Journal of Cellular Biochemistry 47:136–146 (1991)

Genetic and Structural Analysis of G Protein α Subunit Regulatory Domains

Gary L. Johnson, N. Dhanasekaran, Sunil K. Gupta, Joseph M. Lowndes, Richard R. Vaillancourt, and Arnold E. Ruoho

National Jewish Center for Immunology and Respiratory Medicine and Department of Pharmacology, University of Colorado Medical School, Denver, Colorado 80206 (G.L.J., S.K.G., J.M.L.); and Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706 (N.D., R.R.V., A.E.R.)

Abstract Genetic and structural analysis of the α chain polypeptides of heterotrimeric G proteins defines functional domains for GTP/GDP binding, GTPase activity, effector activation, receptor contact and βγ subunit complex regulation. The conservation in sequence comprising the GDP/GTP binding and GTPase domains among G protein α subunits readily allows common mutations to be made for the design of mutant polypeptides that function as constitutive active or dominant negative α chains when expressed in different cell types. Organization of the effector activation, receptor and βγ contact domains is similar in the primary sequence of the different α subunit polypeptides relative to the GTP/GDP binding domain sequences. Mutation within common motifs of the different G protein α chain polypeptides have similar functional consequences. Thus, what has been learned with the Gs and Gi proteins and the regulation of adenylyl cyclase can be directly applied to the analysis of newly identified G proteins and their coupling to receptors and regulation of putative effector enzymes.

Key words: heterotrimeric G proteins, adenylyl cyclase, phospholipases, ion channels, GTP, GDP

Heterotrimeric G proteins couple to plasma membrane-associated receptors and have been shown to regulate adenylyl cyclase, phospholipases A_2 and C, cGMP phosphodiesterase, and selected ion channels [for review see 1–4]. The α subunit of G proteins bind GDP/GTP in a highly conserved guanine nucleotide binding site. In addition to GDP/GTP binding, the G protein α subunit has several critical functions which are encoded within the polypeptides primary sequence. The α chain functions include GTPase activity, interaction with the βγ subunit complex, coupling to receptors, and regulation of effector enzyme and ion channel activity. Functions encoded within all α chain polypeptides, therefore, include GDP/GTP binding, GTPase activity and βγ association. Unique functions for different α chains include receptor selectivity and effector regulation. The distinction among common and unique α chain functions is not absolute, however, because the heterogeneity of βγ complexes may infer a second tier of complexity in receptor selectivity and effector regulation. Since βγ is essential for receptor activation of α subunits [1–7], variable expression of β and γ subunit isoforms may influence the efficiency of receptor catalyzed G protein activation.

The "turn-on" mechanism for G proteins is regulated by both receptors and the βγ subunit complex. Turn-on requires the exchange of GDP for GTP which is catalyzed by hormone activated receptors [1–4,8,9] and the subsequent dissociation of α_{GTP} from βγ. Free α subunits are poor substrates for hormone activated receptors, whereas the $\alpha_{GDP}\beta\gamma$ heterotrimer readily binds activated receptor. The βγ complex associated with α_{GDP} also inhibits GDP dissociation in the absence of activated receptor, ensuring that without hormonal stimulation G proteins remain in a basal inactive state.

The "turn-off" mechanism for G proteins is a function of the GTPase activity intrinsic to the α chain [1–4,8,9]. Once the α_{GTP} complex is formed, the rate of hydrolysis of the bound GTP determines the lifetime of the activated α chain and

Received July 2, 1991; accepted July 9, 1991.

Address reprint requests to Dr. Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206.

regulation of the appropriate effector enzyme or ion channel. Bound GTP is hydrolyzed to GDP returning the α chain to an inactive state. Cycling to an activated α_{GTP} form requires the subsequent interaction with the hormone-stimulated receptor which catalyzes GDP dissociation.

We present here our mutational and structural analysis of α chain domains which are involved in the control of G protein functions described above. Based on the conservation of sequence among the growing list of G protein α subunits, it is now possible to predict mutations that will selectively influence the different α chain domains. The consequence of specific mutations on α chain function is developed in the framework of our understanding of G protein structure and subunit interactions.

Structure of the GDP/GTP Binding Domain

No crystal structure for G protein α chain polypeptides currently exists. Consequently, understanding of the α chain GDP/GTP binding site 3D structure has relied upon the conservation of the GDP/GTP binding domain between G proteins, p21*ras* and EF-Tu, where crystal structure exists for the latter two GDP/GTP binding proteins [10–13]. Table I shows the sequences comprising the GDP/GTP binding domain for G protein α chains, Ha-p21*ras* and EF-Tu. The consensus sequences in domains G-1 thru -4 are signatures for a large number of GDP/GTP binding proteins [8,9].

Mutations in G-1 and G-3 regions of the p21*ras* GDP/GTP binding site which inhibit GTPase activity are transforming and occur at high frequency in many human cancers [14]. In G-1 mutation of *ras* at Gly12 or Gly13 and in G-3 mutation of Ala59 or Gln61 inhibit GTP hydrolysis leading to constitutive activation of the mutant p21*ras* polypeptide [8,9]. In $G\alpha_s$ mutation of Gly49 or Gln227, which respectively correspond to the aforementioned Gly12 and Gln61 in p21*ras*, similarly results in inhibition of GTPase activity and constitutive activation of the mutant α_s polypeptide [15–17]. Functional consequences are also observed resulting from mutation of p21*ras* Gly60 and the corresponding α_sGly226 residues in the G-3 domain as they inhibit the activity of both p21*ras* and $G\alpha_s$, respectively. The crystal structure of p21*ras* clearly shows this glycine functions as a "pivot" for GTP-induced conformational changes in the GDP/GTP binding protein [11–13]. Substitution of this critical pivot-point glycine with other amino acids prevents the polypeptide from assuming an activated GTP conformation.

