Soluble Adenylyl Cyclase from Spodoptera frugiperda (Sf9) Cells

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION*

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J un-ichi Kawabe, Yoshiyuki Toya, Carsten Schwencke, Naoki Oka, Toshiaki Ebina, and Yoshihiro Ishikawa†

From the Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

An insect ovarian cell, Spodoptera frugiperda (Sf9), has been widely used to express recombinant proteins, including adenylyl cyclase, as a host cell in the baculovirus expression system. We report the presence and characterization of a soluble adenylyl cyclase (sAC) distinct from a membrane-bound form of adenylyl cyclase (mAC) that is also present in Sf9 cells. sAC was purified 3,500-fold to near homogeneity; a single band at 25 kDa on SDS-polyacrylamide gel electrophoresis correlated well with adenylyl cyclase catalytic activity. The purified enzyme had a catalytic activity of 0.1 µmol/min·mg and the Kₘ of 0.35 mM for the substrate ATP. In contrast to mAC, sAC was heat-stable. Enzymatic activity of sAC was not stimulated by forskolin and was inhibited by pyrophosphate, noncompetitively inhibited sAC. Our data suggest that the physical and biochemical characteristics of sAC are different from those of mAC in Sf9 cells as well as from those of other known forms of adenylyl cyclase in animal cells; sAC in Sf9 cells may constitute a new member of adenylyl cyclase found in animals.

It is well known that cAMP plays a widespread role in the control of gene expression and the integration of hormonal stimulation (1). Adenylyl cyclase is widely expressed in many organisms from bacteria to animals (2, 3). Although this enzyme invariably converts ATP to cAMP, its other biochemical properties vary among species. In bacteria, adenylyl cyclase exists within the bacterial body to integrate metabolic function (the enterobacterial form). In some bacteria, such as Bacillus anthracis (4), adenylyl cyclase is secreted as a toxin and is activated by calmodulin in the host cell, facilitating the bacterial invasion of the target animal cell (the calmodulin-activated toxin form). In animals, on the other hand, adenylyl cyclase exists in a membrane-bound form to integrate hormonal stimulation. Typically, this membrane-bound adenylyl cyclase has tandem repeats of a six-transmembrane structure and a cytoplasmic catalytic domain similar to a transporter or ion channel (5). Regulation of this membrane-bound form of adenylyl cyclase by the receptor/G protein and the production of cAMP as a second messenger within the cell are the two common features conserved in both non-vertebrates and vertebrates. A calmodulin-sensitive form of adenylyl cyclase from Drosophila, unlike the bacterial form, shares the same membrane topology and the amino acid sequence in the catalytic domain with a calmodulin-sensitive form of adenylyl cyclase found in mammalian brains (type I). This Drosophila adenylyl cyclase is also capable of coupling to the endogenous mammalian G protein when overexpressed in mammalian cells (6). Thus, the properties of adenylyl cyclase in animals, including its structure and its functional interaction with other signal components, are well conserved between non-vertebrates and vertebrates.

The germ cells of animals, however, contain an additional form of adenylyl cyclase that displays physical and functional properties markedly different from those of the membrane-bound form (7–9). This adenylyl cyclase is not stimulated by G protein or forskolin. It is active only in the presence of manganese, but not magnesium, and is unaffected by fluoride or gonadotrophic hormones (10–12). Thus this adenylyl cyclase is regulated differently from the hormonally regulated adenylyl cyclase found in somatic cells; its physical properties are similar to those of adenylyl cyclase found in bacteria, an indication that the adenylyl cyclase in germ cells may be an ancestral form of mammalian adenylyl cyclase. However, attempts to characterize this soluble adenylyl cyclase have been hampered by its limited availability in germ tissues; in the absence of a cultured cell line to study this adenylyl cyclase, sperm has so far provided the only source.

Sf9 cell, an ovarian cell from Spodoptera frugiperda, or fall armyworm, has been widely used as a host cell of baculovirus to express recombinant proteins. We and others have successfully overexpressed the membrane-bound form of mammalian adenylyl cyclase in this cell (13–18). Sf9 cells, like other animal cells, have an endogenous membrane-bound form of adenylyl cyclase that is regulated by GTP and forskolin (16). We have noticed that there is an additional form of adenylyl cyclase that is exclusively expressed in the cytosolic fraction of this cell. In this study, we attempt to answer two questions. First, what are the physical properties of this soluble adenylyl cyclase? Second, how are the biochemical characteristics of this adenylyl cyclase different? Our data suggest that the adenylyl cyclase found in Sf9 cells is similar to the bacterial form of adenylyl cyclase, as well as to the form of adenylyl cyclase found in mammalian germ cells; it may constitute a new member of the adenylyl cyclase family found in animals.

