Expression and Characterization of Calmodulin-activated (Type I) Adenylylcyclase*

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A complementary DNA that encodes a bovine brain, calmodulin-sensitive (type I) adenylylcyclase has been inserted into the baculovirus genome under the control of the strong polyhedron promoter. Expression of the recombinant adenylylcyclase in SF9 cells using recombinant baculovirus increases adenylylcyclase activity in cell membranes to 10–20 nmol·min⁻¹·mg⁻¹ (approximately 0.1% of membrane protein). The catalytic activity of the recombinant adenylylcyclase can be stimulated by Gα, calmodulin, or forskolin, and it can be inhibited by adenosine analogs and by G protein βγ subunits. The specific activity of the purified recombinant protein approximates 5 μmol·min⁻¹·mg⁻¹. This is similar to that of the enzyme purified from bovine brain.

Type I adenylylcyclase has a quasiduplicated structure. There are two membrane-spanning domains, each with six putative transmembrane helices, and there are two presumed nucleotide-binding domains that are about 55% similar to each other. No catalytic activity is detectable when each half of the adenylylcyclase molecule is expressed by itself. However, coexpression of the two halves results in considerable enzymatic activity. Interaction between the two halves of adenylylcyclase may be necessary for catalysis.

Cyclic AMP and the enzymes that synthesize the nucleotide, adenylylcyclases, are found in most organisms and in all mammalian tissues. Two signaling pathways are known to regulate the activity of one or more forms of mammalian adenylylcyclase. Receptors for many hormones and neurotransmitters activate heterotrimeric G proteins¹ that, in turn, either stimulate (Gα) or inhibit (Gγ) adenylylcyclase activity (1). Elevation of intracellular Ca²⁺ concentrations can also activate calmodulin-sensitive forms of the enzyme (2, 3). At least three types of mammalian adenylylcyclase have been distinguished biochemically: a calmodulin-sensitive form of the protein from brain (4–6) and other tissues (2), a calmodulin-insensitive enzyme from nonneural tissues and from brain (7, 8), and a soluble protein from testes that is stimulated by calmodulin but is not regulated by G proteins (9, 10).

Several genes that encode bacterial (11–15) and yeast (16, 17) adenylylcyclases have been cloned and sequenced. We recently purified a membrane-bound, calmodulin-sensitive adenylylcyclase from bovine brain and isolated cDNA clones that encode this protein (arbitrarily designated as type I) (18). Using probes based on the type I sequence, three additional mammalian adenylylcyclases have been cloned subsequently (and named chronologically). Type II adenylylcyclase, cloned from rat and bovine brain cDNA libraries, is found in both brain and lung²; type III is expressed abundantly in olfactory tissue (20); type IV was cloned from a testicular cDNA library.³ Only the type I enzyme appears to be sensitive to calmodulin. Although there are distinct regions of the four proteins that differ markedly in their amino acid sequence, hydropathy analysis suggests that they all share the same general topology with regard to the membrane. Each protein is hypothesized to contain two domains that are associated with the lipid bilayer, each of these consisting of six transmembrane helices. There appear to be two large cytoplasmic domains; the first of these lies between the two sets of membrane-spanning helices, and the second is at the carboxyl terminus (see Fig. 8).

Expression of individual forms of adenylylcyclase and alterations of these proteins offer obvious paths to their more detailed characterization. We describe herein the expression of type I adenylylcyclase in SF9 (fall army worm ovarian) cells using the recombinant baculovirus expression system (21–23). We document the utility of this approach for expression of this complex protein and present the results of its initial characterization.

MATERIALS AND METHODS

Plasmid Construction—For convenience of cloning, a 4.1-kb EcoRI fragment of DNA containing the entire coding sequence of type I adenylylcyclase was transferred from pMKAC (18) to the EcoRI site of pBluescript SK−. The resulting plasmid was linearized by digestion with HindIII and was then digested with exonuclease III and nuclease S1 to delete approximately 100 bp of untranslated sequence from the 5' end of the cDNA (24). After treatment with EcoRI, a mixture of 4 kb of DNA was isolated and cloned into pBluescript SK− (digested with SmaI and EcoRI). Clones were screened by double-stranded DNA sequencing, and one clone (designated pSKACΔ58) was identified as described elsewhere (25). The cloned insert was excised from the plasmid using the appropriate restriction enzyme, purified on a 1-mL Prep-Gene column (Promega Corporation), and quantitated by spectrophotometry.

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¹ The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; Gα, the α subunit of the G protein that stimulates adenylylcyclase; Gβγ, the short form of Gβγ, expressed in E. coli; B-rAC, baculovirus containing the cDNA for bovine type I adenylylcyclase; GTPγS, guanine nucleotide with gamma-phosphate; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, diithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); bp, base pair(s). The protease inhibitors used are: A, 22 mg/liter each of t-1-tosylamide-2-phenylethyl chloromethyl ketone, t-1-chloro-3-tosylamido-7-amino-2-heptanone, and phenylmethylsulfonyl fluoride; B, A plus 3.2 mg/liter each of leupeptin and lamma trypsin inhibitor; C, B plus 0.5 mg/liter aprotinin.

² R. Reed, manuscript in preparation.