Differences in the consequence of additional mutations within the GDP/GTP binding domains, G-3 and G-4 of p21*ras* and $G\alpha_s$, however, distinguish regulatory properties of the two polypeptides (Table II). Mutation of Ala59 to threo-

TABLE I. Sequences Comprising the GDP/GTP Binding Domain

	G-1	G-2	G-3	G-4
EF-Tu	^{13}NVGTIGHVDHGKTTLT	^{50}DNAPEEKARGITINTS	^{76}YAHVDCPGHA	^{131}IVFLNKCD
Ha-p21-*ras*	^{5}KLVVVGAGGVGKSALT	^{32}YDPTIEDSY	^{53}LDILDTAGQE	^{112}VLVGNKCD
$G\alpha_s$	^{42}RLLLLGAGESGKSTIV	^{196}DILRCRVLTGIFE	^{219}FHMFDVGGQR	^{288}ILFLNKQD
$G\alpha_{i2}$	^{35}KLLLLGAGESGKSTIV	^{173}DILRTRVKTTGIVE	^{196}FRLFDVGGQR	^{265}ILFLNKKD
Consensus	GXXXXGKS	DX_nT	DXXG	NKXD
Function	Binding loop for α and β phosphates of GDP and GTP.	Important for binding of Mg^{2+} ion that is coordinated to oxygens of β and γ phosphates; important for GTPase activity.	Invariant aspartate binds the catalytic Mg^{2+} ion thru a H_2O molecule and glycine forms a hydrogen bond with GTP γ-phosphate; glycine functions as a "pivot" for conformational change induced by GTP.	Aspartate forms hydrogen bond with guanine ring and amides of asparagine and lysine stabilize the GDP/GTP binding site by hydrogen bonds to residues in G-1 region.

TABLE II. Consequence of Common Mutations in GDP/GTP Binding Domains of p21*ras* and α_s*

	Domain	GTPase activity	Transformation	Adenylyl cyclase stimulation
	G-1			
p21*ras*	^{12}Gly → Val	Inhibited	++	n.a.
α_S	^{49}Gly → Val	Inhibited	n.a.	++
	G-3			
p21*ras*	^{59}Ala → Thr	Inhibited	++	n.a.
α_S	^{225}Gly → Thr	?	n.a.	Inhibited (behaves as a dominant negative)
p21*ras*	^{60}Gly → any a.a.	Unable to assume GTP conformation	—(inactive)	n.a.
α_S	^{226}Gly → Ala	Unable to assume GTP conformation	n.a.	—(inactive)
p21*ras*	^{61}Gln → Leu	Inhibited	++	n.a.
α_S	^{227}Gln → Leu	Inhibited	n.a.	++
	G-4			
p21*ras*	^{119}Asp → Ala	Decreases affinity for GDP and GTP, GTPase normal	++	n.a.
α_S	^{295}Asp → Ala	Decreases affinity for GDP and GTP, GTPase normal	n.a.	Null

*n.a., *not applicable*; mutations in the G-2 domain have not been compared between p21*ras* and α_S, however, mutation of α_S ^{201}Arg → Cys or Pro inhibits GTPase activity (20); Null, similar to wild-type.

nine in the G-3 domain of p21*ras* is a common transforming mutation resulting from GTPase inhibition. In contrast, mutation at the corresponding residue of Gα_s, Gly225 to threonine, results in loss of adenylyl cyclase activation similar to that observed with mutation of Gly226. The contrast in properties of the Ala59 → Thr and Gly225 → Thr mutations in p21*ras* and α_s indicates the regulation of the two polypeptides by GTP mediated conformational changes involving the G-3 domain are not identical. Similarly, mutation in the NKXD G-4 domain defines a second apparent difference in the guanine nucleotide regulation of p21*ras* and Gα_s. The aspartate residue in the G-4 domain interacts with the C-2 amino group on the guanine ring to stabilize the binding of GDP and GTP. In p21*ras*, replacement of this aspartate (Asp119) with an alanine (Asp119 → Ala) reduced the affinity of both GTP and GDP by a factor of 20 [18]. However, the ability of the p21*ras*Asp119 → Ala mutant to induce transformation of NIH3T3 cells was similar to that of the oncogenic p21*ras* having Gly12 → Val and Ala59 → Thr mutations. The increased transformation potential observed with the reduced affinity for GDP and GTP in the p21*ras*Asp119 → Ala mutant was attributed to an increased dissociation rate for bound GDP. Asp119 in p21*ras* corresponds to Asp295 in the α_s polypeptide. Mutation of Asp295 → Ala in the α_s polypeptide had no influence on the ability to stimulate cAMP synthesis relative to the wild-type α_s chain [19]. Thus, the α_sAsp295 → Ala mutant is not an activated α_s polypeptide in contrast to the results obtained with the α_sGly49 → Val and Gln227 → Leu mutations.

The failure of the α_sAsp295 → Ala mutant to be an activated α_s may result from intrinsic differences in the GTPase regulatory properties of p21*ras* and G protein α chains. The p21*ras* polypeptide has a low intrinsic GTPase activity with a k_{cat} of approximately 0.02 min^{-1}. In the presence of a second gene product, the GTPase activating protein or GAP, the p21*ras* GTPase activity is stimulated to a k_{cat} of about 1 min^{-1}. In contrast, the G protein α chain GTPase activity is not regulated by an independent GAP-like protein, but rather has an intrinsically high GTPase activity (k_{cat} ~ 3–5 min^{-1}) by itself. Thus, even though the GDP dissociation rate may be enhanced in the α_sAsp295 → Ala mutant, the combination of a diminished GTP affinity and the intrinsic high GTPase activity of α_s may prevent the Asp295 → Ala mutation from activating the α_s polypeptide measured by increased adenylyl cyclase activity. This hypoth-

esis is testable by introducing the Asp295 → Ala mutation in the same polypeptide that also encodes a GTPase inhibiting mutation (i.e., Gly49 → Val or Gln227 → Leu). The prediction is that inhibition of the α_s GTPase activity would allow the enhanced GDP dissociation rate to be observed in the measurement of the time course of α_s activation by GTP.

