The enzymatic activity of adenyl cyclase is the key step in regulating the intracellular cAMP concentration upon stimulation of a variety of hormones, neurotransmitters, and other regulatory molecules. There are at least nine distinct mammalian adenyl cyclases which have a similar structure (Fig. 1A) (1-11). This includes two intensely hydrophobic domains (M1 and M2) and two -40 kDa cytoplasmic domains (C1 and C2). The C1 and C2 domains contain sequences (C1α and C2α) that are similar to each other and to other adenyl and guanylyl cyclases (12, 13). Each isoform of adenyl cyclase has its own distinct tissue distribution and unique regulatory properties, providing modes for different cells to respond divergently to similar stimuli (12, 14).

Membrane-bound adenyl cyclases are expressed in small quantities, and the enzyme is labile and difficult to manipulate in detergent-containing solutions. To facilitate biochemical and structural analysis, a soluble adenyl cyclase has been constructed by linking the C1α and C2α domains of type I and type II adenyl cyclases, respectively (15). The resulting protein is sensitive to activation by Gαs-1 and forskolin and to inhibition by P-site inhibitors, indicating the essential roles of C1α and C2α domains for catalysis and regulation. In this paper, we describe the expression and purification of the C1α and C2α domains of type I and type II adenyl cyclase, respectively. Alone, each has no adenyl cyclase activity; however, mixing of the two domains in vitro results in Gαs- and forskolin-activated enzyme activity.

EXPERIMENTAL PROCEDURES

Plasmids—For construction of the expression plasmid vector pProEx-HAH6, the NcoI and EcoRI 4.9-kb fragment of pProEx-1 (Life Technologies, Inc.) was ligated with the phosphorylated linkers (5'-CATGTCACATCACATGCAAGGCGGCGCTACGGATGTG-3') and the 5'-ATTGGCATTAGGGATGAG-3' and 5'-ATTCGCGGACATCGATGCTG-3' linkers. Proper insertion of DNA at the NcoI site of pProEx-HAH6 vector resulted in the expression of a fusion protein that contained both HA1 and hexo-histidine tags at the N terminus (Fig. 1A). The BspHI and HindIII fragments were excised from pTrc-I and pTrc-IIC2-L3 (15). The resulting fragments were ligated with NcoI and HindII-digested pProEx-HAH6 to construct vectors, pProEx-HAH6-I and pProEx-HAH6-IIC2. To construct pProEx-HAH6-IIC2, the BspHI-blunted EcoRI fragment was excised from pUC-IIC2 and ligated into pProEx-HAH6 that was digested with NcoI and Smal (15). To construct pProEx-HAH6-IIC2, an 0.8-kb DNA fragment encoding IIC2 was amplified by 15 cycles of polymerase chain reaction using pProEx-HAH6-IIC2 as the template, Vent DNA polymerase, and two oligonucleotides (5'-CAGGAATTCTAGGAACTGCTTCTTCACAC and 5'-TTGGGTCTTATTTTACTGCTGCGTGA) as the primers. The resulting DNA was digested with EcoRI and HindIII and ligated into pProEx-HAH6 that was digested with the same enzymes. Plasmids pProEx-HAH6-IIC2, -IIC2, -IIC2, -IIC2-IIC2, and -IIC2-IIC2 were used to express C1α, C2α, IIC2, and IIC2, respectively.

Expression of Soluble Adenyl Cyclase—Escherichia coli cells with a plasmid were grown in Luria’s broth containing ampicillin (50 μg/ml) at 30 °C and IPTG to 0.1 mM was added when the culture reached an A600 of 0.4. Cells were harvested at suitable times, centrifuged at 6,000 x g at 4 °C, and frozen. Frozen cells was thawed in 1/10 culture volume of 0.5 mM phenylmethylsulfonyl fluoride, lysozyme to 0.1 mg/ml was added, the cells were sonicated briefly, and the supernatant after centrifugation (150,000 x g for 30 min, 4 °C) was saved. The concentration of proteins was determined using Bradford reagent (Bio-Rad) and bovine serum albumin as standard (16). The proteins were separated by electrophoresis on 13% SDS-PAGE and immunoblot was performed using the ECL system (Amersham). Asites fluid of hybridoma 12CA5 was raised and collected as described (17).

