phenotype (Table 2), all Pin1 mutants, including Y23F, that did not bind phosphoproteins failed to support cell growth (Fig. 4). These results indicate that pSer- or pThr-binding activity of the Pin1 WW domain is required for the protein function in vivo.

We have demonstrated a function of WW domains as pSer- or pThr-binding modules and an essential role for WW domains in mediating protein-protein interactions. Serine or Thr phosphorylation, often on PEST sequences (rich in Pro, Glu, Ser, and Thr), controls the timing of ubiquitination of various proteins, and ubiquitin-protein ligases are responsible for substrate recognition (*4*). Our results indicate that WW domains of ubiquitin ligases may bind pSer- or pThr-containing sequences, thus targeting their catalytic domains to phosphorylated substrates to initiate protein degradation. Proline residues can put kinks into polypeptide chains because they undergo cis or trans isomerization catalyzed by PPIases (*24*). Phosphorylation reduces the isomerization rate of the pSer- or pThr-Pro bond, and Pin1 is a unique enzyme that isomerizes the pSer- or pThr-Pro bond and regulates activity of phosphoproteins (*12*, *13*). The Pin1 catalytic domain neither interacts with protein substrates in vitro, nor performs the essential function of the protein in vivo. The WW domain binds pSer- or pThr-Pro–containing peptides and mediates Pin1 interactions with most substrates. One of its biological functions may be to facilitate the processive isomerization of Pin1 substrates. SH2 domains have similar functions. Some SH2 domains in Tyr kinases preferentially bind pTyr residues phosphorylated by the catalytic domain, thereby increasing the local kinase concentration so that substrates are processively phosphorylated on multiple sites (*25*). Because Pin1 substrates are regulated by phosphorylation of multiple Ser or Thr residues clustered at the regulatory domains (*12–14*, *26*), isomerization of these sites may provide a means to generate coordinate "all-or-none" activity of heavily phosphorylated phosphoproteins.

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15. Pin1 and its mutants were produced, and their binding to phosphoproteins was assayed, as described (*12*, *13*).
16. Cdc25C was synthesized by in vitro transcription and translation in the presence of $^{35}$S-Met and phosphorylated by *Xenopus* interphase or mitotic extracts, and some mitotically phosphorylated samples were dephosphorylated with calf intestine phosphatase (CIP) before binding, as described (*13*).
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18. For peptide competition experiments, various peptides were incubated with GST-Pin1 or -WW domain in a binding buffer (*13*). After 1 hour of incubation, $^{32}$P-labeled (*21*) or nonlabeled mitotic cell extracts were added. Proteins associated with glutathione beads were detected by autoradiography or immunoblotting with MPM-2. To obtain semi-quantitative data, we scanned films of immunoblots at the region of 55 kD, the major Pin1-binding protein, and analyzed data with ImageQuan (ScanJet II CX). Peptide dissociation constants were measured with a fluorescence polarization assay as described (*22*). Peptides were labeled at the $NH_2$-terminus and purified by thin-layer chromatography. Various concentrations of WW domain proteins were incubated with 0.1 μM of the labeled peptides in a binding buffer containing 50 mM Hepes (pH 7.4), 100 mM NaCl, and 2% glycerol, and fluorescence polarization values were obtained with a PanVera 2000 system.
19. HeLa cells were labeled overnight with $^{32}$P-orthophosphate or $^{35}$S-Met as described (*27*). Cells were lysed in lysis buffer with or without phosphatase inhibitors (40 mM glycerol phosphate, 50 mM NaF, 10 mM $NaVO_4$, and 2 μM okadeic acid) (*12*, *13*). For dephosphorylation experiments, three Ser phophatases (CIP, PP1, and PP2A) were added to lysates for 30 min at 30°C in the absence or presence of the above phosphatase inhibitors as described (*27*).
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23. To determine the function of Pin1 mutants in vivo, cDNAs of *PIN1* or its mutants were subcloned into the yeast expression vector YEp451 and transformed into a temperature-sensitive *ptf1* strain YMP2, as described (*28*). Those strains expressing similar amounts of Pin1 proteins were selected, as detected by immunoblotting with the 12CA5 antibody to the inserted $NH_2$-terminal hemagglutinin A epitope tag (*21*). Three to four independent strains were tested for each transformation.
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29. We thank two anonymous reviewers, T. Hunter, B. Neel, and L. Cantley for constructive comments, D. Parker and M. Montony for help with fluorescence polarization assay, and T. Stukenberg, M. W. Kirschner, M. Sudol, J. Kunag, J. Hani, H. Domdey, M. Schutkowski, and G. Fischer for providing reagents. K.P.L. is a Leukemia Society of America Scholar. The work was supported by NIH grants R01GM56230 and R01GM58556 to K.P.L.

