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# Regulation of Adenylyl Cyclases by a Region Outside the Minimally Functional Cytoplasmic Domains*

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**The highly conserved topological structure of G protein-activated adenylyl cyclases seems unnecessary because the soluble cytoplasmic domains retain regulatory and catalytic properties. Yet, we previously isolated a constitutively active mutant of the *Dictyostelium discoideum* adenylyl cyclase harboring a single point mutation in the region linking the cytoplasmic and membrane domains (Leu-394). We show here that multiple amino acid substitutions at Leu-394 also display constitutive activity. The constitutive activity of these mutants is not dependent on G proteins or cytosolic regulators, although some of the mutants can be activated to higher levels than wild type. Combining a constitutive mutation such as L394T with K482N, a point mutation that renders the enzyme insensitive to regulators, restores an enzyme with wild type properties of low basal activity and the capacity to be activated by G proteins. Thus regions located outside the cytoplasmic loops of adenylyl cyclases are not only important in the acquisition of an activated conformation, they also have impact on other regions within the catalytic core of the enzyme.**

G protein-coupled adenylyl cyclases are responsible for the synthesis of the ubiquitous second messenger cAMP. In eukaryotic cells, cAMP regulates a multitude of cellular responses, including cell growth and differentiation, metabolism, and synaptic transmission (1, 2). Modulation of adenylyl cyclase activity is thus responsible for a wide variety of biological and pathological states. Hormones, neurotransmitters, odorants, and chemokines control this activity by interacting with G protein-coupled receptors. These activated receptors stimulate the exchange of GDP for GTP on the $\alpha$-subunit of the heterotrimeric G proteins, and the $\alpha$-subunit dissociates from the $\beta\gamma$ complex. Both the activated G$\alpha$-subunit and the released G$\beta\gamma$ complex can stimulate or inhibit adenylyl cyclase activity. In mammalian cells, at least nine different forms of this enzyme have been cloned, and each subtype has been shown to possess a specific pattern of regulation and expression. Although these adenylyl cyclases are all activated by $G_s\alpha$, they show a distinct response to $G_i\alpha$- and G$\beta\gamma$-subunits, $Ca^{2+}$, $Ca^{2+}$/calmodulin, as well as protein kinase A and C. In *Drosophila*, a large family of these enzymes has also been identified (3). One enzyme has been shown to be involved in learning and memory (4). In the social amoebae *Dictyostelium discoideum*, the expression of a single G protein-coupled adenylyl cyclase, named ACA,[1] is essential for the survival of this lower eukaryote (5).

Adenylyl cyclases share a common topology predicted to consist of two sets of six transmembrane helices and two large cytoplasmic domains (C1 and C2) located C-terminally to each set of helices (2). Each cytoplasmic domain contains a region of homology (designated C1a and C2a) with the cytoplasmic domains of several adenylyl and guanylyl cyclases. The interaction between the two cytoplasmic domains of adenylyl cyclases is necessary for the activation of the enzyme; the catalytic and G protein-mediated activities are retained by separately purifying and mixing the two cytoplasmic domains (6). The availability of such a soluble form of adenylyl cyclases has allowed the completion of crystallographic studies (7, 8). These studies revealed that the C1/C2 catalytic core is a symmetric heterodimer that binds one molecule of $G_s\alpha$, one molecule of forskolin (a hypotensive drug that stimulates all mammalian forms, except type IX), and one molecule of ATP. The active site of the enzyme is located at the C1/C2 interface. It has been proposed that G proteins regulate the activity of the enzyme by inducing a conformational change that reorients the C1 and C2 domains.

In *D. discoideum*, the proper regulation of the G protein-coupled adenylyl cyclase ACA is essential for the early stages of development during which starvation induces cells to aggregate and differentiate into spores atop a stalk of vacuolated cells (9). In this system, cAMP acts like a hormone; after its synthesis, it is secreted and it binds to specific G protein-coupled receptors called cARs (cAMP receptors). Receptor occupancy leads to the activation of several effectors including ACA, and the signal is thereby relayed to neighboring cells. Genetic analysis revealed that receptor-mediated activation of ACA requires, in addition to heterotrimeric G proteins, several cytoplasmic regulators. Among them, CRAC (cytosolic regulator of adenylyl cyclase), a novel soluble protein containing a pleckstrin homology domain, is recruited to the plasma membrane in response to receptor activation and is absolutely required for receptor and GTP$\gamma$S activation of ACA (10). Consequently, *crac*$^-$ cells cannot activate ACA, do not aggregate, and remain as smooth monolayers when starved (11). By using random mutagenesis and phenotypic rescue of *crac*$^-$ cells, we previously isolated a mutant of ACA displaying constitutive activity that required neither receptor activation nor cytoplasmic regulators (12). This mutant harbored a single point mu-

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

tation, Leu-394, located N-terminally to the first cytoplasmic domain just after the first hydrophobic cluster. To determine whether this domain is involved in the formation of an activated conformation of ACA, we mutagenized the amino acid sequence in that region and assessed the consequence of these alterations on the developmental and biochemical phenotypes of *D. discoideum aca*$^-$ and *crac*$^-$ cells expressing the mutants.