The high intrinsic GTPase activity of G protein α chains appears to be a function encoded by amino acid sequences that in α_s surround Arg201 in the G-2 domain, which is the residue ADP-ribosylated by cholera toxin [20]. ADP-ribosylation of α_sArg201 results in inhibition of GTPase activity. Mutation of Arg201 to almost any other amino acid also inhibits GTPase activity [20], even though this region of the α_s polypeptide is not directly involved in the binding of GDP/GTP. It has been proposed that this G protein α chain domain functions as an intrinsic GAP-like sequence [21]. In support of this notion, residues 189–203 within the α_s polypeptide have a limited homology with the putative GAP binding site in p21*ras*. Thus, there are substantial differences in the regulation of G protein α subunits and p21*ras* that are related to the additional amino acid sequence information encoded in the α chains. The increased amino acid sequence encoded in G protein α chains most certainly results in numerous additional intramolecular contacts in their tertiary structure as well as complex interactions with other proteins including receptors and the βγ subunit complex. Table II summarizes the various mutations in the G-1 through G-4 GDP/GTP binding domains and their functional consequence in p21*ras* and α_s.

Functional Properties of α_s/α_{i2} Chimeras

To further identify regions of α chain primary sequence involved in intra- and intermolecular contacts that are important for regulation and impart the unique properties of the different G proteins we generated a series of α_s/α_{i2} chimeras and examined their characteristics. The α_s and α_{i2} polypeptides respectively stimulate and inhibit adenylyl cyclase. The G_i and G_s proteins couple to different receptors, although their βγ complexes are interchangeable [3,22–24], indicating receptor selectivity is a property of the α_s and α_i subunits. Cholera toxin ADP-ribosylates α_s but not α_i, whereas pertussis toxin ADP-ribosylates α_i but not α_s. Thus, appropriate α_i/α_s chimeras have the potential to switch functional

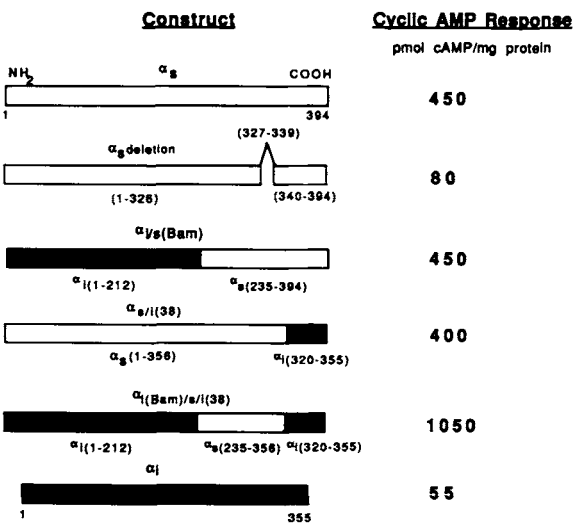


Fig. 1. Identification of the core α_s activation domain using deletion and α_s/α_i chimeras. A map of the deletion and chimeric mutants is shown on the left. Each construct was transiently expressed to similar levels in COS cells as determined by immunoblotting. The ability of each mutant to stimulate adenylyl cyclase (cyclic AMP response) relative to the wild-type α_s polypeptide is shown in the right panel as described previously [15].

domains as well as introduce multiple nonconserved amino acid substitutions within unique domains of the two α chains. The GDP/GTP binding sequences are highly conserved between α_s and α_i (Table I) indicating substitutions of G-1 thru -4 domains between the two polypeptides should have little functional consequence.

Initially three chimeras, where corresponding regions of α_s and α_i were shuttled within cDNAs, were used in expression assays in order to define domains in the α_s and α_i polypeptides responsible for regulation of adenylyl cyclase (Fig. 1). One chimera, referred to as $\alpha_{i/s(Bam)}$, encodes the first 212 residues of α_i and the COOH terminal 160 residues of α_s, yielding an α chain chimera that encodes the first 60% of α_{i2} and the last 40% of α_s. The second chimera, referred to as $\alpha_{s/i(38)}$, has the last 38 amino acids of α_s substituted with the COOH-terminal 36 residues of α_i [15,25,26]. Both mutations were also placed within the same cDNA encoding for the $\alpha_{i(Bam)/s/i(38)}$ chimeric polypeptide. Expression analysis of the three chimeras demonstrated each was a functional α_s polypeptide capable of activating adenylyl cyclase. The $\alpha_{i(Bam)/s/i(38)}$ polypeptide also appeared to be approximately 2-fold greater in ability to activate adenylyl cyclase at similar levels of expression relative to the $\alpha_{i/s(Bam)}$ and $\alpha_{s/i(38)}$ polypeptides, indicating it was a more ac-

tive α_s subunit. Cumulatively, the three chimeras define the α_s activation domain to be encoded within a 122 amino acid core sequence residing within residues Ile235-Arg356 of the α_s polypeptide. Both the NH_2-terminal 60% and COOH-terminal 10% of the α_s polypeptide may be substituted with α_i sequences and maintain the ability of the chimera to activate adenylyl cyclase. Within the core adenylyl cyclase activation domain deletion of an α_s unique 13 residue sequence (G327–Y339) resulted in complete loss of adenylyl cyclase activation. This sequence is absent in all α chain polypeptides characterized to date that do not stimulate cAMP synthesis, indicating this domain is critical for stimulation of adenylyl cyclase activity by the core activation domain.

