Choleragen Catalyzes ADP-Ribosylation of the Stimulatory G Protein Heterotrimer but Not Its Free α-Subunit

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ABSTRACT: The heterotrimeric ($\alpha\beta\gamma$) stimulatory G protein (G_s) mediates activation of adenylylcyclase. G_s is inactive when GDP is bound to the guanine nucleotide binding site of the α-subunit ($G_s\alpha$). G_s can be activated by fluoroaluminate or by binding GTP or GTP analogues, (*e.g.*, GTPγS) in place of GDP. Magnesium ion is also required for the activation of G_s, and $G_s\alpha$ is a substrate for ADP-ribosylation catalyzed by choleragen (CT). G_s activation can also be accompanied by dissociation of $G_s\alpha$ from the $G\beta\gamma$-subunit complex. When dissociated G_s subunits were separated by chromatography, isolated $G_s\alpha$ could not be ADP-ribosylated by CT unless $G\beta$ was added back. RM/1 antiserum against $G_s\alpha$ was used to immunoprecipitate G_s, and the subunit composition of the immunoprecipitate was determined. When G_s was incubated with 2 mM $MgCl_2$, the G_s heterotrimer was immunoprecipitated, and $G_s\alpha$ could be ADP-ribosylated by CT. Activation of G_s with GTPγS or fluoroaluminate in the presence of 2 mM $MgCl_2$ did not cause G_s subunit dissociation nor did it affect the ability of $G_s\alpha$ to be ADP-ribosylated by CT. $MgCl_2$ caused a dose-dependent decrease in the amount of $G\beta$ that coprecipitated with $G_s\alpha$ in the absence as well as the presence of GTPγS or fluoroaluminate. G_s subunit dissociation was accompanied by a corresponding decrease in CT-catalyzed ADP-ribosylation of $G_s\alpha$ regardless of whether or not GTPγS or fluoroaluminate was bound to $G_s\alpha$. If G_s subunits were dissociated with 120 mM $MgCl_2$ and fluoroaluminate, and the concentration of $MgCl_2$ was subsequently reduced to 2 mM, subunit reassociation occurred, and the ability of $G_s\alpha$ to be ADP-ribosylated by CT was restored.

The stimulatory G protein (G_s)[1] mediates activation of the catalytic component of adenylylcyclase by a stimulatory ligand–receptor complex (Gilman, 1987; Birnbaumer, 1990; Simon *et al.*, 1991). Like other members of the heterotrimer ($\alpha\beta\gamma$) G protein family, the α-subunit of G_s ($G_s\alpha$) has a guanine nucleotide binding site, and G_s is not active when GDP is bound to this site. Activation of G_s occurs when a stimulatory ligand–receptor complex facilitates the exchange of GDP for GTP. G_s is returned to its unactivated state by the intrinsic GTPase activity of $G_s\alpha$. The activation of G_s also requires Mg^{2+}, and can be accompanied by dissociation of $G_s\alpha$ from the $\beta\gamma$-subunit complex ($G\beta\gamma$). It has been proposed that Mg^{2+} can cause G_s subunits to dissociate without being activated (Northup *et al.*, 1983); however, some experimental evidence does not support this hypothesis (Codina *et al.*, 1984).

G_s is also the substrate for covalent modification by the bacterial toxin choleragen (Cassel & Pfeuffer, 1978; Gill & Meren, 1978). Choleragen (CT) acts catalytically to transfer the ADP-ribose moiety of NAD to an arginine residue in the guanine nucleotide binding pocket of $G_s\alpha$ (Van Dop *et al.*, 1984). It has been reported that both free $G_s\alpha$ and heterotrimeric G_s are substrates for ADP-ribosylation by CT (Kahn & Gilman, 1984a). However, recombinant $G_s\alpha$ is a poor substrate for CT unless $G\beta\gamma$ is present (Graziano *et al.*, 1987, 1989). Furthermore, removing 28 amino acids from the N-terminus of $G_s\alpha$ appears to inhibit both its interaction with $G\beta\gamma$ and its ability to be ADP-ribosylated by CT (Journot *et al.*, 1991). We conducted these studies to determine if both free $G_s\alpha$ and heterotrimeric G_s are substrates for CT and if the ligand bound to the guanine nucleotide binding site of $G_s\alpha$ had any effect on CT-catalyzed ADP-ribosylation. Our results indicate that only the G_s heterotrimer can be ADP-ribosylated by the toxin regardless of the ligand that is bound by $G_s\alpha$. This makes CT potentially useful for investigating G_s–subunit interactions in biological membranes.

EXPERIMENTAL PROCEDURES

Materials. Anti-$G_s\alpha$ (RM/1) and anti-$G\beta$ (SW/1) antisera, [α-^{32}P]ATP (30 Ci/mmol), [^{32}P]NAD, and ^{125}I-protein A (2–10 μCi/μg) were obtained from DuPont NEN Research Products (Boston, MA). Protein A–Sepharose CL-4B and octyl-Sepharose CL-4B were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). Immobilon P came from Millipore Corp. (Bedford, MA), guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) from Boehringer-Mannheim (Indianapolis, IN), dimyristoyl-L-α-phosphatidylcholine from Sigma Chemical Co. (St. Louis, MO), and choleragen from List Biochemical Laboratories (Campbell, CA). Goat anti-

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[1] Abbreviations: ARF, ADP-ribosylation factor; rARF, recombinant ARF; CT, choleragen; BSA, bovine serum albumin, DTT, dithiothreitol; fluoroaluminate, 10 mM NaF plus 10 μM $AlCl_3$; G_s, stimulatory guanine nucleotide binding protein; $G_s\alpha$, α-subunit of G_s; $G\beta\gamma$, $\beta\gamma$-subunit complex of G proteins; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; solution A, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 10 mM $MgCl_2$; solution B, 30 mM HEPES, pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 150 mM NaCl, and 0.1% Lubrol-PX; solution C, 425 mM potassium phosphate, pH 7.0, 25 mM thymidine, 2.5 mM ATP, 250 μM GTP, and 5 mM $MgCl_2$.

rabbit IgG–horseradish peroxidase conjugate was purchased from Pierce Chemical Co. (Rockford, IL).