## EXPERIMENTAL PROCEDURES

*Cell Lines and Culture Conditions*—The *crac*$^-$, *aca*$^-$, and *g*$\beta^-$ cell lines were generated previously by homologous recombination and maintained in standard HL5 media (5, 11, 13). Transformants with wild type and mutant ACA plasmids were obtained by electroporation (14), selected with 20 $\mu$g/ml G418, grown in shaking suspension to densities of 2–5 $\times$ $10^6$ cells/ml, and harvested for experimental analysis.

*Oligonucleotide-directed Mutagenesis of ACA and Transformations in D. discoideum Cells*—Site-directed mutagenesis was performed using the MORPH Kit (5 Prime $\rightarrow$ 3 Prime, Boulder, CO) with primers encoding a specific codon or a partially degenerate primer at position 1180 of the ACA cDNA. A construct harboring a 7-amino acid deletion (3 amino acids on either side of Leu-394 of ACA and Leu-394) was generated using a primer missing the corresponding nucleotides and containing 15 nucleotides 5′ and 3′ to the deleted domain to ensure proper annealing. Finally, two plasmids with 3 Ala residues inserted 5′ or 3′ of Leu-394 were also obtained using the MORPH Kit. For each of these constructs, a plasmid containing an 800-bp fragment of ACA was used as a template. The mutated ACA cDNA were subsequently cloned into an extrachromosomal *D. discoideum* expression vector (pCP33), which gives high and constitutive expression. The resulting plasmids were electroporated into *aca*$^-$ and *crac*$^-$ cells. Selected plasmids were also transformed into *g*$\beta^-$ cells.

*Phenotypic Screening*—The transformed cells were grown in shaking cultures to a density of 3–5 $\times$ $10^6$ cells/ml, harvested, washed once in DB (5 mM $Na_2HPO_4$, 5 mM $NaH_2PO_4$, pH 6.2, 2 mM $MgSO_4$, 200 $\mu$M $CaCl_2$), resuspended at 1 $\times$ $10^7$ cells/ml, and plated on DB agar (15). The plates were stored at 22 °C, and development was assessed 24 h later. Under normal conditions, 1 $\times$ $10^7$ cells were plated on 35-mm plates. In some experiments, up to 1 $\times$ $10^8$ cells were plated, and the resulting phenotype was assessed 24 h later.

*Immunoblotting*—Western analysis was performed as described previously using a peptide antibody directed against the last 15 amino acids of ACA (16). Detection was performed using enhanced chemiluminescence.

*Adenylyl Cyclase Assays*—Enzyme activity was measured either in vegetative cells, where the basal and unregulated ($MnSO_4$) activities of ACA can be assessed, or in cells starved for 5 h (repeatedly stimulated with 75 nM cAMP), where receptor and CRAC are expressed and the GTP$\gamma$S 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 $\mu$M GTP$\gamma$S and 1 $\mu$M cAMP as described previously (17). For the receptor-mediated activation, the cells were stimulated with 10 $\mu$M cAMP, rapidly lysed at specific time points, and assayed for 1 min at room temperature. Assays on membrane preparations were performed as described previously (12).

## RESULTS

By using site-directed mutagenesis, we replaced Leu-394 with 11 amino acids: 5 non-polar (Ala, Phe, Gly, Ile, and Pro); 3 polar (Asn, Thr, and Tyr); 2 acidic (Asp and Glu); and 1 basic (Arg). These constructs were cloned into an episomal plasmid, downstream of the actin-15 promoter that gives high constitutive levels of expression, and transformed in both *aca*$^-$ and *crac*$^-$ cells. The developmental phenotype of these cell lines on non-nutrient agar is shown in Fig. 1*A*. All substitutions at Leu-394 gave rise to ACA molecules that could complement the *aca*$^-$ cells, and many of the mutants suppressed the aggregation-deficient phenotype of the *crac*$^-$ cells. In addition to the original L394S mutation, L394A, L394I, L394G, L394R, and L394T could all aggregate in the absence of the essential cytoplasmic regulator CRAC. One mutant, L394N, differentiated into weak aggregates and was thus scored as a partial suppressor. Western analysis revealed that each mutant expressed similar levels of ACA in both cell lines, although L394P expressed significantly lower levels of protein and was not further analyzed (Fig. 1*B*). Taken together, these observations suggested that many of the substitutions rendered the enzyme constitutively active.

