Mutation of the G_s Protein α Subunit NH₂ Terminus Relieves an Attenuator Function, Resulting in Constitutive Adenylyl Cyclase Stimulation

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G-proteins couple hormonal activation of receptors to the regulation of specific enzymes and ion channels. G_s and G_i are G-proteins which regulate the stimulation and inhibition, respectively, of adenylyl cyclase. We have constructed two chimeric cDNAs in which different lengths of the α subunit of G_s (α_s) have been replaced with the corresponding sequence of the G_i α subunit (α_i). One chimera, referred to as $\alpha_{i(54)/s}$, replaces the NH₂-terminal 61 amino acids of α_s with the first 54 residues of α_i . Within this sequence there are 7 residues unique to α_s , and 16 of the remaining 54 amino acids are nonhomologous between α_i and α_s . The second chimera, referred to as $\alpha_{i/s(Bam)}$, replaces the first 234 amino acids of α_s with the corresponding 212 residues of α_i . Transient expression of $\alpha_{i(54)/s}$ in COS-1 cells resulted in an 18- to 20-fold increase in cyclic AMP (cAMP) levels, whereas expression of either $\alpha_{i/s(Bam)}$ or the wild-type α_s polypeptide resulted in only a 5- to 6-fold increase in cellular cAMP levels. COS-1 cells transfected with α_i showed a small decrease in cAMP levels. Stable expression of the chimeric $\alpha_{i(54)/s}$ polypeptide in Chinese hamster ovary (CHO) cells constitutively increased both cAMP synthesis and cAMP-dependent protein kinase activity. CHO clones expressing transfected $\alpha_{i/s(Bam)}$ or the wild-type α_s and α_i cDNAs exhibited cAMP levels and cAMP-dependent protein kinase activities similar to those in control CHO cells. Therefore, the $\alpha_{i(54)/s}$ chimera behaves as a constitutively active α_s polypeptide, whereas the $\alpha_{i/s(Bam)}$ polypeptide is regulated similarly to wild-type α_s . Expression in *cyc*⁻S49 cells, which lack expression of wild-type α_s , confirmed that the $\alpha_{i(54)/s}$ polypeptide is a highly active α_s molecule whose robust activity is independent of any change in intrinsic GTPase activity. The difference in phenotypes observed upon expression of $\alpha_{i(54)/s}$ or $\alpha_{i/s(Bam)}$ indicates that the NH₂-terminal moieties of α_s and α_i function as attenuators of the effector enzyme activator domain which is within the COOH-terminal half of the α subunit. Mutation at the NH₂ terminus of α_s relieves the attenuator control of the G_s protein and results in a dominant active G-protein mutant.

G_s and G_i are two related G-proteins which, respectively, stimulate and inhibit adenylyl cyclase (7, 8). The G_s and G_i α subunits (α_s and α_i) are approximately 65% homologous in primary sequence (16), share common $\beta\gamma$ subunits (4), and selectively couple to different receptors (29). Additional activities intrinsic to α_s and α_i include GDP-GTP binding and GTPase activity. Based on homologies with the p21^{ras} protein and mutation analysis of the α_s subunit, the GDP-GTP binding and GTPase domains of G-protein α subunits have been demonstrated to involve four highly conserved regions in the primary sequence of the polypeptide (14). In addition, the COOH-terminal 40% of the α_s polypeptide has been shown to encode the necessary structural information that is critical to both receptor selectivity and adenylyl cyclase stimulation (20). However, the actual contact domains for $\beta\gamma$ interaction and coupling to receptor and effector enzyme remain ill defined.

Intramolecular control of protein function is characteristic of allosterically regulated proteins whose activity is vital in cell function and response to extracellular signals. For example, the glucocorticoid receptor can be envisioned as an NH₂-terminal DNA-binding domain and a COOH-terminal steroid-regulatable inactivator domain (24). Binding of ste-

roid to the inactivator domain releases an inhibition that allows the DNA-binding domain to regulate the transcription of specific genes. Similarly, the NH₂-terminus of protein kinase C encodes a pseudosubstrate sequence which, in the inhibited state, resides in the active site of the enzyme (15). Upon activation by diacylglycerol and calcium, conformational changes in protein kinase C release the pseudosubstrate from the active site, allowing substrate phosphorylation to occur. On the basis of the finding that the COOH-terminal 40% of α_s encodes the receptor selectivity and adenylyl cyclase stimulatory functions (20), we predicted that the α -subunit NH₂ terminus would function as an intrinsic regulator that serves to inhibit G-protein activity in the absence of appropriate receptor stimulation. In the present work, we have used NH₂-terminal chimeras of α_s and α_i to define the role of the α subunit NH₂ terminus in the control of G-protein activity and have determined that mutation in this region in α_s results in constitutive activation of G_s and adenylyl cyclase activity by a mechanism independent of any apparent influence on GTPase activity. The results define the NH₂-terminal moiety of the α subunit as an attenuator of G-protein activity. Thus, the G-protein α subunit has intramolecular control of its activity similar to that observed for the glucocorticoid receptor and protein kinase C.

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MATERIALS AND METHODS

Plasmid construction. The chimeric cDNA for $\alpha_{i(54)/s}$ was constructed from partial restriction enzyme products of the rat α_s and α_{i2} cDNAs (16). Partial *Bam*HI restriction of the 1.56-kilobase-pair (kbp) *Hind*III-*Hind*III α_s cDNA was used to isolate a 1,191-bp fragment encoding codons 62 to 394 and the 3' untranslated region of the α_s cDNA. A 318-bp fragment encoding the 5' untranslated region and the first 54 codons of the *Eco*RI-*Eco*RI 1.75-kbp α_{i2} cDNA was isolated as an *Eco*RI-*Sau*3A digestion product. The chimeric $\alpha_{i(54)/s}$ cDNA was then assembled by ligating the 318-bp *Eco*RI-*Sau*3A α_{i2} fragment and the 1,191-bp *Bam*HI-*Hind*III α_s fragment into pUC12 previously linearized with *Eco*RI and *Hind*III. The identity of the chimeric cDNA was verified by restriction analysis and DNA sequencing. The 1.51-kbp chimeric $\alpha_{i(54)/s}$ cDNA was isolated by partial *Eco*RI-*Hind*III digestion and blunt ended with Klenow fragment, and 12-bp *Hind*III linkers were added by ligation.

