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Identification of CRAC, a Cytosolic Regulator Required for Guanine Nucleotide Stimulation of Adenylyl Cyclase in *Dictyostelium**

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Pamela J. Lilly and Peter N. Devreotes‡

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

As previously reported, guanine nucleotide regulation of adenylyl cyclase activity in the *Dictyostelium* mutant *synag* 7 can be restored *in vitro* by addition of a high-speed supernatant prepared from wild-type cells (Theibert, A., and Devreotes, P. N. (1986) *J. Biol. Chem.* 261, 15121–15125). We have designated this activity CRAC, for cytosolic regulator of adenylyl cyclase. Trypsinization of partially purified material demonstrated that this activity contains a protein. We report here a 50,000-fold purification of this protein using Q and S Sepharose fast flow and P11 cellulose chromatography, followed by sucrose gradient centrifugation and separation on SDS-polyacrylamide gel electrophoresis. Purification of wild-type and mutant supernatants in parallel allowed identification of an 88-kDa protein required for reconstituting activity. A polyclonal antibody was raised against this protein; it stains a band in unfractionated wild-type, but not mutant, supernatants. Immunoblots of fractions from each purification step show that activity and the immunostaining band cochromatograph. We have determined a short N-terminal sequence of the 88-kDa CRAC polypeptide, which matches a portion of the deduced N terminus of a gene, *dagA*, isolated from a mutant similar to *synag* 7. Expression of the *dagA* cDNA in *synag* 7 cells restores both the 88 kDa band and CRAC activity.

Three major components have been implicated in the activation of hormone and neurotransmitter sensitive adenylyl cyclases in mammalian systems: cell surface receptors, G-proteins, and the adenylyl cyclase catalytic subunits. The seven transmembrane domain receptors bind extracellular ligand and in turn activate the heterotrimeric G proteins, catalyzing the exchange of GTP for GDP bound to the α-subunit. The activated G-proteins dissociate into α and $\beta\gamma$ components (2). Both the activated α-subunits and the free $\beta\gamma$-subunit complexes have been implicated as activators of the catalytic subunit (3). Several mammalian adenylyl cyclases have been described, all sharing the topological motif of 12 transmembrane domains and two large cytoplasmic domains (4–6).

G-protein-linked signal transduction pathways play a critical role in cell-cell communication in the cellular slime mold *Dictyostelium discoideum* (7). In a nutrient-rich environment, these amoebae grow exponentially; under starvation conditions, they enter a developmental program. In the initial stages, 3′,5′-cyclic adenosine monophosphate (cAMP),[1] acting as an extracellular signaling molecule, coordinates a complex choreography in which up to a million cells coalesce to form a multicellular structure. Aggregation centers periodically secrete the cyclic nucleotide cAMP, which diffuses outwardly and acts as a chemoattractant. Nearby amoebae also propagate the signal to distal cells by producing and secreting additional cAMP. A reversible adaptation process ensures that the signal is relayed outwardly, resulting in concentric waves of cAMP emanating from the center. The amoebae react to each passing wave with a transient inward step, enabling large numbers to be attracted. cAMP is also involved in later development, which culminates with a mass of spores held aloft by a vacuolated stalk (8).

The components involved in these G-protein-linked signal transduction pathways are highly homologous to their mammalian counterparts. Four cAMP receptors have been identified, comprising a family of seven-transmembrane domain receptors (9–11). Eight G-protein α-subunits have been cloned; each is 45% identical to the others and to mammalian α-subunits (12–14). A single β-subunit, 70% identical to those in other systems, is thought to couple to each of the α-subunits (15). The adenylyl cyclase involved in aggregation, ACA, shares the 12-transmembrane domain topology with mammalian adenylyl cyclases and is regulated by guanine nucleotides (16). These latter observations suggest that the signaling system in *Dictyostelium* may be a relatively direct precursor to the mammalian hormone-activated adenylyl cyclase systems.

Several mutant cell lines in which signaling components have been disrupted have shown that cAR1, Gα2, Gβ, and ACA play vital roles in the developmental program. Mutants lacking each of these components are aggregation deficient and have defects in receptor-mediated adenylyl cyclase activity (15–18).[2] Interestingly, the defect in the *aca*$^-$ cells can be bypassed by providing a physiologically relevant regimen of cAMP stimulation or by mixing the mutant and wild-type cells in synergy tests (16).

A series of mutants, designated *synag*, which display phenotypes similar to *aca*$^-$ have previously been isolated and mapped to several complementation groups.[3,4] While lysates from wild-type cells show marked stimulation of ACA by GTPγS, several of the *synag* mutants are specifically defective in GTPγS stimulation of the enzyme (1, 19). One of these previously described mutants is *synag* 7. In *synag* 7 lysates, the defect in GTPγS stimulation of ACA can be reconstituted by addition of a supernatant from wild-type cells (1). We have designated this recon-

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‡ To whom correspondence should be addressed. Tel.: 410-955-3225; Fax: 410-955-5759.

[1] The abbreviations used are: cAMP, 3′-5′-cyclic adenosine monophosphate; GTPγS, guanosine 5′-*O*-(thiotriphosphate); rpm, revolutions/min; PAGE, polyacrylamide gel electrophoresis.

[2] L. Wu, personal communication.

[3] B. Coukell, personal communication.

[4] P. J. Lilly, R. H. Insall, and P. N. Devreotes, manuscript in preparation.

stituting activity CRAC, for cytosolic regulator of adenylyl cyclase. The reconstituting activity is present in *aca*$^-$ and each of the other known *synag* mutants. Partial purification of wild-type and *synag 7* supernatants in parallel allowed us to identify CRAC activity as an 88-kDa protein present in wild-type cells but absent in *synag* 7. We provide an N-terminal sequence of this novel component essential for activation of adenylyl cyclase.