10 September 1998; accepted 27 January 1999


# Facilitation of Signal Onset and Termination by Adenylyl Cyclase


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The α subunit ($G_{s\alpha}$) of the stimulatory heterotrimeric guanosine triphosphate binding protein (G protein) $G_s$ activates all isoforms of mammalian adenylyl cyclase. Adenylyl cyclase ( Type V ) and its subdomains, which interact with $G_{s\alpha}$, promoted inactivation of the G protein by increasing its guanosine triphosphatase (GTPase) activity. Adenylyl cyclase and its subdomains also augmented the receptor-mediated activation of heterotrimeric $G_s$ and thereby facilitated the rapid onset of signaling. These findings demonstrate that adenylyl cyclase functions as a GTPase activating protein (GAP) for the monomeric $G_{s\alpha}$ and enhances the GTP/GDP exchange factor (GEF) activity of receptors.


Regulators of G protein signaling (RGS proteins) increase the GTPase activity of α subunits of heterotrimeric G proteins and play an important role in the termination and onset of signals mediated by the $G_i$ and $G_q$ families of G proteins (*1–8*). To date, however, no protein that acts as a specific GAP for $G_{s\alpha}$ has been identified. Because all

isoforms of adenylyl cyclase (AC) are activated by $G_{s\alpha}$ (*9, 10*), we hypothesized that AC might serve as a GAP for the G protein. To address this possibility, we determined whether AC could alter the GTPase activity of $G_{s\alpha}$.

We used a purified, soluble, engineered form of type V AC (C1-C2 ACV) whose regulation by G proteins, forskolin, and $Ca^{2+}$ is identical to that of the full-length enzyme (*11, 12*). Addition of purified C1-C2 ACV to $G_{s\alpha}$ that had been incubated with [$\gamma$-$^{32}$P]GTP increased the rate of single-cycle GTP hydrolysis (hydrolysis of prebound [$\gamma$-$^{32}$P]GTP) such that the half-time $t_{1/2}$ for complete hydrolysis of [$\gamma$-$^{32}$P]GTP bound to $G_{s\alpha}$ at 14°C was decreased from 55 ± 3 s to 11 ± 3 s (Fig. 1). Similarly, a protein corresponding to the C2 domain of ACV (amino acids 933 to 1184) also increased the GTPase activity of $G_{s\alpha}$ and decreased the $t_{1/2}$ of single-cycle GTP hydrolysis (Fig. 1). In contrast, the C1 domain of ACV (amino acids 321 to 683), at a concentration 50 times that of $G_{s\alpha}$, did not alter the rate of single-cycle GTP hydrolysis by $G_{s\alpha}$ (Fig. 1). C1-C2 ACV and its C2 domain did not enhance the GTPase activity of $G_{s\alpha}$ by altering its stability because, as assessed by guanosine 5′-*O*-(3-thiotriphosphate) (GTP-$\gamma$-S) binding under conditions that allow maximal and stoichiometric binding of the guanine nucleotide, the stability of the G protein was not changed (*13*). Moreover, the concentrations of C1-C2 ACV and its subdomains used were similar to the concentration of RGS4 required to stimulate the GTPase activity of $G_{i\alpha 1}$ (*5*). Thus, C1-C2 ACV and its C2 domain act as GAPs for $G_{s\alpha}$.