The $\alpha_{i/s(Bam)}$ chimera was generated by using the conserved *Bam*HI site in the α_s and α_{i2} cDNAs that encodes a polypeptide having the first 212 residues of α_{i2} and the last 160 residues of α_s (20). The chimeric $\alpha_{i(54)/s}$ and $\alpha_{i/s(Bam)}$ *Hind*III-*Hind*III cDNAs were then inserted into pCW1-neo (31), and the orientation of the insert was verified by the asymmetric *Bam*HI sites in the cDNAs and vector.

The expression plasmid pCW1-neo (31) is a pUC13 derivative containing the simian virus 40 enhancer, replication origin, and early promoter (*Eco*RI-*Hind*III fragment) and the simian virus 40 splicing and polyadenylation sequences (*Hind*III-*Bam*HI fragment). Insertion into the *Hind*III site allows the expression of cloned cDNA under the control of the simian virus 40 early promoter. In addition, the plasmid carries the Tn5 gene for neomycin resistance under the control of Moloney virus LTR (26). COS-1 cells were transfected with 1 μ g of the appropriate pCW1 plasmid construct by using DEAE-dextran as described by Ausubel et al. (1). Cells were used for cyclic AMP (cAMP) analysis, immunoblots, and cholera toxin labeling 65 h after transfection. Chinese hamster ovary (CHO K-1) cells were transfected by electroporation of 10^7 cells with 10 μ g of plasmid DNA (Bio-Rad Gene Pulser; 25 μ F, 1 kV). G418-resistant CHO cell colonies were isolated and screened for chimeric α_i/α_s expression by both Northern (RNA) (31) and immunoblotting (30, 31) analyses. *cyc*⁻S49 cells were infected with the retroviral vector, pMV7 (20), which contained the $\alpha_{i(54)/s}$ cDNA. Transformants of *cyc*⁻S49 cells infected with retrovirus-packaged constructs were isolated in soft agar containing G418 (20).

Expression analysis of chimeric α_i/α_s chimeras. For immunoblotting, 70 μ g of membrane protein from either COS or CHO cells or 200 μ g of protein from S49 cell membranes was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% polyacrylamide), transferred to nitrocellulose, and probed with antibodies specific for either α_s (20), α_i (9), or β and γ (28). The blots were washed, incubated with ¹²⁵I-protein A, and autoradiographed as described previously (31).

For cholera toxin-catalyzed ADP-ribosylation analysis, COS cell membranes (50 μ g of protein) were incubated with [³²P]NAD and activated cholera toxin as described previously (30). Following the incubation, membranes were precipitated in 10% trichloroacetic acid, pelleted by centrifugation, and rinsed with ethanol. After the membranes had been dissolved in SDS sample buffer and boiled for 5 min, the [³²P]ADP-ribosylated proteins were resolved on 10% poly-

acrylamide gels by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. Autoradiographs were analyzed for quantitation of α -chain bands by using the gel scan attachment for a Beckman DU65 spectrophotometer.

Myristylation of NH₂-terminal α_i/α_s chimeras. COS cells were transfected with appropriate pCW1-cDNA plasmids as described above, and 44 h later [³H]myristic acid (200 μ Ci/ml) was added to the cells in Dulbecco modified Eagle medium containing 10% dialyzed fetal calf serum. The cells were harvested in 20 mM Tris hydrochloride (pH 8.0)–2 mM MgCl₂–1 mM EDTA–1 mM 2-mercaptoethanol–0.02 U of aprotinin per ml–0.1 mM phenylmethylsulfonyl fluoride (low-salt buffer) 4 h later and ruptured by passage through a 20-gauge needle. Nuclei were removed by centrifugation of the homogenate at $500 \times g$ for 5 min at 4°C, and the supernatant was then centrifuged at $100,000 \times g$ for 30 min at 4°C. Pelleted membranes were suspended in low-salt buffer, and 70 μ g of membrane protein was dissolved in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. After electrophoresis, the gels were fixed in 10% acetic acid–10% methanol for 1 h, soaked in Autofluor (National Diagnostics) as specified by the manufacturer, dried, and autoradiographed for 38 h with an intensifying screen.

Other assays. cAMP was measured by using a radioimmunoassay kit from Amersham Corp. (30). Adenylyl cyclase was measured by conversion of [α -³²P]ATP to [³²P]cAMP (31), and binding of [¹²⁵I]ICYC (iodocyanopindolol) was performed by incubation with membranes for 60 min at 30°C followed by rapid filtration and washing as previously described (18).

RESULTS

Structure of NH₂-terminal chimeras. To perturb putative regulatory functions associated with the G_s α -subunit NH₂ terminus, we constructed two chimeras in which different lengths of the NH₂ terminus of α_s were substituted with the corresponding region of α_{i2} (Fig. 1). One chimera, referred to as $\alpha_{i(54)/s}$, has the first 61 amino acids of α_s replaced by the first 54 amino acids of α_{i2} . There are seven more amino acids in α_s than in α_{i2} within this region of the NH₂ terminus, and 16 of the first 34 α_{i2} residues are nonconserved relative to the α_s sequence. The result is a net charge within nonconserved residues of +6 for α_s and +3 for α_{i2} . The last 20 α_{i2} amino acids (Lys-35 to Lys-54) used in the chimera are identical or highly conserved when compared with the α_s sequence.