MATERIALS AND METHODS

Cell Culture and Development—*D. discoideum* strain AX-3 cells were grown in HL-5 at room temperature as previously described (20, 21). PD722 (722), an axenic derivative of *synag* 7,[4] were grown in an identical manner. In large scale preparations, 30 liters of cells at 10^7/ml were harvested using a refrigerated Sharples T-1 continuous flow centrifuge (22). Cells were resuspended in development buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.8), centrifuged again, and resuspended at 3–5 × 10^7/ml in DB. Cells were shaken at 110 rpm for 4 h. The mutant cell line was given 100 nM pulses of cAMP every 6 min after the first hour. They were then diluted 3-fold with DB minus $CaCl_2$ at 22 °C and shaken for 30 min at 200 rpm. The cells were centrifuged again and resuspended at 8 × 10^7/ml in ice-cold sucrose lysis buffer (SLB; 10 mM Tris-HCl, pH 8, 200 mM sucrose, 0.2 mM EGTA). All subsequent steps were carried out at 4 °C.

Supernatants—The developed cells were lysed by releasing the pressure from a Parr bomb after 15 min of equilibration at 1300 pounds/square inch of nitrogen. Lysates were centrifuged at 7000 rpm in a GSA rotor for 20 min. The resulting supernatant was the starting material for the purification protocol. The typical protein concentration was approximately 4 mg/ml.

Column Chromatography—All columns were run using a Gilson eight-channel peristaltic pump. Fractions from multiple columns run in parallel were collected together and assayed for activity. Peak fractions were pooled and used as the starting material for the next purification step.

Initial Q Sepharose Fast Flow Column—Q Sepharose fast flow resin (QSFF, Pharmacia Biotech Inc.) was pre-equilibrated in SLB. The columns were poured in 2.5 × 20-cm Bio-Rad Econo-Columns. A 50-ml column was used for every 450–550 ml of crude supernatant. The sample was applied at a flow rate of 5–9 ml/min (6–10 min/column volume). The flow-through was used as the sample for the next step.

S Sepharose Fast Flow Column—S Sepharose fast flow resin (SSFF; Pharmacia or Sigma) was pre-equilibrated in SLB. Columns of approximately 15 ml were poured in 2.5 × 20-cm Bio-Rad Econo-Columns. The flow-through from the QSFF step was adjusted to 150 mM NaCl using 3 M NaCl in SLB. Between 50 and 100 column volumes of material (*i.e.* 750–1500 ml) were loaded per column at a rate of about 9 ml/min (1.7 min/column volume). Each column was rinsed with 100 ml of 150 mM NaCl in SLB, then 12 ml of 200 mM NaCl in SLB. The column was eluted with a linear gradient, from 200 to 300 mM NaCl in SLB. Ten column volumes (150 ml) of each solution were used per column. One column volume fractions were collected and assayed for activity.

Phosphocellulose Column—Phosphocellulose P11 (Whatman) was prepared according to the manufacturer's instructions. The resin was then equilibrated in SLB and 5-ml columns were prepared in 1 × 20-cm Econo-Columns. The peak fractions from the SSFF column were pooled and adjusted to 175 mM NaCl using SLB. Approximately 32 column volumes (160 ml) were loaded per column. The columns were run at 0.6 ml/min (8.1 min/column volume). Each was rinsed sequentially with 1 column volume each of 200 and 250 mM NaCl in SLB. Each column was then eluted with 7 column volumes (35 ml) of 350 mM NaCl in SLB. Fractions were collected at 1-min intervals for the first 10 min, then at 2-min intervals for the remainder of the elution.

Second QSFF Column—1.5-ml QSFF columns were prepared in 0.7 × 10 cm Bio-Rad Econo-Columns. The peak activity from the P11 column was diluted 10-fold (to about 35 mM NaCl) with SLB (10% glycerol was added as indicated). Typically, 120 column volumes were applied. The samples were applied at 0.6–0.75 ml/min (2–2.5 min/cv) and rinsed with 1 ml of 30 mM NaCl in SLB. Each was eluted with 8 ml of 110 mM NaCl in SLB (10% glycerol as indicated). Fractions of 1/2 column volume were collected.

Sucrose Gradients—Crude supernatant or material from various steps in the purification (0.25 ml) was run on 4 ml 10–20% sucrose gradients containing 10 mM Tris, pH 8, and 0.2 mM EGTA. The gradients were centrifuged at 52,000 rpm for 17.5 h in a SW 60 rotor. Fractions were collected from the gradient, assayed for activity, and analyzed on SDS-PAGE. Glycerol gradients from 16–25% were run in a similar fashion.

Hydroxylapatite Column—Hydroxylapatite resin (Bio-Rad HTP) was equilibrated in SLB. The 0.7 × 10-cm Econocolumns were used to run a 0.25-ml column. Material from the second QSFF step was loaded at 0.3 ml/min. The column was rinsed briefly with SLB, then eluted in steps from 100–400 mM sodium phosphate, pH 7.8. (Elution buffers were prepared by mixing various ratios of 400 mM sodium phosphate buffer with SLB, thus final concentrations of Tris and sucrose also vary).

Trichloroacetic Acid Precipitation—Trichloroacetic acid precipitation of samples was carried out by adding 1/10 volume of 0.14% deoxycholate (sodium salt), incubating 10 min on ice, then adding 1/10 volume 100% trichloroacetic acid followed by vigorous vortexing. The precipitated material was recovered by centrifugation at 13,000 × *g* for 20 min, then resuspending in ice-cold 100% acetone, and centrifuged again (23). Pellets were dissolved in two × Laemmli sample buffer and stored at −70 ° until use.