EXPERIMENTAL PROCEDURES

Cell Culture—We obtained S. frugiperda Sf9 cells from the American Tissue Culture Collection (CRL 1711) or Invitrogen (San Diego, CA).
Cells were grown in Grace medium containing 4% (v/v) fetal bovine serum, penicillin (100 µg/ml), and streptomycin (100 µg/ml). For harvesting, cells were washed three times with ice-cold phosphate-buffered saline and stored at −70 °C until use.

Adenylyl Cyclase Assay—We performed adenylyl cyclase assays by incubation at 30 °C in a reaction buffer containing 20 mM Hepes (pH 8.0), 5 mM MgCl2, 0.1 mM cAMP, 1 mM creatine phosphate, 8 units/ml creatine phosphokinase, and 0.1 mM [α-32P]ATP (about 1 µCi/assay tube), unless otherwise specified. After 20 min, the reaction was terminated by the addition of 2% SDS. We measured the production of cAMP as described previously (13). The protein concentration was determined either by the method described by Bradford (20) or by staining with Amido Black (21) using bovine serum albumin as a standard.

Purification of Soluble Adenylyl Cyclase—All purification steps were performed at 4 °C. We thawed frozen cells (about 5 × 108) in 100 ml of a homogenization buffer consisting 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1% v/v glutaraldehyde, 0.1% v/v Triton X-100, and a protease inhibitor mixture (10 µg/ml leupeptin, 1 µg/ml phenylmethylsulfonlfyl fluoride, 50 units of egg white trypsin inhibitor, 20 µg/ml 1-tosylamido-2-phenyl-ethyl chloromethyl ketone, 20 µg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone, and 2 µg/ml aprotinin). The cells were lysed by nitrogen cavitation (800 p.s.i., 30 min). Cavitated cells were centrifuged at 500 × g to remove cell debris, and the supernatant was further centrifuged at 100,000 × g for 1 h. The resulting pellet was resuspended in the homogenization buffer and used as “the membrane fraction.” The supernatant was passed through a 0.45-µm filter membrane and was used as “the cytosolic fraction.” We also purified adenylyl cyclase protein from this fraction.

The cytosolic fraction was loaded onto a DEAE-MemSep-1500 column (Millipore) at 10 ml/min using the ConSep LC100 system (Millipore). The column was washed with 50 ml of buffer A (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 20 mM NaCl, followed by elution with 15-min gradient from 20 to 200 mM NaCl in buffer A. The adenylyl cyclase activity typically eluted at 100 mM NaCl (Fig. 1A, top). These fractions were collected at a flow rate of 0.1 ml/min. Fractions with adenylyl cyclase activity were pooled in a final KPO4 concentration of 20 mM.

We then loaded the pooled samples onto four hydroxyapatite columns (1 × 5 cm, Bio-Rad), equilibrated with buffer B (20 mM KPO4, pH 7.5, 0.1 mM EDTA, 2 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride). Each column was washed with 10 ml of buffer B (40 mM KPO4), Gravity elution was performed in 5 ml increments with increasing KPO4 concentrations (50, 60, 70, 80, and 100 mM in buffer B). Adenylyl cyclase activity usually eluted between 60 and 70 mM KPO4. These fractions were concentrated by use of a Centricon 30 (Amicon).

The concentrated sample was loaded onto a Sephacryl-200 HR column (1 × 80 cm, Pharmacia Biotech Inc.) equilibrated with buffer A containing 150 mM NaCl. Fractions were collected at a flow rate of 0.1 ml/min. Fractions with adenylyl cyclase activity were pooled in a final KPO4 concentration of 20 mM.

We then loaded the pooled samples onto four hydroxyapatite columns (1 × 5 cm, Bio-Rad), equilibrated with buffer B (20 mM KPO4, pH 7.5, 0.1 mM EDTA, 2 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride). Each column was washed with 10 ml of buffer B (40 mM KPO4), Gravity elution was performed in 5 ml increments with increasing KPO4 concentrations (50, 60, 70, 80, and 100 mM in buffer B). Adenylyl cyclase activity usually eluted between 60 and 70 mM KPO4. These fractions were concentrated by use of a Centricon 30.