³ B. Gao and A. G. Gilman, unpublished observations.
was selected in which all of the putative 5'-noncoding region was removed and a new NcoI site was generated at the initiating methionine residue. To create a better site for initiation of translation (AXXATGG) (25), an EcoRI-blunted NcoI fragment was excised from pSKACΔ58 and ligated into pBluescript SK- that had been cut with EcoRI and EcoRV; this plasmid is designated pSKACΔ58-13. For expression of adenylyl cyclase in human 293 cells and simian COS-m6 cells, a 4.0-kb fragment of DNA was excised from pSKACΔ58-13 with HindIII and XhoI and was ligated into pCMV-5 that had been digested with the same enzymes; this plasmid is designated pCMVAC-N (18). For construction of recombinant baculovirus, the NcoI-EcoRI fragment from pSKACΔ58 was ligated into baculovirus vector pAcC4. For construction of mutant adenylyl cyclases, convenient restriction enzyme sites were chosen to release DNA fragments, and oligonucleotides were ligated with these fragments to create initiation or stop sites. For mutant ΔN, a SalI-EcoRI fragment was isolated from pSKACΔ58. For mutants NMCI, fragments were isolated from pCMVACΔ58 by digestion with EcoRI and partial digestion with RsaI. For mutant MCI, a HindII-EcoRI fragment was isolated from pSKACΔ58. These fragments were ligated with phosphorylated adaptors (sequences listed below for each mutant) and pBluescript SK- that had been digested with XbaI and EcoRI. The mutant ΔN was cloned into the XbaI and EcoRI sites of the baculovirus vector pVL1393. The EcoRI-blunted XbaI fragments of mutants NMCI and MCI were cloned into the Smal and EcoRI sites of baculovirus vectors pVL1392 and pVL1393, respectively.

**Adapter ΔN**

\[
5' \quad C \quad T \quad G \quad A \quad A \quad A \quad A \quad C \quad A \quad T \quad G \quad G \quad G \quad G \quad C \quad T \quad G \quad T \quad G \quad A \quad C
\]

**Adapter NMCI**

\[
5' \quad A \quad C \quad T \quad A \quad A \quad T \quad 3'
\]

**Adapter MCI**

\[
5' \quad C \quad T \quad G \quad A \quad A \quad A \quad A \quad C \quad T \quad G \quad G \quad C \quad T \quad G \quad T \quad G \quad A \quad C
\]

**Cell Culture—Human embryonal kidney (293) cells and simian COS-m6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Full arm waro ovarian (SF9) cells were propagated in suspension in either Grace's or IPL/41 medium with 10% heat-inactivated fetal bovine serum plus 0.1% pluronic polyl F68. For large scale culture (>1 liter), SF9 cells were adapted to IPL/41 medium supplemented with 1% heat-inactivated fetal bovine serum, 0.1% Pluronic polyl F-68, 10 mg/liter cod liver oil, 25 mg/liter Tween 80, 4.5 mg/liter cholesterol, and 2 mg/liter d-tocopherol acetate (25).

**Production of Recombinant Baculovirus**—To generate recombinant baculoviruses, SF9 cells (1 × 10^6) were transfected with 1 µg of wild-type viral DNA and 18 µg of baculovirus vector containing DNA encoding wild-type or mutant adenylyl cyclase; a calcium phosphate precipitation method was used as described (27). The virus was harvested after 6 days and was screened by limiting dilution and dot hybridization (28). Positive clones were then screened with plaque assays. Purification of the recombinant enzyme was accomplished by ammonium sulfate precipitation followed by ammonium sulfate precipitation was usually sufficient to obtain pure virus. Viral titers were estimated by end point dilution (27).

**Antibodies**—Two peptides were synthesized corresponding to amino acid sequences in the C, and C, domains of type I adenylylcyclase: CI-251 (CIEDRLRLEDENKE) and CI-1115 (CGLAPGPPG-AXXATGG) (25), an EcoRI-bluntedNcoI fragment was excised from pCMVAC-58 digested with the same enzymes; this plasmid is designated pCMVAC-N (18). For expression of adenylyl cyclase in human 293 cells and simian COS-m6 cells, a 4.0-kb fragment of DNA was excised from pSKACΔ58-13 with HindIII and XhoI and was ligated into pCMV-5 that had been digested with the same enzymes; this plasmid is designated pCMVAC-N (18). For construction of recombinant baculovirus, the NcoI-EcoRI fragment from pSKACΔ58 was ligated into baculovirus vector pAcC4.

**Expression of Adenylyl Cyclase in SF9 Cells**—SF9 cells were usually infected with 1 plaque-forming unit per cell of baculovirus and were harvested 54–60 h after infection. Cells were lysed and membranes were washed and resuspended in HEME buffer (20 mM NaHepes (pH 8.0), 2 mM MgCl2, 1 mM EDTA) plus protease inhibitors B, as described (18). On some occasions membranes were washed in HEED buffer (20 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM EGTA, 2 mM DTT) containing protease inhibitors B prior to suspension in HEME.