Adenylyl Cyclase Assay—Lysates of the haploid mutant segregant 722 were prepared as described for *synag 7* (1) except that cells were developed for 10.5 h and GTPγS was used in place of Gpp(NH)p. The reconstitution assay was performed essentially as described, except that the assay was typically done for 1.5 min and the concentrations of unlabeled ATP and cAMP were increased to 3 and 5 mM, respectively (1). The cAMP produced was recovered by the method of Salomon (24). Activity is reported per milligram of 722 lysate. Typically, volumes of supernatants were adjusted such that assays were carried out in the linear portion of the reconstitution curve (see below). The same mutant lysate preparation was used throughout any individual purification. Recovery was taken as a fraction of the starting activity for that column. Losses between steps were addressed by comparison to the starting supernatant, held at 0 ° throughout the purification, and assayed in parallel with fractions of the subsequent columns. These losses were minimal until after the second QSFF step.

Antibody Production—Large scale purifications were carried out through the second QSFF step. The peak fractions were concentrated by centrifugation in centricon-30 microconcentrators or by trichloroacetic acid precipitation. The pooled purified sample was run on a preparative SDS-PAGE gel. The gels were either transferred to nitrocellulose or Coomassie Blue and then silver-stained. The band corresponding to that missing in 722 was excised and used as the antigen. Stained gels were sonicated using a Branson sonicator until homogeneous; the band transferred to nitrocellulose was dissolved in a small volume of dimethyl sulfoxide (25). The immunizations contained approximately 20 μg of CRAC protein mixed 1:1 with Freund's adjuvant. A female New Zealand White rabbit was immunized at 4-week intervals, and a low titer antibody was detected at 11 weeks. Three subsequent monthly boosts raised the titer slightly.

The serum generated stained numerous nonspecific bands as well as the blocked nitrocellulose. These difficulties were alleviated by preabsorption and affinity purification. First, the serum was diluted at 1:500 in wash buffer and preabsorbed *versus* nitrocellulose sheets which had been soaked in supernatants prepared from developed 722 cells. Two 10 × 17-cm sheets were used for each 50 ml of diluted serum. Each sheet was incubated for 30 min at room temperature and then discarded. The preabsorbed serum was then affinity purified to yield CRAC-specific antibodies. Preparative gels of material taken from the second QSFF step (or the P11 step) were transferred to Immobilon-P and used as the affinity purification matrix. Affinity purification was carried out essentially as described (26). Briefly, the entire blot was exposed to the preabsorbed serum and strips from the edges of the blot were exposed to secondary antibody and detected by ECL. The strip corresponding to CRAC was excised, cut into small (0.2 × 0.4-cm) pieces, and placed in the bottom of a plastic 50-ml tube. It was rinsed with wash buffer (50 mM Tris, 150 mM NaCl, 0.02% sodium azide, 0.05% Nonidet P-40, 1% bovine serum albumin), and the antibodies eluted by gently vortexing in 2 ml of 0.2 M glycine, pH 2.5, for two min. The solution was neutralized with 1 ml of 1 M KPO_4, pH 9. To ensure maximal recovery, an additional 1 ml of glycine was added, with gentle agitation, for 2 min then neutralized with 0.4 ml of KPO_4. These eluants were pooled and diluted with 7 ml of wash buffer, then used on immunoblots.

Immunoblots—Immunoblots were performed as previously described (27). Samples were separated on 1.5-mm 7.5% SDS-PAGE gels and transferred to either nitrocellulose (Schleicher and Schuell) or Immobilon-P (Millipore). Blots were exposed to primary antibody overnight. ECL was performed using a kit from Amersham Corp.

N-terminal Sequence Analysis—Material carried through the second QSFF step was separated on a 7.5% SDS-PAGE gel which had been aged overnight and prerun for about 3 h. Thioglycolic acid (0.1 mM) was

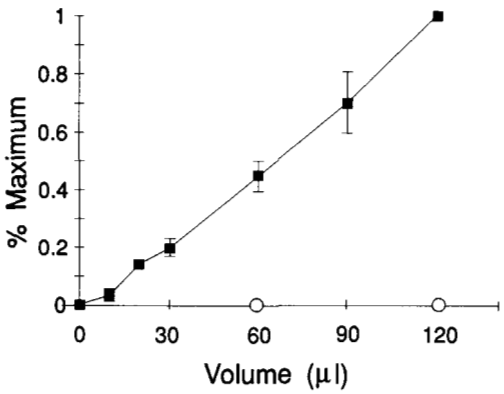


FIG. 1. **Dose curve for reconstitution.** Various amounts of supernatant prepared from wild-type (■) or 722 (○) cells were preincubated for 8 min at 0 °C with 120 µl of 722 lysate, then assayed for 1.5 min at 22 °C. Basal activity was subtracted, and values are reported as a percentage of the activity obtained with 120 µl of supernatant, which was 67.6 ± 29.6 pmol/min/mg, $n = 3$.

included in the upper running buffer (28). The proteins were transferred to Immobilon-P for 4 h and stained as described (29). Two 3 × 7 mm portions of the 88 kDa band were excised and subjected to N-terminal sequencing.

Protein Quantitation—Protein concentration was determined using the Bio-Rad Protein Assay Reagent.

Trypsinization—L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Worthington. Soybean trypsin inhibitor was from Sigma. Trypsinization was carried out in SLB.

RESULTS

Characterization of CRAC Activity—We have previously reported that Mn^{2+}-dependent adenylyl cyclase activity in *synag 7* lysates is similar to that in wild-type cells, suggesting that the mutant contains adenylyl cyclase catalytic subunit (1). Consistently, in immunoblots of *synag* 7 cells, ACA protein is present at near wild-type levels (data not shown). Unlike the adenylyl cyclase activity of the wild-type, which is stimulated 20-fold by GTPγS, that in *synag 7* lysates is nearly insensitive to the nucleotide unless the lysate is supplemented with wild-type cytosol. Cytosol alone had little stimulatory activity, but it stimulated about 8–10-fold in the presence of GTPγS. We attribute this activity to CRAC, which is readily assayed by mixing a supernatant fraction from wild-type cells with a GTPγS-activated lysate from *synag 7* cells (Fig. 1). CRAC activity is present in supernatants of growing as well as developing wild-type cells but is not detectable in supernatants of *synag 7* cells.