The concentrated samples were applied again onto a Sephacryl-200 HR column under the same conditions described above. The fractions with high adenylyl cyclase catalytic activity were pooled (Fig. 1A, middle). The pooled sample was applied onto a Polytron column (1 × 8 cm, Sigma) equilibrated with buffer C (10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 2 mM MgCl2, 1 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride). The column was washed with 20 ml of buffer C at a flow rate of 5 ml/min, and eluted with 20-min gradient from 0 to 2 mM ATP in buffer C (Fig. 1A, bottom). Fractions with high catalytic activity were pooled (2.5 ml), and ATP was repeatedly removed at different dilutions and concentrations by use of a Centricon 30. The active fractions were pooled and stocked at −70 °C.

RESULTS AND DISCUSSION

Cytosolic Adenylyl Cyclase in Sf9 Cells—We first noticed the presence of adenylyl cyclase catalytic activity in the cytosol of Sf9 cells when we purified a recombinant membrane-bound form of adenylyl cyclase (14). This catalytic activity ceased during the purification process, indicating that the physical properties of this putative adenylyl cyclase were different from those of the membrane-bound form of adenylyl cyclase.

We further investigated the adenylyl cyclase catalytic activity present in the cytosol of Sf9 cells. We used the cytosolic fraction obtained by centrifugation at 100,000 × g for 1 h, followed by filtration through a 0.45-µm filter membrane. The pellet was used as the membrane-bound form of adenylyl cyclase.

| Fraction | Adenylyl cyclase activity | Kinetics, Km | μM |
|----------|--------------------------|-------------|-----|
| Basal    | GTP-S                  | Forskolin   |     |
| Cytosol  | 63.8 ± 6.4             | 56.9 ± 5.0  | 68.4 ± 7.7 |
| Membrane | 29.5 ± 7.9             | 84.0 ± 14.7 | 109.0 ± 12.9a |
|          | 550.9 ± 82.5           |             | 138.5 ± 13.8a |

*a p < 0.01 from basal activities.

The abbreviations used are: mAC, membrane-bound adenylyl cyclase; sAC, soluble adenylyl cyclase; GTP-S, guanosine 5'-O-(thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.
kDa is likely to encode sAC. Based upon the intensity of stainwe therefore concluded that this band of molecular mass exclusively correlated with adenylyl cyclase catalytic activity; 20134 molecular mass column with the highest activity showed a single band of molecular mass chromatogram denoted by the (hydroxyapatite columns (not shown), second Sephacryl-200 column under “Experimental Procedures,” and sAC was purified by a chromatographic purification series: DEAE-MemSep column (25), very stable. Indeed, even when the crude cytosolic fraction was left at room temperature overnight, a significant portion of adenylyl cyclase catalytic activity was retained. We did not obtain more than 100% recovery in any step, which indicates that serious overestimation is also unlikely.

Molecular Mass of sAC—To measure the molecular mass of sAC in its native condition, partially purified fractions, after undergoing DEAE-MemSep chromatography, were loaded on a Sephacryl-200 column and the elution profile was obtained using protein molecular mass standards. The activity eluted in a single peak corresponding to a molecular mass of approximately 40 kDa (Fig. 2).

The apparent size difference between SDS-PAGE and gel chromatography may be due to the structural nature of sAC, the formation of dimer in native condition, or a protein-matrix interaction in the gel. This size (40 kDa) was unchanged when fractions from different purification steps, such as the initial crude cytosolic fraction and eluate from the hydroxyapatite column, were similarly analyzed by gel filtration, which indicates that the size difference is not due to degradation of sAC during purification. When eluate from the hydroxyapatite column was subjected to a microfiltration membrane, Centricon 30, with the molecular mass cutoff of 30 kDa, the adenylyl cyclase catalytic activity was completely retained, a finding that suggests the size of sAC in native condition is greater than 30 kDa.

The size of sAC in Sf9 cells is apparently different from that of sAC in mammalian testis, which shows a molecular mass = 52 kDa on SDS-PAGE and 50 kDa by gel filtration (26). sAC in Sf9 cells is much smaller than many bacterial adenylyl cyclases (80–200 kDa) and membrane-bound forms of adenylyl cyclase (100–120 kDa) (3). Thus, sAC in Sf9 cells is likely the smallest adenylyl cyclase so far identified in animal cells, although we await the results of a future cloning study to determine the exact size. The small size of sAC in Sf9 cells is similar to that found in Rhizobium meliloti (20 kDa) (27).