Structural data from cocrystallization of $G_{s\alpha}$ with the C1 domain of ACV and C2 region of ACII demonstrate that the G protein interacts with amino acids in two highly conserved regions within the C2 domain of all isoforms of the enzyme (*14*). Therefore, we monitored the ability of the C2 domain and its two highly conserved regions that consist of amino acids 995 to 1058 and 1091 to 1151 (referred to as C2I and C2II, respectively) to modulate the GTPase activity of $G_{s\alpha}$. Proteins corresponding to the C2, C2I, and C2II regions of ACV were as effective as C1-C2 ACV in decreasing the $t_{1/2}$ of GTP hydrolysis by $G_{s\alpha}$ in the GAP activity assay (Fig. 2A). Notably, purified preparations of either C1-C2 ACV that were devoid of AC activity or C2 protein, which in the presence of the C1 domain of ACV did not have AC activity, failed to increase the GTPase activity of $G_{s\alpha}$ (*15*). Thus, to act as a GAP for $G_{s\alpha}$, AC or its C2 domain apparently must be in a conformation that is active.

These experiments (Figs. 1 and 2) were done in the absence of exogenously added $Mg^{2+}$. Therefore, as shown for the GAP activity of RGS4 and $G_{i\alpha 1}$ (*5*), either the GAP activity of AC and its subdomains is independent of $Mg^{2+}$, or the affinity of the $G_{s\alpha}$-AC subdomain complex for $Mg^{2+}$ is extremely high. Because enzymatically inactive (or boiled) preparations of C1-C2 ACV and its subdomain did not increase the GTPase activity of $G_{s\alpha}$ (*13*), the GAP activity of C1-C2 ACV and its subdomains cannot be attributed to contamination of these proteins by $Mg^{2+}$.

To examine the specificity with which AC augmented the GTPase activity of $G_{s\alpha}$, we monitored the single-cycle GTP hydrolysis of monomeric $G_{i\alpha 1}$. $G_{i\alpha 1}$ inhibits the activity of C1-C2 ACV (*11*) and therefore interacts with the protein. However, the GTPase activity of $G_{i\alpha 1}$ was not altered by interaction with C1-C2 ACV (Fig. 2B). This is further evidence that the effects of soluble AC on $G_{s\alpha}$ are not due to contamination by $Mg^{2+}$, because $Mg^{2+}$ would also augment the GTPase activity of $G_{i\alpha 1}$. Thus, just as RGS2 appears to act as a specific GAP for $G_{q\alpha}$ (*8*), AC acts as a specific GAP for $G_{s\alpha}$. The GAP activity of AC would expedite the conversion of GTP-bound, active $G_{s\alpha}$ to its GDP-bound, inactive form and thereby ensure the rapid termination of signaling.

Because AC facilitates the rapid inactivation of $G_{s\alpha}$, we reasoned that the enzyme

