ADP-Ribosylation by Cholera Toxin: Functional Analysis of a Cellular System That Stimulates the Enzymic Activity of Cholera Toxin Fragment A_1[†]

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ABSTRACT: We have clarified relationships between cholera toxin, cholera toxin substrates, a membrane protein S that is required for toxin activity, and a soluble protein CF that is needed for the function of S. The toxin has little intrinsic ability to catalyze ADP-ribosylations unless it encounters the active form of the S protein, which is S liganded to GTP or to a GTP analogue. In the presence of CF, S·GTP forms readily, though reversibly, but a more permanent active species, S–guanosine 5′-*O*-(3-thiotriphosphate) (S·GTPγS), forms over a period of 10–15 min at 37 °C. Both guanosine 5′-*O*-(2-thiodiphosphate) and GTP block this quasi-permanent activation. Some S·GTPγS forms in membranes that are exposed to CF alone and then to GTPγS, with a wash in between, and it is possible that CF facilitates a G nucleotide exchange. S·GTPγS dissolved by nonionic detergents persists in solution and can be used to support the ADP-ribosylation of nucleotide-free substrates. In this circumstance, added guanyl nucleotides have no further effect. This active form of S is unstable, especially when heated, but the thermal inactivation above 45 °C is decreased by GTPγS. Active S is required equally for the ADP-ribosylation of all of cholera toxin's protein substrates, regardless of whether they bind GTP or not. We suggest that active S interacts directly with the enzymic A_1 fragment of cholera toxin and not with any toxin substrate. The activation and activity of S are independent of the state, or even the presence, of adenylate cyclase and seem to be involved with the cyclase system only via cholera toxin. S is apparently not related by function to certain other GTP binding proteins, including $p21^{ras}$, and appears to be a new GTP binding protein whose physiologic role remains to be identified.

Cholera toxin differs from other ADP-ribosylating toxins in that its catalytic A_1 peptide has little activity in the absence of GTP (Enomoto & Gill, 1980). For several years, we have been investigating this nucleotide requirement. We know that the requisite GTP binds not to the toxin or substrates but to a membranous "S"[1] site, which increases the enzyme activity of the A_1 peptide. This binding of GTP to S is promoted by a soluble cellular protein termed "CF" (Gill & Meren, 1983) which was originally characterized in whole cell cytosol. To better understand the nature of the CF/S system and its effect upon cholera toxin, we have largely purified CF and have developed conditions that allow the assay of partial reactions within the intact membrane while maintaining as much as possible of the original sensitivity to cholera toxin. Nonhydrolyzable GTP analogues have a stable effect on S which allows the CF-directed activation of S to be separated temporally form the assay of the effect of activated S on ADP-ribosylation rates. The two stages of the assay can also be separated physically by solubilizing S after activation with CF and GTPγS and using the solution to support the ADP-ribosylation of nucleotide-free substrates on other membranes.

By these means, we have established that S is indeed a GTP binding protein that is active when its nucleotide site is occupied by GTP or a GTP analogue. The generation of $S{\cdot}G_{nuc}$ can occur without the binding of G nucleotide (G_{nuc}) to the major ADP-ribosylation substrate, G_s. In fact, $S{\cdot}G_{nuc}$ increases the ADP-ribosylation rate in parallel for major and minor protein substrates regardless of whether they bind GTP. Despite earlier suppositions (Gill & Meren, 1983; Kahn & Gilman, 1984), there is no reason to suppose that $S{\cdot}G_{nuc}$ interacts directly with any component of adenylate cyclase. Instead, $S{\cdot}G_{nuc}$ must interact with peptide A_1 of cholera toxin, perhaps to form an enzymically potent complex.

Kahn and Gilman have recently confirmed that the protein cofactor required for cholera toxin stimulation of adenylate cyclase activity is a GTP binding protein (Kahn & Gilman, 1986). We believe that their "ARF" and our S are the same protein, but we cannot agree with their suggestion that ARF/S interacts with G_s. Our data indicate that it interacts with cholera toxin. In the normal cell, it presumably interacts with some other enzyme and changes its activity in some important way. We have investigated several possibilities for the physiologically relevant role of S but remain able to assess the status of S only by its effect on cholera toxin.

Experimental Procedures

The medium used throughout is 130 mM NaCl, 10 mM HEPES, pH 7.3, 0.01% sodium azide, and 0.01 TIU/mL aprotinin. Cholera toxin (List Laboratories, Campbell, CA) was reduced with dithiothreitol, carboxymethylated in the presence of 0.1% SDS, and dialyzed into 0.1% SDS. The stock was stored at a concentration of 200 μg/mL and was generally used at a final concentration of 10 μg/mL, thus giving a final SDS concentration of 0.005%.

For most experiments, we have used pigeon erythrocyte membranes. Washed erythrocytes in 1 volume of medium were lysed by freeze–thaw, and the ghosts were washed once.

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GPP(NH)p, guanylyl imidodiphosphate; $GPP(CH_2)p$, guanylyl β,γ-methylenediphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); CF, cytosolic protein factor required for the activity of cholera toxin; S, membranous guanyl nucleotide site required for the activity of cholera toxin; ARF, ADP-ribosylation factor (Kahn & Gilman, 1984, 1986); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; ADPR, ADP-ribose; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; TIU, trypsin inhibitor unit.

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The red pellet was incubated for 15 min at 37 °C in 2 mM $CaCl_2$ and 3 units/mL micrococcal nuclease, to digest DNA. The digest was stored at this stage at −70 °C. For use, an aliquot was thawed and washed twice in 5 volumes of medium to yield white membranes. We have found by experience that this procedure allows the best retention of S activity.

Bull testis CF was purified to near-homogeneity as described by Wookalis et al. (1987). In outline, the purification begins with the 100000*g* supernatant fraction of fresh bull testis which is passed successively through columns of DEAE-Sephadex, QAE-Sephadex, Bio-Gel P30, and hydroxylapatite. Both the Bio-Gel P30 product and the hydroxylapatite product were used, the final medium being 50 mM NaCl, 10 mM potassium phosphate, and 50 mM HEPES, pH 7.3. By densitometric analysis of stained gels, the product after Bio-Gel P30 chromatography is about 4% pure. Different samples have 10–20 μg/mL of the specific CF protein, whose apparent molecular weight on SDS–polyacrylamide gels is about 20000. The final material is about 40% pure and contains 50 μg/mL CF. Further purification to homogeneity has been achieved by eluting the protein from preparative gels, but the eluted material has a very low specific activity and was not used in the present series of experiments. For heat inactivation, CF solutions were held at 80 °C for 15 min.

