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, Ga2, or Gβ (car1−, ga2−, or gβ− cells). In lysates, GTPγS results in activation of ACA in car1− and, surprisingly, ga2− 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-
other component, designated cytosolic regulator of adenyl cyclase (CRAC), is also required for both receptor- and GTPyS-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 adenyl cyclase activity, even in the presence of GTPyS, but stimulation can be reconstituted by addition of purified CRAC to lysates that have been pre-activated. 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 NH2-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 adenyl 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), cAR1−/− (JB4), go2−/− (myc2), gb−/− (I6W6), 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 into 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 adenyl 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 GTPyS 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 K2HPO4, 2 mM MgCl2). For the 0 time points, lysates were diluted prior to addition of the cytosolic fraction. Samples were centrifuged for 1.5–2 min at ~13,000g in a microfuge at 4°C. After removal of the supernatant, the pellet was resuspended in either 120 μM PM for assay of adenyl 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 × 106/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 adenyl cyclase, CRAC, is essential for both receptor- and guanine nucleotide–mediated activation of adenyl 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 adenyl 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 adenyl cyclase activation, which peaked between 1 and 2

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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 R18 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 adenyl 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 adenyl 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 adenyl cyclase.

We have previously demonstrated that CRAC isolated from wild-type cells can restore GTPγS-activated adenyl 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 adenyl 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 adenyl 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 (R18). 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 adenyl 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 adenyl 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 adenyl 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.

| Source of adenyl cyclase | Addition | Activity (pmol/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.

*Aliquots of lysate were mixed with 120 μl buffer (SLB) or CRAC-containing supernatant (R18), incubated for 5 min, then assayed for adenyl 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 adenyl 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.
Binding of CRAC to membranes occurs in a dose-dependent manner. 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, 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 ga2- cells retained the ability to bind CRAC to its membranes in a GTPγS-dependent fashion, suggesting that Ga2 is not essential to this process. Similar results were obtained in examining carp- cells (data not shown). Conversely, the gb- cells exhibited no CRAC binding in GTPγS-activated lysates. These observations are consistent with the fact that GTPγS can activate ACA in carp- and ga2- cells but not in gb- cells (Kesbeke et al., 1988; Pupillo et al., 1992; Wu et al., 1995). Lysates derived from either wild-type or carp- cells served equally well as controls. Surprisingly, robust CRAC binding was observed in the carp- 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 R18 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-
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 GTPyS 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 GTPyS-dependent, paralleling the requirements for in vitro activation of the adenylyl cyclase.

The receptor- and GTPyS-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 GTPyS. 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 enhanced by incubation with GTPyS; lysates prepared from adapted cells displayed only low activity in the presence or absence of GTPyS.

**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 cyclases types 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). For adenylyl cyclases types II and IV, free Gβγ-dimers can greatly potentiate the stimulatory action of activated Gαs (Gilman, 1987). In vitro, purified activated Gαs directly binds to and stimulates purified adenylyl cyclases (Gilman, 1987). For adenylyl cyclases types II and IV, free Gβγ-dimers can greatly potentiate the stimulatory action of activated Gαs (Gilman, 1987). In vitro, purified activated Gαs directly binds to and stimulates purified adenylyl cyclases (Gilman, 1987).
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, Go2, and CRAC (in the host cells). These results might have been predicted since GTPγS effectively activates ACA in car1 or goα2 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 Go2. 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 NH2-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.

Chemotacticants 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 chemotactic receptor. In support of this notion, goα2 cells that cannot sense cAMP respond normally to the chemotacticant folic acid, while goα cells, which cannot sense folic acid, respond normally to cAMP (Kesbeke et al., 1988; Hadwiger et al., 1994). In contrast, the gb cells fail to respond to either of these chemotactants (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 NH2-terminal PH domain (Insall et al., 1994). Although this organism is evolutionarily distant, the other components of this signal transduction pathway—the chemotactrant 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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