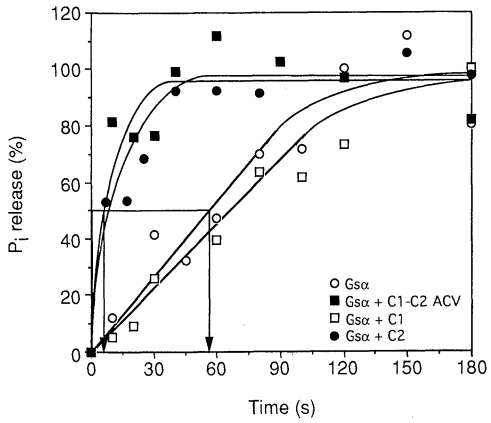


**Fig. 1.** GAP activity of the C1-C2 ACV and its C2 domain for $G_{s\alpha}$. $G_{s\alpha}$ (37 nM) was incubated for 10 min at room temperature with [$\gamma$-$^{32}$P]GTP (100 nM) in medium containing 25 mM Hepes-NaOH (pH 8.0), 0.1 mM EDTA, and 1 mM dithiothreitol (DTT). The temperature was then lowered to 14°C. At time zero, 0.1 mM GTP and the indicated protein (1.1 μM) were added (final volume 1 ml). Portions (100 μl) were withdrawn at the indicated times and free $PO_4$ was measured as described (*16*). Recombinant $G_{s\alpha}$, C1-C2 ACV, and its subdomains were purified as described (*11, 23, 24*). As determined by GTP-$\gamma$-S binding under conditions that allow maximal and stoichiometric binding of the nucleotide to $G_{s\alpha}$ (*23*), the recombinant G protein was 51% active. Symbols: ○, $G_{s\alpha}$ alone; ■, $G_{s\alpha}$ in the presence of C1-C2 ACV; ●, $G_{s\alpha}$ in the presence of C2; □, $G_{s\alpha}$ in the presence of C1 ACV. Arrows indicate $t_{1/2}$ values for complete hydrolysis of GTP bound to $G_{s\alpha}$ under control conditions and in the presence of C1-C2 ACV or its C2 domain. Values are presented as percent of total inorganic phosphate ($^{32}P_i$) released. Each experiment was repeated at least three times. Total $P_i$ released in 100 μl under the various conditions: $G_{s\alpha}$ alone, 50 fmol; $G_{s\alpha}$ + C1-C2 ACV, 48 fmol; $G_{s\alpha}$ + C1, 42 fmol; $G_{s\alpha}$ + C2, 48 fmol.

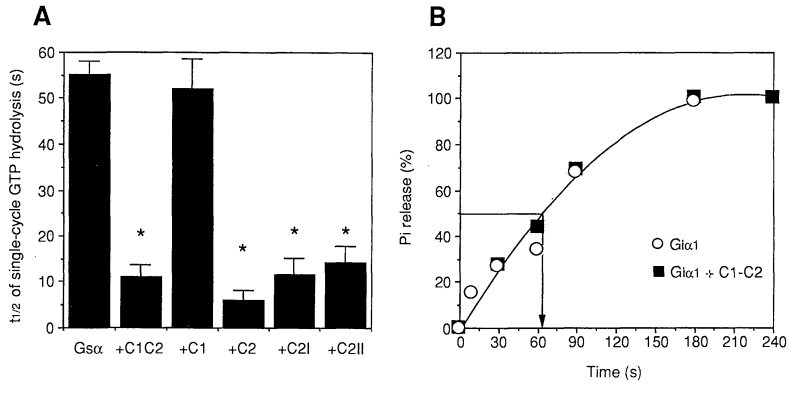


**Fig. 2.** Accelerated hydrolysis of [$\gamma$-$^{32}$P]GTP bound to $G_{s\alpha}$ in the presence of C1-C2 ACV, C2, and its subdomains C2I and C2II. (**A**) $t_{1/2}$ of hydrolysis of GTP bound to $G_{s\alpha}$ in the absence or presence of the indicated protein (each at 1.1 μM). Experimental conditions were as in Fig. 1. Each value is the mean ± SEM of at least four determinations; *$P < 0.001$ for each individual condition compared with $G_{s\alpha}$ alone (Student's unpaired $t$ test). (**B**) Hydrolysis of [$\gamma$-$^{32}$P]GTP bound to $G_{i\alpha 1}$. The single-cycle GTP hydrolysis by $G_{i\alpha 1}$ (54 nM) was measured in the absence or presence of 1.1 μM C1-C2 ACV as described (Fig. 1). $G_{i\alpha 1}$ was expressed in Sf9 cells and purified as described (*25*). Data are representative of three experiments.