A second NH_2-terminal chimera, referred to as $\alpha_{i(54)/s}$, has the first 61 amino acids of α_s substituted with the first 54 residues of α_i (Fig. 2). The $\alpha_{i(54)/s}$ chimera results in the loss of seven unique α_s amino acids, and 16 of the first 34 α_i residues are nonconserved relative to the α_s sequence. The last 20 α_{i2} amino acids within the chimera are identical or highly conserved when compared to the α_s sequence. The $\alpha_{i(54)/s}$ chimera behaves as an activated α_s polypeptide which robustly activates adenylyl cyclase [15,27]. The activated character of the $\alpha_{i(54)/s}$ polypeptide contrasted with that of the $\alpha_{i/s(Bam)}$ construct, which behaves as a functional wild-type α_s. It was shown that the activated character of the $\alpha_{i(54)/s}$ chimera was the result of enhanced GDP dissociation allowing GTP activation of the α chain in the absence of hormonal stimulation. Thus, the NH_2-terminal moiety of α_i and α_s may be interchanged with normal maintenance of intrinsic α_s regulation, but mutation at the extreme α_s NH_2-terminus results in loss of an attenuator function controlling α_s activation of adenylyl cyclase. It was also observed that when the $\alpha_{i(54)/s}$ mutation was placed in the same cDNA as the α_sQ227L point mutation, the resulting adenylyl cyclase activation and cAMP accumulation was additive relative to each mutation alone (Fig. 2). The enhanced rate of GDP dissociation observed with the $\alpha_{i(54)/s}$ polypeptide and the inhibited GTPase activity resulting from the α_sQ227L mutation explains their additivity. By altering the two rate-limiting steps in α chain activation (GDP dissociation and GTPase), a very strong constitutively active α_s polypeptide is observed.

Three additional chimeras were constructed with different regions of α_s substituted with corresponding NH_2-terminal regions of α_{i2} in order to further define the α_s region controlling GDP dissociation (Fig. 3). Expression of the three chimeras, $\alpha_{i(7)/s}$, $\alpha_{i(64)/s}$, and $\alpha_{i(122)/s}$, defined the boundaries within the α_s polypeptide chain that encoded the attenuator function controlling GDP dissociation. The phenotype of the $\alpha_{i(64)/s}$ chimera was similar to $\alpha_{i(54)/s}$ in its enhanced ability to stimulate adenylyl cyclase activity. The $\alpha_{i(7)/s}$ and $\alpha_{i(122)/s}$ chimeras were similar to

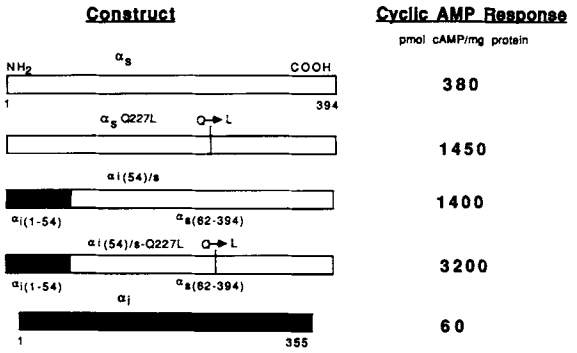


Fig. 2. Activation of cAMP synthesis by expression of the $\alpha_{i(54)/s}$ chimera and its additivity with the GTPase-inhibiting α_sQ227L mutation within the α_s polypeptide. Left panel shows each construct and the right panel shows their ability to stimulate cAMP synthesis when expressed at similar levels in COS cells.

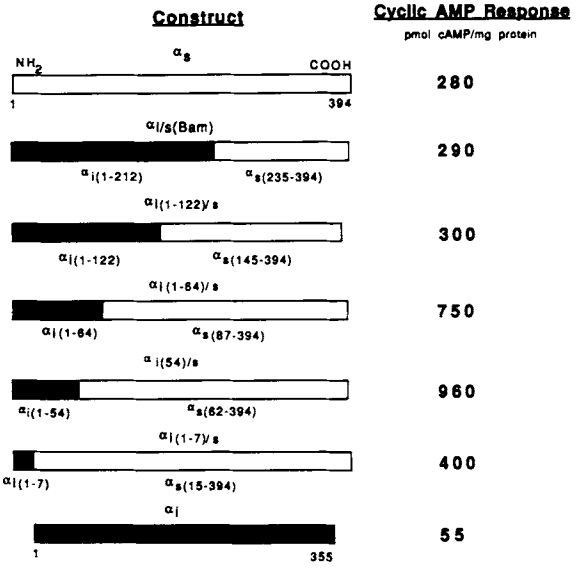


Fig. 3. Mapping of the α_s NH_2-terminus attenuation domain using α_i/α_s chimeras that were transiently expressed in COS cells. Immunoblotting demonstrated similar levels of expression for each construct. Right panel shows the ability of each construct to stimulate cAMP synthesis. The $\alpha_{i(64)/s}$ and $\alpha_{i(54)/s}$ chimeras constitutively activate adenylyl cyclase, whereas $\alpha_{i/s(Bam)}$ and $\alpha_{i(122)/s}$ are similar to wild-type. The $\alpha_{i(7)/s}$ chimera is weakly activated relative to the other chimeras.

wild-type α_s and $\alpha_{i/s(Bam)}$ in their ability to activate adenylyl cyclase. These findings demonstrated that deletion of the unique α_s sequences Leu4–Glu10 and Gly72–Gly86, which are absent in the corresponding region of α_i, are not responsible for the $\alpha_{i(54)/s}$ and $\alpha_{i(64)/s}$ activated phenotype. In addition, the $\alpha_{i(122)/s}$ chimera behaved similarly to α_s and $\alpha_{i/s(Bam)}$ in its ability to activate adenylyl cyclase, indicating that the α_s sequence Glu145–Trp234 is not involved in the phenotypic differences of $\alpha_{i(54)/s}$ and $\alpha_{i(64)/s}$ relative to the $\alpha_{i\ s(Bam)}$ polypeptide. Thus, the domain controlling the rate of GDP dissociation maps to α_s residues Gly15–Pro144. Within this region, residues corresponding to α_s Arg42–Arg62 and α_iLys35–Lys55, which contains the G-1 phosphate binding sequence, are conserved in the two α chains, indicating that these sequences are not involved in the mutant phenotype. The α_s domains involved in the activating mutation must be within the sequences Gly15–His41, Ile62–Glu71, and Glu87–Pro144. The corresponding sequences in α_i are Glu8–Val34 and Ile55–Glu122.