Effects of Salts—Both NaCl and KCl inhibited the catalytic activity of sAC at high concentrations (Fig. 3); the potency of inhibition was similar for both salts at the IC50 value of 500 mM. This inhibition was reversible; catalytic activity recovered completely and rapidly after the salts were extracted. Similar salt-mediated inhibition was reported in the adenylyl cyclase of E. coli. (25).

We also examined the hypothesis that salt inhibition was due to disassociation of the putative homodimer (40 kDa) into monomers (25 kDa). Fractions from 5 to 150 kDa were obtained by gel filtration (Sephacryl-200) in the presence of 500 mM NaCl. When diluted to 50 mM NaCl, the 40-kDa fraction recovered adenylyl cyclase catalytic activity, whereas the 25 kDa fraction did not. Thus it is very unlikely that the inhibition of sAC catalytic activity by salts is the result of disassociation.

Effects of β-Mercaptoethanol—In contrast to the potent inhibitory effect of salts, no change in the catalytic activity of sAC was not detected in the presence of β-mercaptoethanol at 0–50 mM (Fig. 4). Therefore, disulfide links do not seem important in maintaining its catalytic activity.

Heat Stability—sAC was resistant to heat inactivation (Fig. 5). After 1-h incubation at 37 °C, the catalytic activity of sAC

**Fig. 1. Purification of soluble adenylyl cyclase from Sf9 cells.** A, five hundred mg of cytosolic proteins were obtained as described under “Experimental Procedures,” and sAC was purified by a chromatography series: DEAE-MemSep column (top panel), Sephacryl-200 and hydroxyapatite columns (not shown), second Sephacryl-200 column (middle panel), ATP-Sepharose column (lower panel). Fractions of each chromatogram denoted by the horizontal bar were pooled for further purification. Protein concentrations were monitored by absorbance at 280 nm or by the Bradford method. B, active fractions from each step of chromatographic purification were subjected to SDS-PAGE on a 4–20% gradient gel. After purification by ATP-Sepharose column, each fraction was concentrated by a Centricon 30, and applied to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Left, a, cytosol fraction (10 μg); b, DEAE-MemSep pool (7 μg); c, 2nd Sephacryl-200 pool (3 μg). Right, eluates from ATP-Sepharose column. The arrow indicates sAC. Each number corresponds to the fraction number in ATP-Sepharose chromatogram in A.
decreased by 10%, whereas that of mAC decreased 40%; at 55°C for 1 h, mAC was totally inactivated while sAC was mostly (60%) alive.

Effect of Divalent Cations—The presence of manganese was not an absolute requirement to maintain the catalytic activity of sAC in Sf9 cells (Fig. 6). However, the increase of catalytic activity of sAC was manganese and magnesium concentration-dependent and reached its maximum at 1 mM for both cations. The maximal activity was higher by 30% with magnesium than manganese. This property is similar to that of mAC (28), ad-

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**TABLE II**

Purification of soluble adenylyl cyclase (AC) from Sf9 cells

| Step              | Volume | AC activity | Protein | Total AC | Yield | Purification |
|-------------------|--------|-------------|---------|----------|-------|--------------|
| Cytosol           | 94.0   | 31          | 4.880   | 14,129   | 100   | 1            |
| DEAE-MemSep       | 3.2    | 321         | 10.020  | 11,260   | 75    | 11           |
| 1st Sephacryl 200 | 9.0    | 883         | 1.050   | 8,346    | 59    | 29           |
| 2nd Sephacryl 200 | 6.5    | 5,309       | 0.042   | 1,449    | 10    | 172          |
| ATP Sepharose     | 2.2    | 109,893     | 0.001   | 220      | 2     | 3568         |

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**Fig. 2.** Gel filtration of soluble adenylyl cyclase. The partially purified sample (30 μg) after DEAE-MemSep column was applied onto a Sephacryl-200 column. Gel filtration was performed as described under "Experimental Procedures." The loading buffer contained 150 mM NaCl. The fractions were assayed for adenylyl cyclase activity in the presence of 5 mM MgCl₂. Arrows denote the fractions containing the following molecular mass standards: a, thyroglobulin (670 kDa); b, bovine γ-globulin (158 kDa); c, chicken ovalbumin (44 kDa); d, equine myoglobin (17 kDa).

