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Communication

Constitutively Active Adenylyl Cyclase Mutant Requires Neither G Proteins nor Cytosolic Regulators*

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Receptor-mediated G protein-linked adenylyl cyclase systems are universal signal transducers. We exploited the essential role of this cascade in *Dictyostelium* development to screen for random mutations in the catalytic component, ACA. This enzyme is activated by G protein βγ-subunits acting in concert with a novel cytosolic regulator, CRAC. By suppression of the CRAC-null phenotype, we isolated constitutively active versions of the enzyme that require neither exogenous stimuli nor internal regulators. One mutant displayed a 15-fold increase in its V_{max}. It harbors a single amino acid substitution (L394S) affecting a conserved residue located in the first cytoplasmic loop near the N-terminal hydrophobic domain of ACA. The screening procedure can be adapted for isolation of constitutive mutations in mammalian adenylyl cyclases.

G protein-coupled adenylyl cyclases remain in a relatively silent basal state until they receive an activating signal from a surface receptor. Enzyme activity is then rapidly turned on in response to G protein α- or βγ-subunits and a variety of regulators including calmodulin and protein kinases (1). The receptor-G protein-adenylyl cyclase complex is involved in numerous physiological processes including the action of many hormones, metabolism, learning, and memory. Constitutively activating mutations in receptors and G protein subunits have been linked to human diseases (2). However, no defects have yet been identified in the catalytic subunit, adenylyl cyclase. Using the accessible genetics and biochemistry of *Dictyostelium*, we devised a screen to isolate both loss- and gain-of-function mutations in adenylyl cyclase (3). We report here that a single point mutation in adenylyl cyclase is sufficient to produce a constitutively active enzyme suggesting that mutations in the catalytic subunit might explain certain genetic disorders.

Adenosine 3′-5′ monophosphate (cAMP) plays a central role in the aggregation and development of the free living amoebae, *Dictyostelium.* Following its synthesis, the nucleotide is secreted and binds to the surface receptor, cAR1, leading to chemotaxis, increased gene expression, and activation of the adenylyl cyclase, ACA.[1] The latter response relays the cAMP signal to neighboring cells and allows long distance cell-cell communication to take place leading to cell aggregation and differentiation into fruiting bodies (4–6). Genetic and biochemical analyses have demonstrated that receptor-mediated activation of ACA requires the βγ-subunits of the heterotrimeric G protein, G2, and CRAC (cytosolic regulator of adenylyl cyclase). CRAC is an 88-kDa cytosolic protein rich in threonine and serine residues. It contains a PH domain in its N-terminal (7). Following cAMP stimulation, CRAC is translocated to the plasma membrane; this membrane association does not take place in *gβ*⁻ cells (8). Cells lacking the Gβ-subunit or CRAC show neither receptor nor GTPγS activation of ACA, remain as monolayers when plated on non-nutrient agar, and form smooth plaques on bacterial lawns (7–9). The overexpression of wild type ACA in *crac*⁻ cells does not suppress the aggregation-deficient phenotype. Therefore, we used suppression of the *crac*⁻ phenotype to isolate gain-of-function mutations in ACA. We expected to find enzymes possessing high, unregulated activity or displaying supersensitivity to regulators such as G protein βγ-subunits.

EXPERIMENTAL PROCEDURES

Library Construction, Transformation, and Phenotypic Screening—Randomly mutagenized libraries of ACA were constructed using polymerase chain reaction and subcloned into an extrachromosomal expression vector that gives constitutive expression, as described previously (3). The mutagenized region corresponded to five predicted transmembrane helices as well as the C1 domain. The libraries were electroporated into *crac*⁻ cells, and transformants were selected on 24-well plates (7, 10). To screen for gain-of-function ACA mutants, the resulting transformants were spread on *Klebsiella aerogenes* lawns, as described (11). The aggregation-competent clones were selected, transferred into liquid media, and grown in the presence of 20 μg/ml G418. Once grown, development under more stringent conditions was performed by plating washed cells on non-nutrient agar at 22 °C as described previously (12). Western analysis was performed as described previously, using a peptide antibody directed against the last 15 amino acids of ACA. Detection was performed using enhanced chemiluminescence (3). Selected mutants were electroporated into *gβ*⁻ cells (13).

