Chemoattractant and GTPγS-mediated Stimulation of Adenylyl Cyclase in *Dictyostelium* Requires Translocation of CRAC to Membranes

Pamela J. Lilly and Peter N. Devreotes

Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. We have previously reported that activation of adenylyl cyclase by chemoattractant receptors in *Dictyostelium* requires, in addition to a heterotrimeric G-protein, a cytosolic protein, designated CRAC (Lilly, P., and P. N. Devreotes. 1994. *J. Biol. Chem.* 269:14123–14129; Insall, R. H., A. Kuspa, P. J. Lilly, G. Schaulsky, L. R. Levin, W. F. Loomis, and P. N. Devreotes. 1994. *J. Cell Biol.* 126:1537–1545). In this report, we show that in intact cells, chemoattractants promote translocation of CRAC from the cytosolic to the membrane fraction. However, CRAC is not required at the time of receptor stimulation; it can be added to lysates of activated cells. Treatment of membranes with guanine nucleotides creates binding sites for CRAC. These binding sites can be generated in mutants lacking each of the components of the pathway except the β-subunit, suggesting that free or "activated" βγ-subunits may be a part of the binding site. This hypothesis is consistent with previous observations that CRAC contains a pleckstrin homology domain and that the βγ-subunits likely mediate activation of adenylyl cyclase in this system. Thus, CRAC may serve as an adapter, linking the G-protein βγ-subunits to activation of the enzyme. GTPγS cannot generate CRAC-binding sites when the adenylyl cyclase pathway has been adapted by prior chemoattractant stimulation, suggesting that this is a point of downstream adaptation.

A wide variety of hormones and neurotransmitters exert their actions by regulating the activity of adenylyl cyclases and thus levels of the intracellular second messenger, cAMP. In many instances, stimulation and inhibition are mediated by activation of Gs and Gi; the α-subunits of these heterotrimeric G-proteins modulate the activity of multiple isotypes of adenylyl cyclase catalytic units by direct interaction (Gilman, 1987; Birnbaumer, 1992). Recently, the βγ-dimer has been shown to enhance markedly the stimulation of adenylyl cyclase isotypes II and IV by the α-subunits of the G-protein (Tang and Gilman, 1991). In vivo, this type of regulation could lead to augmentation of cAMP levels by receptors linked to Gi, Go, or Gq (Federman et al., 1992). The role of the βγ-dimer in the adenylyl cyclase system adds to the growing list of effectors that are targets for this subunit (Camps et al., 1992; Katz et al., 1992; Clapman and Neer, 1993).

G-protein–linked signal transduction pathways are essential for the developmental program in *Dictyostelium* (Firtel, 1991; Van Haastert and Devreotes, 1993; Devreotes, 1994). Aggregation in this organism is mediated by extracellular cAMP, which acts as a cell–cell signaling molecule and chemoattractant. A cell-surface receptor for cAMP, cAR1, is linked to a specific G-protein, G2. Signaling through G2 leads to activation of both the cytoskeletal components involved in chemotaxis and the adenylyl cyclase required for aggregation, ACA. This latter response produces transient increases in intracellular cAMP; the messenger is secreted, serving as an intercellular signaling molecule that coordinates the chemotactic movements of an assembly of cells.

Although cAR1, the subunits of G2, and ACA are topologically and structurally homologous to their mammalian counterparts, the pathway from cAR1 to ACA is unusual (Klein et al., 1988; Pupillo et al., 1989; Lilly et al., 1993; Pitt et al., 1993; Devreotes, 1994). cAMP does not stimulate ACA activity in mutants lacking cAR1, Gα2, or Gβ (*car1*$^-$, *gα2*$^-$, or *gβ*$^-$ cells). In lysates, GTPγS results in activation of ACA in *car1*$^-$ and, surprisingly, *gα2*$^-$ cells, but not in *gβ*$^-$ cells (Kesbeke et al., 1988; Pupillo et al., 1992; Wu et al., 1995). These observations have led to the hypothesis that the βγ-dimer, rather than the α-subunit of G2, is linked to activation of ACA (Pupillo et al., 1992; Wu et al., 1995). Accordingly, in intact cells stimulated with cAMP, all of the subunits are required for coupling of G2 to cAR1. In lysates, GTPγS can presumably release activated βγ-subunits from other G-proteins, because the cells contain at least eight distinct α-subunits that share a unique β-subunit (Lilly et al., 1993).