Adenylyl cyclase activity was assayed for 10 min in the presence of 10 mM MgCl2, using 5 µg of membrane protein or for 5 min using appropriate aliquots of the purified enzyme (5). Gs was activated for 30 min at 30 °C in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM EGTA, 5 mM MgSO4, and 100 pM GTPγS (34). Unbound GTPγS was removed by gel filtration; GTPγS does not dissociate from Go over the course of several hours if the concentration of free Mg2+ is greater than 1 µM. The stoichiometry of GTPγS binding was usually between 50 and 70%. Calmodulin and/or activated Go was incubated with membranes for 10 min or with the purified enzyme for 2 min at 30 °C prior to assay; forskolin, G-site inhibitors, and/or G protein βγ subunits were added immediately prior to assay. Membranes were incubated for 20 min with 200 µg of carrier DNA (pUC-18) by calcium phosphate precipitation, washed with acetone for 1 h, and pelleted again. Samples were subjected to SDS-PAGE followed by silver staining or immunoblotting and protein concentration was determined by staining with Amido Black (35).

**Calmodulin-activated Adenylyl Cyclase**

Calmodulin-activated adenylate cyclase was partially purified by forskolin-Sepharose chromatography (18). Reactivity of the antisera was initially determined by probing nitrocellulose to which varying amounts of the forskolin-Sepharose eluate had been transferred. Membranes from infected SF9 cells and bovine brain were heated to 80 °C in the presence of 2% SDS and 0.2 mM dithiothreitol for 5 min; they were then treated with 50 µM N-ethylmaleimide for 10 min prior to 50 µM N-ethylmaleimide, and washed sequentially with 100 µM NaCl and 100 µM GTPγS during centrifugation at 80,000 g for 30 min. The supernatant was collected after centrifugation for 30 min at 50,000 × g. The insoluble material was extracted again with the same detergent-containing buffer, and the two supernatants were pooled. NaCl was added to a concentration of 0.5 M, and the detergent extract was then incubated with 3 µl of forskolin-Sepharose for 2 h at 4 °C with gentle rocking. The resin was poured into a column and washed sequentially with 100 µl of buffer A (10 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM MgCl2, 2 mM diethiothreitol, 0.05% Lubrol PX) containing 0.5 M NaCl and 0.05% GtpyS and protease inhibitors C; 100 µl of buffer A with 2 M NaCl plus protease inhibitors A and 10 µl of buffer A with 0.5 M NaCl, 2% dimethyl sulfoxide, and protease inhibitors A. Adenylyl cyclase was finally eluted by incubation of the column for 15 min with 4 ml of 200 µM forskolin in buffer A containing 0.5 M NaCl and protease inhibitors A. This elution procedure was repeated four times. Fractions containing purified adenylyl cyclase were isolated and assayed for enzyme activity (26).

To check the specificity of the purification, a portion of the eluate was precipitated with 15% trichloroacetic acid. The precipitate was collected by centrifugation, washed with acetone for 1 h, and pelleted again. Samples were subjected to SDS-PAGE and subjected to SDS-PAGE followed by silver staining or immunoblotting and protein concentration was determined by staining with Amido Black (35).

**Biological Significance—Metabolic Labeling**

Calmodulin-activated adenylyl cyclase, which is induced by tumor promoters, is inhibited by certain antibiotics. The inhibitory effect of these antibiotics may be due to their ability to bind to the enzyme and/or to interfere with its function. The inhibitory effect of these antibiotics may be due to their ability to bind to the enzyme and/or to interfere with its function.
of Sf9 cells was performed as described (27) with few modifications. Cells were labeled 66 h after infection with 30 μCi of [35S]methionine/0.5 ml in methionine-free Grace’s medium for 4–6 h. Cells were incubated in methionine-free medium for 30–60 min prior to labeling, and medium was changed by sedimentation and resuspension of the cells. Cells were then harvested and lysed in HME buffer containing centrifugation and were analyzed by SDS-PAGE. For analysis of protease inhibitors B. Supernatant and pellet fractions were separated of Sf9 cells was performed as described (27) with few modifications.

RESULTS

Transient Expression of Recombinant Adenylylcytase in COS-m6 and Human 293 Cells—We demonstrated previously that transient expression of recombinant adenylylcytase in COS-m6 cells raised Mn2+-activated adenylylcytase activity modestly from 40 to 130 pmol min−1 mg−1 and forskolin-activated enzymatic activity from 380 to 840 pmol min−1 mg−1 (18). The construct used (pCMVAC) has an ATG sequence 8 base pairs upstream from the putative initiation codon, and the 102-base pair 5′-noncoding sequence is extremely rich in guanine and cytidine residues (91%). In an attempt to facilitate the expression of adenylylcytase we removed all of the 5′-noncoding sequence and constructed a better site for initiation of translation (ATCATGG). Unfortunately, this construct (pCMVACΔ58) was only marginally better. Mn2+-activated adenylylcytase activity was 160 pmol min−1 mg−1 and forskolin-stimulated activity was 1100 pmol min−1 mg−1 after transfection of COS-m6 cells.