As shown in Fig. 1, the amount of GTPγS-stimulated adenylyl cyclase activity increases linearly with increasing amounts of wild-type cytosol, up to an addition of 120 µl. Typically, the wild-type cytosol increased the activity to roughly 50 pmol/min in our standard assay, about 30–50% of the activity observed in a comparable wild-type lysate. In most instances, the same 722 lysate preparation was used throughout each individual purification. Since the activity in wild-type supernatants increased 3–5-fold after 4 h of development, we routinely prepared supernatants from 4-h stage cells.

As shown in Table I, CRAC activity was sensitive to mild trypsin digestion, suggesting it contains a protein portion. The effects of trypsin were prevented by soybean trypsin inhibitor. We also noted that CRAC activity was very sensitive to warming; 50% of the activity in unpurified supernatants disappeared within 20 min at 22 ° (data not shown). At 0 °C, the activity was relatively stable for several days.

CRAC activity behaves as a monodisperse component when analyzed by sucrose gradient velocity sedimentation. A single symmetrical peak of activity was found at 6 S, a value higher than that expected of a G-protein heterotrimer or its individual subunits (data not shown). CRAC activity in unpurified supernatants was not significantly separated from the majority of the protein on the sucrose gradient. The protein profile of wild-type and *synag 7* supernatants, separated on sucrose gradients and analyzed by SDS-PAGE, were identical.

TABLE I
Trypsinization of CRAC activity

Partially purified CRAC in SLB was incubated in the presence or absence of L-1-tosyl-amido-2-phenyethyl chloromethyl ketone-treated trypsin (5 µg/ml) and/or soybean trypsin inhibitor (SBTI 25 µg/ml) for 1 h at 4 °C. Trypsin inhibitor was then added to the sample containing only trypsin and all tubes were incubated an additional 30 min at 4 °C. Adenylyl cyclase activity was then assayed as described under "Materials and Methods," and is reported per milligram of 722 lysate.

Addition	pmol/min/mg
Buffer only	7.08
Buffer, trypsin, and SBTI	7.00
CRAC	15.58
CRAC + SBTI	13.81
CRAC + trypsin, then SBTI	6.45
CRAC + SBTI, then trypsin	13.54

TABLE II
Overall purification

The general purification scheme and average recoveries for each step are presented as the mean and standard deviation from several experiments. Recoveries are presented as a percentage of the initial activity ($n = 6$) or protein ($n = 3$) in the starting supernatant (see "Materials and Methods" and "Results"). Typically, 2 liters of supernatant were used in the fractionations and all assays were carried out in the linear range (see Fig. 1). Initial supernatant is at 4 mg/ml. Because losses between steps are minimal, recovery for any given column can be calculated from the data below.

Purification step	Activity recovered	Protein recovered	Fold purification
	%		
Supernatant	100%	100%	1
QSFF (flow-through)	89 ± 11.9	41.8 ± 9.2	2.1
SSFF (250 mM)	49 ± 19.5	0.54 ± 0.25	91
P11 (350 mM)	16.2 ± 2.8	0.05 ± .03	320
QSFF (110 mM)	4.42 ± 1.8	0.004 ± 0.002	1100
Sucrose gradient	1.3[a]	0.00012[b]	11000[b]
SDS-PAGE	NA[c]	0.000026[b]	50000[d]

[a] Represents the experiment presented in Fig. 6.
[b] Recoveries were estimated from silver-stained SDS-PAGE gels by comparison to a dilution series of standards on the same gel.
[c] NA, not available.
[d] Recovery cannot be assayed in SDS-PAGE gels, but is assumed to be 100%.

Partial Purification of CRAC Activity—The CRAC activity in unpurified supernatants adsorbed to QSFF, but the columns had very low capacity. The first column volume is depleted of activity; subsequently, all of the activity appeared to flow directly through the column. However, over 50% of the protein continued to be removed in a steady-state manner. Ten-column volumes of the crude supernatant could be filtered through a single QSFF column, providing a rapid 2–3-fold purification at 80–100% recovery (Table II).

The QSFF flow-through was then adjusted to 150 mM NaCl and applied to an SSFF. Under these conditions, the CRAC activity bound avidly to this column; up to 200 mg of QSFF flow-through could be applied per milliliter of resin before activity began to appear in the flow-through. The adjustment to 150 mM NaCl was essential. Presumably the higher ionic strength increases the capacity of the resin by preventing retention of weakly interacting components; 98–99% of the protein flows through the column. As illustrated in Fig. 2, CRAC activity was eluted as a sharp peak by a gradient from 200–300 mM NaCl. Recovery ranged from 40–80% with less than 1% of the protein coeluting with the activity, yielding an overall purification of approximately 100-fold (Table II).

