Three Discrete Regions of Mammalian Adenylyl Cyclase Form a Site for Gsα Activation*

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The interaction between the α subunit of G protein Gs (Gsα) and the two cytoplasmic domains of adenylyl cyclase (C1 and C2) is a key step in the stimulation of cAMP synthesis by hormones. Mutational analysis reveals that three discrete regions in the primary sequence of adenylyl cyclase affect the EC50 values for Gsα activation and thus are the affinity determinants of Gsα. Based on the three-dimensional structure of C2-forskolin dimer, these three regions (C2 α2, C2 α3/β4, and C1 β1) are close together and form a negatively charged and hydrophobic groove the width of an α helix that can accommodate the positively charged adenylyl cyclase binding region of Gsα. Two mutations in the C2 α3/β4 region decrease the Vmax values of Gsα activation without an increase in the EC50 values. Since these three regions are distal to the catalytic site, the likely mechanism for Gsα activation is to modulate the structure of the active site by controlling the orientation of the C2 α2 and α3/β4 structures.

Mammalian adenylyl cyclase is the enzyme responsible for integrating multiple extracellular and intracellular signals to generate cAMP and thus activate cAMP-dependent protein kinase and cyclic nucleotide-gated ion channels (1, 2). All nine cloned mammalian and Drosophila rutabaga adenylyl cyclases are stimulated directly by the α subunit of Gs (Gsα),1 and all but type IX are activated by forskolin. Gsα and forskolin bind and activate adenylyl cyclases separately or synergistically when presented together (3, 4). Mammalian adenylyl cyclases are integral membrane proteins consisting of two homologous cytoplasmic domains (C1 and C2), each following a membrane domain (M1 and M2) (1, 2). The C1 and C2 domains form the catalytic core and can be engineered as a Gsα- and forskolin-sensitive soluble adenylyl cyclase, i.e. by mixing of IC1 protein (C1 domain of type I adenylyl cyclase) and IC2 protein (C2 domain of type II adenylyl cyclase) in vitro (5–8). In this paper, we describe mutations at three discrete regions of the soluble adenylyl cyclase, one in the IC1 protein and two in the IC2 protein, that significantly affect Gsα activation with little change in forskolin activation.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Purification of Wild Type and Mutant Forms of IC1 and IC2 Proteins—Plasmids used to express mutant forms of IC1 and IC2 were constructed using site-directed mutagenesis using pProExHAH6-IC1 or -IC2, as the phagemid (9). Oligonucleotides used for mutagenesis contained 10–12 complementary nucleotides flanking each side of the target codon(s) that was replaced with the appropriate codon. Mutations were confirmed by dideoxy nucleotide sequencing of phagemid DNA.

To express wild type and mutant forms of hexohistidine-tagged IC1 and IC2, the plasmids that encoded wild type or mutant forms of IC1 or IC2 were transformed into Escherichia coli BL21(DE3) cells. E. coli cells that harbored the desired plasmid were cultured in T7 medium containing 50 mg/ml ampicillin at 30 °C (10). When A600 reached 0.4, isopropyl-1-thio-β-D-galactopyranoside (100 μM) was added. After 3–4 h, the induced cells were then collected and lysed; IC2 proteins were purified using the nickel nitritrolactric acid column and fast protein liquid chromatography Q-Sepharose column as described (5). The Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis was used to determine the protein peak in the fractions from Q-Sepharose column. The concentration of proteins was determined using Bradford reagent and bovine serum albumin as standard (11). The construction of plasmid H6-pQE60-Gsα and the expression and purification of hexohistidine-tagged Gsα were performed as described (10). Gsα was activated by 30 μM AICl3 and 10 mM NaF, and adenylyl cyclase assays were performed at 30 °C for 20 min (5, 12).

Molecular Modeling of the Interaction between Gsα and Mammalian Adenylyl Cyclase—The Gsα structure was modeled using the sequence alignment and homology-modeling program LOOK version 2.0 (Molecular Applications Group) based on its sequence homology to GTPγS-bound forms of bovine G protein transducin α (13). The same protocol was tested by modeling the structure of Gαi which resulted in a model closely agreeing with GTPγS-bound Gαi structure (the root mean square deviation of the Cα atoms was found to be 1.17 Å) (14). A C2α heterodimer was modeled based on the structure of (IC24)2-forskolin2 (15). Gsα was docked onto the C2α heterodimer using program O (16) and data from the mutational analysis of Gsα and C2α soluble adenylyl cyclase (Ref. 17 and this paper).