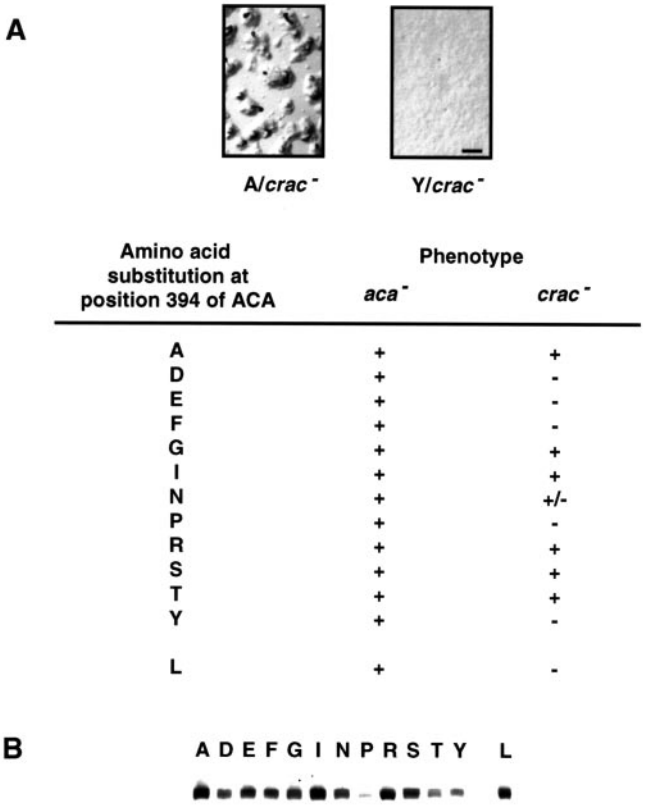


FIG. 1. **Developmental phenotypes of Leu-394-substituted ACA mutants.** *A, top panel* depicts representative developmental phenotypes observed when the ACA mutants, L394A and L394Y, were expressed in *crac*$^-$ cells. The *table* reports the phenotypes for each substitution mutant when expressed in *aca*$^-$ or *crac*$^-$ cells. The + and − signs refer to the capability of the mutants to suppress the aggregation-deficient phenotypes of the parent cell line. The N/*crac*$^-$ cells partially rescued the development and showed only loose aggregates after starvation. Transformants were grown in liquid culture and washed, and 1 $\times$ $10^7$ cells were plated on 35-mm plates at 22 °C on non-nutrient agar. Photographs were taken 24 h after the cells were plated. *Bar* represents 2 mm. *B,* ACA protein expression of the substituted mutants in *crac*$^-$ cells. Western analysis was performed using a peptide antibody directed against the last 15 amino acids of ACA. Detection was performed using enhanced chemiluminescence. *L* represents the wild type sequence of ACA. Identical results were observed when the mutants were expressed in *aca*$^-$ cells.

We next evaluated the intrinsic adenylyl cyclase activity of each mutant by measuring the enzyme activity in the presence of $Mn^{2+}$ (Fig. 2*A*). $Mn^{2+}$, a more potent cofactor than $Mg^{2+}$, will stimulate the enzymatic activity in the absence of G proteins (18). It is thus used to assess the integrity of the catalytic core. As expected, all mutants expressed in either *aca*$^-$ or *crac*$^-$ cells showed high enzyme activities in the presence of $Mn^{2+}$ (results are shown for the mutants expressed in *crac*$^-$ cells only). However, their activities, measured in the presence of $Mg^{2+}$, varied greatly. For a number of mutants, the activity in the absence of $Mn^{2+}$ was close to their activity in the presence of $Mn^{2+}$ (Fig. 2*B*). By comparing the $Mg^{2+}/Mn^{2+}$ adenylyl cyclase activity ratio of the mutants with their developmental phenotype, we observed that a certain level of adenylyl cyclase activity suppressed the aggregation-deficient phenotype of the *crac*$^-$ cells. A substitution leading to a ratio of 0.5 or greater (corresponding to an activity of ~50 pmol/min/mg) was sufficient. The glycine mutant defined the boundary between the two classes