Experimental Treatment of G_s and Isolation of G_s Subunits. G_s was prepared from bovine brain as previously described (Roof *et al.*, 1985) and stored in liquid nitrogen in 50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1.0% cholate. In all preparations, the 45-kDa species of $G_s\alpha$ predominated. The sample used for reconstitution, CT-catalyzed ADP-ribosylation, and immunoprecipitation experiments was estimated to contain 450 μg of G_s/mL. The sample was diluted to adjust the concentration to approximately 40 μg/mL (10 μg/mL for reconstitution assays), while making the solution 20 mM HEPES, pH 8.0, 1 mM DTT, 1 mM EDTA, 0.1% Lubrol-PX, and the desired concentration of $MgCl_2$. Sometimes the solution also included either 100 μM GTPγS or 10 mM NaF plus 10 μM $AlCl_3$ (fluoroaluminate). Sample volumes containing 80 ng (20 ng for reconstitution assays) of G_s were incubated at 30 °C for 2 h. The incubations were done in tightly capped 0.65-mL siliconized polypropylene Microfuge tubes. To minimize solute concentration caused by evaporation, samples were centrifuged briefly in a Microfuge at 15-min intervals to recover condensate that collected on the sample tube walls. Following the incubations, samples were diluted for assay as described below.

The sample used for separation of G protein subunits contained approximately 160 μg of G_s/mL. Thirty-five microliters of this sample was diluted to adjust the concentration to approximately 80 μg/mL and make the solution 25 mM HEPES, pH 8.0, 1 mM DTT, 1 mM EDTA, 1% cholate, 120 mM $MgCl_2$, and 100 μM GTPγS. The sample was incubated at 30 °C for 3 h and then diluted with 11 volumes (770 μL) of 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 109 mM NaCl, and 0.18% cholate. After dilution, the sample was applied to a 1-mL octyl-Sepharose CL-4B column that had been equilibrated with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 10 mM $MgCl_2$ (solution A) containing 100 mM NaCl and 0.25% cholate. The column was washed with 2 mL of solution A containing 300 mM NaCl and 0.25% cholate followed by a 26-mL linear gradient from 170 mM NaCl and 0.45% cholate in solution A to 90 mM NaCl and 1.0% cholate in solution A. The flow rate was approximately 10 mL/h, and 500-μL fractions were collected. Volumes of 133 μL were removed from every other fraction, and the protein was precipitated with 10% trichloroacetic acid using 7 μg of BSA as a carrier protein. The precipitates were washed with ethanol/ethyl ether (50%50 v/v) to remove cholate and dissolved by incubating at 100 °C for 5 min in a solution containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol. Immunoblots were used to identify fractions containing G_s subunits (see below). Fractions containing $G_s\alpha$ were pooled, and the buffer was exchanged for 20 mM HEPES, pH 8.0, 1.5 mM EDTA, 1 mM DTT, and 0.7% cholate with a Centricon 10 filter (Amicon Corp., Beverly MA). Fractions containing Gβ were treated similarly, and the samples were stored at −80 °C until needed for experiments. Subsequently, the $G_s\alpha$ and Gβ subunits were either mixed or incubated separately in a volume of 40 μL that contained 20 mM HEPES, pH 8.0, 1.5 mM EDTA, 1 mM DTT, 0.7% cholate, and 30 μM GDP for 30 min at 30 °C before being ADP-ribosylated by CT as described below.

Reconstitution of S49 Cyc⁻ Adenylylcyclase with G_s. Membranes of the cyc⁻ variant of S49 lymphoma cells which lack $G_s\alpha$ were prepared as described previously (Ross *et al.*, 1977). G_s activity was determined by its ability to reconstitute the adenylylcyclase in these membranes. Alternatively, the membranes were used for the preparation of ADP-ribosylation factor (see below). Reconstitution of S49 cyc⁻ adenylylcyclase was accomplished by modification of a published method (Sternweis *et al.*, 1981). Briefly, G_s treated as described above was diluted to 150 μL, and 30-μL samples were mixed with 10 μL of suspended membranes containing 25 μg of S49 cyc⁻ protein. The resulting mixture was 20 mM HEPES, pH 8.0, 0.01 mM DTT, 1 mM EDTA, 0.03% Lubrol-PX, and 2 mM $MgCl_2$. Samples were incubated for 1 h at 10 °C; then 20 μL of 25 mM phosphocreatine, 5 units of creatine phosphokinase, 150 mM HEPES (pH 8), 0.3% BSA, 2 mM $MgCl_2$, and 2 mM ATP was added. The incubation was continued for 10 min at 30 °C. Ten microliters of water (unstimulated) or 200 μM isoproterenol plus 300 μM GTPγS (isoproterenol-stimulated) was added followed by 20 μL of 25 mM phosphocreatine, 5 units of creatine phosphokinase, 250 mM HEPES (pH 8), 0.2% BSA, 5 mM DTT, 4 mM $MgCl_2$, 0.5 mM ATP, 5 mM theophylline, 5 mM EDTA, and 5 mM cyclic AMP. The samples were then assayed for adenylylcyclase activity immediately or after a 1-h incubation at 30 °C (designated as "preliminary incubation" in Table 1). The assay for adenylylcyclase activity was initiated by adding 10 μL of [α-^{32}P]ATP (1–2 μCi) to bring the total volume to 100 μL. The samples was incubated for 15 min at 30 °C, and the amount of cyclic [^{32}P]AMP was determined as previously described (Salomon, 1979).