Biochemical and genetic analyses have shown that an-

Address all correspondence to P. N. Devreotes, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolf Street, Baltimore, MD 21205. Tel.: (410) 955-3225. Fax: (410) 955-5759.

other component, designated cytosolic regulator of adenylyl cyclase (CRAC), is also required for both receptor- and GTPγS-mediated stimulation of ACA (Theibert and Devreotes, 1986; Snaar-Jagalska and Van Haastert, 1988; Insall et al., 1994; Lilly and Devreotes, 1994). Null mutants lacking CRAC do not produce cAMP in response to agonists and consequently fail to aggregate. Lysates of *crac*$^-$ cells have no adenylyl cyclase activity, even in the presence of GTPγS, but stimulation can be reconstituted by addition of purified CRAC to lysates that have been preactivated. Since the other components, cAR1, the subunits of G2, and ACA are membrane bound, while CRAC is a cytosolic protein, we proposed that CRAC must become associated with membranes during receptor and G-protein activation.

Sequence analysis indicates that CRAC contains 693 amino acids, is hydrophilic, and is not homologous to any known protein (Insall et al., 1994). Interestingly, it contains an NH_2-terminal pleckstrin homology domain (PH-domain). Pleckstrin homology domains are believed to participate in protein–protein interactions; in some instances, these domains have been implicated in targeting their host proteins to the βγ-subunits of G-proteins (Musacchio et al., 1993; Gibson et al., 1994; Touhara et al., 1994). Participation of CRAC in the activation of adenylyl cyclase in *Dictyostelium* may exemplify a novel class of PH-domain containing "adaptors" that link activated βγ-subunits to effectors. In this study we investigated the receptor- and G-protein–stimulated association of CRAC with membranes from wild-type and mutant cells.

Materials and Methods

Cell Culture and Development

Dictyostelium discoideum strains were grown in HL-5 and developed for 5 h in development buffer (DB), as previously described (Watts and Ashworth, 1970; Sussman, 1987; Devreotes et al., 1987). Cell lines used included wild type (AX3), a CRAC overexpressor (RI8), and the null mutants *crac*$^-$ (BW4), *carl*$^-$ (JB4), *gα2*$^-$ (myc2), *gβ*$^-$ (LW6), and *aca*$^-$ (CAP1).

Supernatant Preparation

Supernatant was prepared from vegetative RI8 cells as described (Lilly and Devreotes, 1994), except that cells were lysed by forcing them through a nucleopore filter (5-μm filter) prior to low-speed centrifugation.

Adenylyl Cyclase Assay

GTPγS-stimulated adenylyl cyclase activity was measured as previously described (Theibert and Devreotes, 1986; Lilly and Devreotes, 1994). The activation trap assay was performed as described (Pupillo et al., 1992).

Immunoblots

Immunoblots were performed as previously described (Towbin et al., 1979; Insall et al., 1994). Samples were separated on 7.5% SDS-PAGE gels and transferred to Immobilon-P. The primary antibody was directed against a peptide corresponding to the deduced C terminus of the CRAC protein. An ECL kit (Amersham Corp., Arlington Heights, IL) was used for detection.

Lysate Preparation

Lysates were prepared from 5-h developed cells in the presence or absence of GTPγS and cAMP, as previously described (Theibert and Devreotes, 1986; Lilly and Devreotes, 1994), and frozen in 10% glycerol in dry-ice/ethanol. These were stored at −70°C prior to use.

Reconstitution of in Vivo Activation

The basic activation trap assay was modified to include a brief incubation of the lysates with either buffer or cytosol. Intact cells were treated with 10 μM cAMP and 10 mM DTT, then analyzed at various times after stimulation. At each time point, an aliquot of cells at 1.6×10^8 ml was mixed with an equal volume of 2× lysis buffer (20 mM Tris, pH 8, 2 mM $MgSO_4$) (Pupillo et al., 1992) and lysed by pushing through a 5-μm membrane. Aliquots of the lysate were mixed 1:1 with either buffer (SLB; 10 mM Tris-HCl, pH 8, 200 mM sucrose, 0.2 mM EGTA) or supernatant prepared from cells overexpressing CRAC activity (RI8). The mixtures were incubated on ice for 1 min, then 200-μl aliquots were assayed for adenylyl cyclase activity as described. Assays for each time point were performed in duplicate.