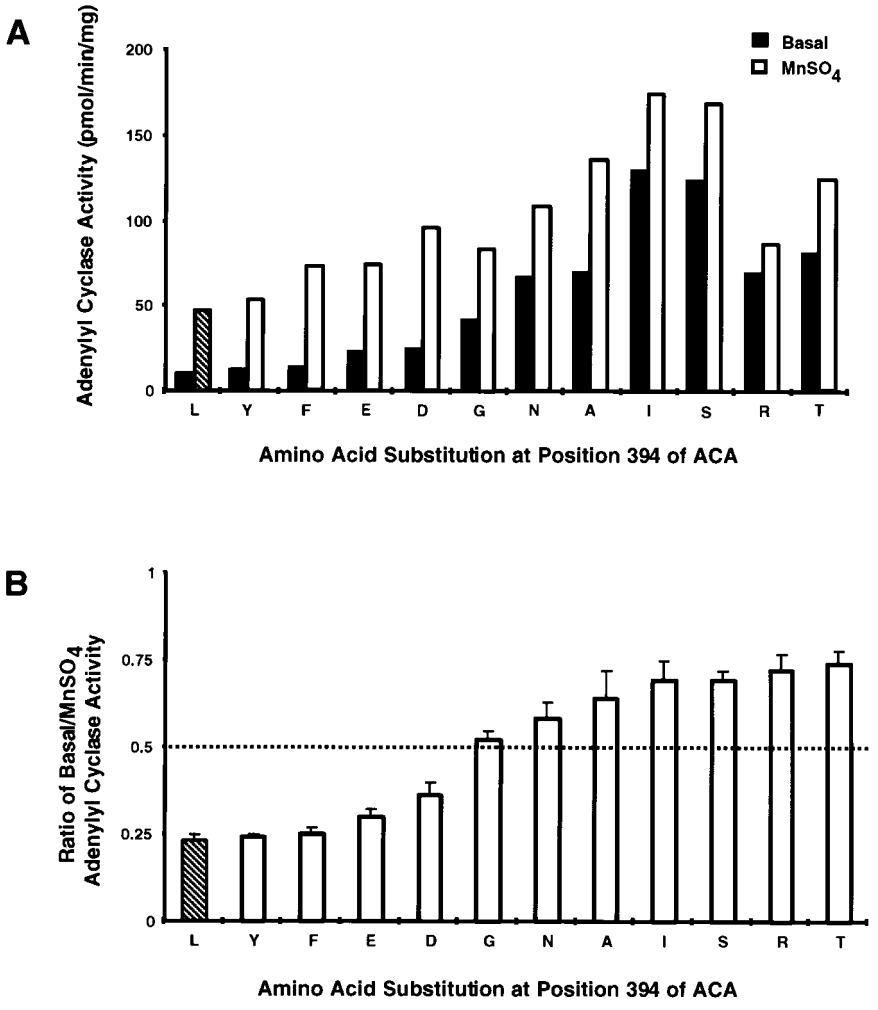


FIG. 2. **Adenylyl cyclase activation of Leu-394-substituted ACA mutants expressed in *crac*$^-$ cells.** *A,* basal and $MnSO_4$-stimulated adenylyl cyclase activity. Mutant cell lines were grown, washed, lysed, and assayed for 2 min with and without 5 mM $MnSO_4$ as described under "Experimental Procedures." The results presented were performed in duplicate and are representative of at least three independent experiments. *B,* cumulative adenylyl cyclase activation expressed as a ratio of Basal/$MnSO_4$ activity. The values presented are the mean ± S.E. of three to eight independent experiments. *L* represents the wild type sequence of ACA.

of mutants. These data show that single point mutations on adenylyl cyclases give rise to enzymes possessing distinct intrinsic activities.

The original mutant, L394S, rescued the aggregation-deficient phenotype of *crac*$^-$ cells by providing a constant high source of cAMP (12). It displayed no significant activation in response to either receptor or GTPγS stimulation in *crac*$^-$ cells, and the same high basal activity was observed in membranes derived from L394S/*gβ*$^-$ cells, confirming that the mutant was not supersensitive to G proteins or cytosolic regulators. The new mutants showed a similar behavior. As expected, when expressed in *crac*$^-$ cells, none showed a response to GTPγS (data not shown). Next, we transformed three representative mutants in *gβ*$^-$ cells and measured the adenylyl cyclase activity in both lysates and membrane preparations. The three mutants retained high basal activity in the absence of functional G proteins (Fig. 3). Moreover, the high activity was measured in both cell lysates and membrane preparations. Interestingly, for both control and mutant enzymes, the $Mg^{2+}$/$Mn^{2+}$ activity ratio was further elevated in membrane preparations compared with cell lysates (Fig. 3). We also observed that many of the constitutively active mutants possessed a residual activation potential because they could be further activated when expressed in *aca*$^-$ cells. In this parental background, where wild type levels of G proteins and CRAC are expressed, the mutants showed a 2–3-fold stimulation in response to GTPγS (Fig. 4). This behavior was also observed with the original L394S mutant. Intriguingly, L394I showed the weakest stimulation in the presence of GTPγS (Fig. 4).

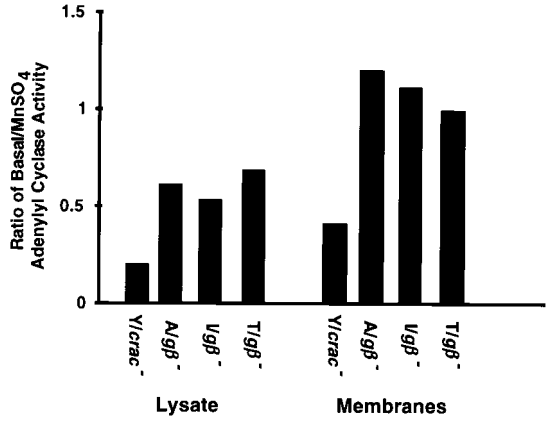


FIG. 3. **Adenylyl cyclase activity of Leu-394 substituted ACA mutants expressed in *gβ*$^-$ cells.** Basal and $MnSO_4$-stimulated adenylyl cyclase activities were measured in lysates and membrane preparations with and without $MnSO_4$ as described under "Experimental Procedures." The results, performed in duplicates, are expressed as a ratio of Basal/$MnSO_4$ adenylyl cyclase activity and are representative of at least two independent experiments.

We next wanted to more generally disturb the N-terminal domain of the C1 loop of ACA. We generated three mutants that harbored the following alteration in the ACA protein: two mutants were designed to have three alanine insertions either N-terminal (AAAL) or C-terminal (LAAA) from the Leu-394 residue, and one had a 7-amino acid deletion (Del) across the