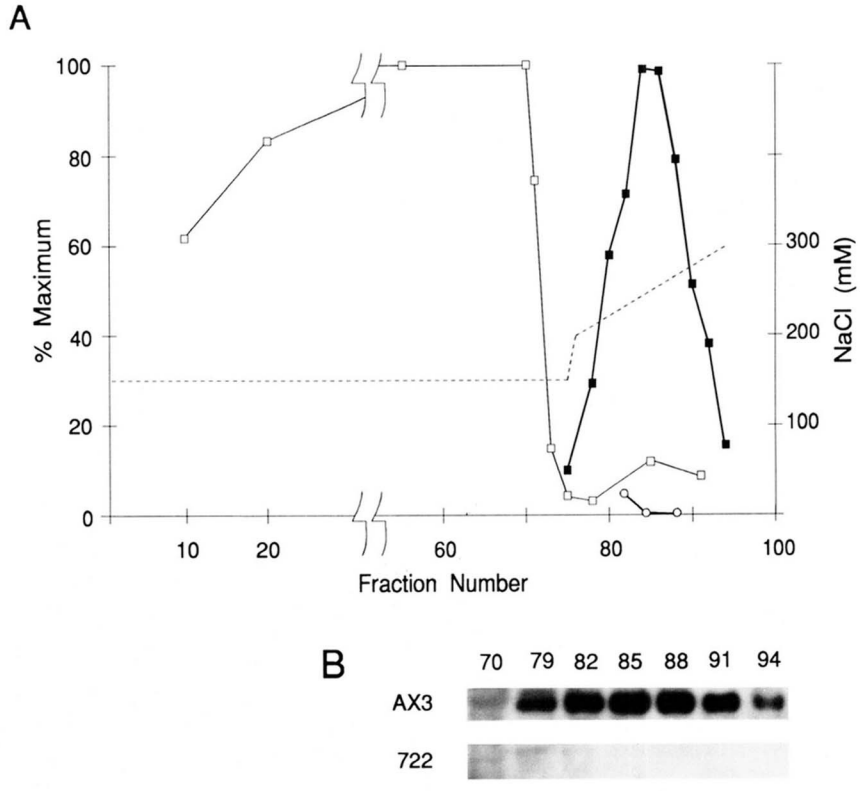


FIG. 2. **S Sepharose fast flow column chromatography.** *A*, Flow-through from QSFF columns was applied to 15-ml SSFF columns. Activity presented here for wild-type samples (■) represents the average of 12 columns and is plotted as a percentage of the peak fraction, which averaged 64.3 ± 53.3 pmol/min/mg in 20 μl of sample. Activity from a parallel fractionation of 722 is also depicted (○). Protein concentrations (□) from a similar column were measured using the Bio-Rad Protein Assay kit (100% = 1.7 mg/ml). Typically, 70 column volumes of material were applied, then rinsed with 6 column volumes of 150 mM NaCl in SLB (- - - -). A brief rinse of 200 mM NaCl was followed by elution with a gradient from 200–300 mM NaCl in SLB. Fractions of 1 column volume were collected. *B*, fractions from wild-type and 722 were blotted using the antibody generated against the 88 kDa band.

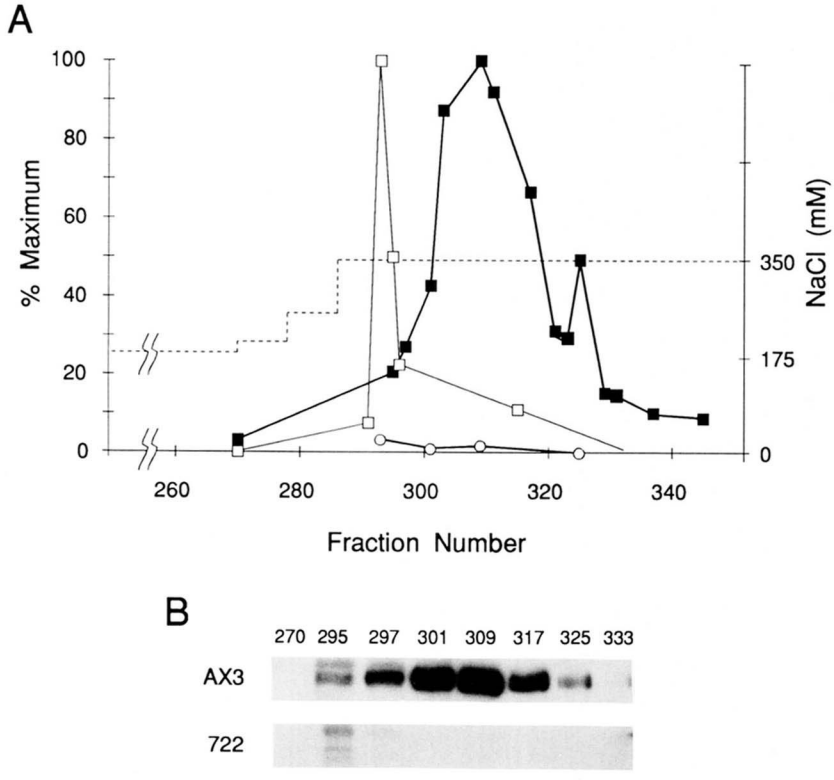


FIG. 3. **Phosphocellulose P11 column chromatography.** *A*, peak fractions from the SSFF column were pooled, diluted to 175 mM NaCl, and applied to 5-ml P11 columns at 0.6 ml/min. Columns were rinsed with 1 column volume each of 200 and 250 mM NaCl in SLB, then eluted with a step to 350 mM NaCl in SLB. Fractions were collected at 1-min intervals for the first 10 min and at 2-min intervals thereafter. Fraction numbers represent 1 min each (0.6 ml/column). The activity is plotted as a percentage of that in the most active fraction and averaged 135.8 ± 96.1 pmol/min/mg in 20 μl of sample. Data represents 11 fractionations from wild-type (■) and a single 722 fractionation (○). Comparable fractions were analyzed for protein concentration (□, 100% = 4 mg/ml). Peak fractions ranged from 0.15 to 0.45 mg/ml. *B*, wild-type and mutant samples from parallel fractionations were blotted with the antibody directed against the 88-kDa protein.

The peak from the SSFF column was pooled, diluted, and applied to a phosphocellulose P11 column. All of the CRAC activity and most of the applied protein adsorbs to the column. The column was eluted with 350 mM NaCl. As seen in Fig. 3, protein eluted slightly before the peak in activity. The peak fractions contained about 35% of the activity and about 10% of the protein applied to the column. The configuration of this column was critical; short, wide columns of the same volume did not separate as well as the long narrow columns used. The overall purification at this point was typically about 350-fold (Table II).