Membrane Association Assay

In 1.5-ml eppendorf tubes on ice, aliquots of lysate prepared in the presence or absence of GTPγS were mixed with various amounts of supernatant prepared from cells overexpressing CRAC protein (RI8). These were incubated for various lengths of time, then diluted with 1 ml of PM (5 mM sodium phosphate, pH 6.1, 5 mM KH_2PO_4, 2 mM $MgCl_2$). For the 0 time points, lysates were diluted prior to addition of the cytosolic fraction. Samples were centrifuged for 1.5–2 min at ~13,000*g* in a microfuge at 4°C. After removal of the supernatant, the pellet was recentrifuged and the residual liquid removed. Triplicate pellets were then resuspended in either 120 μl PM for assay of adenylyl cyclase (duplicate samples) or in 60 μl 2× sample buffer for immunoblot analysis.

Adaptation of Cells to Chemoattractants

Cells developed for 5 h were shaken at 1.6×10^8/ml in PM for 10 min, then stimulated with 10 μM cAMP at 2-min intervals for 15 min. Lysates were prepared immediately (within 20 s) following the first cAMP stimulus, and again after 15 min of stimulation.

Results

CRAC Acts Downstream of Receptor–G Protein Coupling

Previous studies have established that the cytosolic regulator of adenylyl cyclase, CRAC, is essential for both receptor- and guanine nucleotide–mediated activation of adenylyl cyclase (Lilly and Devreotes, 1994). However, the site at which CRAC acts in this signal transduction pathway, which includes cell-surface receptors, G-proteins, and adenylyl cyclase catalytic subunits, has not been determined. Guanine nucleotide inhibition of cAMP binding is normal in *crac*$^-$ cells, indicating that cAR1/G2 coupling is not impaired (Snaar-Jagalska and Van Haastert, 1988). This suggests that CRAC is required downstream of G-protein activation by the receptor. To verify this, we used the *crac*$^-$ cells to ask whether CRAC must be present at the time of cAMP stimulation.

The ability of ACA to be activated by chemoattractants is typically measured using an "activation trap" assay (Pupillo et al., 1992). Intact cells are stimulated with cAMP, then lysed at various times after stimulation, and the instantaneous state of activation of adenylyl cyclase is measured. To determine whether the *crac*$^-$ cells could be activated, the assay was modified to include preincubation in either the presence or absence of a CRAC-containing supernatant prior to assay. As shown in Fig. 1, wild-type cells incubated with buffer exhibited a rapid rise in adenylyl cyclase activation, which peaked between 1 and 2

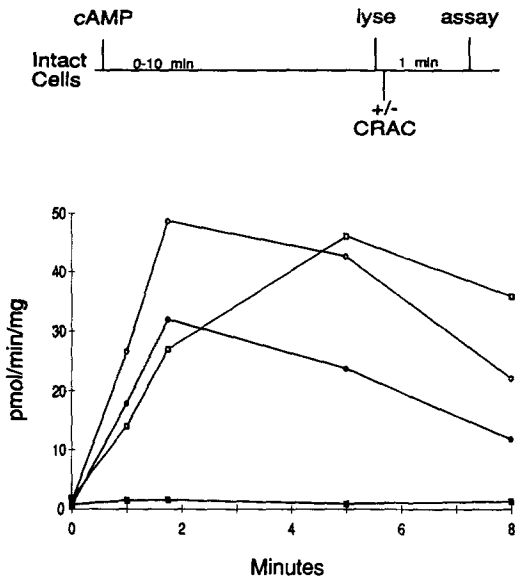

Figure 1. Reconstitution of cAMP-stimulated adenylyl cyclase activation. Both wild-type (*circles*) and *crac*$^-$ (*squares*) cells were shaken at 0°C, treated with 10 mM DTT, then stimulated with 10 μM cAMP for the indicated times. They were rapidly lysed, and aliquots of the lysate were incubated for 1 min, with either buffer (*closed symbols*) or supernatant (*open symbols*) prepared from RI8 cells. Duplicate 1-min assays were performed on each mixture, and the average of these is presented. The results are representative of four independent experiments.