Requirements for Pertussis and Cholera Toxin-Catalyzed ADP-Ribosylation

Pertussis toxin catalyzed ADP-ribosylation of α_i requires the presence of the βγ subunit complex [1–4,6]. The consensus amino acid in α_i-like polypeptides ADP-ribosylated by pertussis toxin is a cysteine four residues from the COOH-terminus. The $\alpha_{s/i(38)}$ chimera, therefore, encodes the pertussis toxin ADP-ribosylation site normally found in the α_i polypeptide. The $\alpha_{s/i(38)}$ chimera is not ADP-ribosylated by pertussis toxin [15], indicating that the 36 amino acid α_i sequence at the COOH-terminus is not sufficient for pertussis toxin recognition of a G protein α subunit polypeptide. In contrast, the $\alpha_{i(Bam)/s/i(38)}$ and $\alpha_{i(64)/s/i(38)}$ chimeric polypeptides were excellent substrates for pertussis toxin-catalyzed ADP-ribosylation, even though they are functional α_s chains in their ability to activate adenylyl cyclase. Thus, sequences within the NH_2-terminal moiety of α_i are required for pertussis toxin-catalyzed ADP-ribosylation of the cysteine four amino acids from the COOH-terminus. Surprisingly, the $\alpha_{i(54)/s/i(38)}$ and $\alpha_{i(122)/s/i(38)}$ polypeptides were found not to be substrates for pertussis toxin. Thus, interaction of the $\alpha_{i(64)/s/i(38)}$ and $\alpha_{i(Bam)/s/i(38)}$ polypeptides with the βγ subunit complex is sufficient to allow pertussis toxin ADP-ribosylation of the G protein. In contrast, $\alpha_{i(54)/s/i(38)}$ and $\alpha_{i(122)/s/i(38)}$, which disrupt the α chain sequence surrounding the NH_2-terminal junction for the $\alpha_{i(64)/s/i(38)}$ chimera, are not pertussis toxin substrates. Cumulatively, the phenotypes of the different chimeras indicate there must be multiple βγ contact sites in the α subunit polypeptide NH_2-terminus, and these sites appear to be disrupted by the $\alpha_{i(54)/s}$ and $\alpha_{i(122)/s}$ chimeric sequences (see Table III for summary).

Obviously, the regulatory properties we assign to the α subunit attenuator domain overlap with the functions regulated by the βγ subunit complex. The functions assigned to the βγ subunit complex include attenuation of GDP disso-

TABLE III. Properties of α_i/α_s Chimeras*

Construct	Adenylyl cyclase	Cholera toxin	Pertussis toxin
α_s	↑	++	—
α_{i2}	↓ (−−)	—	++
$\alpha_{i(7)/s}$	↑	n.t.	—
$\alpha_{i(54)/s}$	↑ (++)	±	—
$\alpha_{i(64)/s}$	↑ (++)	n.t.	—
$\alpha_{i(122)/s}$	↑	—	—
$\alpha_{i\ s(Bam)}$	↑	—	—
$\alpha_{s\ i(38)}$	↑ (+)	—	—
$\alpha_{i(54)/s/i(38)}$	↑ (++)	—	—
$\alpha_{i(64)/s/i(38)}$	↑ (++)	—	++
$\alpha_{i(122)/s/i(38)}$	↑ (++)	—	—
$\alpha_{i(Bam)/s/i(38)}$	↑ (++)	—	++

*A diagram showing the relative contribution of α_s (open bar) and α_{i2} (black bar) is shown for each α_i/α_s chimera. The ability of each α subunit construct to regulate adenylyl cyclase activity is designated inhibition (↓) or stimulation (↑). (+) and (++) refers to the relative ability to stimulatory α subunit mutants to activate adenylyl cyclase in comparison to α_s. For cholera toxin and pertussis toxin (++) and (—) indicates the α subunit polypeptide is or is not a substrate for ADP-ribosylation. (±) refers to a $\alpha_{i(54)\ s}$ being a very poor cholera toxin substrate relative to α_s. n.t. denotes not tested.

ciation from the α subunit, pertussis toxin recognition of α_i-like polypeptides, and its requirement for efficient coupling of receptors to α chain activation. The control of GDP dissociation and attenuation of adenylyl cyclase activation by GTP are lost in the $\alpha_{i(54)/s}$ and $\alpha_{i(64)/s}$ chimeras. ADP-ribosylation by pertussis toxin of the $\alpha_{i(64)/s/i(38)}$ polypeptide was similar to that observed with wild-type α_i, but inhibited in the $\alpha_{i(54)/s/i(38)}$ and $\alpha_{i(122)/s/i(38)}$ chimeras. The $\alpha_{i(54)/s}$ polypeptide is, however, efficiently coupled to the β-adrenergic receptor [27], which requires association with the βγ subunit complex. The $\alpha_{i(54)/s}$, $\alpha_{i(64)/s}$, and $\alpha_{i(122)/s}$ NH_2-terminal mutants, therefore, differentially disrupt two of the three functions assigned to the βγ control of the α subunit polypeptide: attenuation of GDP dissociation and recognition by pertussis toxin.