Activation of S and Immediate ADP-Ribosylation Assay. To a 5-μL pellet of washed nuclease-treated erythrocyte ghosts in a polypropylene tube was added 10 μL of CF solution and GTPγS to 100 μM. After vortexing to suspend the pellet, the mixture was incubated at 37 °C for 15 min. A 4-μL portion of a reagent mixture was then added, giving the following final concentrations: 10 μg/mL carboxymethylated cholera toxin, 5 μM [^{32}P]NAD (30000–60000 cpm/pmol), 10 mM thymidine. ADP-ribosylation was then allowed to proceed for 30 min at 25 °C.

Preparation of Soluble Active S. Activation of S, as above, involved incubating 1 volume of erythrocyte ghosts (typically 100 μL of packed pellet) with 2 volumes of CF solution and 100 μM GTPγS in a polypropylene tube. The membranes were washed twice in 20 volumes of medium, with centrifugation (5 min, 14500*g*), and then suspended in 1 pellet volume of medium containing 0.1% ovalbumin as a protein protectant and 0.3% NP40. After 5 min at 20 °C, the membranes were removed by centrifugation (5 min at 14500*g*). Before assay, it was important to remove the bulk of the detergent, and this was achieved by mixing the extract with the pellet of 0.5 volume of Extractigel (Pierce Chemical Co., Rockford, IL) that had been prewashed in medium containing 0.1% ovalbumin. The slurry was mixed gently for 5 min at 20 °C, and the extract, from which most of the detergent had been adsorbed, was recovered by centrifugation.

ADP-Ribosylation Using Solubilized S. Typically, 10 μL of the detergent extract was added to a 5-μL pellet of washed nuclease-treated erythrocyte ghosts and 4 μL of the same reagent mixture as is used for the immediate ADP-ribosylation assay.

Analysis of ADP-Ribosylated Products. After the ADP-ribosylation reaction, the mixture was diluted in 2 mL of saline, and the membranes were recovered by centrifugation, dissolved in SDS gel sample buffer, and analyzed on 7.5–15% polyacrylamide slab gels containing 0.1% SDS. The gels were stained with Coomassie blue, destained, and dried. After autoradiography, either the whole track or the region that corresponded to G_s (M_r 42000) was excised and counted. The counts in the M_r 42000 region were corrected for nonspecific counts by subtracting the counts in the region below of equal area. From the counts were calculated the femtomoles of ADPR incorporated into total protein or into G_s alone.

Cell Lines. NIH-3T3 cells and Harvey virus-infected NIH-3T3 cells were kindly provided by Naomi Rosenberg and were analyzed initially by James Hope and Fariba Houman. The cells were grown in Dulbecco's modified Eagle's medium plus 10% newborn calf serum. S49 lymphoma cells, the cyc$^-$ variant S49 cells (Bourne et al., 1975), and membranes from the two lines were generously given by Henry Bourne. These cells were grown in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum. For all cells, a pellet was suspended in 1 volume of medium, lysed by freezing and thawing, and separated by centrifugation into particulate and soluble fractions. The CF activities of the cytosolic fractions were assayed by using erythrocyte membranes as sources of S and toxin substrates, as described above. The S activity of whole lysate and the particulate fraction was evaluated by measuring the increase in rate of ADP-ribosylation of the cellular proteins when provided with GTPγS and purified CF. Before gel analysis of the ADP-ribosylated products, the cell particles were recovered by centrifugation and incubated to destroy DNA using 5 units/mL micrococcal nuclease and 10 mM $CaCl_2$, 5 min at 37 °C. Activated S was extracted as described for erythrocytes.

Polyclonal anti-human HA-*ras* was purchased from Oncor Inc. (Gaithersburg, MD). Five microliters of erythrocyte ghosts was incubated for 1 h at 20 °C with 20 μL of the antibody, while a control sample of ghosts was incubated with an irrelevant antibody. The ghosts were washed and assayed for S activity with fresh CF and GTPγS, followed by ADP-ribosylation, as above. In addition, a 5-μL portion of each antibody was incubated with 10 μL (0.1 μg) of CF, and the CF activities of these samples were determined in parallel assays.

RESULTS

Reversible Activation of Membrane-Bound S by GTP and CF. The state of activation of the S site is usually monitored by measuring the rate of toxin-catalyzed ADP-ribosylation of proteins on the same membranes as S. The target protein with the greatest specific rate of ADP-ribosylation is the α subunit of G_s, but, as we have done in Table I, it is equally relevant to include the ADP-ribosylation rates of less rapidly modified protein targets ("secondary substrates") some of which are identified later in this paper. By either measure, the activation of S by GTP (and CF) is rapid. The rate of ADP-ribosylation reaches a maximal level as soon as GTP is added (Enomoto & Gill, 1979) and remains constant until substrates become depleted. Since GTP is effective at supporting ADP-ribosylation, and GDP is not, GTP cannot be rapidly hydrolyzed at the S site. Nevertheless, the GTP-dependent activity of S is entirely lost when the membranes are washed, implying either that GTP is removed during the wash or that slow GTP hydrolysis at the S site is nevertheless significant when replacement of GDP by GTP is prevented.

Conditions for Quasi-Permanent Activation of S by GTP Analogues and CF. In contrast to GTP, poorly hydrolyzed analogues such as GTPγS generate a more permanent active state of S that persists when the membranes are washed. This quasi-permanent activated state takes time to develop, and the kinetics are complicated by a generalized thermal inactivation of S which will be discussed below. A reasonable compromise temperature is 37 °C at which the quasi-irreversible activation is maximal after 10–15 min. This is similar to the behavior which we had observed using unfractionated cytosol as a source of CF (Enomoto & Gill, 1980; Gill & Meren, 1983). The

Table I: Characteristics of CF at Different Stages of Purification[a]

	medium only	testis cytosol	Bio-Gel P30	hydroxyl-apatite
protein concn (mg/mL)	0	26	0.5	1.2
volume used (μL)	10	3	10	1
protein supplied (total μg)	0	78	5	1.2
protein supplied, as CF (μg)	0		0.2	0.5
preincubation conditions	ADP-ribosylated protein (fmol/mg)			
CF, 37 °C	0	170	60	30
CF, 0 °C, GTPγS	30	840	720	1220
CF, 37 °C, GTPγS	60	850	980	1760
heated CF, 37 °C, GTPγS		110	100	60