min poststimulation, then subsequently declined as the cells adapted. This time course is similar to the profile seen in the standard assay, indicating that the incubation does not perturb the normal dynamics of this assay. In contrast to wild-type cells, *crac*$^-$ cells show no response to cAMP stimulation. However, incubation of these lysates with CRAC-containing supernatant restored the activation to wild-type levels. This observation suggests that CRAC need not be present at the time of chemoattractant addition and probably acts downstream of receptor excitation of G-proteins. A slight increase in adenylyl cyclase was observed in wild-type lysates that had been preincubated with CRAC supernatant, which may indicate that wild-type CRAC levels are somewhat limiting in vitro. In several experiments, the time course was prolonged in the reconstituted *crac*$^-$ cells.

GTPγS Creates Binding Sites for CRAC

CRAC is a relatively large hydrophilic protein found in the cytosol of *Dictyostelium* amoebae. All other known components of the adenylyl cyclase activation pathway are associated with the membrane fraction. This suggests that CRAC must exert an effect on the membrane either by changing a membrane component or by becoming physically associated with the membrane. No catalytic function has yet been ascribed to the CRAC protein. We sought to determine whether CRAC becomes membrane associated during stimulation of adenylyl cyclase.

We have previously demonstrated that CRAC isolated from wild-type cells can restore GTPγS-activated adenylyl cyclase activity to lysates prepared from *crac*$^-$ cells (Theibert and Devreotes, 1986; Lilly and Devreotes, 1994). Table I demonstrates that the membrane fraction prepared from cells lysed in the presence of GTPγS can be reconstituted in a similar manner. Thus adenylyl cyclase activity can be reconstituted in membranes as well as in crude lysates, suggesting that preincubation with GTPγS is required to activate the membrane fraction but that free GTPγS can be removed prior to reconstitution with CRAC (also see Snaar-Jagalska and Van Haastert, 1988). Next, we asked whether the reconstitution was stable. Preincubation of supernatant with the activated lysate and subsequent preparation of the membrane fraction resulted in fully reconstituted adenylyl cyclase activity (Table I). This observation suggests that CRAC has either modified or become stably associated with the membrane fraction.

An assay was devised to test whether CRAC did become stably associated with the membranes during reconstitution. Lysates were prepared from *crac*$^-$ cells in the presence or absence of GTPγS. The lysates were incubated with supernatant prepared from cells that were overexpressing CRAC activity (RI8). After 0, 2, or 8 min of incubation, the mixtures were diluted to wash away unbound CRAC and the membrane fractions were pelleted. Assay of these pellets revealed that adenylyl cyclase activity increased as the incubation period lengthened, consistent with the data in Table I. As illustrated in Fig. 2, association of the CRAC protein with the membranes also increased with time of preincubation with supernatant. This observation indicates that during the reconstitution assay, CRAC translocated from the supernatant to the membrane, and that this enabled adenylyl cyclase activation. Similar results were obtained by preparing the membrane fraction first, then incubating with CRAC-containing supernatant and reisolating the membrane portion. Translocation of CRAC occurred only in lysates that had been prepared in the presence of GTPγS. Unactivated lysates prepared in the absence of GTPγS displayed only basal adenylyl cyclase activity, and CRAC did not become associated with the membrane fraction under these conditions. These observations suggest that GTPγS creates binding sites for the CRAC protein on the membrane.

Table I. Comparison of Reconstitution Conditions

Source of adenylyl cylase	Addition	Activity
		pmoles/min-mg
Lysates*	+Buffer	19
	+Supernatant	310
Membranes‡	+Buffer	27
	+Supernatant	371
Membranes of reconstituted lysates§	+Buffer	439

For all experiments, *crac*$^-$ cells were lysed in the presence of GTPγS.
*120 μl aliquots of lysate were mixed with 120 μl buffer (SLB) or CRAC-containing supernatant (RI8), incubated for 8 min, then assayed for adenylyl cyclase activity.
‡Aliquots of lysate were incubated in the absence of supernatant, diluted with 1 ml buffer, and the membrane fraction isolated. The pellets were resuspended in 120 μl buffer or CRAC-containing supernatant and immediately assayed for adenylyl cyclase activity.
§The lysate incubated in the presence of supernatant was diluted with 1 ml buffer, the membrane fraction isolated, resuspended in buffer and immediately assayed. Each treatment was done in duplicate, and the experiment shown is representative of three independent experiments.