The P11 peak was then diluted with SLB and applied to a small QSFF column. At this stage, presumably sufficient protein had been removed to enable more CRAC activity to be bound to the same resin used earlier as a filter. Typically, 100 column volumes containing 2–4 mg of protein were applied per milliliter of resin. Activity was eluted with a step to 110 mM NaCl. As shown in Fig. 4, approximately 10% of the protein was

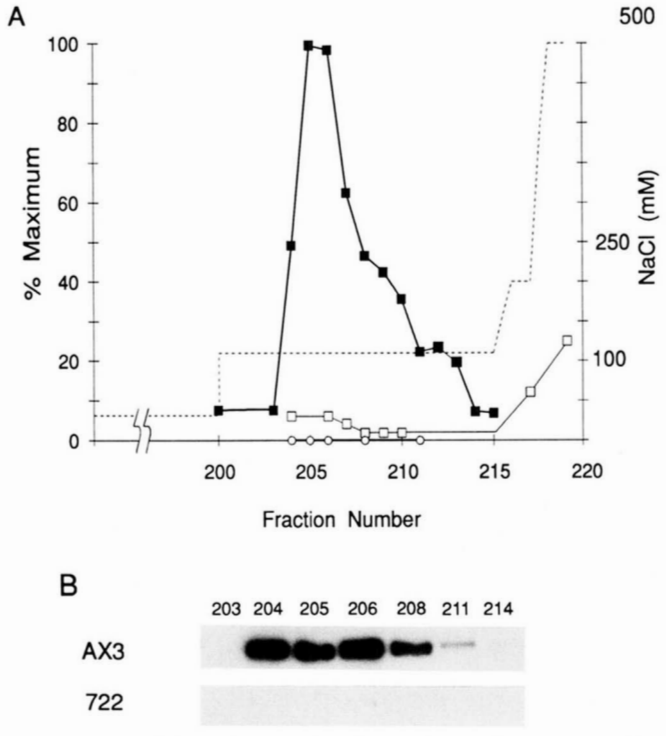


FIG. 4. **Second Q Sepharose fast flow column chromatography.** Pooled fractions from the P11 peak were diluted and applied to 1.5 ml QSFF columns at 0.6 ml/min. Fractions represent 1/2 column volume. Column was rinsed with 1 column volume, then eluted with 7 ml of 110 mM NaCl in SLB. Protein concentration (□) was measured from a similar fractionation. Seventeen wild-type fractionations were averaged and are presented as a percentage of the peak fraction (■), which was 111.8 ± 82 pmol/min/mg in 20 µl of sample. One 722 fractionation is represented (○). *B*, parallel fractionations of wild-type and 722 were blotted with the 88-kDa protein antibody.

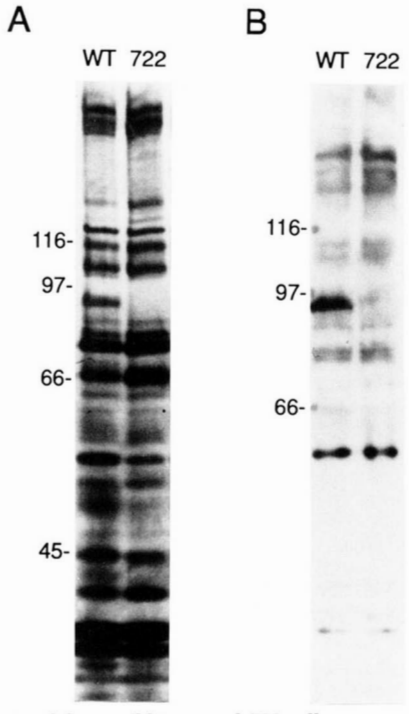


FIG. 5. *A*, cytosol from wild-type and 722 cells were purified in parallel through the QSFF step. Peak fractions from the second QSFF were compared. A band at 88 kDa was present in wild-type but absent in the mutant. *B*, an antibody was raised against this protein and used to stain unfractionated supernatants from both wild-type and 722 cells. The antibody detects a band in wild-type that is absent in the mutant.

eluted with 20–30% of the activity. A majority of the protein can be eluted with 0.5 M NaCl in SLB but contains little CRAC activity. Overall purification at this point was typically 1200-fold.

Identification of an 88 kDa Band Corresponding to CRAC Activity—In several experiments, supernatants isolated from both AX3 and 722 cells were purified in parallel. Since there was no activity in the 722 sample, the fractions corresponding to the active fractions in the parallel wild-type purification were pooled and applied to the subsequent columns. Following the second QSFF step, the material was examined on silver-stained SDS-PAGE gels. Although there were often slight variations in the intensity of several bands, one striking difference between the two samples was consistently observed (Fig. 5*A*). A band of 88 kDa was present in material prepared from wild-type but absent in the mutant.

The amount of protein in the 88 kDa band was estimated by comparison to silver-stained standards run on the same gel. In several experiments, the 88 kDa band was estimated to represent 2–3% of the protein in the lane. This suggests that purification of the 88 kDa band to homogeneity would require 50,000-fold purification.

Following the second QSFF column, the activity becomes unstable to handling and was difficult to further purify. For instance, activity was quickly destroyed by 3–5 s of vortexing at 0 °C. These losses were only partially prevented by inclusion of 10% glycerol in elution of the QSFF. Material eluted without glycerol was carefully applied to a sucrose gradient and, in several experiments, a portion of the activity was retained in the collected fractions. As shown in Fig. 6*A*, the 88 kDa band corresponded to one of two silver-stained bands with sedimentation profiles that match that of the activity. When the QSFF peak was run on a hydroxylapatite column, the activity and the 88 kDa band adsorbed to the column and coeluted at 150–200 mM phosphate (data not shown).