[a] CF was partially purified from bull testis cytosol as outlined under Experimental Procedures and as described in detail by Woolkalis et al. (1987). Up to 10 μL (as shown) of the crude or partially purified CF, or of the same material after heat treatment to inactivate the CF, was added to 5 μL of pigeon erythrocyte membranes. The volume in each case was adjusted to 15 μL by adding CF medium which is 100 mM NaCl, 10 mM potassium phosphate, and 50 mM HEPES, pH 7.3. The quantities of total protein added, and of CF when known, are given in the upper section of the table. The mixtures were supplied with 100 μM GTPγS and were incubated for 15 min at 0 or 37 °C as indicated in the lower section of the table. Membrane proteins were then immediately ADP-ribosylated as described under Experimental Procedures. After gel fractionation, the entire gel tracks, exclusive of poly(ADP-ribose) polymerase in the testis cytosol samples, were excised and counted.

requirement for a 37 °C incubation contrasts with the temperature dependence of the ADP-ribosylation step since cholera toxin's optimal temperature is 25–30 °C. Accordingly, the most effective procedure involves a preincubation of membranes with CF and GTPγS at 37 °C, followed by an ADP-ribosylation reaction at 25–30 °C. The advantage of having a 37 °C stage in addition to the 25 °C incubation is illustrated in Figure 1. GTPγS, Gpp(CH$_2$)p, and Gpp(NH)p have similar effects on S activation. The ED_{50} for each of them, as assayed by subsequent ADP-ribosylation of the same erythrocyte membranes, is between 1 and 3 μM (Figure 2), which is the same value as was previously determined for their effects on adenylate cyclase activation by cholera toxin (Enomoto & Gill, 1980). This value is also obtained as the apparent affinity from double-reciprocal plots. By contrast, these three guanine nucleotides behave very differently toward erythrocyte G_s. Gpp(CH$_2$)p is particularly useful as a discriminator between S and G_s because 3 μM Gpp(CH$_2$)p activates S but not G_s (Figure 2).

Inhibition by Ineffective Nucleotides. Nucleotides which are incapable of generating an active state (GDP and GDPβS) or the quasi-permanent active state (GTP) can block the activation by GTP analogues if present in sufficient quantity. We have pointed out before that 100 mM GDPβS does not affect S activation (Gill & Meren, 1983), but we find now that 1 mM or more of GTP or GDPβS will block the effect of 10 μM GTPγS on S (data not shown). This amount is considerably greater than the amounts that are needed to block the binding of GTPγS to G_s in erythrocyte or other membranes, underlining the fact that nucleotide bindings to S and to G_s are different events.

Role of Cytosolic Factor. Both the reversible and the quasi-permanent activations of S depend in a dose-dependent manner on the proteinaceous cytosolic factor "CF" (Figure 1). Unfractionated cytosol is effective but contains guanine nucleotides, poly(ADP-ribose) polymerase, and in some cases other enzymes that consume NAD, and it is inhibitory to the

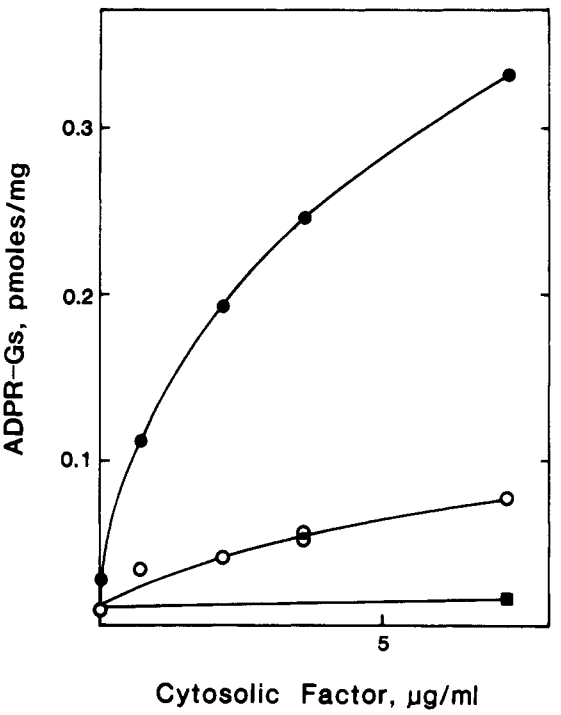

FIGURE 1: Dependence of the S activity on preincubation with CF at 37 °C. Membranes mixed with 100 μM GTPγS and different volumes of CF (10 μg/mL) or with heated CF (■) and buffer to 15 μL total volume were incubated for 15 min at 37 °C (●, ■) or 0 °C (○) and then immediately ADP-ribosylated at 25 °C, as described under Experimental Procedures.

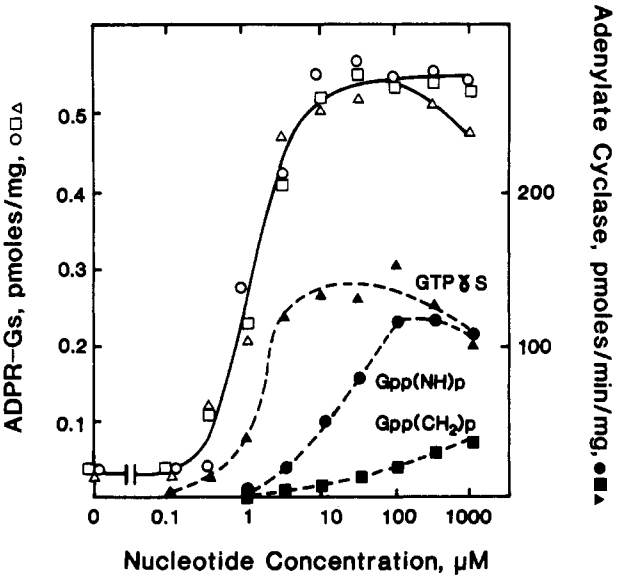

FIGURE 2: Effects of three guanyl nucleotide analogues on the S site and G_s site of pigeon erythrocyte membranes. Each point represents a sample of 5 μL of membranes plus 50 ng of CF, incubated 15 min at 37 °C with the indicated concentration of nucleotide: GTPγS (△, ▲); Gpp(NH)p (○, ●); or Gpp(CH$_2$)p (□, ■). After being washed in 50 volumes of saline, duplicate samples of membranes were assayed for S activity (upper line, open symbols) or adenylate cyclase activity (lower three lines, closed symbols).