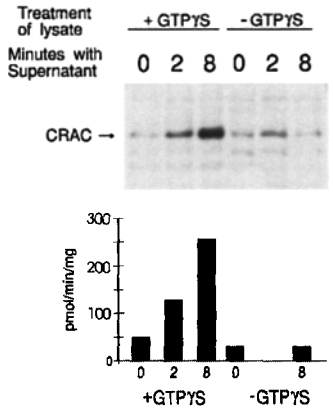

Figure 2. CRAC translocation to *crac*$^-$ membranes in the presence of GTPγS. Lysates were prepared from 5-h developed *crac*$^-$ cells, as described in *Materials and Methods*, in the presence or absence of GTPγS and cAMP. Aliquots of each lysate were incubated with RI8 supernatant for the indicated time, diluted with buffer, and the membrane fraction isolated by centrifugation. Duplicate samples were immunoblotted for CRAC protein (*upper panel*) or resuspended in buffer and analyzed for adenylyl cyclase activity (*lower panel*).

Binding of CRAC to membranes occurs in a dose-dependent fashion. As shown in Fig. 3, as increasing volumes of CRAC supernatant are incubated with a fixed amount of GTPγS-activated membranes, more CRAC protein translocates to the membrane fraction. Similarly, higher doses support increased adenylyl cyclase activity in the assayed membranes. Only ~3–10% of the CRAC in the reconstitution reaction becomes stably associated with membranes. This corresponds to ~1,000–3,000 molecules per cell equivalent (Lilly and Devreotes, 1994). However,

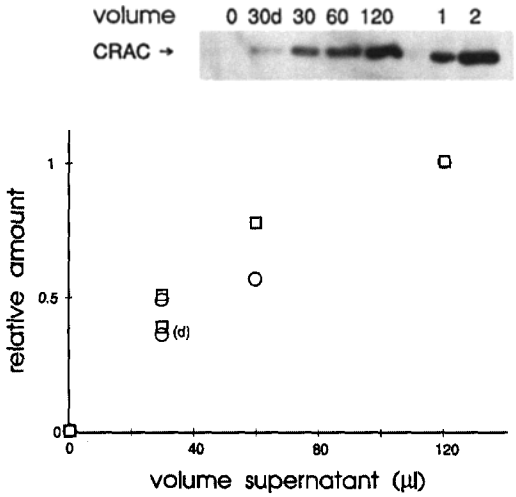

Figure 3. Dose dependence of CRAC association with membranes. The indicated amounts (μl) of CRAC-containing supernatant were incubated with 60 μl of GTPγS-activated *crac*$^-$ lysate. (In the case of 30 *d*, an additional 30 μl of buffer was added along with the supernatant.) After 8 min, the lysates were either assayed or diluted and the membrane fraction isolated. The membrane-associated CRAC protein, visualized by immunoblot, is shown in the upper panel. Lanes labeled 1 and 2 represent 10 and 20 μl of CRAC supernatant directly loaded to gel as a standard. The reconstituted adenylyl cyclase activity, (average from two representative experiments), expressed as a fraction of the maximum level, is depicted in the lower panel (□). The immunoblotted samples were scanned, and the relative amounts of associated CRAC are indicated for comparison (○). The sample labeled 0 on the gel illustrates the amount of "trapped" CRAC from the 60-μl sample.