**Fig. 3.** Effects of salts. The cytosolic fraction of Sf9 cells (5–10 μg) was preincubated at 4°C for 30 min in the presence of increasing concentrations of NaCl (open circle) or KCl (closed circle), followed by adenylyl cyclase assays as described under "Experimental Procedure." When the purified sAC was used, the results were similar. The value of adenylyl cyclase catalytic activity is shown as a percentage of control in the absence of NaCl or KCl. Means ± S.E. from four experiments are shown.

**Fig. 4.** Effects of β-mercaptoethanol. Assays were performed using the cytosolic fraction of Sf9 cells (5 μg) in the presence of β-mercaptoethanol (0–50 mM). The value of adenylyl cyclase activity is shown as a percentage of control in the absence of β-mercaptoethanol. When the purified sAC was used, the results were similar. Means ± S.E. from three experiments are shown.

**Fig. 5.** Effects of heat treatment. The cytosolic fraction (open bar) and membrane fraction (closed bar) of Sf9 cells (5 μg) were preincubated at 37 or 55°C for 60 min, followed by adenylyl cyclase assays. The value of adenylyl cyclase activity is shown as a percentage of control that was preincubated at 0°C. When the purified sAC was used, the results were similar. Means ± S.E. from four experiments are shown.
and adenylyl cyclase from yeast (29), in contrast with the sAC found in mammalian testis, which is stimulated by manganese, but not by magnesium (9–11).

Effects of P-site Inhibitors—Adenosine and its analog, deoxyadenosine, inhibited the catalytic activity of mAC, but not of sAC (Fig. 7). At 100 μM adenosine or deoxyadenosine, 40–50% of catalytic activity was inhibited in mAC while no inhibition was seen in sAC. This finding suggests that sAC, unlike mAC, lacks P-site regulatory sites, although it remains possible that activated sAC is sensitive to P-site inhibition. This is in contrast to mammalian testicular sAC, which is readily inhibited by P-site inhibitors upon stimulation with forskolin (30).

Effects of Other Nucleotides—The inhibition of catalytic activity of sAC by GTP at higher concentrations (≥0.1 mM) was dose-dependent. Kinetics studies revealed that this inhibition by GTP was noncompetitive (Fig. 8) with the K of 0.98 mM.

This inhibition was not unique to GTP; di- or triphosphate analogs of ATP or its related reagents also inhibited sAC. The assays were performed using partially purified adenylyl cyclase in the absence of the ATP regeneration system. At 5 mM, the order of inhibition potency was pyrophosphate >> GTP > GDP = ADP (Table III). AMP, cAMP, and adenosine showed no inhibition. Notably, pyrophosphate, but not monophosphate, inhibited sAC. This finding is similar to that obtained from the adenylyl cyclase in E. coli; P Pi releasing from the enzyme-product complex is slow and a rate-limiting step (25).

Other Effectors—We also examined the effects of calcium/calmodulin, protein kinase C, and nitroprusside; none showed an effect on sAC activity (data not shown). Similarly, no guanylyl cyclase-like activity was detected when cGMP formation was measured by the use of the purified enzyme (data not shown).

The above data suggest that, having such a high enzyme catalytic activity and without any apparent stimulators, sAC is regulated, unlike mAC, in an inhibitory manner, rather than in a stimulatory manner. Candidates for the putative physiological regulator of sAC may include nucleotides and salts, as shown in our study. sAC in Sf9 cells is clearly distinct in physical properties from mAC in Sf9 cells, and has more in

### Table III

| Agents | Adenylyl cyclase activity (% of control) |
|--------|------------------------------------------|
| GTP    | 47                                       |
| GDP    | 79                                       |
| ADP    | 83                                       |
| NADP   | 82                                       |
| AMP    | 99                                       |
| cAMP   | 99                                       |
| Adenosine<sup>a</sup> | 97                                       |
| Pyrophosphate | 9                                        |
| Monophosphate | 97                                      |

<sup>a</sup>Tested at 1 mM.
common with sAC found in mammalian testis. The enzyme catalytic activity was, like that of sAC in testis, stable and nonresponsive to forskolin (26). On the other hand, several biochemical properties of sAC in Sf9 cells, such as pyrophosphate inhibition, sensitivity to salts, and probable lack of P-site inhibition, are similar to those of bacterial adenylyl cyclase. These data suggest that sAC in Sf9 cells is a form of adenylyl cyclase that shares some properties with both bacterial adenylyl cyclase and the mammalian sAC found in germ cells. sAC from Sf9 cells may be a novel animal form of adenylyl cyclase; it may also serve as a good experimental model to study how the cAMP signal is regulated nonhormonally in animal cells.

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