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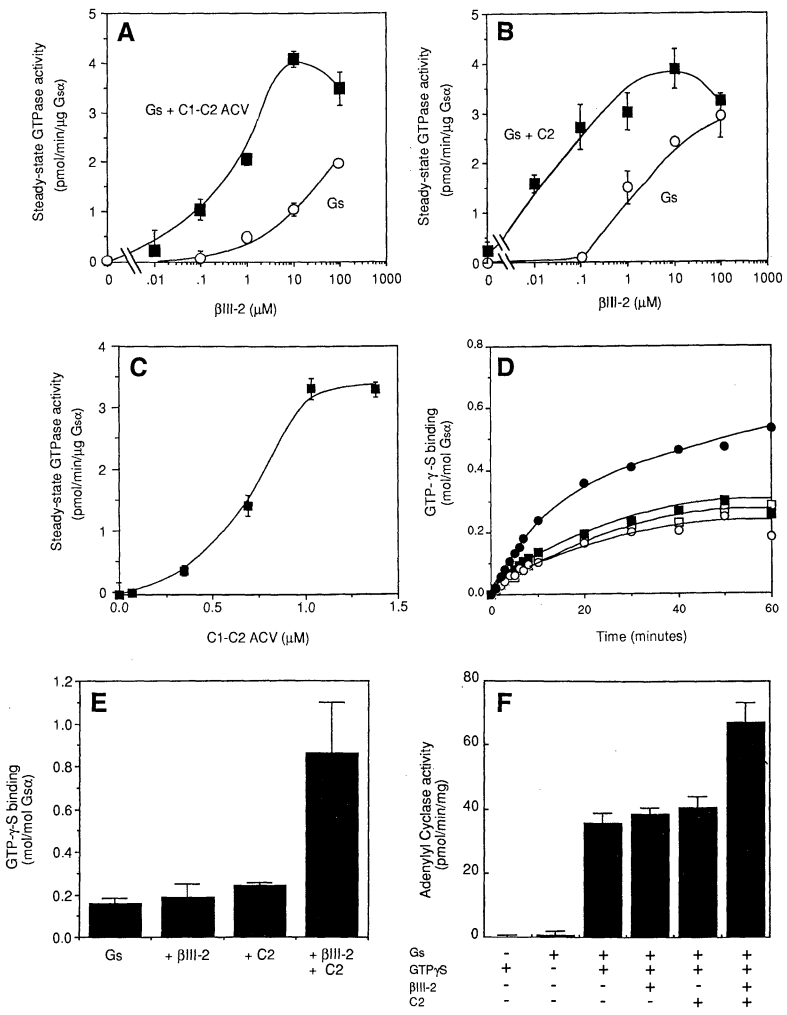


**Fig. 3.** Enhanced βIII-2–mediated activation of heterotrimeric $G_s$ in the presence of C1-C2 ACV and its C2 domain. (**A**) Steady-state GTPase activity of reconstituted $G_s$ (27.7 nM) was monitored in the presence of various concentrations of βIII-2 with (filled squares) or without (open circles) C1-C2 ACV (1.4 μM). Heterotrimeric $G_s$ and $G_i$ were reconstituted by mixing $G_{s\alpha}$ or $G_{i\alpha 1}$ with purified bovine brain βγ subunits ($G_{s\alpha}$:βγ molar ratio, 1:5) and incubating for 1 hour at 4°C (*16*). βγ subunits were purified as described (*26*). The reconstituted G protein was incubated with or without the proteins of interest at 25°C in medium containing 100 nM [γ-$^{32}$P]GTP, 25 mM Hepes (pH 8.0), 100 μM EDTA, 120 μM $MgCl_2$, and 1 mM DTT (final volume 60 μl). Hydrolysis of [γ-$^{32}$P]GTP (100 nM), which was linear for more than 30 min, was monitored in 50 μl of the sample in duplicate after 20 min as described (*16*). Rates of $P_i$ release per microgram of $G_{s\alpha}$ in the heterotrimer are shown as means ± variance; data are representative of two experiments. (**B**) Same as (A), except 1.4 μM C2 was used instead of C1-C2 ACV; data are representative of three experiments. (**C**) Steady-state GTPase activity of $G_s$ was monitored in the presence of 0.1 μM βIII-2 and various concentrations of C1-C2 ACV. Means ± variance are shown; data are representative of two experiments. (**D**) GTP-γ-$^{35}$S binding to $G_s$ (22 nM) alone (□), in the presence of 1.4 μM C1-C2 ACV (○), in the presence of 0.1 μM βIII-2 (■), or in the presence of both C1-C2 ACV and βIII-2 (●) was monitored in buffer containing 100 nM GTP-γ-S, 25 mM Hepes (pH 8.0), 100 μM EDTA, 1 mM DTT, and 150 μM $MgCl_2$ (final volume 750 μl, 50 μl per time point) as described (*16*). Data are presented as moles of GTP-γ-$^{35}$S bound per mole of $G_{s\alpha}$ in the heterotrimer and are representative of four experiments. (**E**) Same as (D), except that the GTP-γ-$^{35}$S binding at only one time point (60 min) was measured and C1-C2 ACV was replaced by C2 protein (1.4 μM). (**F**) After incubations of $G_s$ with GTP-γ-S (100 nM) in the absence and presence of βIII-2 (100 nM), C2 (1.4 μM), or βIII-2 plus C2 for 1 hour under conditions identical to those in (E), 2.2 pmol of $G_s$ was transferred to the AC reaction mixture (final volume 0.1 ml) (*16*) containing membranes (15 μg of protein) of S49 cyc$^-$ cells. AC activity was measured as described (*16*) for 20 min at room temperature. Data are presented as means ± SEM ($n$ = 3).