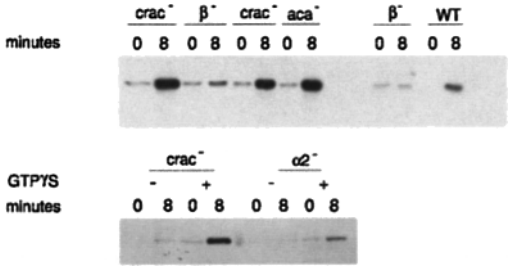

Figure 4. Association of CRAC with membranes from mutant cell lines. Upper panel: Lysates were prepared in the presence of GTPγS from the indicated mutant cell lines at 5 h of development. Initially, 60-μl aliquots of each were incubated with 60-μl CRAC-containing supernatant for 0 or 8 min, diluted with 1 ml buffer, and the membrane fraction pelleted. The isolated membranes were immunoblotted with anti-CRAC antibody. The wt 0-min sample was lost. Lower panel: Lysates were prepared in the presence or absence of GTPγS from *crac*$^-$ and *gα2*$^-$ cells, as indicated, and analyzed for membrane association for CRAC as in the upper panel.

under the conditions used here, saturation was not observed.

To investigate the requirements for GTPγS-stimulated CRAC binding, we analyzed the translocation of CRAC in a variety of mutants in the signal transduction pathway. As illustrated in Fig. 4, the *gα2*$^-$ cells retained the ability to bind CRAC to its membranes in a GTPγS-dependent fashion, suggesting that Gα2 is not essential to this process. Similar results were obtained in examining *car1*$^-$ cells (data not shown). Conversely, the *gβ*$^-$ cells exhibited no CRAC binding in GTPγS-activated lysates. These observations are consistent with the fact that GTPγS can activate ACA in *car1*$^-$ and *gα2*$^-$ cells but not in *gβ*$^-$ cells (Kesbeke et al., 1988; Pupillo et al., 1992; Wu et al., 1995). Lysates derived from either wild-type or *crac*$^-$ cells serve equally well as controls. Surprisingly, robust CRAC binding was observed in the *aca*$^-$ cells. Thus, while CRAC association with the membrane is required to activate ACA, the enzyme does not participate in its association. These observations show that the βγ-subunits are required for generation of CRAC sites in membranes and suggest that they act as a binding site for CRAC. The resulting βγ/CRAC complex may directly activate ACA.

Chemoattractant Stimulation of Cells Induces a Transient Association of CRAC with Membranes

We demonstrated that CRAC becomes associated with the membrane fraction in an in vitro reconstitution assay. Next we tested whether CRAC becomes membrane associated in response to agonist stimulation of intact cells. As shown in Fig. 5, cAMP stimulation of RI8 cells, which overexpress CRAC, elicited a rapid increase in adenylyl cyclase activation, similar to that observed in wild-type cells. Immunoblots of isolated membrane fractions prepared at each of these time points showed a rapid increase in the level of membrane-associated CRAC upon activation. In this experiment, adaptation of the adenylyl cyclase occurred more slowly than it typically does. We are currently investigating whether this effect is due to the overexpression of CRAC.

The role of the adaptation process in regulating the as-

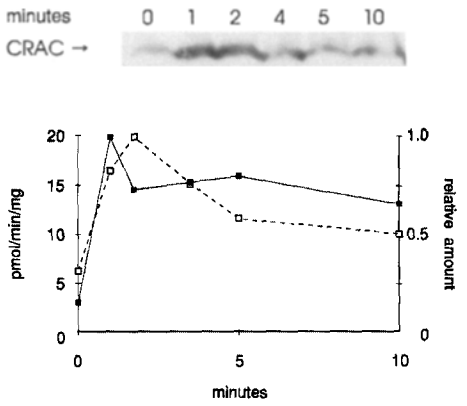

Figure 5. cAMP-stimulated adenylyl cyclase activation. RI8 cells developed for 5 h were shaken on ice at 1.6×10^8, then stimulated with 10 μM cAMP. Aliquots of cells were mixed with an equal volume of lysis buffer and lysed at the indicated time points. Duplicate aliquots of lysate (200 μl each) were assayed for adenylyl cyclase activity (■), and 300 μl lysate was centrifuged to isolate the membrane fraction. Membranes were separated by SDS-PAGE, then immunoblotted with an anti-CRAC antibody. A photograph of the immunoblot is shown in the inset. A corresponding densitimetric scan is shown on the graph (□).

sociation of CRAC with membranes was addressed by comparing the extent of GTPγS-stimulated CRAC binding to wild-type membranes prepared at different times after stimulation. We found that immediately following addition of a cAMP stimulus, CRAC bound readily to membranes prepared from cells in the presence and also, to a significant extent, in the absence of GTPγS. This binding was greatly reduced when cells were pretreated for 15 min to induce adaptation of the response (Fig. 6). Adaptation of the cells was verified by direct assay of the lysates, comparing the adenylyl cyclase activity in the presence or absence of GTPγS. The lysates prepared immediately following stimulation displayed a high activity that was enhanced by incubation with GTPγS; lysates prepared from adapted cells displayed only low activity in the presence or absence of GTPγS.