The second chimera, $\alpha_{i/s(Bam)}$, encodes the first 212 residues of α_{i2} and the COOH-terminal 160 residues of α_s . This chimera, constructed by using the conserved *Bam*HI restriction site, generates an in-frame chimeric polypeptide encoding approximately the first 60% of α_{i2} and the COOH-terminal 40% of α_s . This construct is identical to that described by Masters et al. (20) and was shown to behave as a functional wild-type α_s polypeptide that coupled to β -adrenergic receptors and activated adenylyl cyclase.

COS cell expression of NH₂-terminal chimeras. Transfection of COS-1 cells was used to verify the expression of the $\alpha_{i(54)/s}$ and $\alpha_{i/s(Bam)}$ chimeras as well as the α_s and α_{i2} polypeptides (Fig. 2). COS cells express equivalent levels of the endogenous 45- and 52-kilodalton (kDa) forms of α_s , as determined by immunoblotting (Fig. 2A). The α_s cDNA used in these studies encodes the 52-kDa form of α_s , and transfection of COS cells with pCW1 α_s resulted in a three- to fivefold increase in levels of immunoreactive 52-kDa α_s polypeptide relative to the 45-kDa α_s polypeptide (lane α_s).

FIG. 1. Schematic diagram of the α_i/α_s chimeras. The α_s polypeptide is 394 amino acids, whereas α_{i2} is encoded by 354 residues. The $\alpha_{i/s(Bam)}$ chimera is encoded by the first 212 amino acids of α_{i2} and residues 235 to 394 of α_s . The $\alpha_{i(54)/s}$ chimera is encoded by the first 54 residues of α_{i2} and amino acids 62 to 394 of α_s . The appropriate restriction sites for constructing the chimeras are shown.

Transfection with the pCW1 $\alpha_{i(54)/s}$ construct resulted in expression of a 48-kDa polypeptide, whereas the $\alpha_{i/s(Bam)}$ cDNA codes for a 45-kDa polypeptide. Transfection with the α_{i2} cDNA had no effect on the expression of either the 45- or 52-kDa α_s polypeptides but, as predicted, enhanced the expression of the 41-kDa α_{i2} band (Fig. 2B). Expression of pCW1 without a cDNA insert or containing cDNAs encoding $\alpha_{i(54)/s}$ or $\alpha_{i/s(Bam)}$ had no effect on the level of the 41-kDa α_i polypeptide. Thus, transient expression of each of the α -subunit cDNAs in COS cells resulted in similar three- to fivefold increases in the appropriate immunoreactive polypeptide, with no detectable change in expression of the endogenous α_s or α_i gene products. In addition, none of the α -subunit cDNAs altered COS cell expression of the β or γ subunits during the 65- to 72-h incubation following transfection (Fig. 2C).

Measurement of cAMP levels in transfected COS cells demonstrated that expression of either α_s or $\alpha_{i/s(Bam)}$ polypeptides increased intracellular cAMP levels five- to sixfold over those in control cells (Fig. 3). Surprisingly, expression of $\alpha_{i(54)/s}$ at levels similar to α_s or $\alpha_{i/s(Bam)}$ increased cAMP levels 18- to 20-fold. Expression of α_{i2} slightly decreased cAMP levels, whereas transfection with plasmid without an α -subunit cDNA failed to alter cellular cAMP. Based on the similar levels of expression in transient COS cell assays, the $\alpha_{i(54)/s}$ polypeptide is a more active stimulator of adenylyl cyclase activity than is either $\alpha_{i/s(Bam)}$ or the wild-type α_s polypeptide. In fact, the activation of adenylyl cyclase and increased cAMP levels in COS cells expressing $\alpha_{i(54)/s}$ is similar to that observed with α_s Leu-227 (Fig. 3), a point mutation that inhibits more than 95% of the intrinsic GTPase activity of α_s and constitutively activates adenylyl cyclase (10, 19).

The $\alpha_{i(54)/s}$ polypeptide also couples to β -adrenergic receptors in COS cells (Fig. 3). Subtraction of the cAMP levels observed in COS cells in the absence of isoproterenol from the agonist-stimulated values indicates that the $\alpha_{i(54)/s}$ poly-

FIG. 2. Transient expression of α_i/α_s chimeras in COS cells. (A) Immunoblotting of the various α -subunit constructs expressed in COS cells was performed by using an anti- α_s antiserum raised against the peptide DATPEPGEDPRVTRAK corresponding to residues 323 to 338 of the 52-kDa migrating form of α_s (31). (B) Immunoblot of the same COS cell membrane preparation as in panel A, but probed with an anti- α_i antibody raised against the carboxyl-terminal sequence of α transducin (9). (C) Immunoblot of the membrane preparation used in panels A and B probed with an anti- $\beta\gamma$ antiserum (28). Lanes represent the various transfection conditions with carrier DNA only (mock), pCW1 without a cDNA insert or with the appropriate α_s , $\alpha_{i(54)/s}$, $\alpha_{i/s(Bam)}$, or α_i cDNAs in the pCW1 expression plasmid.

peptide is significantly more active in its coupling to β -adrenergic receptors than is either wild-type α_s or $\alpha_{i/s(Bam)}$. The α_s Leu-227 mutant is also coupled to the β -adrenergic receptor during transient COS cell expression and shows an increased responsiveness to isoproterenol similar in magnitude to that of $\alpha_{i(54)/s}$. These findings indicate that both $\alpha_{i(54)/s}$ and α_s Leu-227 are active α_s molecules that are efficiently coupled to activating receptors and provide a highly robust activation of adenylyl cyclase.