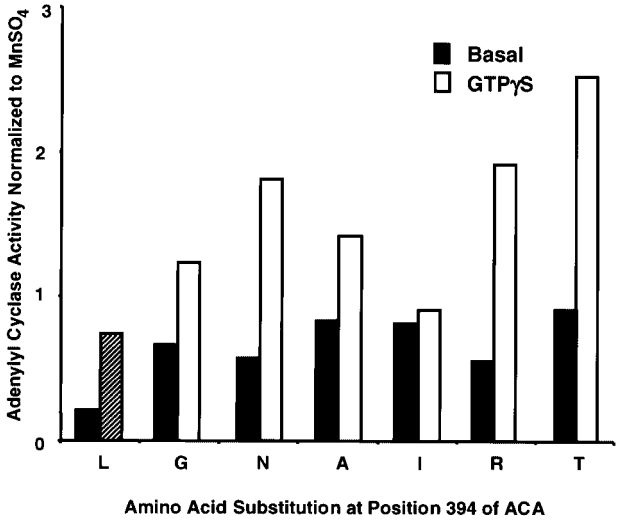


FIG. 4. **GTPγS-stimulated adenylyl cyclase activity in *aca*$^-$ cell lines expressing Leu-394-substituted ACA mutants.** Cells were starved for 5 h and assayed for 2 min at room temperature in the presence of 2 mM $MgSO_4$ (basal) or 40 μM GTPγS plus 1 μM cAMP (see "Experimental Procedures"). The results, performed in duplicate, are expressed as a ratio of the adenylyl cyclase activity/$MnSO_4$ activity and are representative of at least two independent experiments.

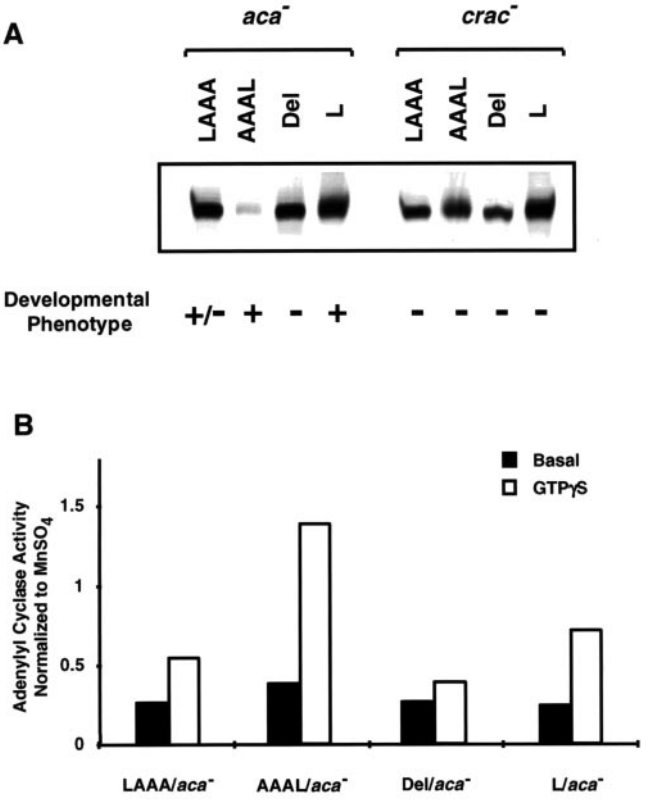


FIG. 5. **Adenylyl cyclase activity of wild type, alanine insertional, and deletion ACA mutants.** *A,* ACA protein expression and developmental phenotypes of the mutants when expressed in *aca*$^-$ and *crac*$^-$ cells. *B,* adenylyl cyclase activity was performed as described in legend of Fig. 4. The results, performed in duplicates, are expressed as a ratio of the adenylyl cyclase activity/$MnSO_4$ activity and are representative of at least five independent experiments. *L* represents the wild type sequence of ACA. LAAA and AAAL designate 3′ and 5′ of Leu-394 alanine insertions. *Del* indicates a mutant of ACA carrying a 7-amino acid deletion spanning the Leu-394 residue.

Leu-394 residue, three residues on either side of the leucine along with the Leu-394 residue. Each construct was transformed in both *aca*$^-$ and *crac*$^-$ cells. Fig. 5*A* shows that each mutant expressed the mutated ACA protein in both cell lines, although the mutants regularly showed lower expression compared with their wild type counterpart. Phenotypic analysis revealed that none of these substitutions suppressed the aggregation-deficient phenotype of the *crac*$^-$ cells, suggesting that they did not display constitutive activity (Fig. 5*A*). When expressed in the *aca*$^-$ background, we observed that the Del mutant acquired a severely impaired conformation. Indeed, although both the AAAL and LAAA mutants could complement the *aca*$^-$ cells, the Del/*aca*$^-$ cell lines remained aggregation-deficient when plated on non-nutrient agar (Fig. 5*A*). These results suggest that this mutation has a deleterious effect on G protein-mediated activation of ACA. Indeed, as shown in Fig. 5*B*, the Del/*aca*$^-$ showed only a weak response to GTPγS. It thus appears that the N-terminal domain of the C1 loop of adenylyl cyclases is critical for the acquisition of an activated conformation.

