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Activating Mutations in the NH_2- and COOH-Terminal Moieties of the $G_s\alpha$ Subunit Have Dominant Phenotypes and Distinguishable Kinetics of Adenylyl Cyclase Stimulation

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Abstract The α subunit polypeptides of the G proteins G_s and G_{i2} stimulate and inhibit adenylyl cyclase, respectively. The α_s and α_{i2} subunits are 65% homologous in amino acid sequence but have highly conserved GDP/GTP binding domains. Previously, we mapped the functional adenylyl cyclase activation domain to a 122 amino acid region in the COOH-terminal moiety of the α_s polypeptide (Osawa et al: Cell 63:697–706, 1990). The NH_2-terminal half of the α_s polypeptide encodes domains regulating βγ interactions and GDP dissociation. A series of chimeric cDNAs having different lengths of the NH_2- or COOH-terminal coding sequence of α_s substituted with the corresponding α_{i2} sequence were used to introduce multi-residue non-conserved mutations in different domains of the α_s polypeptide. Mutation of either the amino- or carboxy-terminus results in an α_s polypeptide which constitutively activates cAMP synthesis when expressed in Chinese hamster ovary cells. The activated α_s polypeptides having mutations in either the NH_2- or COOH-terminus demonstrate an enhanced rate of GTPγS activation of adenylyl cyclase. In membrane preparations from cells expressing the various α_s mutants, COOH-terminal mutants, but not NH_2-terminal α_s mutants markedly enhance the maximal stimulation of adenylyl cyclase by GTPγS and fluoride ion. Neither mutation at the NH_2- nor COOH-terminus had an effect on the GTPase activity of the α_s polypeptides. Thus, mutation at NH_2- and COOH-termini influence the rate of α_s activation, but only the COOH-terminus appears to be involved in the regulation of the α_s polypeptide activation domain that interacts with adenylyl cyclase.

Key words: α_s/α_i, chimeras, GTPase, adenylyl cyclase

INTRODUCTION

G proteins couple specific receptor activation with the regulation of effector enzymes and ion channels [1–3]. G_s and G_{i2} are two related G proteins whose α subunits (α_s and α_{i2}) are 65% homologous in primary sequence [4], share common βγ subunits [5], but selectively couple to different receptors [2,3]. G_s functions to stimulate adenylyl cyclase and the synthesis of cAMP, while G_{i2} functions in part to inhibit adenylyl cyclase. The activation and turn-off of G proteins, such as G_s and G_{i2}, involves two independent mechanisms. G protein activation requires hormone receptor-catalyzed dissociation of GDP from the GDP/GTP binding domain of the α subunit. The subsequent binding of GTP to the α subunit then induces conformational changes that result in βγ dissociation and α_{GTP} regulation of specific effector enzymes. The α chain polypeptide also encodes a GTPase activity which slowly hydrolyzes (kcat ~3–5 min^{-1}) the bound GTP to GDP resulting in the turn-off of the activated complex. In the continued presence of hormone, the system will continue through activation and inactivation cycles. When hormone is removed, the system returns to a basal inactive state because of the GTPase intrinsic to the α subunit polypeptide.

Previously, we demonstrated that the adenylyl cyclase activation domain is encoded within a

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122 amino acid region (Ile235-Gly355) of the COOH-terminal moiety of α_s [6]. The NH_2-terminal moiety of the α_s polypeptide was shown to be involved in regulating the rate of α_s activation by guanine nucleotides, defining this domain as a modulator of GDP dissociation and GTP activation that in part involved interactions with the $\beta\gamma$ subunit complex.

In this report, we have characterized a series of NH_2- and COOH-terminal α subunit chimeras where different regions of α_s have been substituted with the corresponding region of α_{i2} [6–8]. Specific mutations at the NH_2- and COOH-termini of α_s have similar but distinguishable consequences on the regulation of α_s activation and stimulation of adenylyl cyclase by GTP. The findings demonstrate that both the NH_2- and COOH-termini of the α_s polypeptide regulate the "turn-on" mechanism involving GDP dissociation and GTP activation, while only the COOH-terminus selectively regulates the intrinsic activity of the adenylyl cyclase activation domain. The GTPase "turn-off" function is independent of the NH_2- and COOH-terminal domains controlling "turn-on" of the α_s polypeptide.

MATERIALS AND METHODS

The construction and characterization of the $\alpha_{i(54)/s}$, $\alpha_{i/s(Bam)}$, $\alpha_{s/i(38)}$, $\alpha_{i(54)s/i/(38)}$, $\alpha_{i(Bam)/s/i(38)}$, and α_sQ227L mutants has been described previously [6–8]. All cDNA constructs were verified by DNA sequencing and inserted in the Hind III site of the pCW1 expression plasmid [8]. Chinese hamster ovary (CHO) and CHO kin^- (PKA deficient) [9] cells were transfected by electroporation of 10^7 cells with 10 μg plasmid DNA (Bio-Rad Gene Pulser; 25 μF, 1 kV). G418-resistant CHO clones were isolated and screened for chimeric α_{i2}/α_s and α_sQ227L expression by both Northern (RNA) [10] and immunoblotting [6–8] analyses. Clones were chosen for analysis of adenylyl cyclase regulation that expressed similar levels of RNA and α subunit polypeptides as described previously [7,8]. No changes in expression of $\beta\gamma$ complexes or α_i subunits were detected relative to wild-type CHO cells in any of the clones [7,8].