may also alter the initiation of signaling via this G protein. Therefore, we investigated whether AC could alter the rate of onset of signaling through $G_{s\alpha}$. Because the activation of $G_{s\alpha}$ is mediated by the effects of ligand-activated receptors on heterotrimeric $G_s$, all of the following experiments were done with heterotrimeric $G_s$ reconstituted in vitro from purified $G_{s\alpha}$ and βγ subunits in a molar ratio of 1:5 (*16*). We also used the peptide βIII-2, which corresponds to amino acids 259 to 273 of the $\beta_2$ adrenergic receptor and mimics the actions of the active receptor (*16, 17*). Because receptor-mediated activation of heterotrimeric G proteins results in an augmentation of exchange of GTP for GDP on the α subunit, and because the steady-state GTPase activity of G proteins is indicative of this GTP-GDP exchange rate (*5, 17, 18*), we monitored the steady-state GTPase activity of $G_s$.

βIII-2, by enhancing the rate of exchange of GTP for GDP, increased the activation of the heterotrimeric $G_s$ in a concentration-dependent manner (*16, 17*) (Fig. 3A). In the presence of either C1-C2 ACV or the C2 domain of ACV, the sensitivity of $G_s$ to βIII-2 was increased by about two orders of magnitude (Fig. 3, A and B). Neither C1-C2 ACV nor the C2 domain of the enzyme alone altered the steady-state GTPase activity of heterotrimeric $G_s$ (Fig. 3, A and B). Moreover, in the presence of βIII-2 at a concentration (100 nM) that by itself did not increase the steady-state GTPase activity of heterotrimeric $G_s$, the effect of C1-C2 ACV on βIII-2–mediated activation of $G_s$ was concentration-dependent (Fig. 3C). The recombinant $G_{s\alpha}$ preparation was 50% active (Fig. 1). Therefore, the 20-fold (or greater) increase in steady-state GTPase activity observed with either C1-C2 ACV or its C2 domain in the presence of βIII-2 (Fig. 3, A to C) cannot be accounted for by activation of the inactive portion of $G_{s\alpha}$.

The ability of C1-C2 ACV to augment the guanine nucleotide exchange activity of βIII-2 could also be observed by monitoring the binding of GTP-γ-S to $G_s$. These experiments (Fig. 3D) were done in the presence of 50 μM $Mg^{2+}$ and 100 nM GTP-γ-S (*16, 17*). Under these conditions, activation of $G_s$ increases both the rate and extent of GTP-γ-S binding (*16, 17*). Indeed, in the presence of C1-C2 ACV, threshold concentrations (100 nM) of βIII-2 increased the rate and extent of GTP-γ-S binding to $G_s$ (Fig. 3D). C1-C2 ACV itself did not bind any GTP-γ-S and did not modulate GTP-γ-S binding to $G_s$ (Fig. 3D). C1-C2 ACV or its C2 domain in the presence or absence of βIII-2 did not alter the stability of the G protein, as monitored by the stoichiometric binding of GTP-γ-$^{35}$S after 1 hour of incubation under conditions similar to those in Fig. 3D (*13*).