Posttranslational modification of NH₂-terminal chimeras. Previous studies have shown that α_i , but not α_s , is NH₂-terminally myristylated (2). Because of the dramatic influence of NH₂-terminal myristylation on p21^{ras} activity (3), it was possible that the enhanced activity of the $\alpha_{i(54)/s}$ mutant relative to wild-type α_s resulted from differential myristylation. By labeling COS cells that transiently expressed the various cDNA constructs, it was readily apparent that α_{i2} , $\alpha_{i(54)/s}$, and $\alpha_{i/s(Bam)}$ polypeptides, but not α_s , were myristy-

FIG. 3. Measurement of cAMP levels in COS cells transfected with α_i/α_s chimeras. Three days after transfection with the indicated pCW1 α -subunit constructs, COS cells were assayed for intracellular cAMP levels by radioimmunoassay. Ten minutes prior to fixation of the cells, the cAMP phosphodiesterase inhibitor IBMX (0.5 mM) was added to the culture to inhibit cAMP phosphodiesterase in the presence or absence of 1 μ M isoproterenol (ISO), a β -adrenergic receptor-selective agonist. Data represent the mean of triplicate determinations, which varied by less than 10%, and are representative of three independent transfections.

lation substrates (Fig. 4). Since $\alpha_{i/s(BAM)}$ behaves as a functional wild-type α_s molecule in coupling to receptors and adenylyl cyclase activation (Fig. 3) but is myristylated similarly to $\alpha_{i(54)/s}$, the enhanced activity of $\alpha_{i(54)/s}$ cannot be explained simply by a difference in myristylation state between the two NH₂-terminal chimeras.

Cholera toxin ADP-ribosylates Arg-201 in the α_s polypeptide and is a sensitive measure for quantifying functional α_s in plasma membranes. As predicted from the immunoblotting experiment (Fig. 2), increased cholera toxin-catalyzed

FIG. 4. Myristylation of transiently expressed α_i/α_s chimeras. At 44 h after transfection COS cells were incubated with [³H]myristic acid (200 μ Ci/ml) for 4 h and harvested, and membranes were prepared as described in Materials and Methods. Lanes represent an autoradiograph of an SDS-gel of membranes from COS cells transfected with pCW1 without a cDNA insert or with pCW1 containing cDNAs encoding either α_s , α_i , $\alpha_{i(54)/s}$ or $\alpha_{i/s(BAM)}$. Equal amounts of membrane protein (70 μ g) were loaded in each lane.

FIG. 5. Cholera toxin-catalyzed ADP ribosylation of α_i/α_s chimeras. Membranes (50 μ g of protein) from COS cells transfected with the appropriate α -subunit cDNA construct were incubated with [³²P]NAD and activated cholera toxin (30). [³²P]ADP-ribosylated products were resolved by SDS-polyacrylamide gel electrophoresis and identified by autoradiography. The α_s polypeptide is a strong cholera toxin substrate. The $\alpha_{i(54)/s}$ polypeptide, which migrates as a 48-kDa band (arrow), is weakly ADP-ribosylated by cholera toxin. The α_i and $\alpha_{i/s(BAM)}$ polypeptides are not ADP-ribosylated by cholera toxin.

[³²P]NAD labeling of the 52-kDa form of α_s was observed in membranes from COS cells transfected with pCW1 α_s (Fig. 5). In contrast, membranes from pCW1 $\alpha_{i/s(BAM)}$ -transfected COS cells exhibited no increase compared with control levels in cholera toxin-labeled products. Membranes from COS cells transfected with pCW1 $\alpha_{i(54)/s}$ showed a faint [³²P]ADP-ribosylated band corresponding to the $\alpha_{i(54)/s}$ polypeptide. Since the polypeptides encoded by these chimeric cDNAs were readily detected by immunoblotting (Fig. 2), this experiment indicates that $\alpha_{i(54)/s}$ is a very poor substrate and that $\alpha_{i/s(BAM)}$ is not recognized at all by cholera toxin. The ADP-ribosylated residue in α_s is Arg-201 and corresponds to Arg-179 in α_{i2} . Despite the high conservation of primary sequence immediately surrounding α_s Arg-201 and α_{i2} Arg-179, α_{i2} is not a substrate for cholera toxin. Thus, it was not particularly surprising that cholera toxin failed to ADP ribosylate $\alpha_{i/s(BAM)}$, since the arginine residue is contributed by α_{i2} in this construct. However, the poor recognition of $\alpha_{i(54)/s}$ by cholera toxin was unexpected, since only 10% of the primary sequence is contributed by α_{i2} and this sequence is located at the extreme NH₂ terminus. Since the $\alpha_{i(54)/s}$ polypeptide is a functional, highly active α_s molecule, its poor recognition by cholera toxin indicates that a significant change in its tertiary structure has been produced by mutation of the α_s NH₂ terminus.

Stable expression of $\alpha_{i(54)/s}$ and $\alpha_{i/s(BAM)}$ chimeras in CHO cells. To further characterize the phenotype of cells expressing the $\alpha_{i(54)/s}$ chimeric polypeptide, CHO cells were transfected with the wild-type and chimeric constructs and G418-resistant clones were selected (31). Stable expression of the $\alpha_{i(54)/s}$ chimera increased basal cAMP levels two- to threefold in CHO cells, resulting in the constitutive activation of the cAMP-dependent protein kinase (Table 1). In contrast to the results with transient expression in COS cells, stable expression of α_s or $\alpha_{i/s(BAM)}$ had little effect on the cAMP levels or the cAMP-dependent protein kinase activity in CHO cells. Expression of α_{i2} or transfection with control plasmid also had no significant influence on the phenotype of CHO cells, indicating that the constitutive elevation of cAMP levels and kinase activation were unique properties of the chimeric $\alpha_{i(54)/s}$ construct.