Adenylyl Cyclase Activity Measurements

CHO clones were grown to 75% confluence, harvested by scraping, and resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.02 units/ml aprotinin). Cells were ruptured by 50 strokes in a Dounce homogenizer kept in an ice-water bath. Nuclei were removed by centrifugation at 1,500*g* at 4°C for 5 min, and membranes were then pelleted by centrifugation at 31,000*g* for 1 hr, resuspended in lysis buffer, and used for adenylyl cyclase assays. Membranes (25–30 μg/20 μl) were added to 80 μl of reaction mix that contained 50 mM Na-HEPES, pH 8.0, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 10 mM creatine phosphate, 10 units/ml creatine phosphokinase, 0.4 mM [α-^{32}P] ATP (20 cpm/pmol), and either 100 μM GTP, 100 μM GTPγs, 10 mM fluoride ion or H_20. Reactions were incubated for the indicated times at 30°C, stopped by dilution in 1 ml of 1% SDS and ^{32}P-cAMP was purified by chromatography on Dowex-50 and Alumina.

For the preparation of cyc^- S49 membranes the cells were ruptured by nitrogen cavitation (700 psi, 20 min on ice) and then treated as described above for CHO membranes. Reconstitution of cyc^- S49 membranes with cholate extracts of CHO membranes was performed exactly as described previously [8].

Cellular cAMP Determinations

Intracellular cAMP was determined using the cAMP [^{125}I] assay system from Amersham Corp. using the manufacturer's protocol. Cells were incubated for 10 min in fresh Dulbecco's MEM, 10 mM Na-HEPES, pH 7.5, in the presence or absence of 500 μM isobutyl methylxanthine. The medium was rapidly aspirated, the cells rinsed once with ice-cold phosphate-buffered saline, and then quick frozen by placing the dish in a bath of liquid nitrogen. Cyclic AMP was extracted by scraping the cells in 65% ice-cold ethanol, vortexing, then centrifuging to remove protein. The ethanol supernatants were dried and used for cAMP determinations and the pellets solubilized in NaOH for protein analysis.

RESULTS

Five chimeras of α_s and α_{i2} were characterized for their influence on cAMP synthesis in CHO K-1 cells (Fig. 1). For controls, the wild-type α_s and α_{i2} polypeptides were stably expressed in CHO K-1 cells [7,8,10] as well as the α_s mutant having Gln227 mutated to Leu (α_sQ227L), which has been shown to be GTPase deficient and constitutively activate cAMP synthesis [6,11,12]. Expression of the wild-type α_s or α_{i2} had little or no effect on cAMP levels in the presence or

absence of the phosphodiesterase inhibitor isobutyl methylxanthine (IBMX) relative to control CHO cells (Fig. 1B). In contrast, expression of the chimeras $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ markedly stimulated cAMP synthesis. The increased cAMP synthesis was, in fact, similar in magnitude to that observed with the GTPase-deficient α_sQ227L mutant.

No clones were isolated in several independent transfections with the $\alpha_{i(Bam)/s/i(38)}$ or $\alpha_{i(54)/s/Q227L}$ constructs, even though both have been shown to encode functional α_s polypeptides in transient COS cell expression assays [6]. The $\alpha_{i(Bam)/s/i(38)}$ and $\alpha_{i(54)/s/Q227L}$ polypeptides were found to be highly active α_s subunits capable of stimulating cAMP synthesis to levels significantly greater than that observed with $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, or α_sQ227L polypeptides when expressed at similar levels in COS cells. The $\alpha_{i(Bam)/s/i(38)}$ polypeptide has also been shown to be a highly robust constitutively active α_s when expressed in α_s-deficient cyc$^-$ S49 cells (Cathy Berlot, personal communication). The failure to isolate stably expressing $\alpha_{i(Bam)/s/i(38)}$ or $\alpha_{i(54)/s/Q227L}$ CHO clones suggested they had the ability to stimulate cAMP synthesis to levels that growth arrested CHO K-1 cells. In contrast, the α_sQ227L, $\alpha_{i(54)/s}$, and $\alpha_{s/i(38)}$ polypeptides stimulated cAMP synthesis to more modest levels, such that induction of cellular cAMP phosphodiesterase activity could apparently overcome the cAMP-induced growth arrest [13]. Based on the transient COS cell analysis [6], we predicted that $\alpha_{i(Bam)/s/i(38)}$ and $\alpha_{i(54)/s/Q227L}$ were able to stimulate cAMP synthesis to such levels that the induction of cAMP phosphodiesterase was still unable to prevent the growth inhibition induced by the two constitutively active α_s mutants.

To test this hypothesis, CHO kin$^-$ cells deficient in cAMP-dependent protein kinase activity [9] were transfected with the various α_s mutants (Fig. 1C). CHO kin$^-$ cells are not growth arrested by high cAMP levels and cells should express the $\alpha_{i(Bam)/s/i(38)}$ and $\alpha_{i(54)/s/Q227L}$ mutants, if the mutants ability to robustly stimulate cAMP synthesis was the basis for the failure to isolate stably expressing clones from transfected cells having a functional cAMP-dependent protein kinase. Analysis of CHO kin$^-$ clones stably ex-