toxin-catalyzed ADP-ribosylation at high concentrations so that the dose–response curve exhibits a peak. We have achieved significant purification of active CF which eliminates these disadvantages, and the possibility of other interference, and have identified the active material as a single polypeptide chain of about M_r 20 000 (Woolkalis et al., 1987). Activation of S with the purified material is totally dependent upon exogenous guanine nucleotide (Table I). We have not been able

Table II: Dependence of S Activity on the Order of Exposure of Membranes to CF and to Nucleotides

Part A[a]

step I	step II	S act., ADPR G_s (fmol/mg)
none, wash	CF + GTPγS	1105
CF, wash	GTPγS	506
heat-inactivated CF, wash	GTPγS	100

Part B[b]

step Ia	step Ib	step II	S act., ADPR G_s (fmol/mg)
none, wash	0, wash	GTPγS	241
CF, wash	0, wash	GTPγS	494
none, wash	GDP, wash	GTPγS	194
CF, wash	GDP, wash	GTPγS	423

[a] In step I, 5-μL portions of erythrocyte membranes were incubated with 10 μL of CF or heat-inactivated CF for 10 min, 37 °C, and then washed in 300 μL of medium. They were then provided with 10 μL of the ingredients in the step II column (final concentrations 100 μM GTPγS and 7 μg/mL CF) and incubated for 10 min at 37 °C. The S activity of the ghosts was measured immediately. [b] As (A) except that the first incubation was followed by an intermediate incubation (column Ib) with or without 1 mM GDP and a second wash in 300 μL of medium.

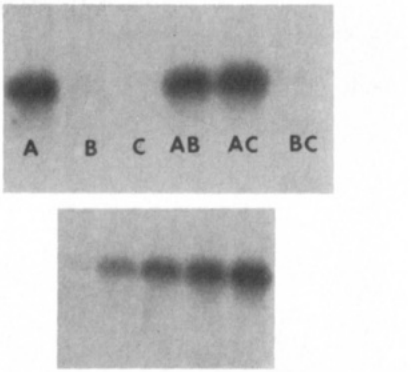

FIGURE 3: Transfer of S activity. Detergent extracts were prepared from donor ghosts and then assayed for S activity on 5 μL of fresh erythrocyte ghosts as described under Experimental Procedures. Autoradiographs of the M_r 42 000 band region are shown. (Upper panel) 10 μL of extract prepared by using both GTPγS and CF (A), GTPγS only (B), CF only (C), or mixtures of 5 μL each of extracts A + B, A + C, and B + C. Lower panel: 0, 1, 3, 5, or 10 μL of extract A was supplied, and medium was added to standardize the volumes.

to purify CF to homogeneity without a major loss of activity, but the most refined active material is about 40% pure on a protein basis. At this stage, after hydroxylapatite column chromatography, different preparations with the same specific activity have only the CF band in common, so the contaminants seem to be silent. It has been particularly useful to find that rather less purified material, taken after the Bio-Gel P30 step (see Experimental Procedures), is indistinguishable in effect from the final product and can be used on a routine basis. The data in Table I show that the Bio-Gel P30 purified material has similar characteristics to the hydroxylapatite-purified protein.

As we had found using whole cytosol as a source of CF (Enomoto & Gill, 1980), the more purified factor is required only at the S activation step, not for ADP-ribosylation itself. It is most effective if provided at the same time as a guanyl nucleotide such as GTPγS. However, with the purified system, we now find a significant effect if membranes are first incubated with CF alone and then washed before being exposed to GTPγS (Table IIA). This is the behavior expected if CF were to act as a nucleotide exchange protein that displaced GDP from S·GDP and left a vacant nucleotide site. S does not become inactive again if the membranes are exposed to GDP (Table IIB) or GDPβS (not shown) after the incubation with CF, but this is not necessarily incompatible with a nucleotide exchange role for CF (see Discussion).

Solubilization of Active S. In order to more clearly distinguish nucleotide effects on S activation from nucleotide effects on ADP-ribosylation itself or on the ADP-ribosylation substrates we set out to solubilize S in an active form and to assay the activated S on nucleotide-free membranes. It is necessary to treat S gently and to work rapidly in the cold, for its activated form readily loses activity. We have found that it is relatively easy to dissolve active S from membranes in detergent, and the conditions selected result in the solubilization of comparatively little membrane protein. Solutions in nonionic detergents such as NP40, Triton X-100, or Zwittergent retain more activity than those in cholate, octyl glucoside, or CHAPS.

The use of 0.3% NP40 allows the maximum extraction of active S with the minimum loss of S activity. We then remove most of the detergent by adsorption to Extractigel prior to assay. Activity is best retained in the presence of a protective protein and in plastic tubes. The active material migrates in the void volume of Sephadex G75 and binds to hydrophobic columns of C>4 (Shaltiel columns; Miles Laboratories, Elkhart, IN). Attempts at further purification have resulted in complete loss of activity.

The active S in these extracts allows the ADP-ribosylation of proteins in "recipient" membranes that have not been exposed to nucleotide or to CF (Figure 3). Control extracts prepared from membranes that had been incubated without GTPγS or without CF are not active. Furthermore, a mixture of the latter two extracts is inactive, although neither is inhibitory (Figure 3). Thus, activity depends on incubation of the donor membranes with both CF and GTPγS. These results also suggest that the active extract does not simply supply small amounts of CF and GTPγS that had been adsorbed to the donor membranes, a conclusion that is supported by the following findings.

An extract prepared from a membrane treated solely with CF is not active when supplemented with GTPγS, even in amounts far exceeding the amount of free GTPγS present in an active extract (see below). Likewise, the full amount of CF used for activation barely raises the activity of an extract prepared from membranes treated only with GTPγS. We cannot detect CF in the detergent extracts by silver staining or by immunoblotting, although the amount of CF originally used to generate the active S was quite detectable.

Our overall conclusions, therefore, are that activated S in the extract is a new species which must be created in the donor membrane *before* detergent extraction and which cannot be generated to any significant extent by mixing CF and GTPγS with detergent extract of control membranes.