Immunoprecipitation of G_s. After experimental treatment of G_s, 40-ng samples were diluted (see figure legends for volume) to make the solution 30 mM HEPES, pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 150 mM NaCl, and 0.1% Lubrol-PX (solution B). For immunoprecipitation, 1 μL of RM/1, a polyclonal antiserum raised against the carboxyl-terminal decapeptide of $G_s\alpha$ (Simonds *et al.*, 1989), was added, and the samples were incubated for 30 min at room temperature. Then 10 μL of a 50% (v/v) suspension of protein A–Sepharose CL-4B in solution B was added, and the incubation was continued for another 30 min at room temperature with mixing to keep the gel suspended. The immune complex was collected by brief centrifugation in a Microfuge, and washed twice with solution B. Precipitated proteins were dissolved by adding 15 μL of solution containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and placing the samples in a boiling water bath for 5 min.

Immunoblots. Proteins from the octyl-Sepharose CL-4B column fractions were separated on a 10% SDS–polyacrylamide gel and transferred by electrophoresis to Immobilon P membranes. Unoccupied binding sites were blocked by incubating the membrane for 30 min at room temperature in a solution of phosphate-buffered saline, pH 7.4, containing 5% nonfat dry milk, 0.25% gelatin, and 10% (v/v) glycerol. RM/1 and SW/1 (Murakami *et al.*, 1992) were used for the detection of $G_s\alpha$ and Gβ, respectively. Membranes were incubated overnight with a mixture of antisera diluted 1:300, washed, and incubated for 1 h with a goat anti-rabbit IgG–horseradish peroxidase conjugate. Incubations with antibodies and washes were all done at room temperature in the same solution used to block the membrane. The immunoblots were developed with chloronaphthol.

For other experiments, G_s subunits were detected in a manner similar to that described above for the octyl-Sepharose CL-4B fractions except that the membranes were blocked in a solution of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.3% Tween 20, the primary antibodies were diluted 1:600, 6 mL of a solution containing 1–2 μCi/mL ^{125}I-protein A was substituted for the secondary antibody, and all incubations

and washes subsequent to the blocking step were done in 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20. Using the autoradiograms as a template, the radiolabeled bands corresponding to $G_s\alpha$ and $G\beta$ were cut from the membranes and assayed for ^{125}I-protein A with a γ emissions spectrometer. The amount of ^{125}-protein A bound to immunoblots was a linear function of the amount of $G_s\alpha$ or $G\beta$ present on the blots. To compensate for variation in sample recovery, the amount of ^{125}I-protein A associated with $G_s\alpha$ was used to normalize the data when calculating the percent heterotrimer. One hundred percent heterotrimer was defined as the amount of $G\beta$ present when G_s was immunoprecipitated from solution containing 20 mM HEPES, pH 8.0, 1 mM DTT, 1 mM EDTA, 0.1% Lubrol-PX, and 2 mM $MgCl_2$ after incubating for 2 h at 30 °C. Incubation of G_s in the same solution at 0 °C had little effect on the amount of $G\beta$ that was immunoprecipitated with $G_s\alpha$.

ADP-Ribosylation Factor. ADP-Ribosylation factor (ARF) is required to support CT-catalyzed ADP-ribosylation of G_s (Enomoto & Gill, 1979; Kahn & Gilman, 1984b). ARF was prepared by suspending S49 cyc$^-$ membranes at a protein concentration of 4 mg/mL in 20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.5% Lubrol-PX with stirring at 4 °C for 1 h. Insoluble material was removed by centrifugation at 100000*g* for 1h, and the soluble fraction which contained ARF was saved. Alternatively, a solution of recombinant ARF (rARF) containing 1 mg/mL in 20 mM Tris, pH 8.0, 0.25 M sucrose, 0.1 M NaCl, 1 mM DTT, 1 mM NaN_3, 1 mM benzamidine, 1 mM EDTA, and 5 mM $MgCl_2$ was obtained as a generous gift from Dr. Joel Moss. Samples of ARF and rARF were stored at –80 °C until needed.

ARF prepared from S49 cyc$^-$ membranes was used for ADP-ribosylation reactions after being diluted 5-fold to reduce the Lubrol-PX concentration to 0.1% without changing the concentration of other components in the solution. ARF is a GTP binding protein, and GTP must be bound to ARF in order for it to support ADP-ribosylation by CT (Kahn & Gilman, 1986). ARF from S49 cyc$^-$ extracts was used for ADP-ribosylation reactions without first incubating it with GTP (solutions used for ADP-ribosylation contained GTP). rARF, on the other hand, was activated with GTP or GTPγS before it was used in ADP-ribosylation reactions. Guanine nucleotide binding was carried out by incubating rARF for 2 h at 30 °C after it was diluted 12.5-fold to make the solution 20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 6 mM $MgCl_2$, 100 mM NaCl, 0.2% sodium cholate, 3 mM dimyristoyl-L-α-phosphatidylcholine, 0.02% BSA, and 100 μM GTP or GYPγS (the concentration of other components in the stock solution of rARF were reduced by 12.5-fold as a result of the dilution). rARF activated with GTP was used for ADP-ribosylation reactions without further treatment. When rARF was activated with GTPγS, the unbound nucleotide was removed by filtration through a Centricon 10 filter using 20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 100 mM NaCl as a wash solution.