We conducted experiments (Fig. 3E) to determine whether the C2 domain of ACV also augments the ability of threshold concentrations of βIII-2 (100 nM) to increase GTP-γ-S binding to $G_s$. Like C1-C2 ACV (Fig. 3D), in the presence of C2 the ability of βIII-2 (100 nM) to increase GTP-γ-$^{35}$S binding to $G_s$ was augmented; neither C2 nor βIII-2 alone significantly altered GTP-γ-S binding to $G_s$ (Fig. 3E). As another approach to monitor $G_s$ activity, we determined the ability of $G_s$ that had been incubated under conditions identical to those in Fig. 3E to increase AC activity in membranes of S49 cyc$^-$ cells, which do not

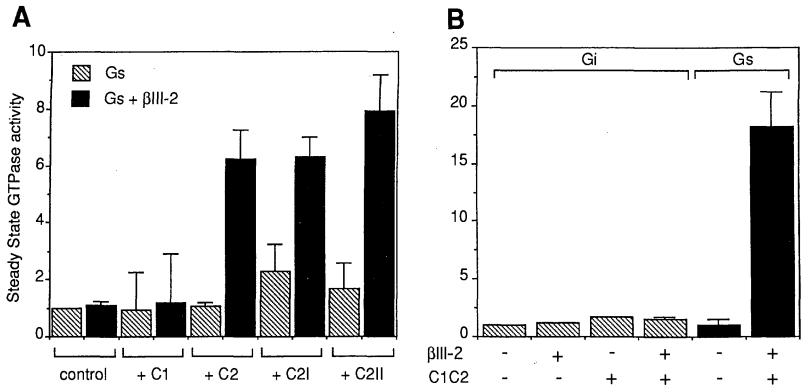


**Fig. 4.** Increased GEF activity of βIII-2 toward $G_s$ in the presence of C2I and C2II regions of ACV, and failure of C1-C2 ACV to activate heterotrimeric $G_i$. (**A**) Steady-state GTPase activity of reconstituted $G_s$ (27.7 nM) in the presence and absence of βIII-2 (0.1 μM) with and without 0.7 μM each of C1, C2, C2I, and C2II was monitored under conditions identical to those in Fig. 3. Data are means ± SEM ($n$ = 3). (**B**) Steady-state GTPase activity of 54 nM reconstituted $G_i$ or 27.7 nM $G_s$ was monitored in the absence and presence of 1.4 μM C1-C2 ACV with and without 0.1 μM βIII-2. Data are means ± SEM ($n$ = 3).

express $G_{s\alpha}$ (*19*). Consistent with the findings for GTP-γ-$^{35}$S binding, βIII-2 in the presence of C2 increased AC activity in membranes of S49 cyc$^-$ cells to a greater extent than with either reagent alone or $G_s$ alone (Fig. 3F).

To demonstrate that the amount of $G_{s\alpha}$ that could activate AC was the same in all incubation conditions shown in Fig. 3F, we incubated $G_s$ as described in Fig. 3E; we then elevated the $Mg^{2+}$ and GTP-γ-S concentrations to 25 mM and 1 μM, respectively, and continued incubations for an additional 45 min at room temperature to permit the maximal binding of GTP-γ-S to $G_{s\alpha}$. Equal portions of the incubations were then mixed with cyc$^-$ cell membranes and assayed for AC activity. The extent to which enzyme activity was stimulated was the same under each experimental condition (*15*). Thus, the differences observed in Fig. 3F cannot be attributed to differences in the amounts of $G_{s\alpha}$ that could activate AC, but rather reflect the different extents of $G_s$ activation in the presence of low concentrations of $Mg^{2+}$ and GTP-γ-S.