To verify the absence of this protein in 722 supernatants, an antibody was raised against the 88 kDa band. Peak fractions from the second QSFF column were pooled, trichloroacetic acid precipitated, and separated on 7.5% SDS-PAGE gels. The 88 kDa band protein was used to immunize a rabbit as described under "Materials and Methods," and a weak titer antibody was generated. As illustrated in Fig. 5*B*, the antibody was able to detect the presence of the 88-kDa protein in unpurified wild-type, but not in 722, supernatants. Immunoblots of fractions prepared throughout the purification show that the immunostaining 88 kDa band and the activity copurified at every stage (see Figs. 2, 3, 4, and 6*B*). The 88 kDa band never appeared in the mutant purification, suggesting that minute amounts are not concentrated during purification.

The 88-kDa protein was prepared for N-terminal sequencing by purifying it through the second QSFF step. Approximately 5 µg was separated on a 7.5% SDS-PAGE gel, transferred to Immobilon-P, and a portion submitted to the Protein Sequencing Facility in the Department of Biological Chemistry. The short N-terminal sequence obtained is shown in Fig. 7. Due to the low amount of protein, low yield was observed for some residues. For some cycles, it was difficult to distinguish between several assignments.

CRAC Activity Depends on the 88 kDa Band—In independent experiments, a mutant similar to *synag 7* was identified by random insertional mutagenesis. The gene disrupted in these mutants, *dagA*, has been cloned and is described in Insall *et al.*[5] Comparison of the N-terminal sequence of the 88-kDa polypeptide with the deduced sequence of the *dagA* gene revealed that,

[5] R. Insall, A. Kuspa, P. J. Lilly, G. Shaulsky, W. F. Loomis, and P. N. Devreotes, manuscript submitted.

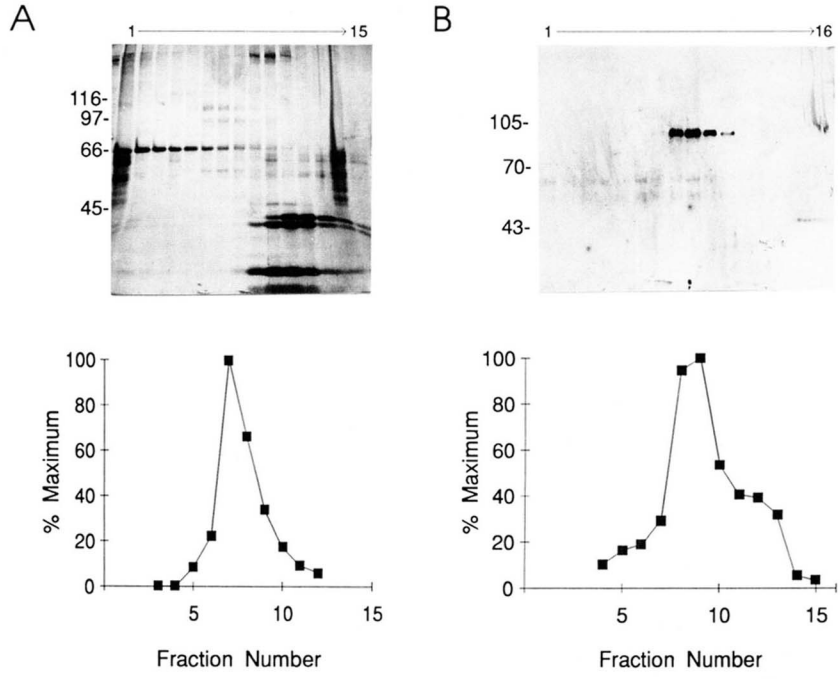


FIG. 6. **Sucrose gradient velocity sedimentation.** *A*, the peak fraction from the second QSFF step was separated on a 10–20% sucrose gradient. Sixteen fractions were collected and 15 of these were trichloroacetic acid precipitated, then analyzed by separation on SDS-PAGE, and silver-stained. Corresponding unprecipitated fractions were assayed for activity and are presented as a percentage of the peak fraction, which was 75.3 pmol/min/mg. *B*, in a similar experiment, 17 fractions were collected from the sucrose gradients. Sixteen of these were trichloroacetic acid precipitated, run on SDS-PAGE, then immunoblotted with the antibody raised against the 88-kDa protein. In the *lower panel*, reconstituting activity of the unprecipitated fractions is plotted as a percentage of the peak fraction, which was 10.5 pmol/min/mg.

1	2	3	4	5	6	7	8
X	(P)$^{+}$	T	E	R	(K)*	(K)*	(E)$^{+}$
	(K)						(S)
							(T)

FIG. 7. **N-terminal sequencing.** Approximately 5 μg of the 88-kDa protein were subjected to N-terminal sequencing and a short N-terminal sequence obtained. $^{+}$ indicates tentative assignments, with the most likely candidate given in the *top line*. * indicates the likely residue, but yield for that amino acid was six times lower than typical for the assigned residues.

1 amino acid from the N terminus, they were identical.[5] When the *dagA* gene is expressed in 722 cells, the aggregation defect is suppressed. Fig. 8 shows that both CRAC activity and the 88 kDa band are overexpressed in these transformants, confirming that the 88 kDa band is required for CRAC activity.

DISCUSSION

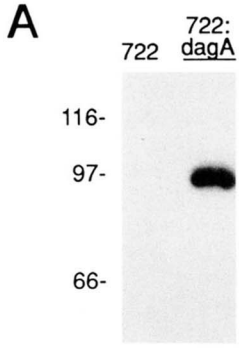
A typical purification of CRAC from a 30-liter culture yields 15 μg of the 88-kDa protein. Based on a fold purification of approximately 50,000 from cell supernatants, we estimate that a cell contains about 7,000 copies of the protein. In the reconstitution assay, CRAC is active at a concentration of less than 100 pM. Experiments in which supernatants prepared from wild-type and 722 cells (which have no activity) were purified in parallel allowed us to identify an 88-kDa protein, present in wild-type but absent from mutant, which is required for CRAC activity.