CT-Catalyzed ADP-Ribosylation of G_s. A stock solution of CT containing 1 mg/mL was prepared as recommended by the manufacturer and stored at 4 °C until needed. To activate CT, 1 volume of toxin was mixed with 4 volumes of solution containing 50 mM HEPES, pH 7.5, 0.25 mg/mL ovalbumin, 169 mM NaCl, and 12.5 mM DTT, and the mixture was incubated for 12 min at 30 °C. Usually ADP-ribosylation was achieved by incubating 40-ng experimental samples of G_s for 1 h at 30 °C with 24 μL of solution C, 5 μM NAD, 2 μCi of [^{32}P]NAD (30 Ci/mmol), 10 μL of S49 cyc$^-$ extract as a source of ARF, and 12 μL of activated CT in a final volume of 60 μL. Solution C was composed of 425 mM potassium phosphate, pH 7.0, 25 mM thymidine, 2.5 mM ATP, 250 μM GTP, and 5 mM $MgCl_2$. For ADP-ribosylation of G_s subunits from the octyl-Sepharose CL-4B column, the incubation included 20 μL each of activated CT, rARF, and a solution containing 300 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 5 mM thymidine, 5 μM NAD, and 2 μCi of [^{32}P]NAD (30 Ci/mmol). ADP-ribosylation was terminated by precipitation with trichloroacetic acid. Precipitated proteins were collected by centrifugation for 15 min in a Microfuge. Sometimes the pellets were washed with a solution of ethanol and ethyl ether (1:1, v/v) to remove cholate. The proteins were then dissolved and separated by electrophoresis on a 10% polyacrylamide gel as described above. Polyacrylamide gels were fixed and stained with Coomassie brilliant blue, dried, and exposed to X-ray film. The amount of [^{32}P]ADP-ribose incorporated into $G_s\alpha$ was estimated with a Betascope 603 blot analyzer (Betagen Corp., Mountain View, CA).

G_s Subunit Reassociation. In some experiments, $G_s\alpha$ was immunoprecipitated, and its ability to reassociate with $G\beta\gamma$ was determined. Immunoprecipitation was carried out as described above except that the concentrations of $MgCl_2$, GTPγS, and fluoroaluminate present in the experiments were maintained during the immunoprecipitation. The pellets containing a complex of G_s, RM/1 antibody, and protein A–Sepharose CL-4B were washed with solution B contained the appropriate ligand for samples of G_s that had been treated with fluoroaluminate or GTPγS. The precipitated protein–Sepharose complex was suspended in 60 μL of solution C, and 20 μL of S49 cyc$^-$ extract or rARF was added. Some samples were ADP-ribosylated by the addition of 10 μL of activated CT and 10 μCi of [^{32}P]NAD (800 Ci/mmol) in a final volume of 150 μL. For these experiments, the CT was activated at twice the concentration used for ADP-ribosylation of soluble G_s. Other samples were used for immunoblots to determine the amount of G_s subunits in the immunoprecipitate. For these experiments, 0.1 μM NAD was substituted for [^{32}P]-NAD, and buffer was substituted for activated CT. Samples were kept suspended by mixing and incubated for 1 h at 30 °C. Following incubations, the immune complex was collected a by brief centrifugation in a Microfuge, and washed twice with solution B. Precipitated proteins were dissolved and separated by electrophoresis as described above. G_s subunits and [^{32}P]ADP-ribosylated proteins were assayed as described above.

Data Presentation. The numerical data presented in figures represent the mean and standard deviation for samples from two or more independent experiments. Each experiment was done 2 or more times with similar results. The data in Table 1 represent the mean and standard deviation for triplicate samples from a representative experiment.

RESULTS

$G_s\alpha$ was dissociated from $G\beta$ by high concentrations of $MgCl_2$ in the presence of GTPγS ($G\gamma$ remains tightly associated with $G\beta$; Gilman, 1987), and the subunits were separated by octyl-Sepharose CL-4B chromatography. On the basis of immunoblots, the $G_s\alpha$ preparation was free of contaminating $G\beta$, but the preparation of $G\beta$ contained 5–10% $G_s\alpha$. Contamination of the $G\beta$ preparation of $G_s\alpha$ was confirmed by its ability to reconstitute S49 cyc$^-$ adenylyl-cyclase. The preparation of $G_s\alpha$ could not be ADP-ribosylated by CT (Figure 1). However, $G_s\alpha$ in the $G\beta$ preparation was

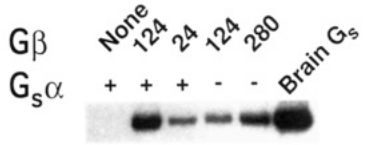

FIGURE 1: CT-catalyzed ADP-ribosylation of the $G_s\alpha$ subunit in the presence and absence of $G\beta$. G_s subunits were separated by octyl-Sepharose CL-4B chromatography as described under Experimental Procedures. Approximately 18 ng of $G_s\alpha$ or the indicated nanogram quantity of $G\beta$ was incubated separately or together as designated in the figure before being ADP-ribosylated by CT. The amounts of $G_s\alpha$ and $G\beta$ should be used for comparison and should not be considered as absolute values since they are based on the amount of starting material and the portion of subunit estimated from immunoblots to be present in the fractions that were subsequently pooled. The figure shows the 45-kDa region of the gel. As discussed under Results, the preparation of $G\beta$ was contaminated with 5–10% $G_s\alpha$, explaining the presence of a radiolabeled 45-kDa band in this preparation.