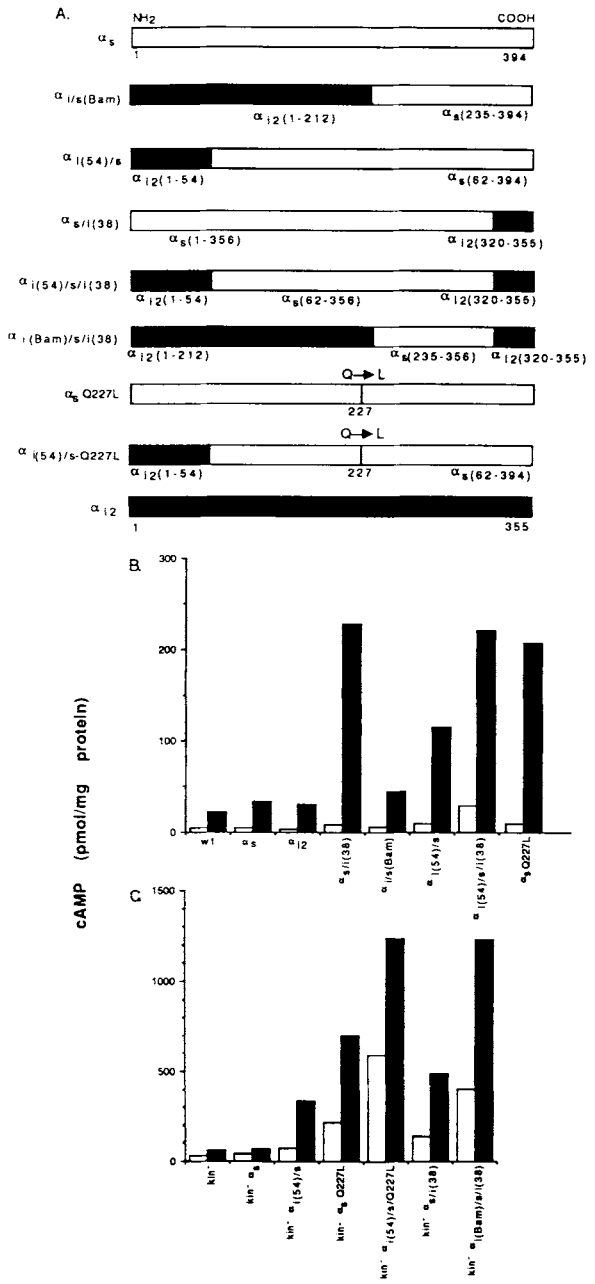

Fig. 1. Activation of cAMP synthesis in CHO and CHO kin$^-$ cells stably expressing α_{i2}/α_s chimeras or the GTPase-deficient α_sQ227L mutant. (**A**) Map of the α_{i2}/α_s chimeras and α_sQ277L point mutant. Numbers refer to the amino acid residues contributed by either α_{i2} (black bar) or the 46.5 kDa α_s (white bar) polypeptides. The α_{i2} polypeptide is 355 amino acids, whereas α_s is encoded by 394 residues. Measurement of cAMP levels in CHO (**B**) or CHO kin$^-$ (**C**) cells stably expressing the designated α_{i2}/α_s chimeras or α_sQ227L mutants. Cells were assayed for intracellular cAMP levels by radioimmunoassay. Cells were incubated for 10 min in the presence (black bar) or absence (white bar) of 500 μM isobutyl methylxanthine, a cAMP phosphodiesterase inhibitor. Cells were then fixed and cAMP extracted with ice-cold 65% ethanol as previously described [8]. The data is the mean of duplicate determinations from two independent experiments that varied by less than 10%. The values obtained are also representative of at least three independent clones isolated for each construct.

pressing the $\alpha_{i(54)/s/Q227L}$ and $\alpha_{i(Bam)/s/i(38)}$ constructs demonstrated that both α_s mutants were able to strongly stimulate cAMP synthesis (Fig. 1C). The levels of intracellular cAMP in clones expressing $\alpha_{i(54)/s/Q227L}$ or $\alpha_{i(Bam)/s/i(38)}$ was significantly greater than that observed with the $\alpha_{i(54)/s}$, α_{sQ227L}, $\alpha_{s/i(38)}$, or $\alpha_{i/s(Bam)}$ mutants.

The phenotypic consequence of the various α_s mutants in CHO kin$^-$ cells was generally similar to that observed with their expression in wild-type CHO cells. Mutants capable of stimulating cAMP synthesis in the wild-type CHO cells also did so in the CHO kin$^-$ cells. In the CHO kin$^-$ cells, however, basal cAMP levels were significantly higher. This results from lower cAMP phosphodiesterase activity and the inability of the cAMP-dependent protein kinase deficient cells to activate or induce phosphodiesterase activity in response to increases in intracellular cAMP [9,20].

The strong activation of cAMP synthesis by the $\alpha_{i(54)/s/Q227L}$ mutant was shown in transient COS cell assays to be additive relative to the cAMP levels observed with expression of the $\alpha_{i(54)/s}$ and α_sQ227L mutants, alone [6]. The basis for the additivity of the $\alpha_{i(54)/s}$ and α_sQ227L mutations is that they independently alter the two rate-limiting steps in α_s activation; the $\alpha_{i(54)/s}$ mutation enhances the rate of GTP activation ("turn-on") and the α_sQ227L mutation inhibits the intrinsic GTPase activity ("turn-off"). A similar additivity of the $\alpha_{i(54)/s}$ and α_sQ227L was observed in CHO kin$^-$ cells when both mutations were placed in the same polypeptide.