We also attempted to express type I adenylylcytase transiently in human 293 cells. These cells are readily transfected, and they efficiently express genes driven by the cytomegalovirus early promoter. They also have relatively low basal adenylylcytase activity: 10–20 pmol min−1 mg−1. Cells transfected with pCMVAC showed a 10–15-fold increase in Mn2+-activated adenylylcytase activity but only a 2–3-fold increase in forskolin-stimulated activity (compared to cells transfected with pCMV5). The effect of expression of type I adenylylcytase on forskolin-stimulated enzymatic activity is less dramatic because the endogenous adenylylcytase of 293 cells is markedly activated by forskolin (30-fold). Again, results were only slightly better when pCMVACΔ58 was used.

The rat HC-1 cell is a hepatoma (HTC) cell variant with little or no detectable adenylylcytase activity. Attempts to express type I adenylylcytase in these cells (transiently or permanently) have failed.

Expression of Adenylylcytase in Sf9 Cells—Recombinant baculovirus (B-rAC) was constructed to contain the cDNA for adenylylcytase under the control of the strong polyhedron promoter, and adenylylcytase activity was then assessed in Sf9 cell membranes at various times after infection with wild-type and recombinant virus (Fig. 1A). Uninfected cells displayed specific activities of 25–30 pmol min−1 mg−1 (Mn2+) or 250 pmol min−1 mg−1 (Mn2+ and forskolin). When cells were infected with wild-type baculovirus, adenylylcytase activity fell to about 10 pmol min−1 mg−1 (Mn2+) or 100 pmol min−1 mg−1 (Mn2+ and forskolin) within 2 to 3 days. For cells infected with B-rAC, however, adenylylcytase activity increased dramatically by 30 h after infection, reaching a max-

Fig. 1. Expression of type I adenylylcytase in Sf9 cells. A, adenylylcytase activity of Sf9 cell membranes at different times after infection with baculovirus: no virus (circles), AcNPV (triangles), and B-rAC (squares). Sf9 cell membranes (5 μg) in HME buffer were assayed for 10 min in either 10 mM MgCl2 and 5 mM MnCl2 (open symbols) or the same plus 100 μM forskolin (filled symbols). B, Western blot analysis of Sf9 cell membranes at different times after infection with B-rAC. Membrane proteins (35 μg) were solubilized, resolved by SDS-PAGE, transferred to nitrocellulose, and stained using affinity-purified rabbit anti-type I adenylylcytase-specific antisera (top, Ab CI-1115; bottom, Ab CI-251). The forskolin-stimulated adenylylcytase activities of the membranes are shown at the top.

Fig. 2. Immunoblots of membrane proteins from bovine brain and Sf9 cells stained with affinity-purified anti-peptide antisera Ab CI-1115 (A) or Ab CI-251 (B). In A, Sf9 cells were infected with B-rAC; the quantities of Sf9 cell membrane protein were 0.4 μg (1), 2 μg (2), and 10 μg (3). Bovine brain membranes (30 μg of protein) are shown for comparison. In B, 30 μg of membrane protein was used from B-rAC-infected Sf9 cells, uninfected Sf9 cells, and bovine brain.
Calmodulin-activated Adenylylcyclase

FIG. 3. Effects of calmodulin (A), GTPγS-rGαs (B), forskolin (C), and Mn2+ (D) on type I adenylylcyclase in Sf9 cell membranes. Sf9 cells were infected with AcNPV (○, ○, △ in A) or B-rAC (●, ●, ▲ in D). Membranes were washed with EGTA to remove endogenous calmodulin and were assayed for 10 min (5 μg of protein). Membranes were incubated with GTPγS-rGαs, or Ca2+ + calmodulin for 10 min at 30 °C prior to assay. In A, assays were performed with either 100 μM Ca2+ (circles) or 100 μM EGTA (triangles). In D, assays were performed in the presence of 300 nM calmodulin (△) or 50 μM EGTA (●).

FIG. 4. Effects of combinations of activators on type I adenylylcyclase activity in B-rAC-infected Sf9 cell membranes. A, calmodulin + GTPγS-rGαs; B, calmodulin + forskolin; C, forskolin + GTPγS-rGαs. Membranes were washed with EGTA and assayed for 10 min (5 μg of protein). Activators were incubated with membranes for 10 min at 30 °C prior to assay. CaCl2 (50 μM) was added when calmodulin was present.

FIG. 5. The effect of P-site inhibitors on type I adenylylcyclase activity in B-rAC-infected Sf9 cell membranes. Assays (10 min, 5 μg of protein) were performed in the presence of 10 mM MgCl2, 5 mM MnCl2, and 100 μM forskolin. A, effects of adenosine (○), 2′-deoxyadenosine (●), 3′-AMP (▲), and 2′-deoxy-3′-AMP (△). B, Lineweaver-Burk analysis and (inset) replots of slopes and intercepts from the double-reciprocal plots of inhibition by 2′-deoxy-3′-AMP; ○, no inhibitor; ●, 1.56 μM; ▲, 15.6 μM; △, 3.1 μM; ▲, 6.25 μM; □, 12.5 μM; ■, 25 μM.