Adenylyl Cyclase Assays—Enzyme activity was measured either in vegetative cells where the basal and unregulated activity ($MnSO_4$) of ACA can be assessed or in cells starved for 5 h (repeatedly stimulated with 50 nM cAMP) where receptor, CRAC, and Gβγ-subunits are expressed and the GTPγS activation of ACA can be measured. Assays for adenylyl cyclase activation were performed for 2 min at room temperature in the presence of 2 mM $MgSO_4$ (basal), 5 mM $MnSO_4$, or 40 μM GTPγS and 1 μM cAMP with and without the addition of exogenous CRAC as described previously (3, 14). For receptor-mediated adenylyl cyclase activation, cells starved for 5 h were stimulated with 10 μM cAMP, lysed at specific time points, and assayed for 1 min. Membranes were prepared as follows: cells were starved for 2 h, washed twice, and resuspended in 10 mM Tris, pH 8, 2 mM EDTA, 200 mM sucrose at 8×10^7 cells/ml, filter-lysed, and spun at 10,000 × *g* for 15 min. The resulting membrane pellet was resuspended in 10 mM Tris, pH 8, 10% glycerol, 2 mM EDTA spun again and finally resuspended in 10 mM Tris, pH 8, 10% glycerol, 2 mM $MgSO_4$, 0.2 mM EGTA at 8×10^7 cell eq/ml. Two hundred μl were assayed with and without 5 mM $MnSO_4$ for 2 min at room temperature.

Plasmid Recovery and Sequence Analysis—Total *Dictyostelium* DNA was isolated as described (15), transformed into competent bacteria,

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[1] The abbreviations used are: ACA, adenylyl cyclase expressed during aggregation; CRAC, cytosolic regulator of adenylyl cyclase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

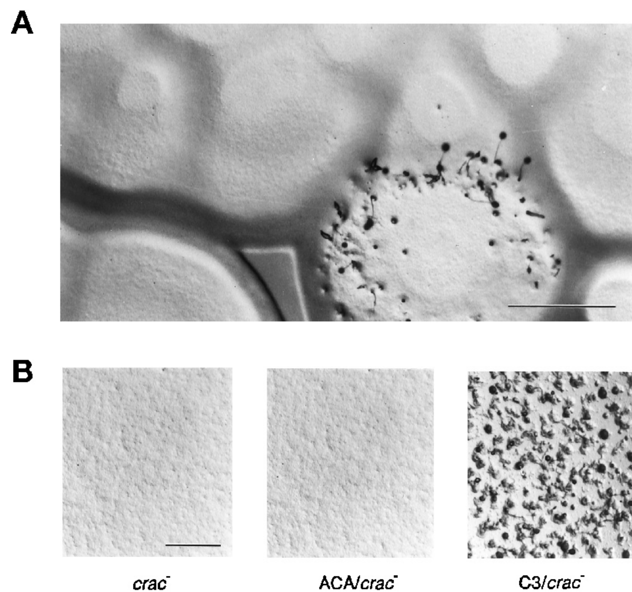

FIG. 1. **Developmental phenotype of ACA mutants.** *A*, representative phenotypic screen. The transformants were mixed with *K. aerogenes* and spread on SM agar plates. This picture was taken 5 days after plating and depicts one *crac*⁻ suppressor in a sea of aggregation-defective clones. *Bar* represents 0.5 cm. *B*, developmental phenotypes of selected transformants on non-nutrient agar plates. Transformants were grown in liquid culture, washed, and plated at 22 °C. C3/*crac*⁻ is a representative of four independently isolated clones. The pictures were taken 24 h after plating. *Bar* represents 0.25 cm in the *crac*⁻ and ACA/*crac*⁻ panels and 0.1 cm in the C3/*crac*⁻ panel.

and DNA was isolated using standard molecular biology techniques. After recapitulation of the phenotype by electroporation into *crac*⁻ or *g*β⁻ cells, the resulting plasmids were sequenced using chain terminator chemistry (DNA Analysis Facility, Johns Hopkins School of Medicine).