RESULTS

Amino Acids in the IC2-α2 Region of IC2 Important in Gsα Activation—We use the sequence comparison to guide the mutagenic mapping of the Gsα binding site (Fig. 1). The IC2α2, but not the IC1α2, protein has weak Gsα- and forskolin-stimulated activity (~1000-fold less than mixed IC1 and IC2 proteins) (18). Thus, the C2α2 domain must include amino acid residues that contribute to binding and partial activation by Gsα and forskolin. Some of these residues are expected to be conserved among the C2α2 domain of mammalian and fly adenylyl cyclases but might not be conserved among the C2α domain of mammalian and fly adenylyl cyclases and the cyclase domains of membrane-bound guanyl cyclases. Fourteen IC2α2 mutants (to either alanine or leucine) at the 13 residues that fit this criterion were constructed, and all of them had relatively normal expression based on immunoblot (Figs. 1 and 2 (mutants IC2α2 C911A, C913A, I919A, and D921A and 10 other mutants not shown). We then tested for Gsα and forskolin activation using E. coli lysates containing the IC2α2 mutant proteins and wild type IC1.

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FIG. 1. Sequence alignment and secondary structure of the C2 domain of types II and IX adenylyl cyclases (ACII C2 and ACIX C2), C1 domain of type I adenylyl cyclase, the consensus sequences of C1 and C2 domains of mammalian and fly adenylyl cyclases (AC C1 or C2 core), and cyclase domains of membrane-bound guanylyl cyclases (Memb GC core) (21). Numbers above and below each row are amino acid residues of type II and type I adenylyl cyclases, respectively. Sequences underlined are absolutely conserved within the family of the indicated groups of cyclases, and sequences in black boxes are conserved among types I-VIII and rutabaga adenylyl cyclases but differ in type IX adenylyl cyclase. Secondary structure is based on the three-dimensional structure of the (IIC2)2 forskolin model (15). The sequences that are mutated in this study are marked by asterisks. Protein sequences include adenylyl cyclases from mammalian and Drosophila melanogaster (AC C1 and AC C2; ACI (GenBankTM accession number M25579), ACII (M80550), ACIII (M55075), ACIV (M80633), ACV (M88649), ACVI (M94968), ACVII (U12919), ACVIII (L26986), ACIX (Z30190), and Rutabaga (M81887)) and membrane-bound forms of guanylyl cyclases (Memb GC; GC-A (J05677), GC-B (M26896), GC-C (M55636), GC-D (L37203), GC-E (L36029), and GC-F (L36030), D. melanogaster (X72800, L35598), and sea urchin (M22444)). The sequence alignment was performed using DNA* MegAlign, J. Hein method (28), with point accepted mutation 250 weight table.

FIG. 2. The expression of IIC2 and IIC1 protein by immunoblot (A) and the purified IIC2 proteins (B). Lysates (10 μg) from E. coli BL21(DE3) cells that expressed IIC1 or IIC2 mutant proteins were prepared 3 h after isopropyl-1-thio-galactopyranoside induction, electrophoresed on 13% SDS-polyacrylamide gel electrophoresis, and immobilblotted with a monoclonal antibody, 12CA5 (A). Purified IIC2 mutant proteins (2 μg) were electrophoresed on 13% SDS-polyacrylamide gel electrophoresis and stained by Coomassie Blue (B). WT, wild type.
protein (Table I). Due to the semiquantitative nature of using *E. coli* lysates, we graded the enzyme activity of the lysates containing IIC2 mutants relative to that containing wild type IIC2 as follows: near normal (+++, >50% of the control), moderately reduced (++, 25–50% of the control), significantly reduced (+, 5–25% of the control), and little or no activation (±, <5% of the control). We expected that mutations at the $G_{\alpha}$-binding site in the IIC2 protein would cause a significant reduction in $G_{\alpha}$ activation but have little or no effect in forskolin activation. Only two of these IIC2 mutants, R913A and D921A, fit these criteria.2

To confirm that IIC2 mutants R913A and D921A had reduced $G_{\alpha}$ activation and to further characterize these mutants, we purified both IIC2 R913A and D921A to homogeneity and tested for their $G_{\alpha}$- and forskolin-activated activity when mixed with purified IC1 protein in vitro (Figs. 2 and 3; Table I). Both IIC2 R913A and D921A had near normal enzyme activity when stimulated by forskolin, whereas they had about a 15-fold reduction in $G_{\alpha}$-stimulated activity (Fig. 3A and B; Table I). In the presence of 10 $\mu$M forskolin, both IIC2 R913A and D921A had relatively normal $V_{\text{max}}$ values but had significantly increased EC$_{50}$ values for $G_{\alpha}$ activity (Fig. 3C and Table I).