The phenotype of the $\alpha_{i(Bam)/s/i(38)}$ polypeptide is more difficult to understand. The $\alpha_{i/s(Bam)}$ polypeptide behaves very similarly to wild-type α_s [7,14], while the $\alpha_{s/i(38)}$ construct is similar to $\alpha_{i(54)/s}$ and α_sQ227L in its ability to stimulate cAMP synthesis. However, placing both the $\alpha_{i/s(Bam)}$ and $\alpha_{s/i(38)}$ mutations in the same polypeptide resulted in a mutant which stimulates cAMP synthesis much greater than that observed with expression of either mutation alone. The findings with the $\alpha_{i(Bam)/s/i(38)}$ mutant suggested that inserting the α_s adenylyl cyclase activation domain within the α_{i2} polypeptide resulted in release of an intrinsic inhibitory function of the α_s polypeptide chain that was greater than that observed with the $\alpha_{s/i(38)}$ mutation alone. Interestingly, placing the $\alpha_{s/i(38)}$ and α_sQ227L mutations in the same polypeptide ($\alpha_{sQ227L/i(38)}$) did not mimic either the $\alpha_{i/s(Bam)/s/i(38)}$ or $\alpha_{i(54)/s/Q227L}$ mutants, but yielded a phenotype based on enhanced cAMP synthesis that was similar to that observed with the α_sQ227L mutant alone (not shown). Thus, it appeared that placing two activating mutations having individually different α_s activation characteristics in the COOH-terminal moiety of the α_s polypeptide was not additive. However, appropriate mutation of the NH_2- and COOH-terminal α_s moieties (i.e., $\alpha_{i(Bam)/s/i(38)}$) was strongly activating. For this reason, the properties of NH_2- and COOH-terminal mutants were examined in relation to their ability to regulate adenylyl cyclase activity in CHO membranes and reconstituted adenylyl cyclase systems in membranes from S49 cyc$^-$ cells deficient in α_s [15].

Adenylyl Cyclase Activity in CHO Membranes Expressing α_i/α_s Chimeras

G_s activation of adenylyl cyclase activity was assayed in membrane preparations from CHO cells expressing different α_i/α_s chimeras (Fig. 2). Activation of G_s was achieved with either the hydrolysis resistant GTP analog GTPγS or aluminum fluoride. Aluminum fluoride occupies the γ-phosphate position in the GDP bound state of G protein α subunits and rapidly activates adenylyl cyclase [16]. GTPγS constitutively activates the α subunit because its γ-phosphate is insensitive to GTPase hydrolysis, whereas GTP activates poorly because it is rapidly hydrolyzed to GDP. The lag time associated with GTPγS stimulation results from the requirement of GDP to dissociate from the α subunit guanine nucleotide binding site, allowing GTPγS to bind and activate the α_s polypeptide. This lag time for GTPγS activation is markedly diminished by hormone-receptor interaction with the G protein which catalyzes the dissociation of GDP from the guanine nucleotide binding site [2,17,18].

Appropriate mutation of either the NH_2- or COOH-terminus of α_s, by substitution with the corresponding region of the α_{i2} sequence, resulted in a marked decrease in the lag time required for GTPγS to activate adenylyl cyclase activity. A similar diminished lag time for GTPγS activation was observed for the $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ chimeras. The $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ mutants are also activating α_s mutants in intact CHO cells, resulting in a constitutive increase in cAMP synthesis (Fig. 1). In contrast, the $\alpha_{i/s(Bam)}$ and α_sQ227L mutants do not show an altered lag