RESULTS AND DISCUSSION

We previously reported the isolation of loss-of-function ACA mutants using phenotypic rescue of *aca*⁻ cells (3). By transforming the same randomly mutagenized ACA libraries into *crac*⁻ cells, we were able to identify gain-of-function mutants. The transformants were spread at high density (1,000/100 mm plate) on *K. aerogenes* lawns (Fig. 1*A*). A first screen of ~2,000 independent transformants yielded only one aggregation-competent clone (named b2). This mutant could also aggregate on non-nutrient agar at high cell densities, but it remained aggregation-less when plated at standard cell densities, a more stringent development condition. A second screen of ~11,000 independent transformants yielded 13 aggregation-competent clones. When plated on non-nutrient agar at standard cell densities, four clones, named c3, c4, c5, and c6, aggregated (Fig. 1*B*). The remaining nine clones displayed a conditional phenotype similar to the one observed for mutant b2. Western analysis of mutant and wild type ACA expressing cells showed they expressed similar levels of ACA (Fig. 2*A*). This indicates that the suppression of the *crac*⁻ phenotype was likely to have arisen from alterations in ACA activity.

Adenylyl cyclase activities of the mutant clones were measured in the presence of $MgSO_4$ and $MgSO_4$ with $MnSO_4$. Wild type ACA is typically activated 5-fold by $MnSO_4$. Mutants c3–c6 displayed high basal activities, ~5-fold greater than wild type, that was barely stimulated further by $MnSO_4$ (Fig. 2*B*). One of the clones with the conditional phenotype was also assayed and displayed an intermediate $MgSO_4$ activity. The $MnSO_4$/$MgSO_4$ ratio for wild type, the weak mutant (b2), and mutant c3 were calculated. A good correlation between the developmental phenotypes and the ratios was observed: mutant b2 showed a slightly lower ratio than wild type (3.7 ± 0.3 compared to 4.9 ± 0.5 for wild type ACA) whereas mutant c3, which suppresses the *crac*⁻ phenotype under all conditions, displayed a much lower ratio (1.3 ± 0.1). Consequently, further analysis focused on mutants c3–c6.