Multiple lines of evidence suggest that this polypeptide is required for CRAC activity. First, the CRAC activity and the 88 kDa band, apparent after the second QSFF column, cochromatograph in two additional chromatographic systems, sucrose gradient velocity sedimentation and hydroxylapatite desorption with phosphate buffer. Second, the antiserum raised against the purified protein stains the 88 kDa band in supernatants of wild-type but not 722 cells. The specifically immunostained 88 kDa band comigrates with CRAC activity throughout the purification procedure. Third, expression of the *dagA* cDNA produces an 88-kDa protein in 722 cells; expression also restores CRAC activity.

The absence of the 88-kDa protein in the mutant supernatant suggests that either 722 cells contain no CRAC protein, or it is rapidly degraded upon lysis of the cells. According to the data presented here, these cells could harbor a defect in the gene encoding CRAC or in a gene necessary for synthesis or activation of the CRAC protein. However, we know that the defect is in the gene encoding CRAC, as we have identified a deletion in the *dagA* locus from 722.[4]

Several features of the CRAC activity deserve further attention. 1) The reconstitution assay was profoundly inhibited by low concentrations of NaCl and phosphate buffer. 2) The protein bound weakly to anion exchangers and strongly to cation exchangers in the pH range from 6 to 9. 3) We have been unsuccessful in our attempts to chromatograph CRAC activity on sizing columns. Use of columns with exclusion volumes greater than 30 kDa resulted in substantial loss of the protein, even when unfractionated supernatants were applied. 4) CRAC activity became extremely unstable in the final stages of purification. The purified material was poorly stabilized by glycerol, polyethylene glycol, dithiothreitol, detergents, or bovine serum albumin. Although instability of the activity may be due to the dilute conditions under which the protein is being held, the failure of bovine serum albumin and glycerol to stabilize it argues against this possibility. The instability and loss on molecular seive chromatography may suggest that the 88-kDa protein requires a cofactor or is a subunit of a larger complex necessary for activation and that these are more easily separated in the purified preparation. Additionally, in several experiments, we have noted that the *s* value appeared slightly higher when unpurified supernatants were sedimented. The availability of recombinant CRAC protein will allow us to explore these phenomena. It is clear, however, that the 88-kDa protein is essential for CRAC activity.

The discovery of an 88-kDa cytosolic protein specifically required for receptor-mediated and GTPγS-dependent activation of adenylyl cyclase is novel. The αβγ subunits of the G-protein heterotrimer are typically not found in supernatants and, although there are a range of sizes of α-subunits, none have been reported to be larger that 55 kDa. Other possible cytosolic regulators of adenylyl cyclase have been reported in the literature. For instance, CAP, a protein copurifying with the yeast adenylyl cyclase, is a 70-kDa cytosolic protein (30). The yeast enzyme is also regulated by *ras* and by GAP, a large cytosolic protein (31, 32). However, it is not clear that the yeast adenylyl cyclase is sensitive to receptor-mediated stimulation and the



B

Source of Cytosol	Adenylyl Cyclase (pmol/min/mg)
None	22
722	21
AX3	124
722:dagA	386

FIG. 8. **Overexpression of the *dagA* cDNA in 722 cells.** *A*, supernatants were prepared from 722 cells and from 722 cells expressing the *dagA* cDNA under a constitutive actin15 promoter (722:*dagA*, the kind gift of Dr. R. Insall) and immunoblotted using the antibody to the 88 kDa protein. *B*, buffer and supernatant prepared from vegetative 722:*dagA* cells or from developed 722 or AX3 cells were analyzed for their ability to stimulate adenylyl cyclase activity. Development is required for 722 and AX3 to induce maximal CRAC production. Data from several experiments were normalized such that buffer alone produced 22 pmol/min/mg lysate.

enzyme differs considerably in topological structure from the common "transporter"-like structure of the *Dictyostelium* and mammalian enzymes (33). The *Dictyostelium* adenylyl cyclase, having a more typical topology and being activated by surface receptors and GTPγS, may provide more relevant clues for the mode of activation in higher systems.

Our evidence suggests that CRAC is needed to mediate activation of the *Dictyostelium* adenylyl cyclase by the G-protein subunit(s). However, no α-subunit analogous to α_s in mammalian systems has been identified. In mutants lacking $G_\alpha 2$, receptor-mediated stimulation of adenylyl cyclase is blocked and in mutants which overexpress an activated $G_\alpha 2$, this response appears to be chronically desensitized (34, 35). However, lysates of the $g\alpha 2^-$ cells display near normal levels of GTPγS stimulated activity *in vitro* (36). These data have led to a hypothesis whereby stimulation is mediated by βγ-subunits (35, 36). In wild-type cells *in vivo*, these would be released from $G_\alpha 2$ by interaction with excited cAR1. *In vitro*, these could be released from any of the eight heterotrimeric G-proteins which become activated by GTPγS. Recent evidence has shown that βγ-subunits can synergize with activated α_s *in vitro* to stimulate mammalian Type II adenylyl cyclase (37).

If our hypothesis is correct, then we would expect that the βγ subunits and CRAC must cooperate in stimulation of ACA. However, CRAC does not appear to be a GTP-binding protein, and evidence suggests that the site of GTPγS activation is in the membrane fraction. CRAC likely plays an intermediate role between the activated G-protein subunit(s) and the adenylyl cyclase catalytic subunit. Consistently, GTP inhibition of binding is retained in *synag 7* cells (38), suggesting that receptor-G-protein coupling is normal. The likelihood of interaction with either G-proteins or the adenylyl cyclase catalytic subunit suggests that a proportion of the CRAC protein must be associated with the membrane or is recruited to the membrane during the activation cycle.

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