Calmodulin-activated Adenylylcyclase

minimum at 2–3 days (5 nmol·min⁻¹·mg⁻¹ with Mn2+ and 14 nmol·min⁻¹·mg⁻¹ with Mn2+ and forskolin). Based on activity, 99% of the adenylylcyclase in cells infected with B-rAC is the recombinant type I enzyme. We estimate that approximately 0.1% of the protein in crude membranes from infected Sf9 cells is the recombinant protein (based on the specific activity of the purified protein of 8 μmol·min⁻¹·mg⁻¹ (5)).

Six peptides that correspond to various hydrophilic regions of type I adenylylcyclase have been synthesized and used to immunize rabbits. To date, we have obtained two antisera that are useful to immunoblot native and recombinant aden-
Forskolin class activity at various concentrations of Pr. Lubrol extract (+0.5 M NaCl) was obtained using a peptide that corresponds to the 20 carboxyl-terminal amino acid residues of the protein. These affinity-purified antisera reacted with a protein with an apparent molecular weight of approximately 110,000 in Sf9 cells infected with B-rAC, and the intensity of the signal correlated reasonably well with the adenylylclase activity of these membranes, particularly during the first few days after infection (Fig. 1B). The subsequent decrement in the intensity of the signals seen with immunoblotting compared to the adenylylclase activity in these membranes suggests that there is proteolysis, but that this does not eliminate catalytic activity. Fragments (36 and 43 kDa) of potential interest were visualized with Ab CI-1115; fragments were not detected with Ab CI-251.

Antibodies were also utilized to detect type I adenylylclase in bovine brain membranes (Fig. 2). A protein that migrated at 110 kDa was detected with both antisera. Type I adenylylclase expressed in Sf9 cells migrated slightly more rapidly than did the bovine brain protein, presumably because of differences in posttranslational modification (e.g., glycosylation). There appeared to be 20–30-fold more type I adenylylclase in Sf9 cell membranes than in bovine brain membranes (based on immunoblotting). This is roughly consistent with the measured enzymatic activities. Thus, adenylylclase activity in membranes from Sf9 cells infected with B-rAC is 4-fold higher than that observed in bovine brain membranes, and we estimate that only 20–30% of the brain enzyme is type I adenylylclase (based on the elution profile from forskolin-Sepharose chromatography) (18).

Activators of Recombinant Type I Adenylylclase—Since at least 99% of the adenylylclase activity in Sf9 cell membranes is contributed by the recombinant enzyme after infection of cells with B-rAC, these membranes offer a unique opportunity to study the properties of this form of adenylylclase in a membrane-bound and reasonably native environment. Most of the enzyme appears to be in the plasma membrane, based on indirect immunofluorescence using permeabilized cells and Ab CI-1115 (data not shown). As anticipated, enzymatic activity is enhanced by the addition of calmodulin, activated G<subiros</sub>, forskolin, or Mn<sup>2+</sup> when compared with assays performed in the presence of Mg<sup>2+</sup> alone (basal activity) (Fig. 3).

The basal activity of adenylylclase in Sf9 cell membranes containing type I adenylylclase decreased by 50–60% after washing with EGTA (a procedure designed to remove endogenous calmodulin (36)). Addition of Ca<sup>2+</sup> and calmodulin together then increased adenylylclase activity about 8-fold to a value of 4 nmol·min<sup>-1</sup>·mg<sup>-1</sup> (Fig. 3A). Half-maximal values were achieved at a calmodulin concentration of 20 nM. Similar maximal activities were observed when Ca<sup>2+</sup> and calmodulin were added to membranes that had not been washed with EGTA (not shown). There was no effect of calmodulin if Ca<sup>2+</sup> was replaced by EGTA during the assay. GTP<sub>yS</sub>·rG<sub>αs</sub> activated type I adenylylclase about 5-fold (Fig. 3B). The half-maximally effective concentration for activated rG<sub>αs</sub> was about 8 nM; again, the maximal activity achieved was nearly 4 nmol·min<sup>-1</sup>·mg<sup>-1</sup>.

The diterpene forskolin interacts with adenylylclase directly and activates the enzyme. When forskolin was added to EGTA-washed Sf9 cell membranes containing type I adenylylclase...
Calmodulin-activated Adenylylcy clase

Adenylylcy clase a maximal specific activity of approximately 4–6 nmol-min⁻¹·mg⁻¹ was observed (although it is notoriously difficult to achieve a stable maximum in the presence of high concentrations of forskolin). Thus, calmodulin, Gₐ₁₁ and forskolin activate type I adenylylcy clase to a similar degree when tested alone.

Although adenylylcy clase activity assayed in the presence of Mn²⁺ also appeared to approximate 4 nmol-min⁻¹·mg⁻¹ (Fig. 1A), this effect was reduced substantially when membranes were washed with EGTA (Fig. 3D). Addition of calmodulin restored the effects of Mn²⁺. Thus, much of the effect of Mn²⁺ appears to be due to a stimulatory effect of Mn²⁺-calmodulin on enzymatic activity.