Although active S is best created in the membrane context, we do not find that it reintegrates into recipient membranes in the assay conditions we use. It appears to be active in solution.

S Activity Is Independent of the Condition or Presence of G_s. Previous experiments and the data in Figure 2 suggest that S activation occurs independently of the nucleotide activation state of G_s. To extent this observation, we searched for chemicals that would selectively render G_s unsuitable as a toxin substrate while permitting the S-dependent ADP-ribosylation of minor substrates. We found several which had this property. The best was the thiol-modifying reagent 3-(chloromercuri)-2-(methoxypropyl)urea (Gill & Woolkalis, 1985), which, as shown in Figure 4, eliminated G_s as a substrate but

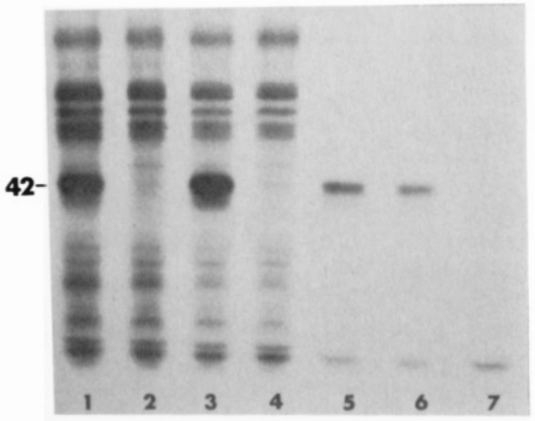

FIGURE 4: One demonstration that there is no transfer of the major toxin substrate G_s. Lane 1 represents control erythrocyte membranes preincubated 10 min at 25 °C in 1% ethanol. For lane 2, similar membranes were preincubated with 0.8 mM 3-(chloromercuri)-2-(methoxypropyl)urea in 1% ethanol to render G_s unsuitable as a cholera toxin substrate. Lanes 3 and 4 show similar material to lanes 1 and 2 after extraction with NP40. Lanes 5 and 6 represent recipient membranes supplied with extracts 1 and 2, respectively, while lane 7 shows 3-(chloromercuri)-2-(methoxypropyl)urea-treated recipient membranes supplied with extract 1. After these treatments, the membranes were subjected to direct [^{32}P]ADP-ribosylation, as described under Experimental Procedures. An autoradiogram is shown.

had little effect on the activation of S or on its activity in solution. Another indication that S does not depend on the integrity of G_s is that the cyc$^-$ variant of S49 lymphoma cells, which lacks the gene for G_s (Harris et al., 1985), and has no ADP-ribosylatable substrate that resembles G_s, is as good a source of active S as is the wild-type cell and its minor substrates are equally well ADP-ribosylated.

Since to have active S we were forced to use impure solutions, we took pains to eliminate the possibility that the extract might also provide toxin substrates from the donor membranes and that donor cell G_s in particular might have been among the proteins eventually ADP-ribosylated. The following results show that little or no substrate transfer to the recipient membranes occurred. Little G_s is dissolved by 0.3% NP40, and most of it can still be shown in the extracted donor membranes by a subsequent ADP-ribosylation (Figure 4). When the G_s of the donor and recipient membranes could be distinguished by size, the donor G_s was found not to be ADP-ribosylated. For example, when the donor membranes were from S49 cells (Figure 5) or brain (not shown), both of which have G_s of two sizes (M_r 42 000 and 46 000/47 000), and the recipients were from pigeon erythrocytes (G_s = M_r 42 000 only), only the M_r 42 000 species became labeled.

Guanyl Nucleotide Interaction with S. Exogenous guanyl nucleotides protect active S in solution against a loss of activity that is otherwise incurred upon heating. This protection is illustrated in Figure 6. In similar experiments, we established the following order of effectiveness as protectants: GTPγS > Gpp(NH)p > GTP ≅ ATPγS ≅ none. We deduce, therefore, that the binding of guanyl nucleotides to S protects it from heat inactivation. The active form of S presumably has GTPγS bound in a manner that is reversed at high temperature unless exogenous nucleotides maintain a fully liganded state. By extension, we suggest that S·GTP is the active form of S normally formed in cells. It has been recently demonstrated that a purified ADP-ribosylation factor ("ARF") with characteristics similar to S is active when bound to GTP and inactive when bound to GDP (Kahn & Gilman, 1986).

We used several techniques to attempt to quantitate the relevant GTPγS, and thereby the active S. We first followed

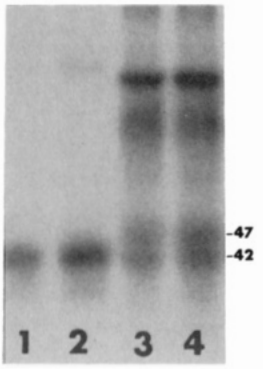

FIGURE 5: [^{32}P]ADP-ribosylated substrates are specific for the recipient membranes, not for the extract. (Track 1) Erythrocyte ghosts ADP-ribosylated in the presence of S supplied as a detergent extract from CF/GTP S-activated S49 lymphoma cell membranes (see Experimental Procedures). (Track 2) Erythrocyte ghosts, erythrocyte extract. (Track 3) S49 membranes, S49 extract. (Track 4) S49 membranes, erythrocyte extract. Marked are the positions of the two forms of G_s, of M_r 47 000 (absent in the erythrocytes) and M_r 42 000.

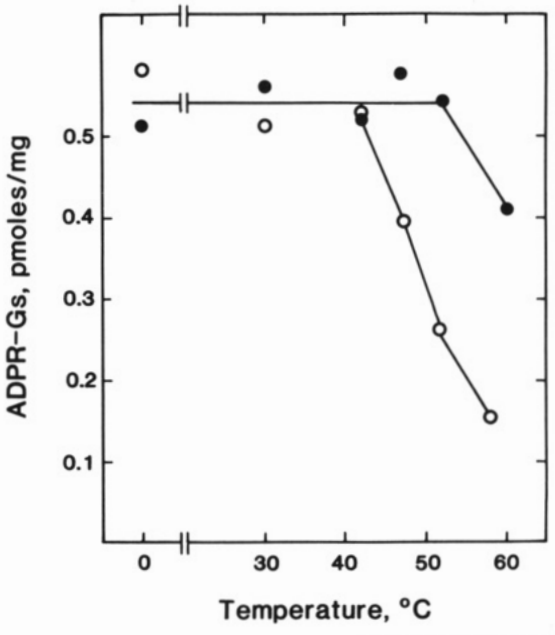

FIGURE 6: Deactivation of active S by heat and protection by GTPγS. Portions of a detergent extract of CF/GTPγS-activated ghosts were held at the indicated temperatures for 10 min, with (●) or without (○) 100 μM GTPγS. The GTPγS concentration was then equalized for all samples. S activities were assayed as described under Experimental Procedures.