ADP-ribosylated by CT. When preparations of $G_s\alpha$ and $G\beta$ were mixed, there was more CT-catalyzed ADP-ribosylation of $G_s\alpha$ than with comparable amounts of either preparation alone. These results were interpreted as meaning that CT-catalyzed ADP-ribosylation of $G_s\alpha$ required the presence of $G\beta$.

To investigate more thoroughly the relationship between G_s subunit interaction and CT-catalyzed ADP-ribosylation of $G_s\alpha$, we developed an immunoprecipitation assay to determine the subunit composition of G_s. RM/1 antiserum against $G_s\alpha$ will form an antigen–antibody complex with soluble G_s (Simonds *et al.*, 1989; Morris *et al.*, 1990). This complex was subsequently precipitated with protein A–Sepharose, and the subunit composition of the immunoprecipitate was determined by assaying immunoblots for $G_s\alpha$ and $G\beta$. When G_s was immunoprecipitated with RM/1 antiserum, $G\beta$ coprecipitated with $G_s\alpha$ (Figure 2). The amount of $G\beta$ that coprecipitated with $G_s\alpha$ was reduced in a dose-dependent manner by concentrations of $MgCl_2$ in excess of 4 mM, indicating that $MgCl_2$ caused G_s subunits to dissociate. G_s is also a substrate for the bacterial toxin CT. The addition of ARF or rARF to the reaction mixture was required for CT to catalyze ADP-ribosylation of G_s in solution. When G_s was incubated with $MgCl_2$, there was a dose-dependent decrease in the amount of [^{32}P]ADP-ribose incorporated into $G_s\alpha$. Quantitative comparison indicated a good correlation between the amounts of G_s heterotrimer and of ADP-ribosylation by CT (Figure 2).

The guanine nucleotide binding site of $G_s\alpha$ can bind a number of different ligands, and ligand binding to the site is influenced by $G\beta\gamma$ (Brandt & Ross, 1985). Consequently, it was important to determine if the ligand that occupies this site might also influence the ability of $G_s\alpha$ to serve as a substrate for CT. To test this possibility, we investigated two such ligands: GTPγS and fluoroaluminate. We first examined their effects on the ability of G_s to reconstitute the adenylylcyclase in $G_s\alpha$-deficient S49 cyc$^-$ membranes. G_s incubated with 2 mM $MgCl_2$ in the absence of GTPγS or fluoroaluminate was able to reconstitute isoproterenol-stimulated enzyme activity in S49 cyc$^-$ membranes (Table 1). In contrast, G_s incubated with 120 mM $MgCl_2$ in the absence of either ligand was unable to reconstitute hormone-stimulated activity, suggesting that G_s had been inactivated. When G_s was incubated with GTPγS or fluoroaluminate, the reconstituted adenylylcyclase activity of S49 cyc$^-$ membranes was increased because of residual ligand binding to G_s (Brandt & Ross, 1985; Northup *et al.*, 1982). Furthermore, both GTPγS and fluoroaluminate protected G_s from the loss of activity that

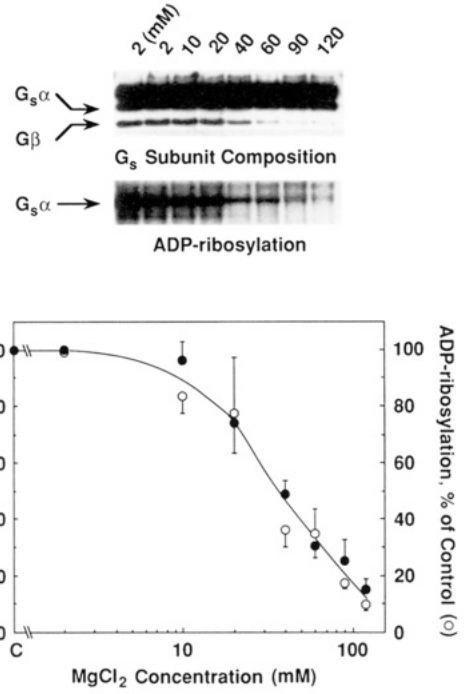

FIGURE 2: Effects of $MgCl_2$ concentration on G_s subunit dissociation and CT-catalyzed ADP-ribosylation of $G_s\alpha$. Samples containing approximately 80 ng of G_s were incubated with the indicated concentrations of $MgCl_2$ as described under Experimental Procedures. Following the incubation, half of each sample was diluted to 180 μL and immunoprecipitated in order to determine the subunit composition of G_s, the other half was ADP-ribosylated with CT. The autoradiograms show representative immunoblots of immunoprecipitated G_s subunits (upper) and [^{32}]ADP-ribosylated $G_s\alpha$ (lower). The samples in the first lane were incubated at 0 °C, and all other samples were incubated at 30 °C with the designated concentrations of $MgCl_2$. The graph shows quantitative data for the G_s heterotrimer (●) and CT-catalyzed ADP-ribosylation (○). There was experimental variation in the amount of radioactivity associated with immunoprecipitated G_s subunits in the control samples. For $G_s\alpha$ and G_β, the counts ranged from 2000 to 4000 cpm and from 1000 to 2000 cpm, respectively.

occurred when it was incubated with 120 mM $MgCl_2$ alone. On the basis of these observations, we concluded that GTPγS and fluoroaluminate could interact with G_s regardless of the $MgCl_2$ concentration present during the incubations.