The reduced effect of forskolin in EGTA-washed membranes (compare Figs. 1A and 3C) suggests that there might be synergistic effects between activators of type I adenylylcy clase; such interaction between forskolin and Gₐ₁₁ and between Gₐ₁₁ and calmodulin have been described previously (37, 38). Greater than additive activation of type I adenylylcy clase is seen when either GTPγS·Gₐ₁₁ or forskolin is tested in the presence of calmodulin (Fig. 4). Interestingly, there is little effect of activated Gₐ₁₁ in the presence of maximally effective concentrations of forskolin, despite the fact that this interaction is prominent with at least certain other types of adenylylcy clase (37, 39) (and has been demonstrated with the type II and type IV proteins in Sf9 cell membranes).²⁻³ Forskolin does not activate adenylylcy clase in the presence of maximally effective concentrations of calmodulin plus GTPγS·Gₐ₁₁ (not shown).

**Inhibitors of Recombinant Type I Adenylylcy clase**—Bovine brain adenylylcy clase is inhibited directly by adenosine and certain of its analogs (see Ref. 40 for review). These compounds have been termed P-site inhibitors, since an intact purine ring is an important structural requirement; 2'-, or 5'-deoxy and 3'-phosphoryl compounds are the most potent. Inhibition is not competitive with respect to metal-ATP, and stimulated forms of adenylylcy clase are more sensitive to inhibition than is basal activity. P-site inhibitors were tested for their effects on type I adenylylcy clase activity in Sf9 cell membranes (Fig. 5). As expected, forskolin-stimulated adenylylcy clase activity was more sensitive to inhibition than was basal activity (not shown). The order of potency was 2'-deoxy-3'-AMP > 3'-AMP > 2'-deoxyadenosine > adenosine, as described previously for the bovine brain enzyme (41). Changes of both slopes and intercepts of Lineweaver-Burk plots were a linear function of inhibitor concentration, consistent with prior observations that inhibition of enzymatic activity was noncompetitive with respect to Mg²⁺-ATP.

The G protein βγ subunit complex can inhibit adenylylcy clase activity; multiple mechanisms have been proposed (1). Inhibition that is apparently not caused by association of βγ with Gₐ₁₁ has been ascribed to interaction between βγ and calmodulin (42). Such inhibition was noted to be most prominent with the calmodulin-stimulated form of the enzyme and

**Table II**

**Activation of purified type I adenylylcy clase**

Purified enzyme was incubated with activators for 2 min in the presence of 0.025% Lubrol PX. Assays were performed for 7 min.

| Activator                  | Adenylylcy clase μmol-min⁻¹·mg⁻¹ |
|----------------------------|---------------------------------|
| GTPγS (10 μM)              | 0.9                             |
| GTPγS·rGₐ₁₁                 | 0.8                             |
| Ca²⁺ (50 μM) + calmodulin (312 nM) | 3.1                             |
| Forskolin (100 μM)         | 2.6                             |

**Fig. 8. Adenylylcy clase activity of truncated constructs.** A model of type I adenylylcy clase is shown at the top. Cells were harvested 60 h after infection. Assays were performed without activators (basal) or with 50 nM GTPγS·rGₐ₁₁, 50 μM Ca²⁺ + 160 nM calmodulin, or 100 μM forskolin.

**Fig. 7. Purified type I adenylylcy clase.** A, silver-stained gel (SDS-PAGE) of approximately 600 ng of adenylylcy clase purified from Sf9 cell membranes by forskolin-Sepharose chromatography. Eluates 1 and 2 are the first two elutions from the column. B, immunoblot of approximately 600 ng of eluates 1 and 2 using Ab CL-1115.
were resolved by absence of the preparation that was used.

Three types of truncated adenylylcyclase constructs have been made. In the first, ACAN, the first 52 amino acid residues of the N region have been removed. Membranes from SF9 cells that were infected with baculovirus containing this cDNA (B-rACAN) displayed an elevated level of adenylylcyclase activity that was stimulated by Gₛ, calmodulin, and forskolin (Fig. 8). However, the specific activity of adenylylcyclase was only 10% of that observed with B-rAC-infected cells. When the level of synthesis of this mutant protein was evaluated by either biosynthetic labeling (Fig. 9A) or immunoblotting (not shown), expression was found to be about 10 times greater than that observed with wild-type adenylylcyclase. Inactivated Bγ did not inhibit adenylylcyclase activity.

Purification of Recombinant Type I Adenylylcyclase—Procedures for the purification of recombinant type I adenylylcyclase were based on those described previously for the bovine brain enzyme (18), with minor modifications. The efficiency of solubilization of recombinant adenylylcyclase with Lubrol PX is about 50–60% (Table I), which is significantly less than that for the bovine brain enzyme. About 80% of this activity bound to forskolin-Sepharose after 4–6 h at 4 °C. Extensive washing of the column was critical for removal of contaminating proteins. Recovery of adenylylcyclase activity was only about 10% of the total. The highest specific activity of the protein recovered in the eluates was 4.2 μmol·min⁻¹·mg⁻¹. This is slightly lower than (but comparable to) that observed with the purified brain protein (8 μmol·min⁻¹·mg⁻¹) (5). As expected, the purified protein migrated as a species with a molecular weight of approximately 110,000, as judged both by silver staining and immunoblotting (Fig. 7).