While this research was in progress, the three-dimensional structure of the IIC2-forskolin complex was solved, and the structure revealed that Arg-913 and Asp-921 were located on the amphiphatic $\alpha$2 helix (Fig. 1) (15). To test the effect of mutations at the conserved residues located at the hydrophilic surface of $\alpha$2, IIC2 mutants E910A, L914A, N916A, E917A, and D924A were constructed and tested for their activity in response to $G_{\alpha}$ and forskolin activation (Table I; Figs. 2 and 3). Similar to IIC2 mutants R913A and D921A, the lysates containing IIC2 E910A, L914A, N916A, and E917A had significantly reduced or little $G_{\alpha}$ activation but only moderate reduc-

![Table I](image-url)
Amino Acids in the IIC\(_{2}\) α3/β4 Region of IIC\(_{2}\) Important in \(G_{s}\) Activation—In contrast to the sensitivity of other mammalian and fly adenylyl cyclases to both \(G_{s}\) and forskolin, type IX enzyme is activated by \(G_{s}\), but not by forskolin (19). We hypothesize that the crucial residue(s) for forskolin binding is missing in the C\(_{2}\) domain of type IX enzyme. Sequence comparison among the C\(_{2}\) domains reveals that eight amino acid residues are absolutely conserved among type I-VIII and rutabaga adenylyl cyclases but differ in type IX enzyme (Fig. 1). Five of them (Gln-880, Ser-881, Ser-942, Ser-990, and Asn-992) have been mutated to alanine and tested for their activation by \(G_{s}\) and forskolin. Fortuitously, another region that affects \(G_{s}\) activation was revealed. Lysates containing mutant IIC\(_{2}\) N992A had near normal forskolin activation but a significantly reduced \(G_{s}\) activation. Lysates containing mutant IIC\(_{2}\) S990A had near normal \(G_{s}\) activation and tested for their activation by \(G_{s}\) and forskolin (Figs. 2 and 3; Table I). All four mutants had about a 10-fold reduction in \(G_{s}\) activation and less than a 2-fold reduction in forskolin activation. Similar to IIC\(_{2}\) R913A and D921A, all four mutants had significant increases in EC\(_{50}\) values for \(G_{s}\) activation. These data indicate that six amino acid residues, Glu-910, Arg-913, Leu-914, Asn-916, Glu-917, and Asp-921, of IIC\(_{2}\) are involved in \(G_{s}\) activation.

Fig. 4. Biochemical analysis of IIC\(_{2}\) mutants that have mutations in the α3/β4 region. Adenylyl cyclase activity of purified IIC\(_{2}\) mutant activated by forskolin (A), \(G_{s}\) (B), and \(G_{m}\) in the presence of 10 μM forskolin (C) is the same as described in Fig. 3. WT, wild type.

Fig. 5. Biochemical analysis of IIC\(_{1}\), F293A. Adenylyl cyclase activity of purified IIC\(_{1}\)-F293A activated by forskolin (A), \(G_{s}\) (B), and \(G_{m}\) in the presence of 10 μM forskolin (C) is the same as described in Fig. 3. WT, wild type.

To further characterize IIC\(_{2}\) S990A, N992A, and S990A/N992A, the three mutant proteins were purified to homogeneity and tested for \(G_{s}\) and forskolin activation (Figs. 2 and 4; Table I). IIC\(_{2}\) S990A was normal in forskolin activation, whereas IIC\(_{2}\) N992A and S990A/N992A had only a slight reduction in forskolin activation (Table I and Fig. 4A). Interestingly, IIC\(_{2}\) S990A had about 3-fold-enhanced \(G_{s}\) activation, whereas IIC\(_{2}\) N992A had 4-fold-reduced \(G_{s}\) stimulation (Table I and Fig. 4B). The \(G_{s}\) activation of double mutant IIC\(_{2}\) S990A/N992A was nearly normal, presumably due to compensation by the two mutations (Table I and Fig. 4B). When simultaneously stimulated by \(G_{s}\) and forskolin, IIC\(_{2}\) N992A had a lower \(V_{\text{max}}\) value but relatively normal EC\(_{50}\) value (Table I and Fig. 4C). In contrast, IIC\(_{2}\) S990A had a decrease in both EC\(_{50}\) and \(V_{\text{max}}\) values; the decrease in EC\(_{50}\) could explain the apparent higher \(G_{s}\) activation when assayed only with \(G_{s}\) (Table I and Fig. 4C). Double mutant IIC\(_{2}\) S990A/N992A had a near-normal \(V_{\text{max}}\) value and a slightly increased EC\(_{50}\) value. The three-dimensional structure of IIC\(_{2}\)-forskolin reveals that Ser-990 is the only residue that joins the α3 and β4 regions of IIC\(_{2}\); thus, it might play a pivotal role in controlling the relative orientation between α3 and β4 of IIC\(_{2}\) (Fig. 1). How the change from Ser-990 to Ala alters both EC\(_{50}\) and \(V_{\text{max}}\) values for \(G_{s}\) activation remains elusive.