In the presence of the phosphodiesterase inhibitor methyl isobutylxanthine (IBMX), the constitutive increase in cAMP

TABLE 1. cAMP levels and protein kinase activity in stable CHO cell transfectants^a

Clone	cAMP level (pmol/mg of protein) ^b		cAMP-dependent protein kinase activity (pmol/min per mg of protein) ^c		
	-IBMX	+IBMX	Basal	+cAMP	Activity ratio ^d
Wild type	3.9	21.3	74.9	1,763	0.04
neo-1	5.4	12.0	82.9	1,410	0.06
neo-4	5.1	19.8	152.9	2,283	0.07
neo-7	6.3	19.2	151.2	2,092	0.07
α_s -5	5.2	38.6	214.9	2,036	0.10
α_s -14	4.4	31.6	259.3	2,098	0.12
α_i -2	3.9	30.4	139.7	1,710	0.08
α_i -13	6.1	25.4	241.0	2,079	0.12
$\alpha_{i/s(Bam)}$ -4	6.5	63.6	214.8	2,058	0.10
$\alpha_{i/s(Bam)}$ -15	6.4	33.0	211.3	2,326	0.09
$\alpha_{i/s(Bam)}$ -16	5.8	21.1	209.2	1,948	0.11
$\alpha_{i/s(Bam)}$ -17	6.7	58.4	295.8	2,036	0.14
$\alpha_{i(54)/s}$ -10	7.5	110.2	999.8	2,340	0.43
$\alpha_{i(54)/s}$ -12	9.3	119.1	1158	2,369	0.49
$\alpha_{i(54)/s}$ -13	11.2	125.2	836.6	1,953	0.43
$\alpha_{i(54)/s}$ -15	10.3	103.1	1026	1,856	0.55

^a CHO cells were transfected by electroporation by using the appropriate pCW1 construct. Independent G418-resistant clones (1×10^6 to 3×10^6 cells per 60-mm dish) expressing the plasmid α -subunit transcripts were incubated for 10 min at room temperature in the absence or presence of 500 μ M IBMX.

^b Cellular cAMP was extracted with 1 ml of ice-cold 60% ethanol and assayed with a radioimmunoassay kit from Amersham.

^c cAMP-dependent protein kinase activity was assayed as described previously (30, 31).

^d The activity ratio is the cAMP-dependent protein kinase activity measured in the absence of added cAMP divided by the maximal kinase activity determined in the presence of 3 μ M cAMP.

synthesis between the different clones was more pronounced, being four- to fivefold greater in the various $\alpha_{i(54)/s}$ -expressing CHO clones relative to α -, $\alpha_{i/s(Bam)}$ - or α_i -transfected cells. The significantly enhanced cAMP levels in the presence of IBMX are indicative of increased cAMP phosphodiesterase activity in the $\alpha_{i(54)/s}$ -chimera-expressing clones. This phenomenon has been observed in other cell types, in which cAMP analogs or hormones have been used to chronically activate cAMP-dependent protein kinase (5). Thus, the constitutive activation of adenylyl cyclase activity in CHO cells results in compensatory increases in cAMP phosphodiesterase activity in an attempt to blunt the elevated intracellular cAMP levels. In addition to the increase in cAMP phosphodiesterase activity observed with $\alpha_{i(54)/s}$ -expressing clones, there was a modest 1.1- to 1.3-fold increase in β -subunit expression as determined by immunoblotting and densitometry (data not shown). CHO clones transfected with wild-type α_s or $\alpha_{i/s(Bam)}$ showed no change in β -subunit expression. However, even with the enhanced cAMP phosphodiesterase activity and modest increase in β -subunit expression, the CHO cells expressing $\alpha_{i(54)/s}$ exhibited constitutively activated cAMP-dependent protein kinase activity. These clones have shown the constitutively elevated cAMP levels and kinase activation for more than 100 cell doublings. Thus, the $\alpha_{i(54)/s}$ chimera, which was a highly active α_s polypeptide in transient COS cell assays, behaves as a dominant constitutively active α_s molecule in stable CHO cell transfectants.

Adenylyl cyclase regulation in CHO cell transfectants. Membranes from the CHO clones expressing $\alpha_{i(54)/s}$ exhibit a significantly altered GTP γ S regulation of adenylyl cyclase activity relative to wild-type α_s - or $\alpha_{i/s(Bam)}$ -expressing clones (Fig. 6). The $\alpha_{i(54)/s}$ phenotype is manifested in adenylyl cyclase assays as a significant decrease in the time required to achieve maximal adenylyl cyclase activation by the hydrolysis-resistant GTP analog GTP γ S. This decreased lag in achieving maximal GTP γ S activation was observed in multiple independent $\alpha_{i(54)/s}$ clones but was not observed in $\alpha_{i/s(Bam)}$ - or wild-type α_s -transfected clones. The diminished

FIG. 6. Adenylyl cyclase activity in membranes from $\alpha_{i(54)/s}$ -, $\alpha_{i/s(Bam)}$ -, and wild-type α_s -expressing CHO clones. Cyclase activity was measured by using 25 μ g of membranes from the appropriate clones in the presence of 100 μ M GTP γ S. Extrapolation of the GTP γ S-stimulated V_{max} indicated that maximal adenylyl cyclase activity was achieved within 2 min for the $\alpha_{i(54)/s}$ clone and 6 min for $\alpha_{i/s(Bam)}$ and wild-type α_s CHO clones. The diminished lag time to achieve maximal GTP γ S-stimulated cyclase activity with $\alpha_{i(54)/s}$ expression was observed in three independent $\alpha_{i(54)/s}$ -expressing clones compared with two $\alpha_{i/s(Bam)}$ or wild-type α_s CHO clones. A single representative clone for each phenotype is shown.

lag time associated with $\alpha_{i(54)/s}$, but not wild-type α_s or $\alpha_{i/s(Bam)}$, is most simply explained by an accelerated rate of GDP dissociation, allowing a faster GTP γ S binding and adenylyl cyclase activation.