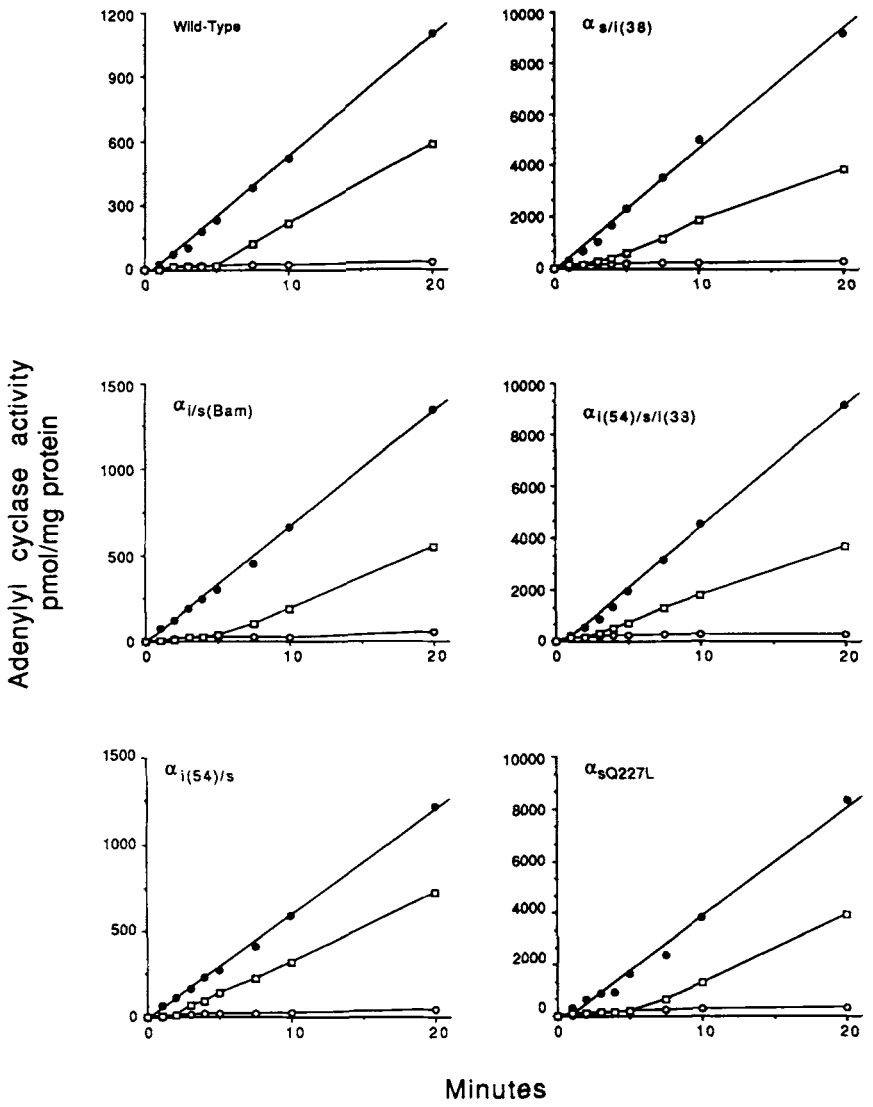

Fig. 2. Time course of fluoride and GTPγS-stimulated adenylyl cyclase activity in CHO membranes expressing the indicated α_{i2}/α_s chimeras or α_sQ227L mutant. Adenylyl cyclase activity was measured by using 25 μg of membranes from the appropriate clones in the presence of 10 mM fluoride ion (closed circles), 100 μM GTPγS (open squares), or H_2O control (open circles). The data is representative of three experiments from two independent membrane preparations for each clone. A single representative clone is shown for each construct, but similar results were obtained for different independent clones expressing each construct. The lag time to reach maximal adenylyl cyclase activity was 5–5.5 min for wild-type, $\alpha_{i/s(Bam)}$, and α_sQ227L expressing clones and 1.5–2 min for $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ expressing clones.

time for GTPγS activation relative to control CHO cell membranes. This finding indicated that the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ mutants similarly mimicked the action of hormone receptors in accelerating the rate of GDP dissociation and GTPγS activation of adenylyl cyclase. Thus, these two mutants influence the control of the "turn-on" mechanism of G_s. The GTPase-deficient α_sQ227L mutant, which inhibits the "turn-off" mechanism for α subunits had no effect on the rate of GTPγS activation, indicating the "turn-on" and "turn-off" of α subunit activation is controlled by independent domains of the polypeptide.

To verify that the mutations introduced in the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ polypeptides were directly influencing the properties of the mutant α_s subunit, CHO membrane detergent extracts were reconstituted with S49 cyc^- membranes which do not express the α_s polypeptide [15]. The cyc^- membrane reconstitution assay directly measures the

properties of the α_s polypeptide in the detergent extract, because the solubilized adenylyl cyclase from CHO membranes is denatured, while the solubilized G_s readily reconstitutes with the membranes and functionally regulates the endogenous cyc$^-$ membrane adenylyl cyclase [19]. Figure 3 shows that the reconstituted $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ mutants have a decreased lag time for GTPγS activation of adenylyl cyclase, whereas α_sQ227L and $\alpha_{i/s(Bam)}$ are similar to extracts from membranes expressing only the wild-type α_s polypeptide. This result unequivocally demonstrates that the altered rate of GTPγS activation is related to the mutations introduced at the extreme NH_2- and COOH-termini of the α_s polypeptide. Secondary changes in CHO cells, such as altered adenylyl cyclase expression, are excluded in the reconstitution assay. Furthermore, we have previously demonstrated that there were no changes in either βγ or α_i expres-