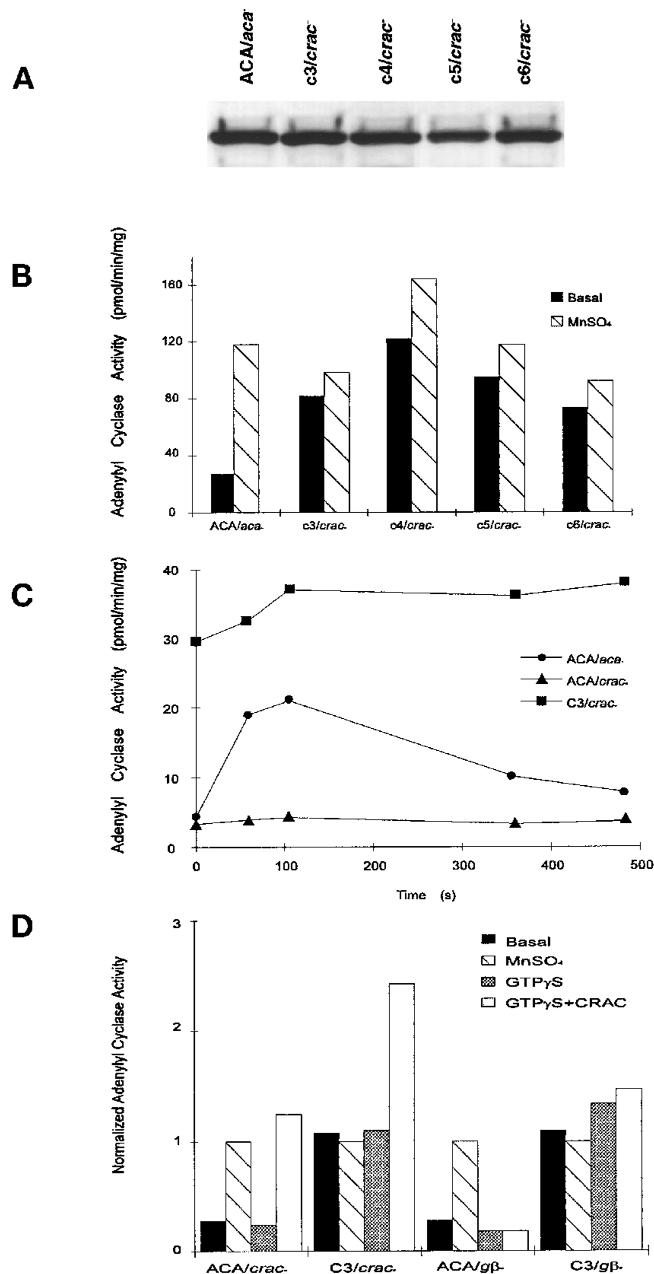

FIG. 2. ***In vitro* and *in vivo* adenylyl cyclase activation of ACA mutants.** *A*, ACA protein expression of mutants c3–c6. Western analysis was performed using a peptide antibody directed against the C-terminal end of ACA. *B*, basal and $MnSO_4$-stimulated adenylyl cyclase activity. For *A* and *B*, wild type ACA expressed in *aca*⁻ cells was used as a control. Identical results are obtained in *crac*⁻ cells expressing ACA. *C*, adenylyl cyclase activity following cAMP stimulation of selected cell lines. *D*, GTPγS-stimulated adenylyl cyclase activity in *crac*⁻ and *g*β⁻ cell lines expressing wild type or mutant ACA molecules. The results are expressed as a ratio of the adenylyl cyclase activity/$MnSO_4$ activity. The absolute $MnSO_4$ activities are as follows: 30.3, 64.1, 23.7, 40.7 pmol/min/mg of protein for ACA/*crac*⁻, C3/*crac*⁻, ACA/*g*β⁻, and C3/*g*β⁻, respectively. See "Experimental Procedures" for details. The results presented were performed in duplicate and are representative of at least three independent experiments.

The plasmids from the mutants were recovered and transformed into fresh *crac*⁻ cells. The retransformed clones dis-

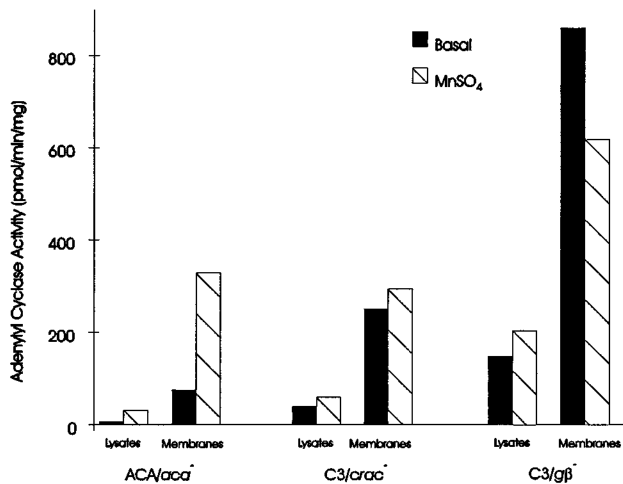

FIG. 3. **Basal and unregulated adenylyl cyclase activity of wild type ACA and mutant C3 in cell lysates and membrane preparations.** See "Experimental Procedures" for details. The results presented were performed in duplicate and are representative of at least two independent experiments.