Expression of $\alpha_{i(54)/s}$ in *cyc*⁻S49 lymphoma cells. The *cyc*⁻S49 mouse lymphoma variant is deficient in α_s expression at both the mRNA and protein levels (11). Expression of the $\alpha_{i(54)/s}$ chimera in *cyc*⁻S49 cells reconstituted a functional hormone-stimulated adenylyl cyclase system whose responsiveness to isoproterenol was similar to that of the wild-type S49 cell line (Fig. 7). Immunoblotting demonstrated that the level of $\alpha_{i(54)/s}$ polypeptide expression was only about 10 to 20% of the level of 45- and 52-kDa α_s polypeptides expressed in the wild-type clone (Fig. 7A). Despite its low level of expression, the similar level of cAMP accumulation in response to isoproterenol in the $\alpha_{i(54)/s}$ -*cyc*⁻S49 clone relative to wild-type S49 cells is consistent with an increased intrinsic activity of the chimera compared with the wild-type gene product (Fig. 7B).

Kinetic analysis of adenylyl cyclase activation in membranes from $\alpha_{i(54)/s}$ -*cyc*⁻S49 and wild-type S49 cells indicated that the intrinsic GTPase activity of the chimeric polypeptide was similar to that of wild-type α_s (Fig. 7C). Measurement of the time course of propranolol-induced inhibition of isoproterenol-stimulated adenylyl cyclase activation permitted estimation of the rate constant for the GTPase turn-off mechanism (k_{off}) (19). Evaluation of the k_{off} by using this indirect method has been shown to be a reliable measure of the k_{cat} for the intrinsic GTPase reaction of α_s and different α_s mutants (10). The wild-type α_s polypeptides (52- and 45-kDa forms) in S49 cell membranes exhibit a k_{off} of approximately 5 to 6 min⁻¹. A similar value was estimated for the $\alpha_{i(54)/s}$ -*cyc*⁻S49 clone, indicating that the GTPase activity of the chimera is not affected by the NH₂-terminal mutations. In contrast, the α_s Leu-227 mutation, whose GTPase k_{off} is approximately 0.05 min⁻¹ (19), inhibits the rate of GTP hydrolysis by more than 95% (10). These findings indicate that the molecular basis for the enhanced intrinsic activity of the $\alpha_{i(54)/s}$ chimera is not altered GTPase activity but is related to an accelerated rate of GDP dissociation and GTP binding as measured by the diminished lag time required for GTP γ S activation of adenylyl cyclase (Fig. 6).

The $\alpha_{i(54)/s}$ polypeptide was shown to be activated by isoproterenol in both COS cells (Fig. 3) and $\alpha_{i(54)/s}$ -*cyc*⁻S49 cells (Fig. 7). Normal interaction of the $\alpha_{i(54)/s}$ polypeptide with β -adrenergic receptors was further substantiated by competition binding experiments with [¹²⁵I]ICYP. GTP caused a nearly 10-fold decrease in the affinity of isoproterenol for binding to the β -adrenergic receptor in membranes from wild-type S49 cells (Fig. 8). The $\alpha_{i(54)/s}$ polypeptide also regulated, in a GTP-dependent manner, the affinity of the β -adrenergic receptor for the agonist isoproterenol. The magnitude of the GTP-induced affinity shift in the $\alpha_{i(54)/s}$ -*cyc*⁻S49 clone was similar to that observed with the wild-type membranes. Thus, the $\alpha_{i(54)/s}$ chimera is functional in activation of adenylyl cyclase and regulation of receptor affinity for agonists, indicating that the NH₂ terminus is involved in the intramolecular control of α_s activity and is apparently distinct from the α_s domains that directly interact with receptor and adenylyl cyclase.

DISCUSSION

The α_s and α_{i2} polypeptides are approximately 65% homologous in amino acid sequence (16). Differences in amino

acid homology between α_s and α_{i2} are fairly evenly distributed in the NH₂- and COOH-terminal halves of the polypeptides, with the highly conserved guanine nucleotide-binding and GTPase domains of the α subunits exhibiting the greatest homology. Common functions of α_s and α_{i2} include interaction with adenylyl cyclase and association with a common pool of $\beta\gamma$ subunits (4, 7, 22). However, α_s and α_{i2} selectively couple to different receptors. The $\alpha_{i/s(Bam)}$ chimera, first described by Masters et al. (20), behaves as a functional wild-type α_s polypeptide with respect to α_s receptor selectivity and adenylyl cyclase activation. $\alpha_{i/s(Bam)}$ therefore defines the COOH-terminal 40% of α_s as encoding cyclase activation and receptor selectivity. The $\alpha_{i(54)/s}$ chimera also maintains the functions of adenylyl cyclase activation and coupling to the β -adrenergic receptor, similar to $\alpha_{i/s(Bam)}$, because the COOH-terminal moiety of $\alpha_{i(54)/s}$ is encoded by the wild-type α_s sequence. In contrast to $\alpha_{i/s(Bam)}$, the $\alpha_{i(54)/s}$ polypeptide behaves as a dominant α_s mutant whose activity is constitutively enhanced. The difference between $\alpha_{i(54)/s}$ and $\alpha_{i/s(Bam)}$ is α_s residues 62 to 234, present only in $\alpha_{i(54)/s}$, and α_{i2} residues 55 to 212, present in the $\alpha_{i/s(Bam)}$ chimera. Within the domain defined by these respective sequences, there are 76 nonconserved amino acids. In addition, there is a 15-amino-acid insertion in the α_s sequence (Gly-72 to Gly-86) that is differentially spliced at the mRNA level to account for the 52- and 45-kDa forms of α_s . This sequence (Gly-72 to Gly-86) is not essential for wild-type G_s activity (21). Finally, at the extreme NH₂ terminus of α_s there are seven unique residues (Leu-4 to Asn-6 and Asp-10 to Arg-13) that are absent in α_{i2} and therefore absent in the $\alpha_{i/s(Bam)}$ and $\alpha_{i(54)/s}$ polypeptides.