Purification of Soluble Adenyl Cyclase—All steps of the purification were performed at 4 °C in a cold room. Supernatant of E. coli lysate (from 4 liters, harvested 4 h after IPTG induction) was applied directly to a 20-mL Ni-NTA column (Qiagen) that was equilibrated with Tris-HCl, pH 8.0, 0.5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and lysozyme to 0.1 mg/ml was added. The column was washed with 500 mM NaCl and 100 ml of 200 mM Tris-β-mercaptoethanol, containing 500 mM NaCl and 100 ml of 200 mM Tris-β-mercaptoethanol, containing 20 mM imidazole (pH 7.0). The column was then eluted with 100 ml of 200 mM Tris-HCl, pH 8.0, containing 150 mM imidazole (pH 7.0). The eluate was concentrated by ultrafiltration (Amicon, positive pressure ultrafiltration device, PM 10 membrane) and then diluted 2-fold with 1/10 culture volume of Tris-HCl, pH 8.0, 0.1 mM EDTA, and 1 mM N20-500 containing 100-500 mM NaCl; P, 0.1 mM phenylmethylsulfonyl fluoride, I20-150 containing 20-150 mM imidazole, pH 7.0.
pressed IC1IIC2 had increased forskolin-stimulated adenylate cyclase activities from both E. coli lysates that contained IC1IIC2; the peak of IC1IIC2 was determined using the lysates containing IC1. Recombinant protein, IC1 was eluted at about 350 mM NaCl and IC1IIC2 at about 200 mM NaCl. The peak fractions of IC1 and IC1IIC2 were then separated by electrophoresis on 11% SDS-PAGE and analyzed for purity by Coomassie Blue staining. The purest fractions of IC1 were then applied to a Pharmacia Superdex 200 HR10/30 gel filtration column that had been equilibrated with T20E1D1 and peak activity was collected. Purified IC1, 12CA5, and IC1IIC2 were concentrated in a Centricon 10 microconcentrator (Amicon) and stored at −80°C (protein concentration ≥1 mg/ml).

**RESULTS AND DISCUSSION**

Expression of IC1IIC2, IC1, and IIC2—A soluble adenylyl cyclase was constructed by linking the conserved cytoplasmic domains from type I (C1) and type II (C2) adenylyl cyclases (15). The resulting protein, IC1IIC2, could be activated by Gs, forskolin, and the activated enzyme could be inhibited by P-site inhibitors. IC1IIC2 was tagged with hexa-histidine and the HA1 epitope at the N terminus to facilitate the detection and purification (Fig. 1A). Hexa-histidine allowed affinity purification using immobilized metal affinity chromatography (e.g. using Ni-NTA resin), and the HA1 epitope permitted the detection of recombinant protein using monoclonal antibody made to a bacterial lysate (from BL21-DE3 cells) containing IC1IIC2 (300 μg in T20D1N100) was applied under the same conditions. Adenylyl Cyclase Assay—Activity was assayed in a 100-μl final volume for 10–20 min at 30°C in the presence of 10 mM MgCl2 (18). Recombinant IC1, IIC2, and IC1IIC2 were premixed on ice for 10 min before assay. Recombinant Gαs was purified and activated as described (19, 20). Membranes of Sf9 cells that expressed IM1C1-(1–570), IM1C1-(571–1193), IM1C1-(1–848), IM1C2, and IM2C2 were prepared as described (21).}

**Fig. 1. Properties of soluble type I-type II adenylyl cyclase constructs expressed in E. coli.** A, the top shows a model of mammalian adenylyl cyclase with each of its regions labeled. ACI = type I enzyme; ACII = type II enzyme. Below are shown the constructs used in this work. The first four include, at their amino termini, a hexahistidine and a HA1 epitope tag with a short linker to create the EcoRI and Ncol restriction sites. B, adenylyl cyclase activities and protein expression of the IC1IIC2 construct in protease-deficient E. coli strains, BL21DE3 and SG22094. Samples (10 μl) were taken for enzyme assay and immunoblot assay with either 12CA5 or C2-1077 antibodies at the indicated times after IPTG induction. C, adenylyl cyclase activities for a mixture of 10 μl of a bacterial lysate (from BL21DE3 cells) containing the components at the top that were obtained after the indicated hours of induction with IPTG and 10 μl of an lysate (from BL21-DE3 cells) containing either IC1 or IC1. The immunoblot shows the amount of the component at the top after the indicated hours of induction by IPTG. p.i. = post-IPTG induction. Adenylyl cyclase activity is shown as nmol/min/mg. Data are representative of two experiments.
that expressed IC1 or IIC2 (expected molecular mass as 27 and 31 kDa, respectively), indicating that the IC1 and IIC2 protein were stable, soluble proteins. Adenylyl cyclase activities of E. coli lysates that expressed either IC1 or IIC2 were not different from those of lysates of E. coli that carried the control vector (0.01 nmol·min⁻¹·mg⁻¹). However, mixing of the lysates, each expressing one of these constructs, resulted in high enzyme activity (2.9 nmol·min⁻¹·mg⁻¹, Fig. 1C). The enzyme activity correlated generally with expression (monitored by immunoblot) of IC1 and IIC2 from cells (Fig. 1C).