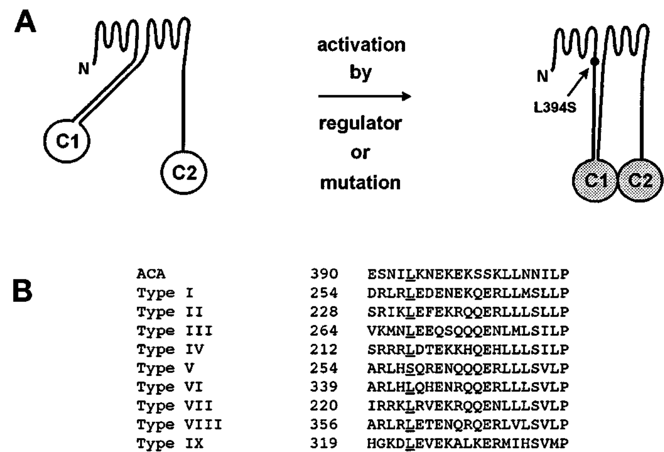

FIG. 4. **Topology and sequence analysis of the C3 ACA mutant.** *A*, diagram showing a model of the conformation change proposed to occur following enzyme activation or mutagenesis. The position of the single amino acid change present in C3 is indicated as a *black circle* (L394S). The conserved C1 and C2 domains are depicted as *circles*; catalytically competent domains are *shaded*. Sequence analysis was performed using chain terminator chemistry (DNA Analysis Facility, Johns Hopkins School of Medicine). *B*, amino acid alignment of ACA and mammalian adenylyl cyclases within the mutated region of C3. The mutated Leu-394 residue of ACA is *underlined*. The corresponding position in the mammalian enzymes is also *underlined*. The nine mammalian enzymes used in the alignment are as follows: bovine type I (25), rat type II (26), rat type III (27), rat type IV (28), rat type V (16), rat type VI (16), mouse type VII (29), rat type VIII (30), and mouse type IX (31). Alignment was obtained using DNAstar with clustal V algorithm.

played the suppressed *crac*⁻ phenotype with 100% efficiency, and each of the mass cultures displayed high basal and unregulated adenylyl cyclase activities (data not shown). Sequence analysis of the four isolated plasmids revealed that all harbored the same single L394S mutation, suggesting that they were siblings originating from a common transformation or PCR event. Consequently, further biochemical analysis was performed on one of these clones (C3).

Stimulation of adenylyl cyclase following chemoattractant receptor (cAR1) activation in intact cells was assessed by rapid lysis and brief assay at different time points after the addition of exogenous cAMP (Fig. 2*C*). In *aca*⁻ cells expressing ACA (ACA/*aca*⁻) receptor stimulation led to a rapid rise in enzyme activity followed by a slow return to basal levels; in ACA/*crac*⁻ cells, there was no activation. On the other hand, in *crac*⁻ cells expressing the mutant enzyme (C3/*crac*⁻), there was very high activity and no significant response to receptor stimulation. These observations suggest that the mutated enzyme rescues the *crac*⁻ phenotype by being constitutively activated.

An *in vitro* GTPγS stimulation assay confirmed these results. As expected, both ACA/*crac*⁻ and C3/*crac*⁻ cells were insensitive to GTPγS activation (Fig. 2*D*). The wild type enzyme activity was stimulated 5-fold when exogenous CRAC was added with the GTPγS. On the other hand, the high activity of the mutant enzyme was increased only 2-fold. This increase brought the activity of C3 to higher levels than the maximally stimulated wild type enzyme. Thus, C3 displays higher than peak wild type activity in the absence of stimuli and can be superactivated by regulators.

If C3 is truly a constitutive mutant, it should maintain its high activity in the absence of the G protein $\beta\gamma$-subunits, which in wild type cells are essential for receptor and GTPγS stimulation (9). To address this possibility and rule out that C3 was not hypersensitive to low levels of free G$\beta\gamma$-subunits present in unstimulated cells, we measured activities in a *g*β^- background (Fig. 2*D*). The results clearly show that the absence of the G protein β-subunit has little effect on the activity of C3. Its $MnSO_4$/$MgSO_4$ ratio remained at ~1.0, and the addition of GTPγS or GTPγS + CRAC elicited no further activation. The same high activity was measured in membrane preparations derived from either C3/*crac*⁻ or C3/*g*β^- cells demonstrating that the mutant was not hypersensitive to a yet unknown cytosolic regulator (Fig. 3). These results strongly suggest that C3 is a constitutive adenylyl cyclase mutant.

Steady state kinetic analyses revealed that in the absence of GTPγS or CRAC, C3 displays a 15-fold greater V_{max} when compared to the wild type enzyme (data not shown). The wild type enzyme displayed average K_m and V_{max} values of 0.2 mM and 20 pmol/min/mg of protein, while the mutant adenylyl cyclase had average K_m and V_{max} numbers of 0.3 mM and 282 pmol/min/mg of protein. Since ACA/*crac*⁻ and C3/*crac*⁻ cells express similar levels of protein, the mutation clearly leads to an increase in the turnover number of the enzyme. In wild type adenylyl cyclases, the addition of regulators such as G proteins, calmodulin, and CRAC elicits increases primarily in V_{max}. Our data show that similar changes can be achieved by subtle mutations.