To address this point further, we designed an enzyme that contained, in addition to a constitutive mutation, a substitution that rendered the enzyme insensitive to G protein activation. In a previous screen designed to isolate loss-of-function mutants of ACA, we identified several mutants that remained aggregation-deficient when expressed in *aca*$^-$ cells. These mutants displayed normal $Mg^{2+}/Mn^{2+}$ adenylyl cyclase activity ratios but showed no response to GTPγS stimulation and were unable to enter development when plated on non-nutrient agar (16). One of these uncoupled mutants harbored a single point mutation (K482N) in the C1 loop of ACA. We introduced the L394T substitution, the strongest constitutive mutant, in the K482N background to construct the double mutant. The double mutant was electroporated in *aca*$^-$ cells, and the developmental phenotype of the resulting transformants was assessed. Western analysis revealed that the L394T,K482N/*aca*$^-$, and the Leu-394/*aca*$^-$ cells expressed similar levels of ACA (data not shown). Under normal plating conditions, the mutant cells did not rescue the aggregation-deficient phenotype of the *aca*$^-$ cells. However, when plated at higher density, the L394T,K482N/*aca*$^-$ cells did enter development to form small mounds and fruiting bodies, a result never observed with *aca*$^-$ or K482N/*aca*$^-$ cells (Fig. 6*A*). The presence of K482N altered the basal activity of the L394T mutation. Under our standard measurement conditions, L394T,K482N displayed a $Mg^{2+}/Mn^{2+}$ adenylyl cyclase activity ratio of 0.3 compared with $> 0.7$ for the L394T mutant. On the other hand, L394T,K482N exhibited a significant activation in the presence of GTPγS (Fig. 6*B*). This activation, which was never observed in K482N/*aca*$^-$ cells, was also detected when the adenylyl cyclase activity was measured *in vivo* after receptor stimulation (Fig. 6*C*).

## DISCUSSION

Throughout evolution, the topology of G protein-coupled adenylyl cyclases has been remarkably conserved. *D. discoideum*, *Drosophila*, and mammals all express enzymes that are predicted to display two sets of 6 transmembrane domains followed by a large cytoplasmic loop (Fig. 7). Although it has been proposed that some adenylyl cyclases are responsive to transmembrane potential, the exact function of the transmembrane spans has remained largely unknown (19). Our data show that regions linking the transmembrane domains to the cytoplasmic loops are important for proper regulation of the enzyme. The crystal structure of the soluble adenylyl cyclase with $G_s\alpha$ revealed that the α-subunit of G proteins binds to a crevice on the outside of the C2 loop on residues mainly located on the α2 helix, as well as to residues on the N-terminal portion of the C1 loop (8). The Leu-394 residue of ACA that is mutated in this study is located ~35 amino acids upstream of this N-terminal

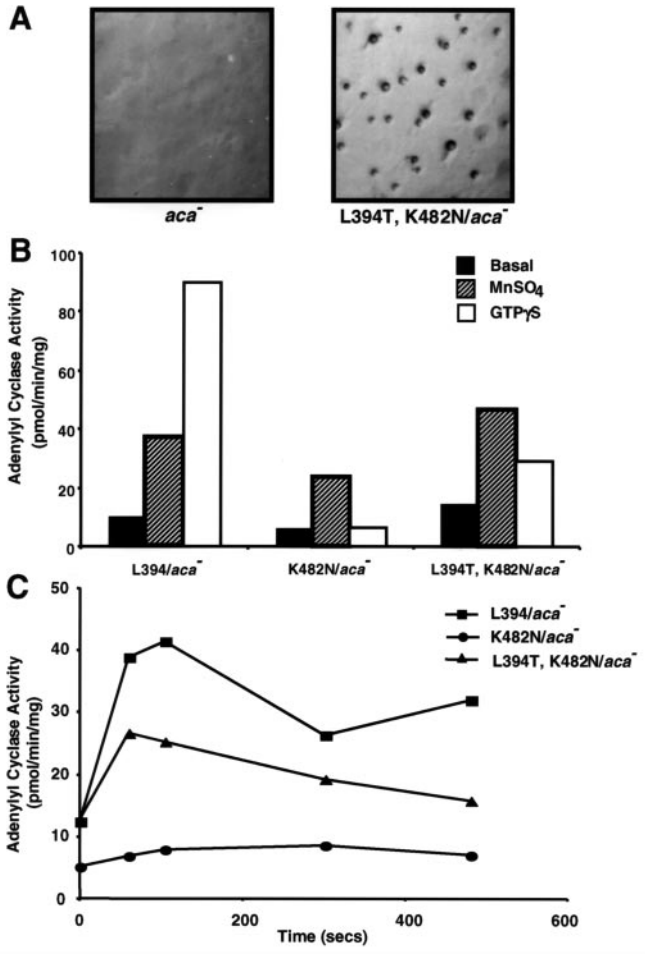


FIG. 6. **Developmental and biochemical phenotype of L394T,K482N/*aca*$^-$ cells.** *A, aca*$^-$ and L394T,K482N/*aca*$^-$ cells were grown in liquid culture and washed, and $1 \times 10^8$ cells were plated on non-nutrient agar on 35-mm plates at 22 °C. Photographs were taken 24 h after the cells were plated. *B,* basal, $MnSO_4$, and GTPγS-stimulated adenylyl cyclase activity in L/*aca*$^-$, K482N/*aca*$^-$, and L394T,K482N/*aca*$^-$ cells. The assays were performed as described under "Experimental Procedures." The results were performed in duplicate and are representative of at least three independent experiments. *C,* receptor-mediated activation of adenylyl cyclase in L/*aca*$^-$, K482N/*aca*$^-$, and L394T,K482N/*aca*$^-$ cells. Cells were stimulated with 10 μM cAMP, rapidly lysed at specific time points, and assayed as described under "Experimental Procedures." The results were performed in duplicate and are representative of at least two independent experiments.