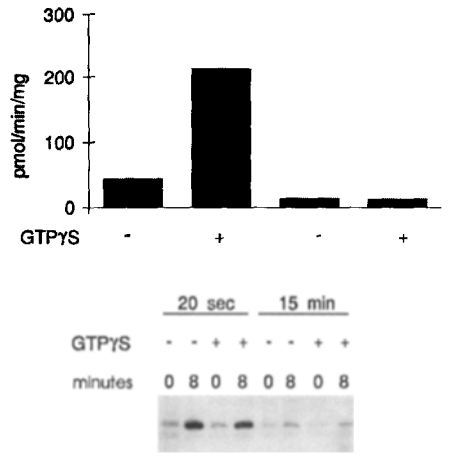

Figure 6. CRAC does not associate with membranes from adapted cells. Using wild-type cells, lysates were prepared within 20 s following a cAMP pulse and again after 15 min of persistent stimulation. GTPγS was included in the lysis buffer where indicated. The upper panel shows the direct assay of the adenylyl cyclase activity immediately after lysis. The lower panel shows membrane-associated CRAC protein in naive (20 s) vs adapted (15 min) cells after 0 or 8 min of incubation with CRAC-containing supernatant.

Discussion

The mechanisms by which hormones activate adenylyl cyclases have been the subject of intense investigation for nearly 25 years (Birnbaumer, 1992). In the generally accepted paradigm, excited surface receptors catalyze guanine nucleotide exchange, leading to the release of Gαs, which stimulates the catalytic unit (Gilman, 1987). In vitro, purified activated Gαs directly binds to and stimulates purified adenylyl cyclases (Gilman, 1987). For adenylyl subtypes II and IV, free Gβγ-dimers can greatly potentiate the stimulatory action of activated Gαs (Tang and Gilman, 1991). Cotransformation experiments have shown that this enhancement can operate in vivo, and a variety of observations suggests that it may be physiologically significant (Federman et al., 1992). Calmodulin also confers calcium sensitivity to certain subtypes (Tang and Gilman, 1991). There has been little evidence suggesting a requirement for other novel components.

Biochemical and genetic analyses of the receptor-mediated activation of the aggregation-stage adenylyl cyclase, ACA, in *Dictyostelium* have recently led to the discovery of a novel component of the system. Previous evidence suggested that CRAC acts downstream of receptor/G-protein coupling. Its deletion does not interfere with GTP inhibition of high-affinity agonist binding to cAR1, an indicator of cAR1/G2 interaction (Snaar-Jagalska and Van Haastert, 1988). It is required for GTPγS activation of the enzyme in vitro, an assay that does not require cAR1 (Theibert and Devreotes, 1986). Consistent with this, we have demonstrated that CRAC can reconstitute receptor-mediated activation of the enzyme when it is supplied to lysates prepared from cAMP-stimulated CRAC null mutants. Thus, CRAC appears to act as an adaptor between the activated G-protein and the adenylyl cyclase.

Since all the known components of this system are membrane proteins, whereas CRAC fractionates as a cytosolic component, CRAC is expected to become associated with the membrane for activation to occur. Indeed, we have shown that during receptor-mediated activation of adenylyl cyclase, there is an increase in the amount of CRAC that co-sediments with membranes. Furthermore, we have shown that CRAC becomes stably associated with membranes during an in vitro reconstitution assay. The association of CRAC with the membranes is time and GTPγS dependent, paralleling the requirements for in vitro activation of the adenylyl cyclase.

