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

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ABSTRACT

Forskolin potently activates all cloned mammalian adenylyl cyclases except type IX by interacting with two homologous cytoplasmic domains (C1 and C2) that form the catalytic core. A mutational analysis of the IIC2 protein (C2 domain from type II adenylyl cyclase) and forskolin analogs suggests that Ser942 interacts with the 7-acetyl group of forskolin. The C1/C2 complex has only one forskolin, one ATP, and one binding site for the \( G_s \) subunit of the G protein that stimulates adenylyl cyclase. The Ser942 mutation defines which forskolin in the (IIC2/forskolin)2 structure exists in the C1/C2 complex. Thus, the forskolin-binding site is close to the \( G_s \)-binding site but distal (15–20Å) from the catalytic site. Mutation from Leu912 of IIC2 protein to tyrosine or alanine severely reduces \( G_s \) 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 \( G_s \) 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 forskolii 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). Mammalian adenylyl cyclases consist of two homologous cytoplasmic domains (C1 and C2), each following one transmembrane domain (M1 and M2) (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 IIC2/forskolin dimer, which resembles that of the C1/C2 complex, has been solved recently (Zhang et al., 1997; Yan et al., 1997a) (Fig. 1). The IIC2 structure consists of a \( \beta\beta\alpha\beta\beta\alpha \) 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 C1/C2 complex binds only one \( G_s \), one ATP, and one forskolin molecule based on equilibrium dialysis of the C1 and C2 domains of type V and type II adenylyl cyclases, respectively (Dessauer et al., 1997). The \( G_s \)-binding site of adenylyl cyclase has been mapped to a region formed by the \( \alpha2 \) and

ABBREVIATIONS: \( G_s \), the \( \alpha \) subunit of G protein that stimulates adenylyl cyclase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
α3/β4 region of C2 domain and the amino terminus of the C1 domain. The Gsα-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 (IIC2/forskolin)2 structure has two forskolin molecules, which lie in the hydrophobic pocket in the ventral cleft of the IIC2 dimer interface. Nine of 13 residues in the C2 domain involved in the binding of forskolin to the IIC2 dimer are conserved in the C1 domain. It remains to be determined which of two forskolin molecules in (IIC2/forskolin)2 binds at the C1/C2 complex and whether the interaction between forskolin and the IIC2 dimer can serve as a model to study how forskolin binds to the C1/C2 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 IIC2 were constructed as described except for those used to express IIC2-L912A and IIC2-L912Y, which were done using Quickchange (Stratagene, La Jolla, CA) (Yan et al., 1997a). A plasmid used to express IXC1 protein was constructed by performing polymerase chain reaction using the primers ATTACCATGGGGCAAAGATCTGGAAGTAGAG and TGGGAAGCTTGAATTAATAATCTTCATCAGGCTGTC, pSK-AC9 as the template, and Vent 2 DNA polymerase. The 1.3-kilobase polymerase chain reaction product was isolated from agarose gel, digested with EcoRI and HindIII, and cloned into pProEx-HAH6 that had been digested with the same enzymes, resulting in the construct pProEx-HAH6-IXC1. For the expression of IXC2, the 1.4 kb NcoI/XhoI fragment was cut out of pSK-AC9 and ligated to pProEx-HAH6 that was digested with the same enzyme. Because the IXC2 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 IXC2, resulting in pProExHAE6-IXC2 (Kunkel, 1985). Kunkel’s method was used to construct IXC2 mutants from pProExHAE6-IXC2 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 C1 and C2 protein from E. coli.** The expression of wild-type and mutant forms of hexo-histidine-tagged IC1 and IIC2 has been described previously (Yan et al., 1996). The conditions for expressing hexo-histidine-tagged IXC1 and IXC2 wild-type and mutant proteins in E. coli BL21(DE3) cells were the same for expressing IC1 and IIC2 (Yan et...
al., 1996). Both IXC₁ and IXC₂ proteins were purified using the Ni-NTA column and Q-sepharose column and IXC₁ was further purified by Superdex 200 column. The conditions and buffers used in purification of IXC₁ and IXC₂ were similar to the purification of IC₁ and IIC₂ (Yan et al., 1996). The Gₛ-a-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).