Purified recombinant type I adenylylcyclase is not activated by GTPγS, indicating that the preparation is free of Gₛ. The enzyme can be activated by Gₛ.GTPγS, Ca²⁺ + calmodulin, and forskolin (Table II). Although the solubilized enzyme was inhibited by Bγ prior to purification, we were unable to observe an effect of Bγ on the purified preparation of adenylylcyclase. We are currently attempting to elucidate the mechanism of this effect of Bγ.

Characteristics of Truncated Forms of Adenylylcyclase—As mentioned above, the four types of mammalian adenylylcyclase that have been cloned to date appear to be similar structurally (see Fig. 8, top). For convenience of discussion, we have divided the structure into seven regions. The amino-terminal domain is designated N, while the two membrane-spanning domains are designated M₁ and M₂. These regions differ markedly from one type of adenylylcyclase to another. The cytoplasmic domains are designated C, C₁a, C₁b, and C₂a. C₁a and C₂a are about 55% similar to each other, and they are also similar to the corresponding domains of other mammalian adenylylcyclases and to the catalytic domains of membrane-bound guanylyl cyclases (43, 44). The C₂b regions are similar between type IV and type IV adenylylcyclases; however, the C₂ regions of types I and III differ from each other and from types II and IV. C₂b is largely absent in types II and IV and differs in the type I and III enzymes.

Three types of truncated adenylylcyclase constructs have been made. In the first, ACAN, the first 52 amino acid residues of the N region have been removed. Membranes from SF9 cells that were infected with baculovirus containing this cDNA (B-rACAN) displayed an elevated level of adenylylcyclase activity that was stimulated by Gₛ, calmodulin, and forskolin (Fig. 8). However, the specific activity of adenylylcyclase was only 10% of that observed with B-rAC-infected cells. When the level of synthesis of this mutant protein was evaluated by either biosynthetic labeling (Fig. 9A) or immunoblotting (not shown), expression was found to be about 20-fold greater than that observed with wild-type adenylylcyclase. Thus, it is possible that the ACAN mutant has an extremely low specific activity; alternatively, improper folding and/or orientation of the mutant protein in the membrane may account for at least some of its low activity.
and NM,C,-s (short) consist of the N, M1, and C1. regions and part of C2, the long and short versions of this construct terminate 10 and 43 amino acid residues prior to the beginning of M2, respectively. The M2,C2 construct begins in region C1, 46 amino acid residues prior to M2, and goes to the carboxyl terminus. Expression of these truncated proteins in S9 cells resulted in little or no change in adenylylcyclase activity. However, coinfection of cells with recombinant baculovirus encoding NM,C1 and M2,C2 resulted in the appearance of a substantial level of adenylylcyclase activity that is stimulable by activators (particularly GTP$\gamma$S-G,, and forskolin) and is inhibitable by 2'-deoxy-3'-AMP and G protein $\beta$-subunits (data not shown). Similar results were obtained if the C2 region of M2,C2 was removed (data not shown). Biosynthetic labeling studies indicate that the level of expression of NM,C1 and M2,C2 proteins is high (roughly 10-fold greater than that for wild-type adenylylcyclase when NM,C1 and M2,C2 are expressed simultaneously, Fig. 9A).

The major products of AC3N and M2,C2, migrate as doublets during SDS-PAGE (89 and 104 kDa for AC3N and 52 and 55 kDa for M2,C2 (Fig. 9, A and B)). The apparent molecular weights of NM,C1 and NM,C1-s are 62,000 and 55,000, respectively (Fig. 9A). All of these species migrate as if their molecular weights are 5,000-10,000 units smaller than predicted; the reason for this deviation is unknown. Products that are presumed to result from proteolysis of these truncated forms of adenylylcyclase are also evident in Fig. 9A and by immunoblotting (not shown).

S9 cells expressing the truncated forms of adenylylcyclase were treated with tunicamycin to determine if the doublet seen with M2,C2 was a result of differences in N-linked glycosylation. Indeed, only the lower band of the M2,C2 doublet was apparent when protein was synthesized in the presence of tunicamycin (Fig. 9B). The sizes of the NM,C1-s and NM,C1-s products were not changed by tunicamycin. There are four consensus sites for N-linked glycosylation in the sequence of type I adenylylcyclase. Our model of the orientation of the protein in the bilayer suggests that only one of these (in the M2 region) would be utilized. Results obtained with tunicamycin are consistent with this model.

**DISCUSSION**

Adenylylcyclase is a complex enzyme. It exists normally at very low concentrations (0.01-0.001% of membrane protein) and is labile and difficult to manipulate in detergent-containing solutions. The existence of multiple forms of the protein confounds interpretation of experiments performed with native membranes, particularly with preparations from sources as complex as brain. These considerations speak to the desirability of expression of individual forms of adenylylcyclase, which can then be studied in situ or after purification to homogeneity. However, expression of complex, intrinsic membrane proteins in quantities sufficient for biochemical characterization has often been difficult. The use of recombinant baculovirus permits expression of quantities of adenylylcyclase that are adequate for several needs. The activity of the recombinant protein exceeds that of the endogenous enzyme by 100-fold, permitting an uncluttered view of the adenylylcyclase of interest. Large numbers of S9 cells can be grown, and it may be possible to scale the system for production of milligram quantities of protein. However, it would certainly be desirable to increase the level of expression of adenylylcyclase that has been obtained to date.