portion of the C1 loop (Fig. 7). In fact, important biochemical differences between the soluble and native forms of adenylyl cyclases have been observed. First, the affinity of the soluble adenylyl cyclase for $G_s\alpha$ is significantly reduced, ~50-fold, compared with the native enzyme (20, 21). Second, the effects of $G\beta\gamma$-subunits on the chimeric soluble preparations do not recapitulate what has been observed with the purified full-length enzymes (22, 23). Finally, the maximal stimulated activity is ~10-fold higher for the soluble enzyme (21, 22). Moreover, the F400Y mutation in the type V molecule, which showed increased basal activity and sensitivity to $G_s\alpha$ and forskolin, did not alter the enzymatic activity of the soluble construct bearing the same substitution (24, 25). It has been shown that the C1b domain of adenylyl cyclases (which is absent in these constructs) possesses regulatory properties (26–31). Particularly, the type VI enzyme has a cAMP-dependent protein kinase phosphorylation site within its C1b region that dampens $G_s\alpha$-mediated activation (32). In addition, glycosylation of the extracellular domains of the type VI enzyme has recently been shown to be important for catalytic activity (33). It is thus possible that some of the differences observed between the native and soluble forms of adenylyl cyclases could be explained by the lack of these domains in the soluble constructs. Our data support and extend these observations by defining yet another region of the native structure that is critical for activation.

Hatley *et al.* (25) isolated mutants of the type II enzyme that rescued the cyclase-null *Saccharomyces cerevisiae* strain. All mutations mapped to the cytoplasmic loops of the enzyme. To investigate the biochemical defect of the mutants, the substitutions were engineered in the type V C1/type II C2 soluble construct. Of 13 mutants analyzed in this context, only a few displayed substantial changes in adenylyl cyclase activity when compared with the wild type control. Because the activity of the mutants was not measured in the context of the native type II enzyme, the lack of change in basal activity in most of the mutants analyzed may be due to the absence of the transmembrane domains or, alternatively, to the supersensitivity of the yeast screen.

We have shown that the activity of native adenylyl cyclases can be dramatically modulated by substituting Leu-394 of ACA. This residue is conserved in eight of nine of the mammalian adenylyl cyclases (Fig. 7) (12). Substituting Leu-394 of ACA with a variety of other residues gives rise to mutants possessing high basal activity. Intriguingly, the substitutions lead to graded ranges of high basal activity suggesting that each mutant acquires a conformation that progressively reproduces the active state of the enzyme and that the Leu-394 position is critical in the formation of an activated conformation of adenylyl cyclases. This type of graded effect has also been observed when the Ala-293 position of the $\alpha_{1B}$-adrenergic receptor is mutated to other amino acids (34). A closer analysis of the nature of the amino acids leading to the broad spectrum of intrinsic adenylyl cyclase activation reveals no particular allegiance regarding charge, size, or hydrophobicity. Surprisingly, the subtlest mutation (L394I) provides significant constitutive activity. It thus seems that changes in the local conformation is responsible for the acquired high activity. Consequently, we do not expect that substitutions of this particular residue in other adenylyl cyclases will lead to constitutively active enzymes. Indeed, the mammalian type V enzyme harbors a serine at the corresponding Leu-394 position and does not display unusually high basal activity. We propose that changes in the N terminus region of the C1 loop will lead to enzymes that display high basal activity. A screen similar to the one we developed for ACA will be required to identify such mutations in mammalian enzymes (12).

Mutations localized to the N terminus of the C1 loop of ACA also give rise to enzymes that are non-responsive to G proteins. In a previous study, we isolated a mutant that harbored two point mutations (L405S and F421S) adjacent to the Leu-394 position (Fig. 7). This mutant showed normal enzymatic activity in the presence of $MnSO_4$. However, it was devoid of any G protein-mediated activation (16). This behavior is identical to the one observed with the Del mutant engineered in the present study. Interestingly, the AAAL mutant had the opposite phenotype displaying increased G protein-mediated activation compared with the wild type enzyme. Taken together, these mutants, which all harbor mutations in a discrete region outside the soluble domains, underscore the importance of this region on the activation potential of adenylyl cyclases.

Structural information shows that the C1/C2 heterodimer structure is based on highly organized interactions between the two domains. It has been suggested that extensive hydrogen bonding between the $\beta$4-$\beta$5 loops of C2 and the $\beta$2-$\beta$3 loops of