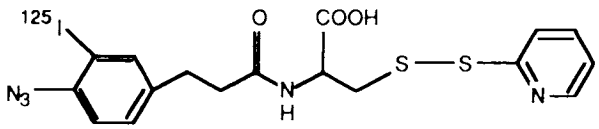
In contrast to pertussis toxin, cholera toxin recognition of α_s does not require the βγ subunit complex. The amino acid in α_s ADP-ribosylated by cholera toxin is Arg201, a site in the middle of the polypeptide whose flanking primary sequence is conserved in α_i [16,17]. Interestingly, $\alpha_{i(54)/s}$ is a poor substrate and $\alpha_{i/s(Bam)}$ is not recognized by cholera toxin. Introduction of the COOH-terminal sequence in the $\alpha_{s/i(38)}$ chimera completely inhibited cholera toxin-catalyzed ADP-ribosylation of the mutant α_s polypeptide. Thus, mutation at both the NH_2- and -COOH-termini disrupted recognition by cholera toxin even though the mutant α_s polypeptides are functionally capable of activating adenylyl cyclase. The multiple nonconserved mutations introduced in the NH_2- and COOH-terminal chimeras must therefore introduce intramolecular changes in the structure of the mutant α_s polypeptides resulting in diminished or inhibited cholera toxin catalyzed ADP-ribosylation of Arg201 by cholera toxin.

Analysis of G Protein α Chain Tertiary Structure

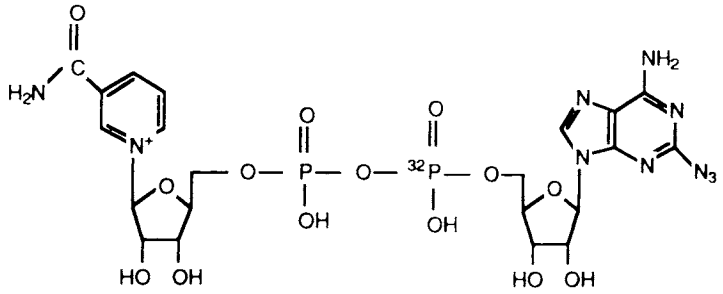
We have also attempted to define the organization of functional domains described above which are independent of the GDP/GTP binding site in the tertiary structure of the G protein α chain. One approach our laboratories has taken is the use of radiolabeled photoactivatable heterobifunctional cross-linking reagents to probe the tertiary structure of G protein α chains [28,29]. Two reagents that have proven particularly useful for α chain structural analysis are [^{125}I]-N-(3-iodo-4-azidophenylproprionamido)-S-(2-thiopyridyl) cysteine referred to as ACTP and 2-azido-[^{32}P]NAD (see Fig. 4 for structures). ^{125}I-ACTP presents a SH-reactive group, a radioiodinated phenylazide moiety, and a cleavable disulfide linkage. Of the eight cysteines in the retinal G protein (G_t or transducin) α chain (α_t, which is a member of the α_i-like subfamily of G proteins), ^{125}I-ACTP rapidly and specifically derivatized only two sulfhydryls. The cysteines derivatized by ^{125}I-ACTP were Cys210 which is between GDP/GTP binding domains G-2 and G-3 and Cys347 which is the fourth amino acid from the COOH-terminus. Azido-^{32}P-NAD was used in combination with pertussis toxin which catalyzed the ADP-ribosylation of Cys347 near the α_t COOH-terminus, which is one of the two cysteines also derivatized by ^{125}I-ACTP. Modifications of either cysteine by ACTP or ADP-ribosylation of Cys347 reversibly disrupted α_t function, measured by the ability of rhodopsin to catalyze guanine nucleotide exchange.

Even though both Cys210 and Cys347 were labeled by ^{125}I-ACTP, as determined by protein sequencing, derivatization was no greater than 1 mol of SH/mol of α_t. This stoichiometry was maintained even with a 200-fold molar excess of ^{125}I-ACTP relative to α_t. ^{125}I-ACTP did not derivatize either the β or γ subunits. It appeared that derivatization of one sulfhydryl (either Cys210 or Cys347) excluded labeling of the second reactive sulfhydryl. The exclusion of derivatization of the second sylfhydryl could occur by either steric hindrance, if the two cysteines are in close proximity, or by conformational changes of the α subunits induced by derivatization of the first sulfhydryl, making the second cysteine inaccessible. Interestingly, ADP-ribosylation of Cys347 also inhibited the ability of ^{125}I-ACTP to derivative Cys210 in the α_t polypeptide. The observation that ^{125}I-ACTP modification of either Cys347 or Cys210 inhibited rhodopsin catalyzed GTPγS binding to α_t as well as ADP ribosylation of Cys347 suggested that the two Cys residues were close to the receptor interaction site and/or the GDP/GTP binding region. The phenotype of the $\alpha_{i/sBam}$ chimera [26], receptor-α subunit uncoupling by ADP-ribosylation of Cys347 [30], as well as the inhibition of rhodopsin binding with α_t-COOH terminus specific monoclonal antibodies [31] agrees well with the fact that Cys347 is in the receptor interaction domain.

Both ^{125}I-ACTP and ^{32}P-NAD have photoactivatible azide moieties that upon activation can cross-link the derivatized cysteine to an adja-



N - (3- ^{125}iodo-4-azidophenylpropionamido)-S-(2'- thiopyridyl)-cysteine (^{125}I-ACTP):



[adenylate-^{32}P]2-AzidoNAD^{+}

Fig. 4. Structures of ^{125}I-ACTP and ^{32}P-azido NAD.

cent polypeptide domain. Following the reduction of the disulfide of ^{125}I-ACTP and Cys210 or 347, or hydrolysis of the thioglycosidic bond between Cys347 and 2-azido-^{32}P-ADP-ribose using mercuric acetate, the labeled moiety will be transferred to the azide-inserted domain of the α chain polypeptide or associated βγ subunits. Using ^{125}I-ACTP, the photoactivated azide derivative at Cys347 inserted into the α_t polypeptide near the Cys210 residue. This indicated that the derivatized Cys347 residue of α_t was oriented towards the α_t polypeptide encoding the region surrounding Cys210. In contrast, the Cys210 derivative is at the surface of the α chain tertiary structure oriented away from the protein. Similar findings were observed with photoinsertion analysis of the azido-^{32}-P-ADP-ribose derivatized Cys347 residue. Photoactivation of the azide followed by cleavage of the ADP-ribose glycosidic bond resulted in transfer of the label to an α_t fragment derived from the center of the polypeptide by proteolysis with endoproteinase arg-C. These results indicate that with both azido-^{32}P-NAD and ^{125}I-ACTP the derivatized α_t COOH-terminus is oriented towards the middle of the polypeptide with photoinsertion occurring near the G-2 and G-3 domains involved in guanine nucleotide exchange.