Toward this end we note that there has been a negative correlation between the amount of adenylylcyclase protein synthesized during our attempts at expression and the intrinsic activity of the specific protein under consideration. Thus, AC3N, which retains most of the structural features of the native protein, has poor activity but accumulates to levels of more than 1% of membrane protein. NM,C,-s and M2,C2 are also inactive and accumulate to high concentrations; however, when they are expressed together (with resultant activity), the level of expression decreases substantially (see Fig. 9). Perhaps these considerations also explain our failure to achieve a more efficient transient expression system by modification of the 5' end of the adenylylcyclase cDNA in the pCMV constructs (pCMVAC versus pCMVACΔ58). Excessive production of cyclic AMP may inhibit transcription and/or translation. Inclusion of cyclic AMP analogs in the S9 cell culture medium did inhibit expression of adenylylcyclase, although millimolar concentrations of chlorophenylthio cyclic AMP were required to observe this effect. We attempted to control concentrations of cyclic AMP in S9 cells by coinfection of these cells with separate viruses encoding both adenylylcyclase and a low $K_m$ cyclic nucleotide phosphodiesterase (cDNA kindly provided by Dr. Michael Wigler, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). Although cyclic nucleotide phosphodiesterase activity increased markedly (to 100 nmol/min/mg), adenylylcyclase activity increased by only 2-fold at best.

Type I adenylylcyclase expressed in S9 cells displays all of the properties known to be characteristic of calmodulin-sensitive adenylylcyclase purified from brain, and certain of these properties are clearly distinct from those of other adenylylcyclases. We note in particular that the enzyme can be stimulated equally by calmodulin and Gs, that calmodulin acts synergistically with other activators (Gs or forskolin), and that there is no synergistic interaction between Gs, and forskolin. Most interesting, perhaps, is the effect of G protein $\beta$-subunits. Inhibition of calmodulin-stimulated enzymatic activity was most notable, as previously described (42). However, we cannot ascribe this effect to interaction between $\beta$- and calmodulin, as mentioned above.

Discussion of interactions of G protein $\beta$-subunits with effectors such as adenylylcyclase, ion channels, and phospholipases has been controversial (45-47). The inhibitory effect of $\beta$- on a purified preparation of calmodulin-sensitive, bovine brain adenylylcyclase was noted previously (5). However, the effect was lost after further manipulation of the protein with alterations of detergent, and it was tentatively ascribed to contamination of adenylylcyclase with Gs, We, too, have failed to detect inhibitory effects of $\beta$- on purified preparations of type I adenylylcyclase. Experiments in progress are designed to determine if an additional protein is necessary to observe this effect.

The concentration of $\beta$- required to inhibit adenylylcyclase is high relative to the concentrations of G protein $\alpha$-subunits required to activate the enzyme or to affect channel function. (Although $\beta$- and Gs, were effective in the same concentration range in the experiments described above, recombinant (Escherichia coli-derived) Gs, was utilized. This protein has an affinity for adenylylcyclase that is approximately 20-fold lower than that of native Gs, (34)). Nevertheless, $\beta$- is a relatively abundant protein complex, particularly in brain, and its total concentration exceeds that of Gs, by a substantial margin (>100-fold in brain). For Gs, to serve as the physiological activator of adenylylcyclase, the stimulatory effect of Gs, must, of course, dominate the inhibitory effect of $\beta$- It will be interesting to determine if individual forms of $\beta$- differ in their ability to inhibit adenylylcyclase and if individual forms

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4 T. Nguyen, W.-J. Tang, and A. G. Gilman, unpublished observations.
of adenyl cyclase are equally susceptible to this effect. Preferential effects of βγ on the calmodulin-stimulated activity of the type I enzyme suggest that different adenyl cyclases may respond to βγ in unique ways.

The quasisymmetrical structure of adenyl cyclase with its “duplicated” C1a and C2a domains is curious. Although these regions do not have sequences that are characteristic of classical nucleotide-binding domains, we assume that at least one of them must have this function. There is a weak homology between C1a and C2a and the catalytic domain of yeast adenyl cyclase, and there is strong similarity between these regions and the catalytic domains of several membrane-bound guanylyl cyclases. Thus, we separated these regions by expression of NMaC2 and MaC2 in an attempt to locate the catalytic domain of adenyl cyclase. The results suggest that interactions between these regions may be necessary for catalysis, but they clearly do not rule out phenomena such as interactions necessary for proper folding of the molecules. Nevertheless, other observations are consistent with the possibility that interactions between C1a and C2a play a role in catalysis. Notably, kinetic analysis suggests that the inhibitory P-site and the catalytic site are separate but interacting domains (48); it may be possible to assign C1a and C2a to these respective functions. The adenyl cyclases of Bacillus anthracis and Bordetella pertussis have duplicated A-type nucleotide-binding consensus sequences, and mutational analysis indicates the importance of these sites for catalysis (49–51). Soluble guanylyl cyclase is a heterodimer. Each of its subunits has a region that is homologous to C1a and C2a, and the subunits must be expressed concurrently in order to observe catalysis (19). Perhaps the membrane-bound guanylyl cyclases, with but one such domain, function as homodimers.

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