Proteins corresponding to the C1 region of ACV failed to confer on βIII-2 (100 nM) the ability to increase the steady-state GTPase activity of $G_s$ (Fig. 4A). However, the C2I and C2II regions of ACV increased the ability of βIII-2 to activate $G_s$ to the same extent as did the C2 domain (Fig. 4A). In the experiments shown, the half-maximally effective concentration of C2 (0.7 μM) was compared with the same concentration of C2I and C2II; hence, the extent of GTPase enhancement with C2 is lower than that seen with the maximally effective concentration of this protein.

AC appeared to act as a specific enhancer of the GTP/GDP exchange factor (GEF) activity of βIII-2 toward $G_s$. In experiments with heterotrimeric $G_i$, βIII-2 did not enhance the steady-state GTPase activity (Fig. 4B). Moreover, C1-C2 ACV, by itself or in the presence of βIII-2, did not alter the steady-state GTPase activity of $G_i$ (Fig. 4B).

ACV, its C2 domain, and the C2I and C2II regions within C2 (comprising 64 and 61 amino acids each, respectively) act as GAPs for monomeric GTP-bound $G_{s\alpha}$. This selective GAP activity of AC toward active $G_{s\alpha}$ would ensure the rapid termination of signaling upon cessation of activation of the G protein by receptors. On the other hand, AC and its C2, C2I, and C2II regions enhance the GEF activity of the β adrenergic receptor–mimetic peptide toward heterotrimeric $G_s$. In an intact system, the GEF-enhancing activity of AC would ensure that in the presence of small amounts of active receptors (at low concentrations of ligand), the GTP/GDP exchange on $G_{s\alpha}$ within the heterotrimer is augmented by the effector. Moreover, the GEF-enhancing activity of AC would permit the rapid onset of signaling through $G_s$ and sensitizes the system such that the amount of active receptor required to activate $G_s$ is one-hundredth that required for activation in the absence of AC. This latter feature of AC would serve to amplify signaling in the presence of low concentrations of ligand or active receptor.

Besides acting as GAPs, RGS proteins may also enhance the onset of signaling (*20*). However, RGS-mediated augmentation of $G_{i/o}$ activation has not been documented. Likewise, although effectors such as phospholipase Cβ-1 and the γ subunit of cyclic guanosine monophosphate phosphodiesterase have been shown to act as GAPs for $G_{q\alpha}$ (*21*) and $G_{t\alpha}$ (*22*), respectively, no effector has yet been shown to act either as a GEF or GEF enhancer for the α subunit of any heterotrimeric G protein. Our results show that a single molecule (AC) can act both as a GAP on monomeric active G protein and as a GEF enhancer on a trimeric G protein.

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13. Monomeric $G_{s\alpha}$ or trimeric $G_s$ were incubated under conditions similar to those in Fig. 1 for 3 min, Fig. 3, A to C, for 20 min, or Fig. 3D for 1 hour, respectively. Thereafter, $MgCl_2$ and GTP-γ-$^{35}$S were added to final concentrations of 25 mM and 1 μM, respectively, and incubations were continued for another 45 min at room temperature to permit the stoichiometric binding of GTP-γ-$^{35}$S (*23*). GTP-γ-$^{35}$S binding was determined as described (*16*). Under all experimental conditions, the $G_{s\alpha}$ bound equal (and stoichiometric) amounts of the GTP-γ-$^{35}$S, indicating that the stability of the G protein was not altered (*15*).
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27. We thank T. Curran, R. Iyengar, and Y. Zheng for critical reading of the manuscript, A. G. Gilman for the $G_{s\alpha}$ cDNA, and Y. Ishikawa for the canine ACV cDNA. Supported by NIH grants HL48308 (T.B.P.) and HL59679 (T.B.P.) and an American Heart Association, TN Affiliate postdoctoral fellowship (to K.S.). This report is dedicated to the memory of Dr. Martin Rodbell.

14 August 1998; accepted 28 January 1999