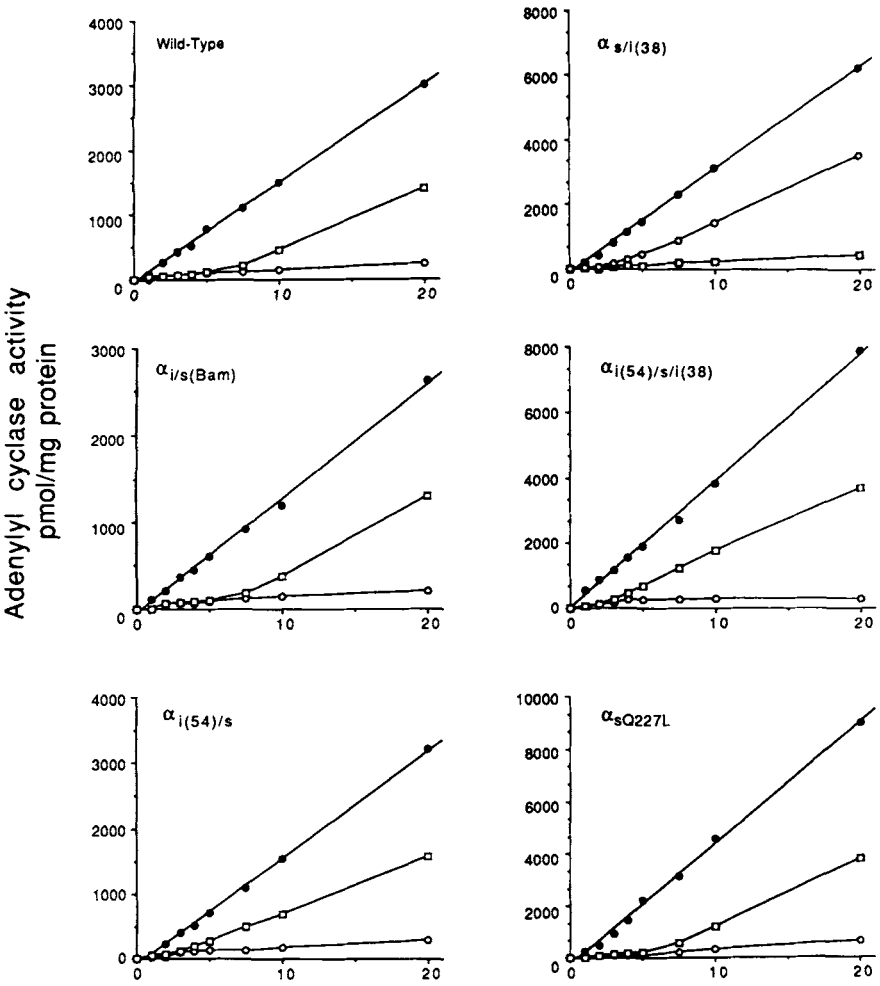

Fig. 3. Reconstitution of α_{i2}/α_s chimeric and α_sQ227L mutants with cyc$^-$ S49 cell membranes. Membranes (5 mg/ml) from CHO clones expressing the indicated constructs were solubilized on ice in 1% sodium cholate. Five micrograms of protein from the 100,000 x g cholate extract supernatants was mixed with 30 μg cyc$^-$ S49 membranes for 10 min on ice. Adenylyl cyclase activity was then assayed, as described in Materials and Methods, in the presence of 10 mM fluoride ion (closed circles), 100 μM GTPγS (open squares), or buffer alone. Reconstitution with heat-inactivated cyc$^-$ S49 membranes gave activities similar to background indicating that catalytic adenylyl cyclase was not contributed by the cholate extract in the assay. Values represent means of duplicate determinations which varied by less then 10% and are representative of two independent membrane preparations and experiments. One representative clone is shown for each construct, but the kinetics of adenylyl cyclase activation is representative of reconstitution assays from at least two independent clones for each mutant. Note the difference in the cAMP synthesis (pmol/mg) on the ordinate for the different clones. The lag time to reach maximal adenylyl cyclase activity was 4.75–5 min for the wild-type, $\alpha_{i/s(Bam)}$, and α_sQ227L reconstituted membranes and 0.5–2 min for the $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ reconstituted membranes.

sion in any of the clones stably expressing mutant α_s polypeptides [7,8,10].

To further support the finding that mutation of the NH_2- and COOH-terminal α_s sequences defined by the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ mutations affected the control of "turn-on" and not "turn-off" of the α_s subunit, a kinetic analysis of the intrinsic α_s GTPase activity was determined in reconstituted cyc$^-$ S49 membranes (Fig. 4). Measurement of the time course of propranolol-induced inhibition of isoproterenol-stimulated adenylyl cyclase activation has proven to be a reliable estimation of the rate constant for the GTPase "turn-off" mechanism (k_{off}) [7,11]. This method has been shown to yield accurate measurements of the k_{cat} for the intrinsic GTPase reaction of α_s and different α_s mutants [11,12]. Reconstitution of wild-type CHO G_s in cyc$^-$ S49 membranes results in a k_{off} of 5–6 min^{-1} for propranolol-induced inhibition of isoproterenol-stimulated