Reconstituted adenylylcyclase activities were higher when G_s was activated by GTPγS or fluoroaluminate in the presence of 120 mM $MgCl_2$ compared with activation in the presence of 2 mM $MgCl_2$ (Table 1). The differences were not due to variation in the amount of $G_s\alpha$ incorporated into S49 cyc$^-$ membranes (data not shown) but were caused by differences in the amount of ligand bound by $G_s\alpha$ (Northup *et al.*, 1982; data submitted for publication elsewhere). When the adenylylcyclase assay was preceded by a "preliminary incubation" of reconstituted S49 cyc$^-$ membranes, the concentrations of GTPγS and fluoroaluminate were reduced almost 230-fold. However, samples of G_s that had been treated with GTPγS were still activated (Table 1). The persistent activity was due to high-affinity binding of GTPγS by G_s (Northup *et al.*, 1982; Brandt & Ross, 1985). On the other hand, G_s activation by fluoroaluminate did not persist when the ligand was diluted, suggesting that fluoroaluminate dissociated from G_s during the preliminary incubation. We also observed that the preliminary incubation increased the hormone response of S49 cyc$^-$ membranes reconstituted with previously unactivated G_s. Presumably this was a consequence of allowing additional time for hormone binding to its receptor and GTPγS activation of G_s. As a result, the disparity between isoproterenol-

Table 1: Effects of $MgCl_2$, GTPγS, and Fluoroaluminate on G_s Activity[a]

treatment	adenylylcyclase act. [pmol of cAMP (mg of S49 cyc⁻ membrane protein)⁻¹ (5 min)⁻¹] after			
	no preliminary incubn		1-h preliminary incubn	
	none	isoproterenol + GTPγS	none	isoproterenol + GTPγS
2 mM $MgCl_2$				
no ligand	14 ± 3	90 ± 3	2 ± 3	740 ± 40
fluoroaluminate	81 ± 2	180 ± 10	7 ± 2	960 ± 20
GTPγS	1050 ± 10	1010 ± 10	1580 ± 10	1820 ± 60
120 mM $MgCl_2$				
no ligand	13 ± 4	14 ± 2	1 ± 1	17 ± 3
fluoroaluminate	570 ± 30	570 ± 7	26 ± 7	1120 ± 30
GTPγS	4600 ± 200	3810 ± 30	3550 ± 30	3600 ± 100

[a] G_s was treated with the indicated concentration of $MgCl_2$ in the presence and absence of fluoroaluminate or GTPγS and used to reconstitute S49 cyc⁻ membrane adenylylcyclase as described under Experimental Procedures. As indicated in the table, some samples were subjected to a 1-h preliminary incubation with (+) or without (–) isoproterenol plus GTPγS.

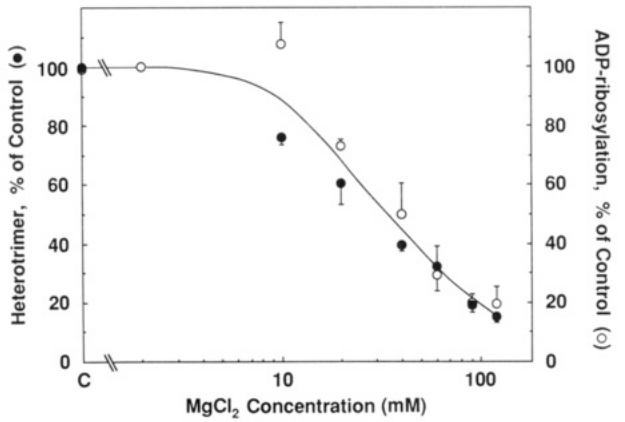

FIGURE 3: Effects of $MgCl_2$ concentration on G_s subunit dissociation and CT-catalyzed ADP-ribosylation of $G_s\alpha$ in the presence of GTPγS. Details are the same as those described in the legend to Figure 2, except the samples of G_s were incubated with the indicated concentration of $MgCl_2$ in the presence of 100 μM GTPγS.

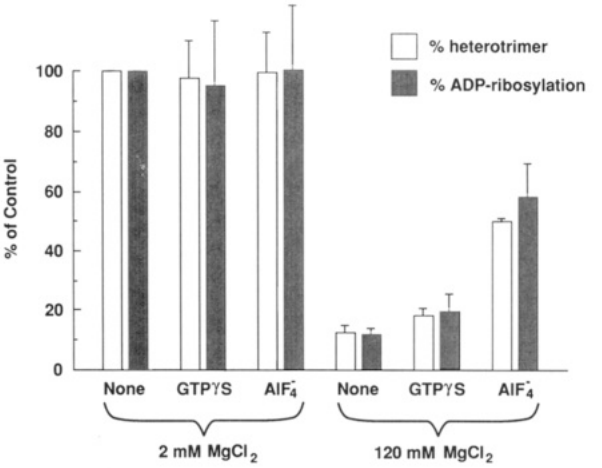

FIGURE 4: G_s subunit dissociation and ADP-ribosylation in the presence of $MgCl_2$ with or without GTPγS or fluoroaluminate. Samples containing approximately 80 ng of G_s were incubated with the indicated concentration of $MgCl_2$ in the presence or absence of fluoroaluminate or GTPγS as described under Experimental Procedures. Following the incubation, half of each sample was diluted to 60 μL and immunoprecipitated to determine the subunit composition of G_s (open bars). The other half of each sample was ADP-ribosylation with CT (stippled bars).

stimulated adenylylcyclase activity in membranes reconstituted with unactivated G_s and those reconstituted with ligand-activated G_s was reduced.