Interestingly, the azido-^{32}P-ADP-ribose labeled α_t Cys347 also demonstrated photoinsertion into the γ polypeptide of the βγ subunit complex. In fact this photoinsertion can be localized within the COOH-terminal 44 amino acid cyanogen bromide fragment of the γ subunit. In contrast, insertion into the β or γ subunit was not observed with ^{125}I-ACTP derivatized α_t. However, if photoactivated ^{125}I-ACTP derivatized $\alpha\beta\gamma_t$ was specifically bound to activated rhodopsin, followed by photoactivation of the azide moiety, the photomoiety was transferred (presumably the ACTP derivatized on Cys210) to the NH_2-terminal 14 kDa tryptic peptide of the β subunit. Together, the findings demonstrate that the COOH-terminus of the γ subunit is in proximity with the extreme α_t COOH-terminus and also $\alpha\beta\gamma_t$ undergoes a conformational change when bound to activated receptor. The structural change induced by receptor repositions the NH_2-terminus of the β subunit and the middle of the α_t polypeptide allowing azide insertion. Because Cys210 is between GDP/GTP binding domains G-2 and G-3, this suggests that the receptor

induces changes in subunit interactions involved in accelerating GDP dissociation, an α_t function controlled by $\beta\gamma$. This is significant because the α chain COOH-terminus appears to be a major receptor contact site which is oriented towards the middle of the α polypeptide and $\beta\gamma$ complexes are required for efficient receptor catalyzed GDP-GTP exchange. The findings also place the γ subunit, specifically the COOH-terminal half of the γ subunit, in closer proximity to the α_t COOH and α_t NH_2 termini. Additional support of this structural relationship between the COOH-terminal domain of γ_t (γCys36/37) and the NH_2 terminal domain of β_t (βCys25) has been reported by Bubis and Khorana [32] using o-phenanthroline oxidation of these two cysteines and localization by peptide sequencing.

It was also found that the tertiary structure of G_t was stabilized by strong noncovalent interactions between different domains; for example, treatment of G_t with trypsin alone was not sufficient to release the peptides from $G\alpha_t$. Only the addition of SDS following trypsinization released the characteristic tryptic fragments as evidenced by molecular sieve chromatography and crosslinking studies. In fact, if trypsinization of the ACTP derivatized native $G\alpha_t$ was performed *prior* to photocrosslinking with ^{125}I-ACTP, the COOH-terminus Cys347 domain was still crosslinked to the 12 kDa GDP/GTP region indicating that the trypsinized $G\alpha_t$ retained its native structure despite proteolytic cleavage. Thus, the NH_2- and COOH-termini are not free but are tightly associated with other regions of the α_t core structure.

A schematic of the predicted subunit arrangements allowing efficient receptor-catalyzed guanine nucleotide exchange is shown in Figure 5. The molecular size of the photocrosslinking reagents used to probe α_t tertiary structure indicates that the α_t subunit NH_2- and COOH-termini and core motif must be 2 nm or less from the β_t NH_2-terminus and the α_t COOH-terminus. This provides an intermolecular structural association of these subunits necessary for receptor activation of the G protein.

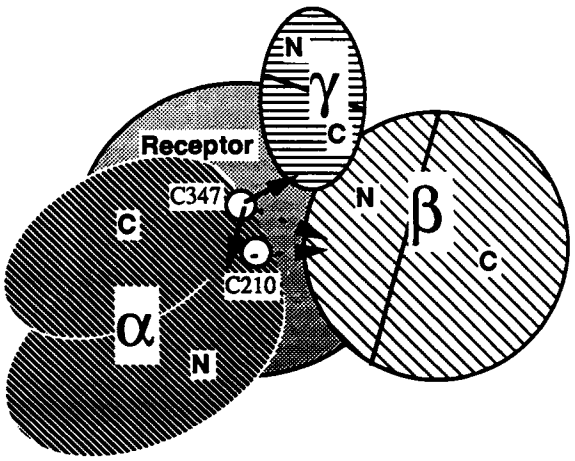


Fig. 5. Model defining the proposed domain interactions of G $\alpha\beta\gamma$ and the receptor. N and C refers to the NH_2- and the COOH-terminal domains respectively. Both the NH_2- and COOH-terminal halves of the α subunit are folded towards the middle of the molecule and are interacting with receptor. The COOH-terminal Cys347 of α is in close proximity to three major domains: (a) Cys210 in the middle of the α_t polypeptide, (b) the COOH-half of the γ subunit, and (c) the NH_2-terminal 14-kDa peptide of β. The open circles indicate the Cys residues. The solid arrows indicate the label transfer from αCys347 to the internal domain of α_t using ^{125}I-ACTP and ^{32}P-azido-NAD as well as label transfer from αCys347 to the γ subunit using ^{32}P-azido NAD. The dashed arrows indicate "conformation-dependent" label transfer from αCys210 to the NH_2-terminal domain of the β subunit using ^{125}I-ACTP which occurs when ^{125}I-ACTP derivatized G_t binds to light activated rhodopsin.