the distribution of label after exposing erythrocyte membranes to 100 μM [^{35}S]GTPγS and CF. The radiolabeled nucleotide is an effective activator. Much of the GTPγS is bound initially and is released from membranes only slowly. The detergent extract containing active S typically contains about 0.5–1 μM GTPγS, but the amount does not differ significantly between extracts prepared with or without CF and which are respectively active or inactive. The number is reduced by more extensively washing the donor membranes before preparing the detergent extract, a treatment that proportionately reduces the S activity of the extract: the removal of about 200 bound GTPγS molecules ultimately results in the transfer of 1 fewer ADP-ribose residue. Thus, only a small fraction of this GTPγS can possibly be relevant to the S activity. The same excess of nonspecifically bound nucleotide may account for our inability to affinity label any erythrocyte membrane protein in a CF-dependent manner using 8-azido[^{32}P]GTP, [^{32}P]-azidoanilido-GTP, [^{32}P]GTP bisaldehyde, or [^{32}P]GTP itself

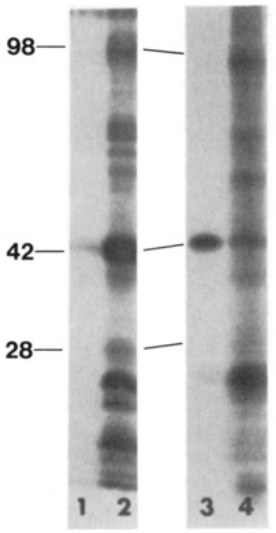

FIGURE 7: ADP-ribosylation patterns of erythrocyte ghost proteins incubated in the presence of (1) GTPγS, (2) GTPγS + CF, (3) detergent extract containing active S, or (4) detergent extract and 5% poly(ethylene glycol). The sizes marked represent G_s (M_r 42000) and two secondary substrates discussed in the text.

(UV irradiated). These reagents also failed to label G_s and G_i in the erythrocyte membranes, which are present at about 1500 and 300 copies per pigeon erythrocyte, respectively. [^{32}P]GTP, with UV light, labeled only the much more abundant proteins α- and β-tubulin. We therefore conclude that S is of low or moderate abundance in pigeon erythrocyte membranes.

General Properties of ADP-Ribosylation Supported by Soluble Activated S. The soluble active S has its maximal effect on the ADP-ribosylating activity of cholera toxin as soon as it is added and without any further requirement for a preincubation at 37 °C; in fact, its activity declines at 37 °C. Evidently, the extract already contains whatever active material is formed at 37 °C and can only suffer thermal inactivation upon further 37 °C incubation. By contrast, if any of the detergent extracts in Figure 3 is supplemented with both CF and GTPγS, the extra activity which these factors confer still requires an incubation with the recipient membranes at 37 °C, just as if they were supplied without extract. In other respects, the reaction using detergent-extracted S is identical with that supported by CF and GTPγS. The optimal temperature for the toxin reaction is still 25–30 °C, and the most readily ADP-ribosylated protein is still G_s. If the cholera toxin activity is increased in any manner [in Figure 7, lane 4, this was achieved by adding 5% poly(ethylene glycol) which we have discovered to be a nonspecific stimulant of cholera toxin], some minor proteins are then ADP-ribosylated by using the extracts, and these are the same, and in the same proportion, as those labeled by using GTPγS and CF directly.

The activation of S proceeds equally well at any salt concentration between 0 and 1 M. On the other hand, salt must be provided for the ADP-ribosylation step. This explains why cholera toxin is ineffective in the absence of cations and why it often exhibits an increase in activity when the salt concentration is raised above isotonicity. Magnesium is not needed for S function.

Differences between S and Other GTP Binding Proteins. S is widely distributed in nature—we have even detected it in yeast—and it obviously did not evolve solely to interact with cholera toxin. In pursuit of its normal role, we searched for similarities between S and known GTP binding proteins, to investigate both whether S was already known in another context and, if not known, whether it might perform a function similar to that of a known GTP binding protein. The results were all negative.

Table III: Pertussis Toxin Catalyzed ADP-Ribosylation of G_i in Erythrocyte Membranes Does Not Affect Subsequent Cholera Toxin Catalyzed ADP-Ribosylation of G_s[a]

first incubation	ADP-ribosyl G_s (fmol/mg) control	pertussis toxin
second incubation, cofactors supplied with cholera toxin		
none	25	54
CF (10 μg/mL)	30	32
GTPγS (100 μM)	168	181
CF and GTPγS	1132	1322

[a] Pigeon erythrocyte ghosts were incubated with 10 μg/mL pertussis toxin (List Laboratories, Campbell, CA) (activated by incubation with 100 mM dithiothreitol), 1 mM ATP, and 100 μM NAD (unlabeled). Parallel experiments using [^{32}P]NAD confirmed that this treatment ADP-ribosylates erythrocyte G_i completely. Control ghosts had dithiothreitol without pertussis toxin. The ghosts were washed and incubated with cholera toxin, [^{32}P]NAD, and the cofactors shown in the first column. The table records the extent of ADP-ribosylation during this second incubation.

Unlike G_s, the S activity of membranes is not affected by 10 mM sodium fluoride (with or without aluminum ions), forskolin, or catecholamines. Isoproterenol plus GTPγS reduces the availability of G_s as a substrate for cholera toxin (D. M. Gill and J. L. Coburn, unpublished experiments), but the S activity of membranes treated with this combination, as judged by the rate of ADP-ribosylation of protein substrates other than G_s, or from the activity of a detergent extract, is normal.

It is also unlikely that S is G_i, or that it interacts with G_i, for the following reasons: (1) The S activity of membranes from different tissues does not vary in proportion to the content of any pertussis toxin substrate. (2) Quantitative ADP-ribosylation of G_i using pertussis toxin has no effect on the cholera toxin reaction rate with CF, with GTPγS, or with both (Table III). (3) The activation of S has no effect on the rate of the pertussis toxin catalyzed ADP-ribosylation of G_i in the same membranes. We also note that S is present in membranes that lack G_o and thus S cannot be G_o.