\(^{3}\) All five mutants expressed normally, based on immunoblot. Lysates containing IIC\(_{2}\), Q880A and S881A had near normal and moderately reduced activation by \(G_{s}\) and by forskolin, respectively. Lysates containing IIC\(_{2}\), S942A had near normal activation by \(G_{s}\) but moderately reduced activation by forskolin. Purified IIC\(_{2}\) S942A had 5-fold reduction in forskolin stimulation but normal \(G_{s}\) activation (S.-Z. Yan, Z. H. Huang, and W.-J. Tang, unpublished data).

\(^{4}\) EC\(_{50}\) and \(V_{\text{max}}\) values were calculated from the fitted curve (Table I).
To further examine the region containing Ser-990 and Asn-992, we constructed and tested six more alanine-scanning IIC2 point mutants in the Asn-987–Lys-995 region. Two more amino acid residues, His-989 and Phe-991 were shown to be involved in Gs activation. Lysates containing IIC2 H989A and F991A had a significantly reduced Gs activation but had near normal or moderately reduced forskolin stimulation, respectively (Table I). Similar results were observed when the purified mutant proteins were used (Table I and Fig. 4). When the mutants were stimulated by Gsa and forskolin simultaneously, IIC2 H989A exhibited a lower Vmax value but relatively normal EC50 value. When the same assay was applied to IIC2 F991A, a significant increase in EC50 value was observed; due to low enzyme activity, the Vmax value of this mutant could not be determined. It is worth noting that two IIC2 mutants in this region (at the α3/β4 region, IIC2 S990A and N992A) had reductions in Vmax values but had little increases in EC50 values (Fig. 4C); this is in contrast to the IIC2 α2 mutants that all have increased EC50 values.

**F293 at the N Terminus of IC1 Is Important in Gsa Activation**—The C1 and C2 domains of mammalian adenylyl cyclase have ~25–50% identity, and there is a high degree of sequence conservation between dimer interface residues in C1 and C2 based on the interaction of the IIC2 dimer in the (IIC2)2-forskolin2 crystal structure (15). Thus, the interaction between C1 and C2 domains might be similar to that of the IIC2 dimer in (IIC2)2-forskolin2 crystal structure. Since the α2 region of the IIC2 protein is close to the interface of the IIC2 dimer, we asked whether Gs could interact with the amino acid residue(s) located at the C1 domain near the α2 helix of the IIC2 protein in order to facilitate the interaction between the C1 and C2 domains. The contact of the IIC2 dimer in IIC2-forskolin model predicts that the sequences at the proposed N terminus of IC1 are likely candidates (Fig. 6A). Truncation analysis revealed that the IC1 mutant, Δ271–292, a deletion of amino acid residues 271–292, had normal Gs activation and forskolin stimulation (Table I). We then constructed and tested the Gsa- and forskolin-stimulated activity of four IC1 mutants, F293A, H294A, S305A, and L307A, that have a mutation in the N-terminal region of IC1. Only one IC1 mutant, IC1 F293A, exhibited little Gs activation but retained a near normal forskolin stimulation when either E. coli lysate containing IC1, F293A or purified IC1 protein mutant (Figs. 2 and 5; Table I) were used. When stimulated by Gsa and forskolin, a significant increase in the EC50 value of mutant IC1 F293A was also observed (Fig. 3). We also tested the conserved amino acid residues in the putative β4/β5 region, which is adjacent to the putative N terminus of IC1, and found that none of the mutants exhibited a preferential

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**Footnotes:**

4 When a lysate containing mutant IIC2 N987A was assayed, significant reduction in both Gsa and forskolin activation was observed. However, purified IIC2 N987A exhibited near normal forskolin activation but somewhat reduced Gs activation. Such a discrepancy is likely due to the lower expression of IIC2 N987A (though not shown by immunoblot in Fig. 2). Lysates containing IIC2 K998A and K995A had near normal Gsa and forskolin activation, and lysate containing IIC2 D993A had moderately reduced Gs and forskolin activation. Mutant IIC2 F994A is described in Footnote 2.