Predictions of G Protein α Chain Polypeptide Structure and Regulation

Conservation of the G-1 thru -4 sequences indicates the GDP/GTP binding domain tertiary structure will be quite similar for p21*ras* and G protein α subunit polypeptides. Consistent with similar GDP/GTP binding site structure is the similar consequence of specific mutations in domains G-1 and -3 which inhibit GTPase activity p21*ras* (Gly12 → Val, Gln61 → Leu) and α_s (Gly49 → Val, Gln227 → Leu). However, significant differences exist in the regulation of p21*ras* and G protein α subunit GTPase activity and selected mutations which inhibit GTPase activity or enhance GDP dissociation in p21*ras* (Ala59 → Thr, Asp119 → Ala) are inhibitory or null (Gly225 → Thr, Asp295 → Ala) in the α_s polypeptide. These differences are related, at least in part, to sequences outside of the GDP/GTP binding domain which are highly divergent in p21*ras* and G protein α chains and influence the regulatory properties of the polypeptides.

Analysis of α_i/α_s chimeras, the consequence of specific point mutations, and the influence of ADP-ribosylation of α_s and α_i by cholera and pertussis toxins define functional domains of the G protein α chain outside of the GDP/GTP binding site. Figure 6 shows a schematic of these domains. The $\alpha_{i/s(Bam)}$ chimera, which functions

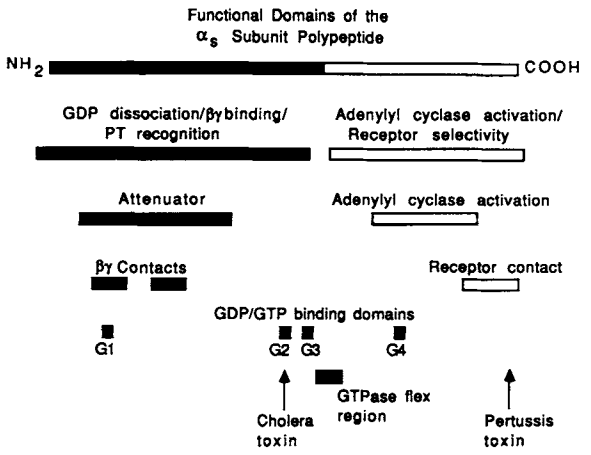


Fig. 6. The $\alpha_{i/s(Bam)}$ chimera functionally divides the α_s polypeptide roughly in half. The NH_2-terminal moiety encodes functions for controlling GDP dissociation, βγ binding, and pertussis toxin (PT) recognition. The COOH-terminal moiety encodes adenylyl cyclase activation and receptor selectivity. Each of these domains in the NH_2- and COOH-terminal halves of the polypeptide are further defined by chimera and mutation analysis (see text). G1–4 refers to the GDP/GTP binding domains and GTPase flex region is where a major conformational change is mediated by GTP binding. Arrows point to sites of ADP-ribosylation by cholera and pertussis toxins.

as a wild-type α_s polypeptide, roughly divides the polypeptide in halves. The COOH-terminal moiety of α_s encodes major effector activation and receptor contact sequences. The NH_2-terminal moiety of α_s controls βγ subunit interactions and GDP dissociation. Because the attenuator function of α_i and α_s are common, the NH_2-terminal half of α_s may be substituted with the corresponding α_i moiety. However, disruption of the attenuator domain, such as in the $\alpha_{i(54)/s}$ and $\alpha_{i(64)/s}$ chimeras results in an activated α_s polypeptide. The NH_2-terminal moiety of α_i and α_s also encodes the GAP-like function intrinsic to G protein α chains.

Our structural analysis of the α_i-like α_t polypeptide places the COOH-terminus in an orientation towards the middle of the molecule near the G-2 and G-3 GDP/GTP binding domains. In addition, several lines of evidence indicate that the NH_2- and COOH-termini are in close proximity to one another [33,34]. The close proximity of the α chain polypeptide termini places the α subunit receptor recognition domain near the attenuator and βγ regulatory domains and receptor catalyzed guanine nucleotide exchange is mediated by receptor contacts with both βγ and α subunits [7,28,29]. Orientation of the α subunit receptor contact site towards the GDP/GTP binding pocket, and the close association of the NH_2-terminal βγ regulatory domain to the α chain COOH-terminus, would provide a structure whereby receptor interaction with βγ and α subunits could readily transmit conformational changes that alter interactions with GDP.

Changes in the ability of cholera and pertussis toxin to ADP-ribosylate different α_i/α_s chimeras support the prediction that the NH_2- and COOH-termini are important regulators of core α subunit function and structure. Mutations at either end of the α_s polypeptide primary sequence dramatically influence the ability of cholera toxin to ADP-ribosylate Arg201 in the middle of the polypeptide primary sequence. In addition, appropriate NH_2-terminal sequences are required for pertussis toxin catalyzed ADP-ribosylation of the cysteine four amino acids from the α_i COOH terminus. Since the α_s and α_i polypeptides bind common βγ subunits, the changes in cholera and pertussis toxin recognition observed in the different mutants must involve changes in intramolecular α chain interactions. Thus, the NH_2- and COOH-termini of G protein α subunits function as key modulators of the core regulatory domains including attenuator, GDP/GTP binding and effector activation sequences. Because of the conservation in structure among all G protein α chains identified to date, it is predicted that mutations within the GDP/GTP binding domain, attenuator, and NH_2- and COOH-terminal modulator sequences will have similar functional consequences. Thus, it is now possible to readily design constitutive active and dominant negative mutations which can be engineered into any G protein α chain cDNA for analysis of function using standard gene transfer techniques.

ACKNOWLEDGMENTS

This work was supported by NIH grants GM 30324 (G.L.J.), DK 37871 (G.L.J.), and GM 33138 (A.E.R.) and the American Heart Association. JML is supported by NIH postdoctoral fellowship HD 07437 and RRV is an Advanced Predoctoral Fellow of the Pharmaceutical Manufacturers Association Foundation.

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