We asked whether S might be similar in biological function to transducin. The latter stimulates a cyclic GMP phosphodiesterase when activated by GTPγS and light, but we could find no similar activity of S. Thus, activation of S by incubation with CF and GTPγS has no effect on the cyclic GMP phosphodiesterase (or cyclic AMP phosphodiesterase) activity of pigeon erythrocyte membranes.

The products of mammalian and viral *ras* genes (p21) are M_r 21 000 membrane proteins that bind and slowly hydrolyze GTP (Scolnik et al., 1979). However, lysates of NIH-3T3 cells infected with Harvey virus had the same sensitivity to cholera toxin as lysates of uninfected NIH-3T3 cells (see Experimental Procedures) even though they have about 20 times more $p21^{ras}$. The CF activity of the virally infected cells was also unchanged from control cells. Further, an antibody directed against $p21^{v\text{-}Ha\text{-}ras}$ that also reacts with cellular $p21^{ras}$ had no effect on the S activation of cholera toxin (see Experimental Procedures). These results imply that S differs from $p21^{v\text{-}Ha\text{-}ras}$ and probably also from the closely related cell-encoded *c-ras* proteins.

It is likely that S is the same protein as the membranous ADP-ribosylation factor ARF (Kahn & Gilman 1984, 1986) which is necessary for the action of cholera toxin on G_s and

which has now been shown to bind GTP. Purified ARF has a molecular mass of 21 000 daltons. Thus, even if S/ARF is not precisely a *ras* protein, its properties suggest that it could be one of the *ras*-related proteins whose functions are not yet established (see Discussion).

ADP-Ribosylation and the Identity of Certain Secondary Substrates. While G_s is by far the preferred substrate for cholera toxin, a variety of other proteins are also ADP-ribosylated. These "secondary substrates" include both soluble and particulate cellular proteins (Gill & Meren, 1978). Most of them are abundant proteins that can be related to specific stained bands on a gel. They are ADP-ribosylated at specific rates several orders of magnitude lower than that of G_s, but those that are most abundant can contribute significantly to the total ADP-ribosyl product.

The ADP-ribosylation of such secondary substrates requires the activation of S in exactly the same way as does the ADP-ribosylation of G_s (Figure 7, lanes 1 and 2).

We have identified two of the secondary substrates of pigeon erythrocyte ghosts. The first protein overlies band III, M_r 98 000, in SDS gels and after isoelectric focusing. The anion transporting protein, which is the major component of the stained band III, has the useful diagnostic property of forming large aggregates when it is boiled in SDS gel sample buffer. No other stained band in erythrocyte membranes does this. The [^{32}P]ADP-ribosylated 98K protein forms aggregates exactly in parallel with the anion channel and moves in concert from the M_r 98 000 position to the top of an SDS–polyacrylamide gel, suggesting that it is the identical protein.

The other identified radioactive product is coincident on SDS gels with the avian erythrocyte-specific high-mobility group nuclear protein of apparent M_r 28 000 (Figure 7). Both the Coomassie blue stained protein band and the radioactive band have the property of precipitating near the top of the gel if the operating voltage exceeds 200–300 V. Under all operating voltages, the stained protein and the radioactive product distribute alike.

Discussion

Effect of S. The membrane protein that we call S is a GTP binding protein that, when active, increases the catalytic activity of cholera toxin. S itself becomes activated upon incubation with a guanine nucleotide and CF. The active derivative probably has the guanine nucleotide bound since GTPγS protects the detergent-solubilized form of active S against loss of activity when heated, but the GTPγS must be held reversibly since it can be removed by extensive washing. S is not sufficiently abundant in pigeon erythrocytes to be demonstrated in the membranes or in detergent extracts either by guanyl nucleotide binding or by labeling with a variety of analogues of GTP.

The activation of S affects the activity of cholera toxin in a general way and in no way depends on the particular substrate. Activated S affects only the rate of ADP-ribosylation, not the amount of toxin substrate available for reaction. The simplest interpretation is that activated S interacts directly with the enzymic A_1 fragment of cholera toxin and increases its ADP-ribosylating activity toward all proteinaceous substrates. An alternative possibility is that A_1 interacts with S, becomes activated, and remains active upon dissociation from S, in which case S might act catalytically [compare Kahn and Gilman (1984)].

Nature of the Cholera Toxin Substrates. It is clear that the binding of GTP to cholera toxin substrates is not required for their ADP-ribosylation. G_s can be readily modified without having any nucleotide bound, and full activation of G_s with GTPγS actually hinders ADP-ribosylation. Indeed, there is no particular reason to focus on GTP binding proteins as toxin substrates. The two secondary substrates that we have identified in pigeon erythrocyte ghosts, the membrane anion channel and a nuclear high-mobility group protein, are not GTP binding proteins. We have calculated from published data that, with the exception of G_s, GTP binding proteins are not especially favored as cholera toxin substrates (Gill & Woolkalis, 1985). Under our optimum conditions, we find only negligible ADP-ribosylation of tubulin. There is no reason a priori to suspect that the secondary substrates yet to be identified will be found to bind GTP.

Active S is as necessary for the ADP-ribosylation of all the diverse secondary substrates as for G_s (Figure 7), and the original assumption that S should interact with the toxin substrates is no longer tenable. The GTP requirement for cholera toxin is adequately explained as an effect via S on the toxin's intrinsic activity rather than as an effect on substrates.

Role of CF. Membrane-bound S becomes active upon incubation with CF and GTPγS at 37 °C. The active form generated under these conditions is presumably S·GTPγS. We have considered whether CF might act as a G nucleotide exchange protein akin to prokaryotic Ts (Weissbach et al., 1971; Kaziro, 1978), eukaryotic IF2-linked release factor (Panniers & Henshaw, 1983; Salmans et al., 1984), rhodopsin (Fung & Stryer, 1980), and G_s- and G_i-linked hormone receptors. If S were inactive when occupied by GDP, the role of CF could be to displace GDP and to allow the entry of GTP. Thus, S·GDP → S, caused by CF, might be followed by the spontaneous formation of S·GTP.