The $\alpha_{i/s(Bam)}$ chimera, which exhibits wild-type α_s activity, divides the α_s polypeptide into two large domains (20). The COOH-terminal 40% of the molecule can be assigned receptor selector-effector activator functions (Fig. 9). The constitutively active behavior of the $\alpha_{i(54)/s}$ chimera indicates that the NH₂-terminal moiety of α_s functions as a regulable attenuator domain. Mutation of the NH₂ terminus of α_s results in the loss of attenuator function and enhanced α_s activity. Loss of attenuator control of an α_s polypeptide promotes enhanced intracellular cAMP synthesis and the decreased lag time required for GTP γ S to maximally activate adenylyl cyclase activity in cell membrane preparations. The attenuator control of the NH₂-terminal moiety of α_s is independent of the GTPase activity intrinsic to the polypeptide, which functions as a shutoff mechanism for the activated α subunit by hydrolyzing bound GTP to GDP (10, 19, 30).

The attenuator function assigned to the NH₂-terminal moiety of α_s is normal in the $\alpha_{i/s(Bam)}$ chimera because the entire α_{i2} attenuator domain is intact. In contrast, the $\alpha_{i(54)/s}$ chimera leads to mutations that disrupt the attenuation function of the NH₂-terminal moiety. The mutations in α_s that result from the $\alpha_{i(54)/s}$ construct are within the first 41 residues of the α_s polypeptide. This suggests that the extreme NH₂ terminus of α_s functions as a modulator of the attenuator activity within this domain. The $\alpha_{i(54)/s}$ phenotype mimics G-proteins that have been activated by receptors, since agonist-activated receptors accelerate the rate of GDP-GTP exchange at the α -subunit guanine nucleotide-binding site (25, 27). In membrane preparations, hormone-activated receptors markedly diminish the lag time for GTP γ S activation of adenylyl cyclase (25), similar to what is observed with the $\alpha_{i(54)/s}$ chimera. In a sense, receptors interact with G-proteins and relieve the attenuator function, allowing enhanced GDP dissociation.

FIG. 7. Expression of the $\alpha_{i(54)/s}$ chimera in *cyc*⁻S49 lymphoma cells. (A) Immunoblotting of membrane proteins from wild-type S49 cells or $\alpha_{i(54)/s}$ -transfected *cyc*⁻S49 cells shows that wild-type cells express both the 52- and 45-kDa α_s polypeptides, whereas *cyc*⁻S49 cells expressing $\alpha_{i(54)/s}$ demonstrate an immunoreactive 48-kDa band characteristic of the behavior of the chimeric polypeptide (Fig. 2). *cyc*⁻S49 cells used for $\alpha_{i(54)/s}$ expression do not express the α_s gene product. (B) Wild-type, *cyc*⁻S49, and *cyc*⁻ $\alpha_{i(54)/s}$ S49 cells (10^6 /ml) were incubated for 10 min with the indicated combinations of no addition (basal), 500 μ M IBMX, 10 μ M isoproterenol, or 100 μ M forskolin. After 10 min, the cells were rapidly chilled and centrifuged, and the cell pellets were fixed in 70% ethanol. cAMP levels after each incubation were determined by radioimmunoassay. Values represent the means of triplicate determinations that varied by less than 10% and are representative of two independent experiments. (C) GTPase activity of α_s polypeptides in membranes from wild-type S49 and *cyc*⁻ $\alpha_{i(54)/s}$ cells was estimated by determining the rate at which propranolol inactivated isoproterenol/GTP-stimulated adenylyl cyclase activity. At 0 min, adenylyl cyclase was stimulated with 10 μ M isoproterenol plus 100 μ M GTP (—). At 5 min one-half of each incubation was mixed with 10 μ M propranolol (---), 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 wild-type membranes returned to basal levels, which is due to the GTPase shutoff mechanism intrinsic to the α_s polypeptide (10, 19). The GTPase activity of the *cyc*⁻ $\alpha_{i(54)/s}$ coupled adenylyl cyclase system was similar to that for the wild-type α_s -regulated adenylyl cyclase.

The α_s attenuator control that is absent in the $\alpha_{i(54)/s}$ polypeptide is characteristic of the inhibitory functions ascribed to the $\beta\gamma$ subunit complex, which has been shown in reconstitution assays with purified α_s to inhibit GDP release and attenuate α_s activation of adenylyl cyclase (12). However, the $\alpha_{i(54)/s}$ phenotype cannot be explained simply as a

loss of $\beta\gamma$ interaction with the α subunits. It is well documented that $\beta\gamma$ is essential for coupling of the α subunit to receptor. In the absence of the $\beta\gamma$ complex the catalysis of α -subunit GDP/GTP exchange is less than 5% of that observed in the presence of $\beta\gamma$ (6, 17). It has also been shown that both α and $\beta\gamma$ subunits have contact sites on the

FIG. 8. GTP-regulated affinity of isoproterenol binding to the β -adrenergic receptor. Membranes (10 μ g of protein) were incubated with 375 pM [125 I]ICYP (2×10^5 cpm) in the presence of various concentrations of isoproterenol with (●) or without (○) 100 μ M GTP. Membranes from wild-type (WT) and *cyc*- $\alpha_{i(54)/s}$ S49 cells showed a similar GTP-mediated shift in K_d for isoproterenol, indicating that $\alpha_{i(54)/s}$ is coupled to the β -adrenergic receptor with similar efficiency as the wild-type α_s polypeptide.

cytoplasmic surface of appropriate receptors (17). Our findings unequivocally show that the $\alpha_{i(54)/s}$ polypeptide is very efficiently coupled to receptors, measured by the ability of isoproterenol to activate adenylyl cyclase and the GTP regulation of agonist affinity for the β -adrenergic receptor. Both of these receptor-coupled functions require the association of $\beta\gamma$ with the α subunit. Thus, the $\alpha_{i(54)/s}$ polypeptide must be associated with the $\beta\gamma$ complex. We cannot dismiss the possibility that the inhibitory function, but not the receptor-coupling function, is altered in the interaction of $\beta\gamma$

with the $\alpha_{i(54)/s}$ polypeptide relative to wild-type α_s . We are presently attempting to map $\beta\gamma$ contact sites on α_i and α_s polypeptides to determine whether an altered $\beta\gamma$ regulation of GDP dissociation is responsible for the $\alpha_{i(54)/s}$ phenotype.