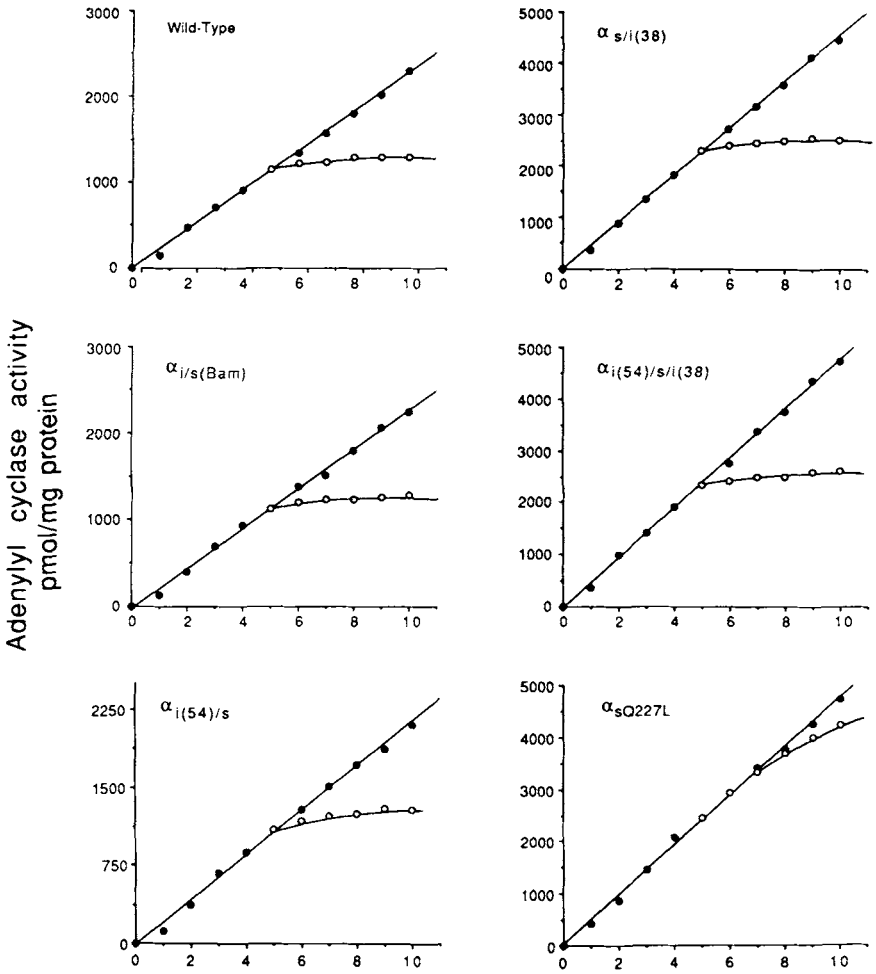

Fig. 4. GTPase activity of G_s proteins from CHO cell membranes expressing α_{i2}/α_s chimeric and α_sQ227L mutants. Cholate extracts from membranes prepared from CHO clones expressing the designated constructs were reconstituted with cyc$^-$ S49 membranes as described in the legend to Figure 3. GTPase activity was estimated by determining the rate at which propranolol inactivated isoproterenol/GTP-stimulated adenylyl cyclase activity [7,11]. At 0 min, adenylyl cyclase was stimulated with 10 μM isoproterenol plus 100 μM GTP (closed circles). At 5 min, one-half of each incubation was mixed with 10 μM propranolol (open circles), while the other half of the incubation received buffer alone. Isoproterenol-stimulated, GTP-dependent adenylyl cyclase activity remained linear over the 10 min incubation. However, when propranolol was added, the isoproterenol stimulation was rapidly inhibited and the adenylyl cyclase activity in the membranes reconstituted with extracts from CHO membranes expressing wild-type, $\alpha_{i/s(Bam)}$, $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ polypeptides rapidly returned to basal levels, due to the GTPase shut-off function intrinsic to the α subunit polypeptide. In contrast, the adenylyl cyclase activity of the GTPase-deficient α_sQ227L mutant remained activated after propranolol inhibition of the isoproterenol stimulation. The GTPase activity of the G_s proteins from CHO membranes expressing the α_{i2}/α_s chimeras is similar to that for wild-type α_s, whereas the α_sQ227L mutant GTPase activity is inhibited greater than 95%.

TABLE I. Adenylyl Cyclase Regulation in CHO Transfectants*

Clone	Lag time to achieve GTPγS-stimulated V_{max}	Adenylyl cyclase V_{max}		GTPase k_{off}
	minutes	pmol/min/mg prot		min^{-1}
CHO membranes		Fluoride	GTPγS	
Wild-type	5	146	92	—
$\alpha_{i/s(Bam)}$	5.5	141	85	—
$\alpha_{i(54)/s}$	2	192	85	—
$\alpha_{s/i(38)}$	2.5	269	169	—
$\alpha_{i(54)/s/i(38)}$	1.5	385	185	—
α_sQ227L	5	429	257	—
Reconstitution with cyc⁻ S49 membranes				
Wild-type	4.8	53	30	5–6
$\alpha_{i/s(bam)}$	5	94	48	5–6
$\alpha_{i(54)/s}$	2	70	40	5–6
$\alpha_{s/i(38)}$	1.5	438	190	5–6
$\alpha_{(54)/s/i(38)}$	0.5	457	171	5–6
α_sQ227L	5	458	276	.05–.07

*Membranes from the designated CHO cell clones were prepared and assayed directly or solubilized in 1% sodium cholate, which denatures catalytic adenylyl cyclase. Equivalent amounts of protein in the extracts containing the G proteins were reconstituted into cyc⁻ S49 membranes. CHO membranes and the reconstituted cyc⁻ S49 membranes were assayed for fluoride and GTPγS-regulable adenylyl cyclase activity. GTPase k_{off} measurements were performed with cholate extracts reconstituted with cyc⁻ membranes by determining the rate of propranolol inhibition of isoproterenol-stimulated adenylyl cyclase activity in the presence of GTP as described in the legend to Figure 4.

adenylyl cyclase activity. In contrast, reconstituted extracts from α_sQ227L expressing CHO cell membranes demonstrated a k_{off} of approximately 0.05 min^{-1}. The slow k_{off} for the α_sQ227L mutant is a consequence of its inhibited GTPase activity [6,11,12]. The adenylyl cyclase assays with reconstituted membrane extracts from $\alpha_{i(54)/s}$, $\alpha_{s/i(38)}$, and $\alpha_{i(54)/s/i(38)}$ expressing CHO cell membranes all demonstrated a k_{off} similar to that observed with the wild-type α_s polypeptide (5–6 min^{-1}). Cumulatively, the findings clearly demonstrate that the molecular basis for the activating NH_2- and COOH-terminal mutations is different from that of the α_sQ227L mutant whose GTPase activity is inhibited.

Table 1 summarizes the lag time and V_{max} for fluoride and GTPγS-stimulated adenylyl cyclase activity and the k_{off} for the GTPase activity of CHO cell clones expressing the different α_s mutants. A rather striking observation is apparent in this analysis. Whereas both NH_2- and COOH-terminal mutations affected the "turn-on" rate for GTPγS stimulation of adenylyl cyclase activity, COOH-terminal mutations dramatically increased the V_{max} for adenylyl cyclase stimulation relative to NH_2-terminal mutations. This was true not only for the COOH-terminal $\alpha_{s/i(38)}$ mutant, but also for the α_sQ227L single amino acid mutation in the COOH-terminal moiety of the α_s polypeptide. The increased adenylyl cyclase V_{max} observed with activating mutations within the COOH-terminal half of the α_s polypeptide was apparent in membranes prepared from CHO cells, as well as when detergent extracts from these membranes were reconstituted with cyc⁻ S49 membranes. Thus, mutations within the NH_2- and COOH-terminal moieties of α_s, which functionally stimulate cAMP synthesis in intact cells, are distinguishable by their properties of adenylyl cyclase activation in membrane preparations. This finding is consistent with our previous observations that the adenylyl cyclase activation domain is localized within a 122 amino acid region in the COOH-terminal moiety of the α_s polypeptide and the NH_2-terminal moiety functions primarily in controlling GDP dissociation and βγ interactions [6,7]. Only mutations in the COOH-terminal region affect the adenylyl cyclase activation function of the α_s polypeptide.