The receptor- and GTPγS-stimulated association of CRAC with membranes suggests that, rather than activating CRAC, the treatments generate specific binding sites for CRAC. Several additional lines of evidence support this hypothesis. First, the membranes can be pretreated with GTPγS, washed, and subsequently incubated with CRAC with similar reconstitution results (Table I; Snaar-Jagalska and Van Haastert, 1988). Second, whether the source of CRAC is stimulated or unstimulated cells, it is equally active. Third, the amount of CRAC associated with membranes in vitro parallels the extent of activation

of the enzyme, and both membrane association and activation depend on the concentration of added CRAC. With the currently available CRAC preparations, we were unable to add sufficient amounts to achieve saturation of the binding sites. Nevertheless, the amounts of CRAC becoming associated with membranes in vivo and in vitro were similar.

The specific association of CRAC with membranes was independent of cAR1, Gα2, and CRAC (in the host cells). These results might have been predicted since GTPγS effectively activates ACA in *carl*$^-$ or *gα2*$^-$ lysates, and it enables *crac*$^-$ lysates to be reconstituted by CRAC (Kesbeke et al., 1988; Pupillo et al., 1992). Because CRAC is absolutely essential for adenylyl cyclase activation, it was surprising that the stimulated association of CRAC with membranes did not require ACA. This observation implies that while CRAC might directly interact with ACA to activate it, ACA does not serve as its membrane binding site. In contrast, deletion of the β-subunit completely abolished the capacity of the membranes to associate with CRAC. This suggests either that βγ-subunits serve as the CRAC-binding sites or that the creation of binding sites depends very closely on βγ-subunits.

While cAR1 was not required for GTPγS-dependent association of CRAC with membranes or activation of ACA, cAR1 occupancy could regulate the availability of CRAC binding sites. In vivo, persistent occupancy of cAR1 caused a transient increase in the stable association of CRAC with membranes in subsequently prepared lysates. The transient association parallels the activation of ACA. Pretreatment of cells with cAMP also influenced the capacity of GTPγS to generate CRAC-binding sites in membranes. Brief pretreatment (20 s) enhanced the stimulatory effect of GTPγS, while prolonged pretreatment (15 min) nearly completely attenuated its action. These observations closely parallel the receptor-mediated regulation of the capacity of GTPγS to stimulate ACA in vitro. Thus, adaptation of this pathway may occur by down-regulation of CRAC-binding sites.

Based on these and other observations, we propose the following working model for the receptor-mediated activation of adenylyl cyclase. In vivo, persistent occupancy of cAR1 elicits a transient activation of G2 that leads to release and "activation" of βγ-subunits. In vitro, GTPγS achieves the same release and activation; it is independent of cAR1 and Gα2. In either case, the process creates a binding site for CRAC, causing it to translocate to the membrane where it activates ACA. The pleckstrin homology domain within the NH_2-terminal of CRAC may bind to the activated βγ-subunits and mediate the translocation. CRAC alone or in association with the βγ-subunits may activate the ACA.

Chemoattractants lead to numerous responses besides activation of adenylyl cyclase (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). It is possible that for some of these responses, the variety of α-subunits serves to specify the activation of the βγ-subunits by different chemoattractant receptors. In support of this notion, *gα2*$^-$ cells that cannot sense cAMP respond normally to the chemoattractant folic acid, while *gα4*$^-$ cells, which cannot sense folic acid, respond normally to cAMP (Kesbeke et al., 1988; Hadwiger et al., 1994). In contrast, the *gβ*$^-$ cells fail to respond to either of these chemoattractants (Wu et al., 1995). If βγ-subunits are a major transducer of signals to effectors, the "activation" of βγ-subunits, reflected in the transient increase in apparent CRAC-binding sites, may be of wider significance than simply in activation of adenylyl cyclase.

CRAC is a novel protein, unrelated to G-protein subunits, that contains an NH_2-terminal PH domain (Insall et al., 1994). Although this organism is evolutionarily distant, the other components of this signal transduction pathway—the chemoattractant receptor (cAR1), the G-protein subunits (α and β), and the catalytic subunit (ACA)—closely resemble their mammalian counterparts (Klein et al., 1988; Pupillo et al., 1989; Lilly et al., 1993; Pitt et al., 1993). Therefore, it is likely that a CRAC homologue is present in mammals, perhaps in specialized cells such as leukocytes that carry out chemotaxis and phagocytosis (Devreotes and Zigmond, 1988) or in other instances where βγ-dimer signaling is particularly important.

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