Purification of IC1 and IIC2—IC1 could be purified by Ni-NTA (Fig. 2A). The enriched IC1 could be further purified using a FPLC Mono Q column and Superdex 200 gel filtration. The 30-kDa protein was the adenylyl cyclase (indicated by arrow and verified based on enzyme activity and immunoblot, Fig. 2, A and C). A 29-kDa protein was a major contaminant. The recovery of forskolin-stimulated adenylyl cyclase activity was only 5%, and the yield was 50 μg from each liter of E. coli culture.

The same procedure did not succeed in the purification of IIC2. The majority of the adenylyl cyclase activity from IIC2 did not bind to Ni-NTA, probably due to proteolysis or masking of the hexo-histidine tag. When lysates containing IC1 were mixed with lysates containing IIC2 and applied to Ni-NTA column, most of adenylyl cyclase did not bind to the column (data not shown). This indicated that the binding between IC1 and IIC2 was not strong enough for copurification of IC1 and IIC2.

To purify IIC2, we used IIC2Δ3, a construct that deleted 36 terminal amino acids of IIC2, residues that are not conserved among mammalian adenylyl cyclases. HA1 and hexo-histidine-tagged IIC2Δ3 protein was expressed as a soluble protein, based on immunoblot, and formed Gs-dependent adenylyl cyclase when mixed with lysate containing IC1 in vitro (Fig. 1C). IIC2Δ3 could be purified by Ni-NTA and, after subsequent chromatography on FPLC-Mono Q, 95% pure protein (29 kDa) was obtained (Fig. 2B). Its identity was confirmed by immunoblot (Fig. 2C). The recovery of adenylyl cyclase activity was about 35%, and the yields for IIC2Δ3 proteins were 2 mg from each liter of E. coli culture.

Characterization of Purified IC1 and IIC2Δ3—Purified IC1 and IIC2Δ3 proteins by themselves had little enzyme activity (Table I). Mixing of IC1 and IIC2Δ3 proteins resulted in Gs- and forskolin-stimulated activity (Table I, Fig. 3, A and B). As it did for IC1IIC2, GTPγS-Gs activated synergistically with forskolin in activating mixed IC1 and IIC2Δ3, while 2’d-3’-AMP inhibited the activity (Fig. 3, C and D). NaCl inhibited the activity of mixed IC1 and IIC2Δ3 (IC50 = 300 mM, not shown). The highest turnover number for adenylyl cyclase activity of mixed IC1 and IIC2Δ3 (when activated by Gs and forskolin simultaneously) was 8.2 s⁻¹, similar to rates of the purified native and recombinant type I adenylyl cyclase (19, 25, 26).

Thus, the purified soluble adenylyl cyclase has the proper catalytic and regulatory properties and could be used as a
model system for the biochemical and structural analysis of mammalian adenylyl cyclase.

Increased concentrations of IIC2-Δ3 markedly increased adenylyl cyclase activity when added to a fixed amount of IC1 (6 mM) (Fig. 4A). The half-saturable concentration (EC50) of IIC2-Δ3 for forskolin- and Gαs-GTPγS-activated activity was 0.8 and 1.3 μM, respectively. When Gαo and forskolin were used together, EC50 of IIC2-Δ3 fell about 10-fold to 0.08 μM. This suggested that the synergistic effects of Gαo-GTPγS and forskolin on enzyme activity reflected an increase in the affinity of IC1 and IIC2-Δ3 for each other.