Previously, we described a similar constitutively activated α_s polypeptide by mutation at the COOH terminus (31). The COOH-terminal mutant, referred to as $\alpha_{s/i(38)}$, had the last 38 amino acids of α_s replaced by the last 36 residues of α_{i2} . The $\alpha_{s/i(38)}$ mutant constitutively elevated cAMP levels in CHO cells, and its influence on adenylyl cyclase regulation was

FIG. 9. Model defining the proposed regulatory domains of the α_s and α_i polypeptides. The COOH-terminal moiety (C) functions in determining the specificity for receptor coupling and activation of adenylyl cyclase, whereas the NH₂-terminal moiety (B) regulates GDP dissociation and behaves as an attenuator of the COOH-terminal effector activating function. The extreme NH₂ terminus (A) modulates the attenuator activity of domain B, and the extreme COOH terminus (D) modulates the activator domain C. Mutation of either A or D, as is observed in the α_i/α_s chimeras (i.e., $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$) results in loss of modulator function of domains B or C, causing a constitutively activated α subunit (see text for detailed discussion).

similar to but distinguishable from the NH₂-terminal chimera described here. The $\alpha_{s/i(38)}$ chimera exhibited a diminished lag for GTP γ S activation of adenylyl cyclase similar to that of $\alpha_{i(54)/s}$ (Fig. 6) but also demonstrated a marked three- to sixfold increase in $V_{\max}$ for GTP γ S-stimulated cyclase activity. The similarly diminished lag time for $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ chimeras to activate adenylyl cyclase in the presence of GTP γ S indicates that the NH₂ and COOH termini of the α -subunit polypeptide have overlapping regulatory functions. However, the marked increase in $V_{\max}$ for adenylyl cyclase stimulation of GTP γ S observed only with the COOH-terminal mutant indicates that this region of the molecule directly influences cyclase activation by the α_s -GTP γ S complex.

Putative tertiary structural properties of the α_s and α_i subunits, drawn by analogy with the p21ras polypeptide, provide a basis for understanding the NH₂- and COOH-terminal mutations (i.e., $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$, respectively) and the mechanism by which they constitutively activate the G protein, G_s. The tertiary structure of p21ras deduced from X-ray crystallography (23) roughly defines the molecule in two halves, or domains, with the guanine nucleotide-binding site in proximity to the cleft between the halves. The crystal structure also shows that the NH₂- and COOH-terminal ends of the molecule are in close proximity to each other. A similar model for a prototypical α subunit of a G protein has recently been proposed (14), and there is physical evidence that the NH₂- and COOH-terminal ends of α_i , the retinal G-protein α subunit, are in close proximity to one another in the tertiary structure of the polypeptide (13). Figure 9 depicts the proposed halves of the α_s molecule with the NH₂ and COOH termini in close proximity. The GTP-binding site would lie in proximity to the groove between the NH₂- and COOH-terminal halves, similar to that for p21ras. The COOH-terminal domain functions as the receptor selector-effector activator as defined by the $\alpha_{i/s(Bam)}$ chimera, and the NH₂-terminal domain functions as an attenuator as determined by the $\alpha_{i(54)/s}$ mutant. The termini of the α_s subunit function as modulators of the two domains to control both activation and attenuation. When this control is lost, as in the $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$ chimeras, the result is a constitutively activated α_s molecule. We propose a model (Fig. 9) in which the attenuator function (B) is regulated by the NH₂ terminus (A) and the selector-activator (C) is regulated by the COOH terminus (D). Substitution of an α_s modulator (A or D) with an α_i modulator (A' or D') results in the loss of B or C regulation (i.e., $\alpha_{i(54)/s}$ and $\alpha_{s/i(38)}$). However, substitution of (A + B) with (A' + B') linked to (C + D) results in the wild-type phenotype (i.e., $\alpha_{i/s(Bam)}$). Our structural hypothesis develops the notion of modulator domains within G-protein α chains. We do not intend to suggest that these have to be discrete "domains" independent of other determinants within the tertiary structure of the polypeptide, but, rather, that mutation within these sequences dramatically alters the modulation of α -chain activation.

One prediction of this model is that mutations in the modulator domains, which we have shown to not influence the GTPase activity of the polypeptide, should be additive with mutations, such as α_s Leu-227, which dramatically inhibit the GTPase. Introduction of the Leu-227 mutation into $\alpha_{i(54)/s}$ resulted in an α subunit which elevated cAMP levels in different cell types after gene transfection to levels that were essentially additive of the two individual mutations (S. Osawa et al., unpublished data). This finding indicates that the double mutation $\alpha_{i(54)/s}$ Leu-227 provides a strong dominant constitutively active α_s polypeptide which has lost

both GTPase shutoff and attenuation of GDP dissociation. Like the various point mutations that have greatly increased the understanding of the GTP-binding/GTPase domains, these constructs should prove invaluable in studies of the regulatory attenuator domains intrinsic to G protein α subunits and their control by activated receptors.

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