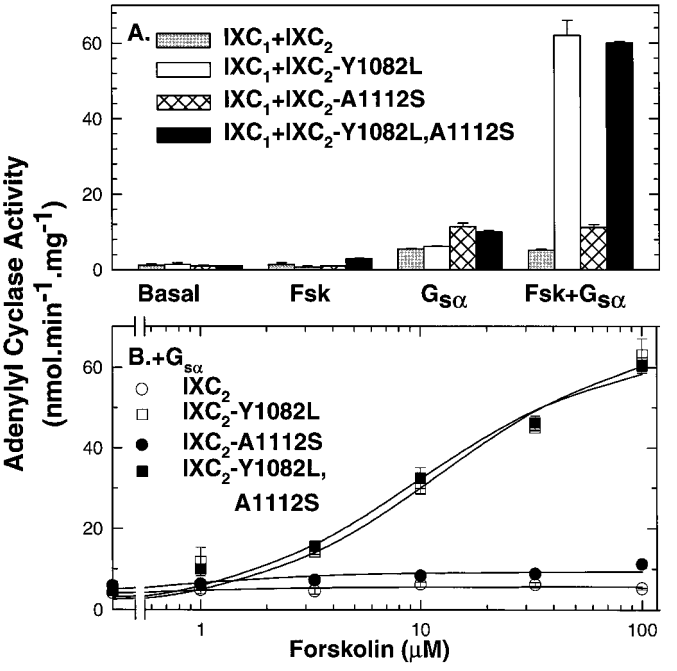

Fig. 5. Characterization of IXC_1, IXC_2, and IXC_2 mutant proteins. A, Enzyme activities of purified IXC_2 or IXC_2 mutant proteins (0.8 μg) mixed with IXC_1 (1.8 μg) with no activators (*basal*), 2.2 μM $G_{s\alpha}$ ($G_{s\alpha}$), 100 μM forskolin (Fsk), and 0.2 μM $G_{s\alpha}$ + 100 μM forskolin (*Fsk*+$G_{s\alpha}$). B, Enzyme activities of purified IXC_2 and IXC_2 mutant proteins (0.8 μg) mixed with IXC_1 (1.8 μg) with 0.2 μM $G_{s\alpha}$ and the indicated forskolin concentration. The enzyme assay data (mean ± standard deviation) are representative of at least two experiments.

As described above, mutations at Ser942 and Leu912 could affect the forskolin-stimulated activity of IC_1/IIC_2 complex. The corresponding position for Ser942 and Leu912 of type II adenylyl cyclase is Ala1112 and Y1082, respectively, in type IX adenylyl cyclase. Thus, we tested whether IXC_1+IXC_2 proteins could become forskolin-sensitive by changing Ala1112 to serine, Y1082 to leucine, and both simultaneously. All IXC_2-A1112S, IXC_2-Y1082L, and IXC_2-Y1082L, A1112S were expressed in similar amounts compared with that of wild-type IXC_2 and could be purified to near homogeneity (Fig. 2, B and C). Mixed IXC_1 and all three IXC_2 proteins were stimulated by $G_{s\alpha}$ using either *E. coli* lysates or purified proteins (Fig. 5A). Purified IXC_2-Y1082L, A1112S protein mixed with IXC_1 had weak but detectable forskolin stimulation, whereas both IXC_2-Y1082L and IXC_2-A1112S proteins mixed with IXC_1 did not (Fig. 5A). In the presence of submaximal concentration of $G_{s\alpha}$, both IXC_2-Y1082L and IXC_2-Y1082L, A1112S, when mixed with IXC_1, had 10- to 15-fold stimulation by forskolin, whereas wild-type IXC_2 had no observable forskolin stimulation (Fig. 5). Thus, Tyr1082 to leucine mutation converts soluble type IX adenylyl cyclase into forskolin-sensitive enzyme.

Discussion

Soluble adenylyl cyclases derived from membrane-bound adenylyl cyclases have proven to be an excellent tool for studying the biochemical properties of adenylyl cyclase. The C_1 and C_2 domains from their natural combination or from

[4] IXC_1 and IXC_2 proteins contain aa 320–741 and aa 1011–1353 of mouse-type IX adenylyl cyclase, respectively.

[5] IXC_1 protein was not purified further because it was rather unstable and the enzyme activity was not preserved by quick freezing, even with 20% glycerol.

different isoforms (chimeric C_1/C_2) can form functional soluble enzymes (Tang and Gilman, 1995; Yan *et al.*, 1996; Whisnant *et al.*, 1996; Dessauer and Gilman, 1996; Scholich *et al.*, 1997; Yan *et al.*, 1997a; Sunahara *et al.*, 1997).[6] The three-dimensional structure of the IIC_2 dimer/forskolin has been solved, providing a structural model of the catalytic domain of adenylyl cyclases (Zhang *et al.*, 1997). Our mutational analysis for the $G_{s\alpha}$ activation site of IC_1/IIC_2 protein indicates that the structure of the IIC_2 dimer is a reasonable representation of the IC_1/IIC_2 protein (Yan *et al.*, 1997a). In this paper, we show that the IIC_2/forskolin model has successfully predicted the essential roles for Ser942, Tyr899, and Leu912 in forskolin sensitivity in either the IC_1/IIC_2 or IXC_1/IXC_2 model, indicating that the forskolin-binding region at the C_2 domain predicted from the IIC_2/forskolin model is reasonably accurate. Forskolin binds to the site that is close to $G_{s\alpha}$, which allows forskolin to synergistically enhance $G_{s\alpha}$ activation. Although the forskolin binding site is 15–20Å away from ATP-binding site, forskolin does affect ATP binding. Forskolin stimulation in the absence of manganese ion increases the K_m value of Mg-ATP 10-fold for the native and recombinant type I, II, V, and rutabaga adenylyl cyclases; the molecular mechanism remains elusive (Tang *et al.*, 1995).

Our result shows that Ser942 of type II adenylyl cyclase modulates the enzyme's affinity for forskolin. Tyr1082 plays an active role in preventing type IX adenylyl cyclase from being sensitive to forskolin, although Ala1112 (the residue corresponding to Ser942 of type II enzyme) may also be involved. It is interesting to note that the type IX enzyme homolog from *Drosophila melanogaster* is forskolin-sensitive and the corresponding Tyr1082 and Ala1112 of *D. melanogaster* type IX enzyme are leucine and serine, respectively (Iourgenko *et al.*, 1997). These facts lead to several questions. Why are the hydrophobic forskolin pockets conserved among eight isoforms of mammalian adenylyl cyclases (type I to XIII) and several fruit fly adenylyl cyclases? Why does mouse-type IX enzyme have a different forskolin-binding pocket and its fruit fly homolog does not? One obvious answer is the existence of endogenous lipophilic compound(s) that can mimic the function of forskolin; if such a molecule exists, it remains to be discovered.

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[6] The soluble enzymes from the C_1 and C_2 domains of type II, VII, and VIII enzymes have also been constructed successfully (S-Z Yan, Z-H Huang, RS Shaw, and W-J Tang, unpublished observations).

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Send reprint requests to: Dr. Wei-Jen Tang, Department of Pharmacology & Physiological Science, University of Chicago, MC 0926, 947 East 58th Street, Chicago, IL 60637. E-mail: tang@drugs.bsd.uchicago.edu