DISCUSSION

The adenylyl cyclase activation domain lies within α_s residues Ile 235-Gly 355 [6]. This sequence may be substituted for the corresponding region within the α_{i2} polypeptide, generating the $\alpha_{i(Bam)/s/i(38)}$ chimera whose stable expression in CHO kin⁻ cells (Fig. 1) or transient expression in COS cells [6] causes a marked activation of adenylyl cyclase activity. Neither the NH_2-terminal 60% nor COOH-terminal 10% of the α_s

polypeptide is required for adenylyl cyclase activation. When the α_s activation domain is placed within the context of the α_{i2} polypeptide, the intrinsic regulation of the α_s polypeptide is lost and the $\alpha_{i(Bam)/s/i(38)}$ chimera behaves as a strong constitutively activated α_s mutant. Thus, the phenotype of the $\alpha_{i(Bam)/s/i(38)}$ chimera is basically a stronger version of the $\alpha_{s/i(38)}$ chimera, a polypeptide which also constitutively activates adenylyl cyclase.

It is clear from our results that appropriate mutation of the extreme NH_2-terminus (i.e., $\alpha_{i(54)/s}$ chimera) also constitutively activates the α_s polypeptide. We have shown that the mechanism by which $\alpha_{s/i(38)}$ and $\alpha_{i(54)/s}$ become activated is different from point mutants such as α_sQ227L. The $\alpha_{s/i(38)}$ and $\alpha_{i(54)/s}$ mutants have an enhanced rate of GTPγS activation, but normal GTPase activity. In contrast, the α_sQ227L mutant has an inhibited GTPase activity, but a GDP dissociation and GTP activation rate similar to the wild-type α_s polypeptide. The common phenotypic consequence of an enhanced rate of GTPγS activation of adenylyl cyclase activity in membranes and constitutively elevated cAMP synthesis in intact cells for the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ mutants is related to their overlapping functions. Structural data for G protein α subunits indicates that the NH_2- and COOH-termini of the polypeptide are in proximity to each other and oriented toward the middle of the molecule [20]. Both genetic and structural data also indicate that the α subunit NH_2-terminus has an attenuator function controlling the rate of GDP dissociation that involves, in part, an association with the βγ subunit complex [6,21,22]. The COOH-terminus is known to be a major contact site for receptor interaction [23]. The close proximity of the α chain polypeptide NH_2- and COOH-termini is consistent with the demonstration that receptors have contact sites for βγ, as well as the α subunit COOH-terminus, and that βγ is absolutely required for efficient guanine nucleotide exchange catalyzed by activated receptors [1,2,18,24]. It is evident from our findings that the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ mutants both markedly diminish the lag time required for GDP dissociation and GTP binding, yet they are not functionally equivalent. The $\alpha_{s/i(38)}$ mutant stimulates adenylyl cyclase activity in the presence of either fluoride ion or GTPγS greater than that observed with the $\alpha_{i(54)/s}$ chimera. As observed in Figure 1, there is also a somewhat higher level of cAMP synthesis in clones expressing $\alpha_{s/i(38)}$ relative to $\alpha_{i(54)/s}$, even though their relative levels of expression are similar [7,8,10].

The increased V_{max} for adenylyl cyclase activation observed with the $\alpha_{s/i(38)}$ chimera was also apparent with the GTPase-deficient α_sQ227L mutant. The increased efficiency with which the α_sQ227L polypeptide activates adenylyl cyclase was also observed when this mutant was expressed in cyc$^-$ S49 cells [11] or recombinantly in *E. coli* and subsequently reconstituted with adenylyl cyclase [12]. By analogy with the GDP- and GTP-induced conformations of the p21 ras protein, whose three dimensional structure has been defined [25–27], the mutation of α_s Gln227→Leu appears to stabilize the conformational change that is induced by the binding of GTP [6,11,12,27]. Stabilization of the GTP activated conformation is a probable mechanism for the enhanced activation properties of the α_sQ227L mutant compared with the wild-type α_s polypeptide. The enhanced activation properties of the $\alpha_{s/i(38)}$ chimera, however, is different from that of the α_sQ227L mutant because the GTPase turn-off function of $\alpha_{s/i(38)}$ is not inhibited. Rather, the mutation of the COOH-terminus relieves an inhibition of the α_s activation domain independent of the intrinsic GTPase activity of the α subunit polypeptide. The ability of COOH-terminal mutations to influence the α_s activation domain is not unexpected because the COOH-terminus is oriented towards the α subunit activation domain within the polypeptide tertiary structure [20,28] and is the major contact site for activated receptors [23], which function to enhance GDP dissociation allowing activation by GTP. The proximity of the COOH-terminus to the α_s activation domain could, therefore, influence tertiary conformations to alter the intrinsic properties of the α_s polypeptide.

Thus, structural changes induced by the COOH-terminal $\alpha_{s/i(38)}$ mutations apparently influence two functions: the rate of GDP dissociation and the intrinsic activation potential of the fluoride ion and GTPγS activated α_s polypeptide, measured by the regulation of adenylyl cyclase stimulation. In contrast, activating NH_2-terminal mutations such as the $\alpha_{i(54)/s}$ chimera influence the rate of GDP dissociation and interactions with the βγ subunit complex. These findings demonstrate that the NH_2- and COOH-termini are modulators of α_s activation with overlapping but distinguishable regulatory functions. The results substantiate an α_s structure

where the adenylyl cyclase activation domain is localized within the COOH-terminal moiety, the NH_2-terminal moiety is involved in $\beta\gamma$ regulatory functions, and the ends of the polypeptide are involved in modulating $\beta\gamma$ interactions, receptor contact, and the activity of the α_s activation domain.

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