Purified IC1 or IIC2-Δ3 were subjected to gel filtration on Pharmacia Superdex 200 using T20E1D1N500 (g), IC1 and IC2 (300 mM) as the buffer. A major peak of adenylyl cyclase activity consistent with a globular 30-kDa protein was observed when expressed alone; half of the size for the enzyme activity of lysates containing IC1 and IC2. When IC1 alone was applied, a major peak of adenylyl cyclase activity consistent with a globular 55 kDa was observed; the shift from 30- to 55-kDa protein was due to the lower concentration of NaCl. This suggested that IC1 might exist as a dimer or as a nonglobular protein at lower salt concentrations (100 mM). When the mixture of IC1 (5 μg) and IIC2-Δ3 (50 μg) was tested, a peak of adenylyl cyclase activity consistent with 45-kDa proteins was observed. The shift in elution profile suggested that IC1 and IIC2-Δ3 did interact. The low apparent molecular mass (45 kDa instead of the expected 60 kDa) could be accounted for by dissociation of the complex of IC1 and IIC2-Δ3 and/or an unusual shape of the complex.

Complementation of IC1 and IIC2-Δ3 by the Halves of Adenylyl Cyclases—Although IC1 and IIC2 did form a complex with adenylyl cyclase activity, we failed to detect enzyme activity when two cytoplasmic domains from type I enzyme (IC1 and IC2) were used (linked or a mixture of IC1 and IC2) (not shown). To investigate the relative affinity of IC1 for either IC1 or IIC2, we tested the ability of IC1 to complement the carboxyl-terminal half of type I or type II enzyme (IM1C1 and IM2C2) (Fig. 4C). As reported previously, the truncation mutants of type I and type II adenylyl cyclases that consisted of either the aminoterminal half (INM1C1-(1–570) and INM1C1-(1–484)) or the carboxyl-terminal half (IM2C2 and IM2C2) of the protein had no detectable adenylyl cyclase activity when expressed alone; however, coexpression of the amino- and the carboxyl-terminal
halves resulted in G_{a} and forskolin-regulated adenylyl cyclase activity (Fig. 1A) (21). IC_{1} and IIC_{2}\Delta 3 each had no adenylyl cyclase activity alone, and SF9 cell membranes containing the amino- or carboxyl-terminal halves of adenylyl cyclase had enzymatic activities that were similar to the control cell membranes (that containing \beta-galactosidase) (Fig. 4, B and C). When IIC_{2}\Delta 3 was added to SF9 cell membranes containing either INM_{2}C_{1}\cdot(1-570) or INM_{2}C_{1}\cdot(1-484), there was up to a 40-fold increase in forskolin-stimulated adenylyl cyclase activity (Fig. 4B). In contrast, there was no increase in enzyme activity when IIC_{2}\Delta 3 was mixed with SF9 cell membranes containing IM_{2}C_{2}. When IC_{1} was reconstituted with SF9 cell membranes containing IIM_{2}C_{2}, there was up to a 20-fold increase in forskolin-stimulated adenylyl cyclase activity (Fig. 4C). However, only a 3-4-fold increase in enzyme activity was observed when IC_{1} was reconstituted with SF9 cell membranes containing IM_{2}C_{2}. The EC_{50} of IC_{1} to reconstitute adenylyl cyclase activity of IIM_{2}C_{2} was at least 10-fold lower than that of IM_{2}C_{2}.

There has been considerable speculation about the roles of the transmembrane domains of adenylyl cyclases (12). The transmembrane domains target adenylyl cyclase to the plasma membrane for interaction with, and thereby regulation by, G proteins. Our studies indicate that the two cytoplasmic domains of mammalian adenylyl cyclases do not appear to have high affinity for each other. EC_{50} for IIC_{2} to complex with IC_{1} is 0.8 and 1.3 \mu M in forskolin- and G_{a}-stimulated activity, respectively.

Affinity between two natural linked cytoplasmic domains (IC_{1} and IC_{2}) is at least 10-fold less than that between IC_{1} and IIC_{2}. Thus, the transmembrane domain (M_{2}) could link and facilitate the interaction of the two cytoplasmic domains by creating a high local concentration. It remains to be determined whether the transmembrane domains have additional functions, such as altering the interaction between two cytoplasmic domains for regulations or serving as pore structures.

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