Adenylyl cyclases are predicted to consist of two sets of six transmembrane helices and two large conserved cytoplasmic domains (C1 and C2) (1). The single amino acid residue difference between ACA and C3 is located at the beginning of the first cytoplasmic domain just after the first hydrophobic cluster (L394S) (Fig. 4*A*). Comparison of nine mammalian adenylyl cyclases (type I-IX) shows that L394 is a conserved residue, eight of the enzymes have a leucine at that position (Fig. 4*B*). Surprisingly, type V adenylyl cyclases from various species have a conserved serine at this amino acid position (16–18). We are not aware that type V adenylyl cyclases display unusually high activities. Moreover, the type II and type VI adenylyl cyclases, which have a leucine at the mutated position, show very different basal adenylyl cyclase activities (19). Thus, this particular substitution may not activate all adenylyl cyclases.

Our results show that mutations can mimic the effects of G proteins in stimulating high enzymatic activity in adenylyl cyclases. Tang *et al.* (20, 21) have demonstrated that both halves of the symmetrical molecule are needed for catalysis and suggested that C1 is a regulatory domain. Our results indicate that a mutation within the region linking C1 to the first set of hydrophobic domains results in the acquisition of an activated conformation. Tang and Gilman (22) and Yan *et al.* (23) recently provided evidence that a soluble enzyme containing only

the tethered C1 and C2 cytoplasmic regions of the type I and type II mammalian enzymes can still be activated by $G\alpha_s$* and forskolin. Interestingly, this heterotrimeric molecule does not include the region corresponding to the one altered in mutant C3 (22, 23). Thus, the L394S mutation is probably affecting a site distinct from that for G protein binding, it may induce a conformational change that mimics activation by G proteins.

The random mutagenesis approach we developed to isolate both loss- and gain-of-function adenylyl cyclase mutants is very informative (3). We found that the mutation yielding a constitutive activity was present in only a few of 10,000 transformants, implying that the other random amino acid substitutions in this pool do not yield constitutively active enzymes. Since our mutagenesis was not saturating, it is possible that other amino acid substitutions may yield a similar phenotype. We previously demonstrated that mutations in the region just C-terminal to the Leu-394 position result in enzymes that remain catalytically active but are resistant to G protein activation (3). The isolation, from a population of randomly mutagenized molecules, of both G protein-resistant and constitutively active mutants that map to a similar region of the C1 loop suggest that it is critical for adenylyl cyclase activation in its native form. We propose that this segment acts as a hinge allowing for the association of the two cytoplasmic loops and formation of an activated conformation (Fig. 4*A*).

The constitutive mutant C3 suppresses the aggregation-deficient phenotype of the *crac*$^-$ cells because it provides a constant high source of cAMP. We devised our screen based on the observation that ACG, an adenylyl cyclase with an unusually high basal activity, suppresses the phenotype of both *aca*$^-$ and *crac*$^-$ cells (24).[2] Taken together, these observations suggest that any adenylyl cyclase that generates an activity equivalent to or greater than that produced by the C3 mutant will rescue both *aca*$^-$ and *crac*$^-$ cells (see Fig. 2*B*). With respect to this, we have expressed the type II mammalian adenylyl cyclase in *Dictyostelium aca*$^-$ cells. As expected, the *Dictyostelium* heterotrimeric G proteins did not efficiently couple to the type II mammalian enzyme, and the transformed cells remained aggregation-deficient. However, forskolin stimulation led to a 50-fold increase in enzyme activity corresponding to the activity measured in *crac*$^-$ cells expressing C3.[3] Thus, it follows that mutations in the type II enzyme that lead to high, unregulated activity will cause the *aca*$^-$ or *crac*$^-$ cells to aggregate. Consequently, this powerful nonbiased random mutagenesis approach can be directly applied to isolate constitutively active forms of mammalian adenylyl cyclases.

[2] C. A. Parent and P. N. Devreotes, unpublished observation.

[3] C. A. Parent, G. S. Pitt, and P. N. Devreotes, manuscript in preparation.

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