5 Based on immunoblot, IC, F293A, H294A, and S305A had normal expression. IC1, H294A and S305A had significantly reduced and little Gs- and forskolin-stimulated activity, respectively. IC1, L307A had more than a 10-fold reduction on expression based on immunoblot and also had no detectable enzyme activity even when Gs and forskolin was used.
tial reduction in Gs activation. These data indicate that the conserved Phe-293 at the C2 domain is crucial in Gs activation. In addition, the data provide support for the idea that the structure of the C2 dimer is valid in examining the structure of the C1C2 heterodimer.

**DISCUSSION**

The mutagenesis based on the sequence comparison of adenyllyl and guanylyl cyclases and the molecular structure of IIC2 has revealed that 10 amino acid residues (Glu-910, Arg-913, Leu-914, Asn-916, Glu-917, Asp-921, His-989, Ser-990, Phe-991, and Asn-992) within two regions (α2 and α3/β4) of IIC2 are essential for Gs activation. Although these two regions of IIC2 proteins are 68 amino acids apart in the primary sequence, they are in close proximity in the structure of the IIC2-forskolin dimer (Fig. 6, A and B). The Gs binding site is separate from but close to the proposed G protein βγ binding site (N terminus of α3) of type II adenyllyl cyclase, which is consistent with ability of the Gβγ to synergize the Gs activation of type II enzyme (Fig. 6A) (20–22). The putative Gs binding site forms a negatively charged and hydrophobic groove 10 × 10 × 15 Å, capacious enough to bind an α helix (Fig. 6, B and C). This negatively charged groove could attract the positively charged surface formed by the putative adenyllyl cyclase binding region of Gs (α2/β4 (switch 2), α3/β5, and α4/β6) (13, 14, 17) (Fig. 6, B and C). It is also worth noting that the sequences at the C1 α2 region are reasonably conserved among the Gs-sensitive adenyllyl cyclase (types I, V, and VI) but not other isoforms; thus, it may be the determinant for Gs binding, a site independent of that for Gz (23).

The complex of C1 and C2 domains are necessary for potent activation by Gs. Although how the C1 domain interacts with the C2 domain remain elusive, we hypothesize that their interaction is similar to the contact of the IIC2 dimer based on the following observations: the relative high degree homology of C1 and C2 domains, the sequence conservation at the dimer interface of C1 and C2 domains based on the structure of IIC2 dimer, and the success of the C1C2 model to predict the importance of the N terminus of C1 domain for Gs activation. The C1C2 model was constructed using the homology modeling based on the (IIC2-forskolin)2 structure (Fig. 6). The model shows that the putative adenyllyl cyclase binding regions of Gs can dock well to the negatively charged groove that is its presumed site in adenyllyl cyclase (Fig. 6B); the validity of this model remains to be tested experimentally.

How does Gs activate adenyllyl cyclase? Based on mutational analysis, we hypothesize the following events leading to the activation of adenyllyl cyclase by Gs. The greatest effects on EC50 values for Gs activation map to the C2 α2 helix, suggesting that in the first step, Gs binds to adenyllyl cyclase with an energetic driving force provided primarily by the α2 helix of the C2 region. The sensitivity to mutation at Phe-293 demonstrates a potential role for Gs in bridging the C1 and C2 domains and promoting their juxtaposition in a catalytically productive manner. The observation that mutation of the α3/β4 of C2 can alter the Vmax for Gs-stimulated catalysis suggests that this region is an allosteric linker between the Gs binding site and the active site. Indeed, both α2 and β4 directly participate in forming the ventral cleft-containing active site. The Gs binding regions occur on portions of the two longest α helices in the cyclase structure. The α2 and α3 helices provide 30- and 37-Å long lever arms, respectively, such that a modest change in their mutual orientation at the Gs binding site could be converted scissorwise into a large change in the structure of the active site, leading to catalytic activation. The determination of the molecular structure of C1C2 and GsC2Cz is in progress, and the solution will yield valuable insight into how Gs activates adenyllyl cyclase.

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