**Adenyl cyclase assay.** The purification of hexo-histidine-tagged Gₛ was performed as described previously (Lee et al., 1994). Gₛ was activated by 50 μM AlCl₃ and 10 mM NaF, and adenyl cyclase assays were performed at 30° for 20 min (Yan et al., 1996; Salomon et al., 1976).

**Results**

Ser942 of IIC₂ protein is important in interacting with forskolin. We have constructed forskolin- and Gₛ-sensitive soluble adenyl cyclase from the C₁ domain of type I enzyme and the C₂ 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 adenyl cyclases except type IX are potently activated by forskolin (Premont et al., 1995a; Yan et al., 1996). Sequence analysis reveals that eight amino acids in the C₂ domain are absolutely conserved among forskolin-sensitive type I to VIII enzyme, and rutabaga adenyl 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 C₂ domain of type II enzyme (IIC₂), we found that E. coli lysates containing mutant IIC₂-S942A had relatively normal stimulation by Gₛ, but had moderately reduced activation by forskolin (not shown). This difference is not caused by low expression because immunoblot analysis indicated that lysate containing IIC₂-S942A had similar amounts of IIC₂ compared with wild-type IIC₂ (not shown). Mutant IIC₂-S942A was purified to homogeneity (Fig. 2A). The purified protein was tested for its Gₛ and forskolin activation when the purified IC₁ protein was added. IIC₂-S942A had nearly normal Gₛ activation but about a 6-fold reduction in forskolin activation (Fig. 3A, B). In the presence of submaximal Gₛ, the maximal forskolin-stimulated activity of IIC₂-S942A was similar to that of wild-type IIC₂ (Fig. 3C). The 6-fold reduction in forskolin activation of IIC₂-S942A may be related to the apparent reduced affinity indicated by the 6-fold increase in EC₅₀ value (0.3 and 1.7 μM for wild type IIC₂ and IIC₂-S942A, respectively; Fig. 3C). Gₛ activation of the IC₁/IIC₂ complex can be greatly enhanced by forskolin; thus, the reduction in forskolin activation should result in reduced Gₛ activation. As expected, IIC₂-S942A did have reduced Gₛ activation when submaximal forskolin (2 μM) was present (Fig. 3D).

The hydroxyl groups at the 1α- and 9α-positions and the acetoyl group at the 7β-position of forskolin are crucial for forskolin activation of adenyl cyclase (Sutkowski et al., 1994). The forskolin analog 1-deoxyforskolin did not activate IC₁-IIC₂ 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 membrane-bound adenyl cyclase (Sutkowski et al., 1994).

When mixed with IC₁, activation of IIC₂-S942A by 9-deoxyforskolin was reduced about 3–7-fold compared with that of wild-type IIC₂, similar to the observed reduction in activation by forskolin. In contrast, the activation of IIC₂-S942A by 7-deacetylforskolin was only about 2–3-fold less than that of wild-type IIC₂. This corroborates the hydrogen bonding between the 7β-acetyl group of forskolin and the hydroxyl group of Ser942 observed in the IIC₂/forskolin crystal structure (Zhang et al., 1997).³

The (IIC₂/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 Gₛ activation. IIC₂-S942P had 25–30% reduction in Gₛ activation when either lysate containing IIC₂-S942P or purified mutant protein was used (Fig. 3B). In contrast to the moderate reduction in Gₛ stimulation,

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³The 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 IIC₂/forskolin dimer.
IIC2-S942P had a 20- to 40-fold reduction in forskolin and 9-deoxyforskolin stimulation, substantially more than that of IIC2-S942A. IIC2-S942P had about 25% reduction in maximal forskolin stimulation when activated with submaximal Gs (Fig. 3C). The reduction in forskolin activation is obvious in the EC50 values, not in the Vmax values (Fig. 3C; the EC50 values for wild-type IIC2 and IIC2-S942P were 0.3 and 5.8 μM, respectively, and Vmax values were 2.5 and 2.1 mmol/min/mg, respectively). In the presence of forskolin (2 μM), IIC2-S942P had significant reduction in Gs activation, presumably because of the drastically reduced forskolin activation of IIC2-S942P mutant (Fig. 3D).