The case may be slightly more complex, however. We showed (Table IIB) that the sequential exposure of membranes to CF, GDP, and GTPγS was little worse than the sequential exposure to CF, no nucleotide, and GTPγS, whereas one might have expected in the former case that vacant sites would be reoccupied by GDP and thereby inactivated again. However, it is not necessary to assume that GDP can stably reoccupy a vacant site on S. Once the original GDP has dissociated, S might change its conformation so as to disfavor GDP rebinding. Alternatively, CF might remain associated with S and prevent any stable occupation by GDP. This is precisely the behavior of EFTs when it catalyzes GTP for GDP exchange on EFTu. Ts is believed to remain complexed with Tu in the absence of nucleotide, or in the presence of GDP, and only to leave when GTP is bound. Thus, the sequence of reactions is

$$\text{Ts} + \text{Tu·GDP} \rightarrow \text{Ts·Tu} + \text{GDP} \rightarrow \text{Tu·GTP} + \text{Ts}$$

(Kaziro, 1978). Quantitative measurements of the binding properties of CF may test whether a similar sequence of reactions describes the CF/S interaction, for example:

$$\text{CF} + \text{S·GDP} \rightarrow \text{CF·S} + \text{GDP} \rightarrow \text{S·GTP} + \text{CF}$$

Inspection of Figure 1, for example, shows that a relatively large amount of CF is needed to activate S in a preincubation, more consistent with a stoichiometric than with a catalytic effect.

Possible Nature of S. We have found S activity in over 20 vertebrate tissues and cell lines and also in yeast. Cholera toxin affects nearly all vertebrate cells, and many invertebrate cells too, and the reaction is always found to depend upon GTP if examined carefully. It must be inferred that both CF and S are widespread in nature. In either case, we anticipate that S will play an analogous role in the normal condition, serving to activate some ordinary cellular enzyme in a GTP-dependent manner.

We have looked for relationships between S and known GTP binding proteins. Evidence presented earlier (Gill & Meren, 1983) and in this paper leaves no doubt that S and G_s are different. The activation of S by a hydrolysis-resistant GTP analogue requires CF and is relatively unaffected, or is even improved, by adding 100 μM GDPβS in addition to 10 μM GTPγS. It is only slightly inhibited by EDTA and then only slowly, and it has an immediate onset. The activation of G_s, on the other hand, is unchanged by CF, is greatly stimulated by isoproterenol (for erythrocytes) or by prior treatment with isoproterenol plus GMP, is blocked completely by 100 μM GDPβS and by EDTA, and has a 1–2-min lag in its onset. Possibly because GTP is hydrolyzed almost as soon as it is bound to G_s, GTP alone is a poor activator of the erythrocyte cyclase, but it is an excellent activator of S in the same membranes (Enomoto & Gill, 1979). If, therefore, S is a GTPase, the rate of GTP hydrolysis must be slow in relation to the rate of replacement of GDP by GTP.

To this set of differences from G_s, we can now add that S is more readily extracted into detergent and remains functional in solution. It survives treatment with certain chemicals that inactivate G_s. The affinities of S for various guanine nucleotides differ from those of G_s (Figure 2).

In addition, we have reported experiments that distinguish S from G_i, G_o, and $p21^{ras}$. Unlike transducin, S activation does not alter cyclic nucleotide phosphodiesterase activities.

Thus, S appears not to be one of the better known GTP binding proteins. Recently, new GTP binding proteins of yet unknown function have been isolated (Evans et al., 1986), and the existence of others has been inferred from genetic evidence (Madaule & Axel, 1985). It is possible that S is one of these.

Kahn and Gilman (1984) described a membrane protein "ARF" that support ADP-ribosylation but which at the time seemed quite different from S because it seemed not to bind GTP. Recently, however, these authors have concluded that ARF does bind GTP after all (Kahn & Gilman, 1986), and it now seems very likely that ARF and S are the same. As purified from cholate extracts of brain membranes, ARF is a M_r 21 000 protein. It has a bound GDP that can be replaced slowly by GTP, and this is consistent both with our findings and with our suggested role for CF.

Assuming that S and ARF are indeed the same, it is instructive to compare the properties of the membrane-bound protein with purified protein. Kahn and Gilman did not report experiments using CF and, perhaps in consequence, obtained very slow GTP binding (Kahn & Gilman, 1986). They found that ARF bound GTP maximally in 2 h of incubation (no CF present) when the protein was supplied with phospholipid vesicles (composed of dimyristoylphosphatidylcholine), 1 mM $MgCl_2$, and high salt (0.8 M NaCl). We had previously noted that S activation required magnesium ions but in the membrane environment the responsible magnesium dissociates only slowly from S and the activation of S can be significantly prevented only by preexposing the membranes to EDTA (Gill & Meren, 1983). Not surprisingly, the provision of extra phospholipid does not alter the behavior of S that is already part of a membrane. On examination, we found that no amount of phospholipid changed the requirement for CF or the effect of the activated S upon cholera toxin. High salt is not needed in the membrane environment and does not reduce the effect of CF (see Results), nor does high salt affect the limited rate of S activation that we can measure in the absence of CF. Thus, the high salt which Kahn and Gilman found necessary to detect ARF activity in solution may simply rectify some artifactual change during the solubilization of ARF and neither reflects nor compensates for the absence of CF.

Kahn and Gilman measured ARF function indirectly by measuring the consequential change in adenylate cyclase activities after reincorporating ADP-ribosyl G_s into cyc^- membranes. Because they did not measure ADP-ribosylation itself, we find it difficult to know how the activity of their purified ARF compares with that of our activated S. We have been concerned throughout our work to maximize the S activity and thereby to parallel as closely as possible events that occur in the cell. Our experience is that any manipulation makes S more difficult to activate and we suspect that purified material may reflect the natural situation poorly. For example, small amounts of the detergents that solubilize S prevent exogenous CF from functioning (D. M. Gill, J. L. Coburn, and M. Woolkalis, unpublished results). It remains to be seen whether a way can be found to allow S and CF to interact in solution, but for now at least S is best activated in the membrane environment.

Finally, we must disagree with the opinion of Kahn and Gilman of S's role. Because they assay ARF by its effect on G_s activity, and were thus unable to observe the ADP-ribosylation of secondary substrates, these authors stress possible relations between ARF and G_s (Kahn & Gilman, 1986). However, our data indicate that this interaction is less likely than a direct activation of cholera toxin by ARF/S.

Registry No. GDP, 146-91-8.

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