We compared the $MgCl_2$-induced dose-dependent dissociation of G_s subunits in the presence and absence of GTPγS (Figure 3 vs Figure 2) and found no significant shift in the curves. Furthermore, even though GTPγS was bound by G_s, $MgCl_2$ still caused a dose-dependent decrease in the ability of CT to ADP-ribosylate $G_s\alpha$. Quantitative comparison of the amount of G_s heterotrimer and the extent of CT-catalyzed ADP-ribosylation showed that they were well correlated (Figure 3). There was also good agreement between the amount of G_s heterotrimer and the extent of ADP-ribosylation when G_s was treated with fluoroaluminate (Figure 4). This was true regardless of the concentration of $MgCl_2$ present during the incubations. When G_s was incubated with 2 mM $MgCl_2$, the amounts of G_s heterotrimer and of CT-catalyzed ADP-ribosylation were not significantly different in the presence and absence of fluoroaluminate. When samples of G_s were incubated with 120 mM $MgCl_2$, however, there was more heterotrimer and more CT-catalyzed ADP-ribosylation when fluoroaluminate was present during the incubation than when it was absent. On the basis of the data presented of Figure 4, a similar tendency was observed for samples G_s treated with GTPγS. More heterotrimer was present in the immunoprecipitate when G_s samples were treated with 120 mM $MgCl_2$ in the presence of GTPγS than in its absence (19.5 ± 3.6% with GTPγS vs 13.6 ± 2.6% without GTPγS), and there was more CT-catalyzed ADP-ribosylation (19.7 ± 6.6% with GTPγS vs 10.1 ± 2.3% without GTPγS). However, these differences were not highly significant, and they were not observed when the volume used for immunoprecipitation was increased (see Figures 2 and 3) or when the immunoprecipitation procedure was otherwise altered (see Figure 5 below).

We investigated why the amount of G_s heterotrimer recovered in immunoprecipitates (as well as the extent of CT-catalyzed ADP-ribosylation of $G_s\alpha$) was increased when G_s was treated with 120 mM $MgCl_2$ in the presence as compared with the absence of ligands that activate G_s. We considered two likely explanations for these observations: Either 120 mM $MgCl_2$ caused less G_s subunit dissociation in the presence of these ligands, or G_s subunits may have reassociated during the assays for CT-catalyzed ADP-ribosylation and G_s heterotrimer. The latter possibility arises because the $MgCl_2$ concentration during these assays was reduced to 2 mM, a concentration of $MgCl_2$ that did not promote G_s subunit dissociation and consequently may have allowed for subunit reassociation. An experiment was designed to test these possibilities. G_s was treated with 2 or 120 mM $MgCl_2$ with or without fluoroaluminate or GTPγS, and the immunoprecipitation was done without changing the experimental conditions. The amount of $G_s\alpha$ precipitated was similar

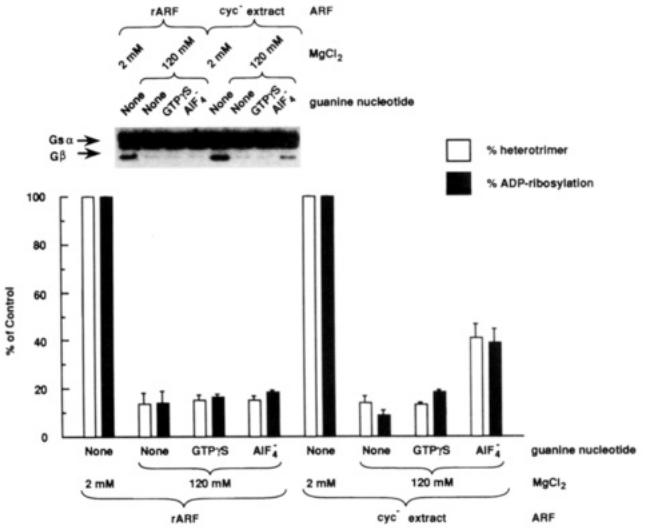

FIGURE 5: Subunit reassociation following treatment with $MgCl_2$ in the presence or absence of GTPγS or fluoroaluminate. Samples of G_s were incubated with the indicated concentration of $MgCl_2$ in the presence or absence of GTPγS or fluoroaluminate as described under Experimental Procedures. Samples were immunoprecipitated in the presence of the same concentrations of $MgCl_2$, GTPγS, and fluoroaluminate used for experiments. Immunoprecipitated samples were subsequently mixed with either S49 cyc⁻ membrane extracts (containing Gβγ) or rARF (lacking Gβγ), and after incubation as described under Experimental Procedures, the precipitate was assayed for G_s subunit composition (open bars) and ADP-ribosylation by CT (stippled bars). The autoradiogram shows the results of a typical subunit composition assay.

regardless of the $MgCl_2$ and ligand concentrations present during the immunoprecipitation, suggesting that the presence of these components did not adversely affect the procedure (Figure 5). The immunoprecipitated samples were then resuspended in buffer containing 2 mM $MgCl_2$ in the presence of rARF or a detergent extract of S49 cyc⁻ membranes. Although the S49 cyc⁻ extract was originally intended as a source of ARF to support ADP-ribosylation (see Experimental Procedures), it also contained Gβγ. The rARF had no Gβγ. When G_s was immunoprecipitated from buffer containing 120 mM $MgCl_2$ and the precipitate was subsequently assayed for G_s subunit composition in the presence of rARF, there was very little Gβ associated with the immunoprecipitate. When the S49 cyc⁻ extract was substituted for rARF during the assays, there was more G_s heterotrimer and a comparable increase in CT-catalyzed ADP-ribosylation for samples treated with fluoroaluminate in the presence of 120 mM $MgCl_2$ when compared with samples that had not been treated with fluoroaluminate. By contrast, this phenomenon was not observed when G_s subunits were treated with 120 mM $MgCl_2$ in the presence of GTPγS.