Based on equilibrium dialysis of the VC1/IIC2 complex, there is only one forskolin in the C1/C2 complex (Dessauer et al., 1997). Our data show that S942 of IIC2 is essential in interacting with the 7-acetyl group of forskolin. Thus, the forskolin molecule binds the site that is close to the Gs-binding site but distal to the ATP-binding site (Fig. 1). Based on the (IIC2/forskolin)2 model, IIC2-S942A or IIC2-S942P should not have significantly reduced activation by 7-deacetylforskolin relative to wild-type IIC2. However, we observed 2- and 10-fold reductions when IIC2-S942A and IIC2-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 C1/C2 complex and the IIC2 dimer. Further experiments are required to resolve this discrepancy.

Mutations near the forskolin-binding site affect both Gs and forskolin activation of C1/C2 complex. We constructed and analyzed two sets of IIC2 mutants that had mutations surrounding forskolin (Fig. 1). Tyr899 is conserved among all the mammalian adenylyl cyclases. Based on the IC1/IIC2 model using (IIC2/forskolin)2 structure, Tyr899 is located at the C1/C2 interface and is in contact with 13-methylenyl and 13-methyl groups of forskolin and Trp421 of C1 protein (Fig. 1). Thus, mutation at Tyr899 of IIC2 is likely to affect both interaction with IC1 and forskolin. We constructed a IIC2 mutant with the mutation of Tyr899 to Leu, and IIC2-Y899L was expressed normally (not shown). As expected, the purified IIC2-Y899L protein had substantially higher reduction in forskolin stimulation (~40-fold) than in Gs activation (6-fold) compared with wild-type IIC2 (Fig. 4, A and B). Submaximal Gs 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 Gs-binding site. To our surprise, mutation of Phe898 to Leu drastically reduced both Gs and forskolin activation of IIC2-F898 (Fig. 4, A and B). IIC2-F898 seemed to have reduced affinity to forskolin depicted by the increased EC50 value of forskolin activation in the presence of submaximal Gs (Fig. 4C). The purified IIC2-F898L, Y899L mutant had substantially higher reduction in forskolin stimulation (~40-fold) than in Gs activation (6-fold) compared with wild-type IIC2 (Fig. 4, A and B). Submaximal Gs 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 Gs-binding site. To our surprise, mutation of Phe898 to Leu drastically reduced both Gs and forskolin activation of IIC2-F898 (Fig. 4, A and B). IIC2-F898 seemed to have reduced affinity to forskolin depicted by the increased EC50 value of forskolin activation in the presence of submaximal Gs (Fig. 4C). The purified IIC2-F898L, Y899L mutant had substantially higher reduction in forskolin stimulation (~40-fold) than in Gs activation (6-fold) compared with wild-type IIC2 (Fig. 4, A and B). Submaximal Gs 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 Gs-binding site. To our surprise, mutation of Phe898 to Leu drastically reduced both Gs and forskolin activation of IIC2-F898 (Fig. 4, A and B). IIC2-F898 seemed to have reduced affinity to forskolin depicted by the increased EC50 value of forskolin activation in the presence of submaximal Gs (Fig. 4C).
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 adenyl cyclases but differ in the forskolin-insensitive type IX enzyme. Based on the (IIC2/forskolin)2 structure, Leu912 is located at the interface of C1/C2 complex and 6Å away from forskolin. Thus, it is involved in coordinating the binding of Tyr899 of IIC2 and Trp421 of IC1 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 IIC2 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 IIC2 to alanine or tyrosine and both IIC2-L912A and IIC2-L912Y could be expressed and purified similarly to wild-type IIC2 (Fig. 2A). When mixed with IC1, both IIC2-L912A and IIC2-L912Y had > 100-fold reduction in Gαs-stimulated activity. Both mutant proteins showed little forskolin stimulation unless Gαs (0.2 μM) was present (Fig. 4, B and C).