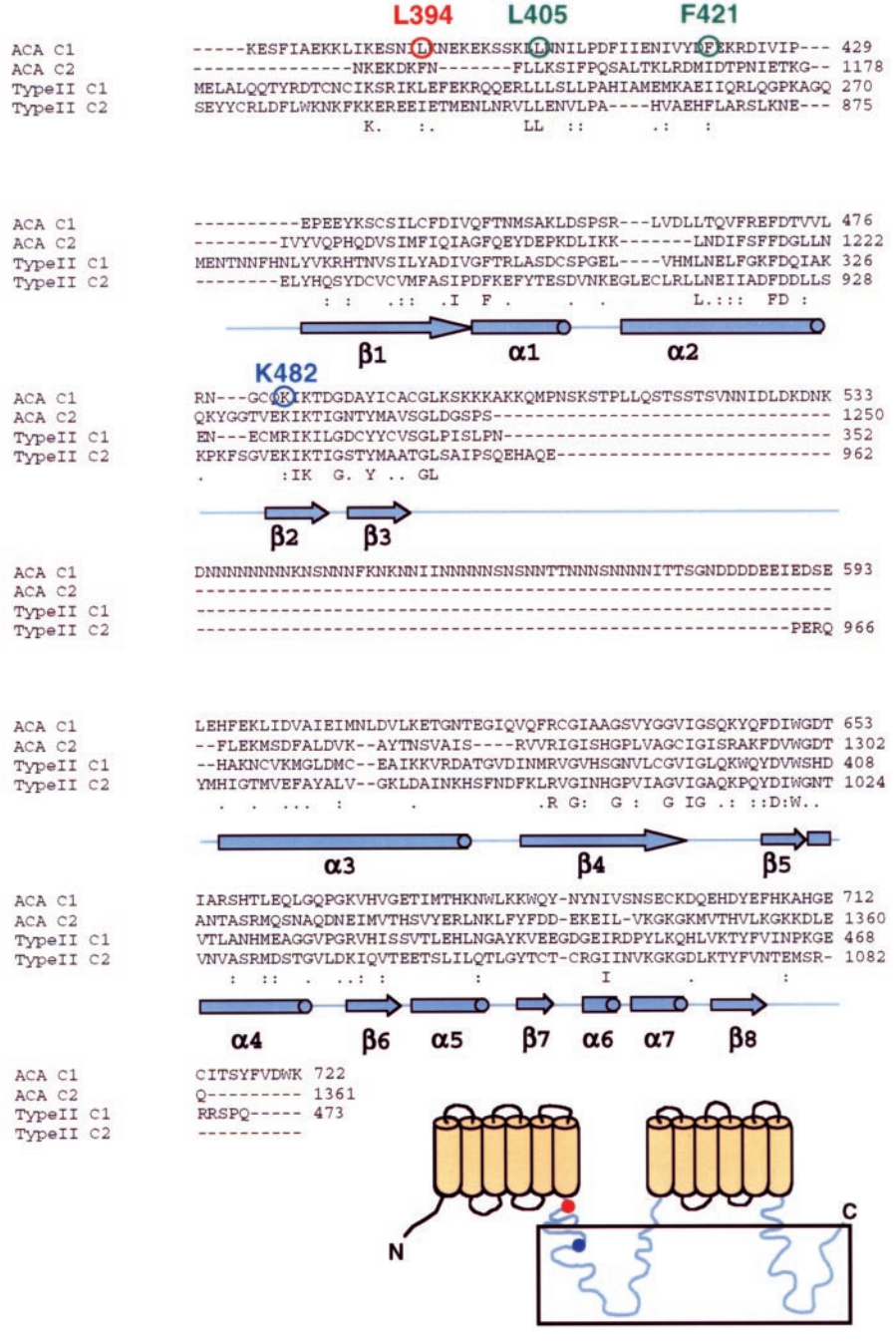


FIG. 7. **Sequence analysis of the location of the ACA mutants.** The C1 and C2 domains of *D. discoideum* ACA and the mammalian type II adenylyl cyclases were aligned using ClustalW analysis. The amino acid position of the sequences is shown at the *end* of each line. The secondary structure of the C1 loop (8) is drawn *below* the consensus sequence in *blue*. The Leu-394 and Lys-482 residues are shown in *red* and *blue*, respectively. The L405S and F421S mutations are shown in *green*. The putative native structure of adenylyl cyclases is illustrated in the *bottom left corner*. The *boxed* region represents the sequence contained within the soluble construct.

C1 exist and that alterations in these interactions have deleterious effects on enzymatic activity (8). The ACA K482N mutant, which is specifically devoid of G protein-stimulated activity, harbors a point mutation in the highly conserved $\beta$2 loop of C1 (Fig. 7). Mutations of this exact residue in mammalian adenylyl cyclases also give rise to defective enzymes (35, 36). The addition of the constitutive mutation L394T to the K482N substitution biochemically and phenotypically suppresses the defects of the K482N mutant. The L394T,K482N/*aca*$^-$ cells regain the capacity to aggregate, respond to GTP$\gamma$S, and show a significant response to receptor stimulation. As with the original K482N/*aca*$^-$ mutant, these results again show that the Lys-482 residue is not essential for the catalytic activation of adenylyl cyclases. The fact that the constitutive mutation can suppress the loss-of-function defect shows that the two sites, although distant, influence each other. Intriguingly, whereas the double mutant regains the capacity to be activated by G proteins, it does not exhibit the constitutive activity of the L394T mutant. It thus appears that the high basal activity of the L394T mutant is somehow lost in the context of the double mutant. Although additional experiments are required to understand the molecular mechanism explaining this interesting behavior, the results presented in this paper definitely show that a region located outside the soluble construct of adenylyl cyclases is pivotal in the acquisition of an activated conformation.

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