DISCUSSION

G_s, which is responsible for mediating stimulation of adenylylcyclase by hormones and neurotransmitters, is an important and arguably the best studied signal-transducing protein. Among the historical milestones in the study of G_s were the discovery that conditions which led to the activation of this heterotrimeric protein could also cause the α-subunit to dissociate from the Gβγ-subunit complex (Sternweis *et al.*, 1981) and the finding that the α-subunit was the substrate for ADP-ribosylation by the bacterial toxin CT (Cassel & Pfeuffer, 1978; Gill & Meren, 1978). The former discovery provided the basis for the most widely accepted mechanistic model of how G_s activates the catalytic subunit of adenylylcyclase and how the activity of adenylylcyclase is regulated. The latter finding, in addition to providing insight into the pathophysiology of cholera, established a method for tagging G_s. Subsequent investigations suggested that $G_s\alpha$ was a substrate for CT both when it was a part of the G_s heterotrimer and when it was dissociated from Gβγ as a result of being activated by GTP analogues or by fluoroaluminate (Kahn & Gilman, 1984a). However, recent evidence has suggested that free $G_s\alpha$ may not be a substrate for CT (Journet *et al.*, 1991). In our own laboratory, we have observed that when $G_s\alpha$ was separated from Gβ it could not be ADP-ribosylated by CT and that adding back Gβ restored the ability of $G_s\alpha$ to serve as a substrate for the toxin.

We developed an immunoprecipitation assay that enabled us to distinguish between heterotrimeric and dissociated G_s. Using this assay together with experimental conditions that allowed us to control G_s subunit dissociation, we have been able to investigate the nature of the CT substrate. The subunits of G_s were dissociated in a dose-dependent manner by $MgCl_2$. Although previous studies have suggested that subunit dissociation is a consequence of G_s activation, we have found that activation is not required to cause subunit dissociation. When $G_s\alpha$ was dissociated from Gβγ by $MgCl_2$, it was no longer ADP-ribosylated by CT, suggesting that the G_s heterotrimer but not free $G_s\alpha$ is the substrate for the toxin. However, it has been reported (Codina *et al.*, 1984; Northup et al., 1982, 1983) and we have observed that G_s loses its ability to activate adenylylcyclase when its subunits are dissociated in the absence of activating ligands that bind to the guanine nucleotide binding site of $G_s\alpha$ (see Table 1). Thus, denaturation may account for the failure of CT to ADP-ribosylate $G_s\alpha$ following dissociation from Gβγ in the presence of high concentrations of $MgCl_2$. Alternatively, the ligand that is bound to $G_s\alpha$ may affect ADP-ribosylation by CT. Since Gβγ can influence ligand binding to $G_s\alpha$ (Brandt & Ross, 1985), the ability of the latter to serve as a substrate for CT may not be a direct consequence of Gβγ interaction with $G_s\alpha$ but an indirect consequence of its influence on the ligand that is bound to it. In the same vein, it is possible that ADP-ribosylation of $G_s\alpha$ may depend upon both interaction with Gβγ and the ligand that is bound to the guanine nucleotide binding site.

In an attempt to address these possibilities, we decided to investigate the effects of two ligands, fluoroaluminate and GTPγS, on G_s subunit dissociation and CT-catalyzed ADP-ribosylation. We observed that both ligands were able to activate G_s regardless of the $MgCl_2$ concentration. Furthermore, G_s subunit dissociation did not occur in the presence of either ligand when the concentration of $MgCl_2$ was low (2 mM). However, subunit dissociation did occur when the concentration of $MgCl_2$ was high (120 mM). We concluded that GTPγS and fluoroaluminate could bind both to the free $G_s\alpha$ subunit and to the G_s heterotrimer. We found that the G_s heterotrimer could still be ADP-ribosylated by CT but despite ligand binding the free $G_s\alpha$ subunit could not be made by a substrate for the toxin.

When G_s subunits were dissociated in the absence of activating ligands, they were apparently unable to reassociate. However, if dissociation was carried out in the presence of fluoroaluminate, subunit reassociation occurred even if the fluoroaluminate concentration was maintained during the reassociation phase of experiments (data not shown). Although some subunit reassociation may also have occurred when GTPγS was bound to $G_s\alpha$, it was less than 10% under the experimental conditions used in these studies, and was

much less than the reassociation that occurred when G_s subunits were dissociated in the presence of fluoroaluminate. These observations could explain the results of other investigators who have observed that G_s activated with fluoroaluminate was a substrate for CT but G_s activated with GTPγS was not even though the conditions for activation by both ligands were shown to cause G_s subunit dissociation (Kahn & Gilman, 1984a). Rapid reassociation of subunits may have occurred for fluoroaluminate-activated G_s but not for GTPγS-activated G_s during CT-catalysed ADP-ribosylation. In contrast, reassociation may not have been possible under conditions used to determine the subunit composition of G_s.

Our results indicate that the G_s heterotrimer but not free $G_s\alpha$ is ADP-ribosylated by CT regardless of the ligand that is bound to the guanine nucleotide binding site of $G_s\alpha$. The importance of this study lies not only in identifying the substrate for CT but also in its ramifications for investigating the role of G_s subunits in the stimulation of adenylylcyclase by hormones and neurotransmitters. As mentioned early in this discussion, the predominant model proposes that when G_s is activated its subunits must dissociate in order to stimulate the catalytic subunit of adenylylcylase. Most of the evidence that supports this model is based on studies of the behavior of G_s and other G proteins in solution. It is not clear that what applies to G_s in solution can be extended to biological membranes. In addition, recent investigations have suggested that G_s subunit dissociation is not required for stimulation of adenylylcyclase (Marbach *et al.*, 1990; manuscript submitted for publication). Since CT can distinguish between the G_s heterotrimer and the free $G_s\alpha$ subunit, the toxin is a potentially useful tool to monitor G_s subunit interactions during adenylylcyclase stimulation in membrane.

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