**Tyr1082-to-leucine mutation converts type IX adenyl cyclase to be forskolin-sensitive.** Type IX adenyl 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 C1 and C2 domains from type IX adenyl cyclase (IXC1 and IXC2, respectively) and tested whether they could form the functional enzyme and exhibit the proper biochemical properties.4 IXC1 and IXC2 proteins were both tagged with the influenza hemagglutinin epitope, and immunoblot analysis showed that IXC1 and IXC2 proteins had the expected 50- and 42-kDa size (Fig. 2B). Small molecular-weight proteins were seen in the lysates containing IXC1 and IXC2 proteins, presumably proteolytic products. E. coli lysates containing either IXC1 or IXC2 protein alone had no detectable adenyl cyclase activity. However, significant Gαs-stimulated enzyme activity was detected when the two lysates were mixed together (not shown). Lysates containing IXC1 and IXC2 protein did not exhibit forskolin sensitivity with or without Gαs (not shown). This result is consistent with the observation that type IX adenyl cyclase is Gαs-sensitive, but is insensitive to stimulation by forskolin (Premont et al., 1996).1

IXC2 protein was expressed in at least 100-fold greater quantity than IXC1 protein based on immunoblot analysis. IXC2 protein could be purified to near homogeneity with the use of a Ni-NTA column and then a Q-sepharose column. The yield of IXC2 protein was about 6 mg/liter of E. coli culture (Fig. 2C). The purification of IXC1 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 IXC1 protein, which was only about 20% pure, could be obtained from a liter of E. coli culture (Fig. 2C).1 Thus, the specific activity of the purified IXC1 and IXC2 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 IXC1 and IXC2 proteins could be activated by Gαs but not by forskolin (Fig. 5A).

As described above, mutations at Ser942 and Leu912 could affect the forskolin-stimulated activity of IIC1/IIC2 complex. The corresponding position for Ser942 and Leu912 of type II adenyl cyclase is Ala1112 and Y1082, respectively, in type IX adenyl cyclase. Thus, we tested whether IXC1+IXC2 proteins could become forskolin-sensitive by changing Ala1112 to serine, Y1082 to leucine, and both simultaneously. All IXC2-A1112S, IXC2-Y1082L, and IXC2-Y1082L, A1112S were expressed in similar amounts compared with that of wild-type IXC2 and could be purified to near homogeneity (Fig. 2, B and C). Mixed IXC1 and all three IXC2 proteins were stimulated by Gαs, using either E. coli lysates or purified proteins (Fig. 5A). Purified IXC2-Y1082L, A1112S protein mixed with IXC1, had weak but detectable forskolin stimulation, whereas both IXC2-Y1082L and IXC2-A1112S proteins mixed with IXC1 did not (Fig. 5A). In the presence of submaximal concentration of Gαs, both IXC2-Y1082L and IXC2-Y1082L, A1112S, when mixed with IXC1, had 10- to 15-fold stimulation by forskolin, whereas wild-type IXC2 had no observable forskolin stimulation (Fig. 5). Thus, Tyr1082 to leucine mutation converts soluble type IX adenyl cyclase into forskolin-sensitive enzyme.

**Discussion**

Soluble adenyl cyclases derived from membrane-bound adenyl cyclases have proven to be an excellent tool for studying the biochemical properties of adenyl cyclase. The C1 and C2 domains from their natural combination or from

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1 IXC1 and IXC2 proteins contain aa 320–741 and aa 1011–1353 of mouse-type IX adenyl cyclase, respectively.

2 IXC1 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 isofoms (chimeric C1/C2) 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\(_{as}\) 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\(_{as}\), which allows forskolin to synergistically enhance G\(_{as}\) 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 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).

References


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