0026-895X/93/020255-09$03.00/0

MOLECULAR PHARMACOLOGY, 44:255-263

G Protein Amino-Terminal α_{i2}/α_s Chimeras Reveal Amino Acids Important In Regulating α_s Activity

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Received March 16, 1993; Accepted June 2, 1993

SUMMARY

G_s and G_{i2} are heterotrimeric G proteins that stimulate and inhibit, respectively, the activity of a common effector, adenylyl cyclase. The G_s and G_{i2} α subunit polypeptides, α_s and α_{i2}, are 65% homologous in primary sequence. A series of α_{i2}/α_s chimeras and α_s point mutations were used to map sequences in the α_s polypeptide that regulate α_s activity. An amino-terminal region controlling the activation of α_s was determined to reside within residues Lys-25 to Glu-101. Amino-terminal α_{i2}/α_s chimeras that disrupt this region in α_s result in an activated α_s. In contrast, replacement of this entire α_s sequence with the analogous α_{i2} sequence produces a chimera whose activity is similar to that of the wild-type α_s polypeptide. The regulation of α_s activation by the amino-terminal sequence is independent of the intrinsic GTPase function. Inhibition of α_s GTPase function by the mutation Gln-227 to leucine is additive with the amino-terminal chimera mutations. These mutations appear to independently alter the two rate-limiting steps in activation of the G protein α subunit, i.e., GTP hydrolysis and GDP dissociation, allowing subsequent GTP binding. Within this region of α_s, Arg-42 is just amino-terminal to the G-1 sequence comprising part of the GDP/GTP binding pocket. The G-1 sequence interacts with the α- and β-phosphoryl groups of GDP and GTP. Mutation of α_s Arg-42 to lysine has modest effects on α_s activation, but when placed in the background of the glutamine to leucine mutation the $\alpha_{sR42K+Q227L}$ mutant polypeptide stimulates cAMP synthesis significantly more than observed with α_{sQ227L} expression. Specific mutations in the amino terminus, therefore, have the ability to enhance α_s activation by influencing the rate of adenylyl cyclase activation, which is independent of GTPase activity.

Within the GTPase family of proteins, the members referred to as G proteins provide a signal transduction coupling mechanism for many cell surface receptors (1, 2). G proteins are responsible for regulating an intracellular effector, such as an ion channel or an enzyme, in response to an activated receptor (3, 4). G protein α subunits bind GDP and GTP. Receptors coupled to specific G proteins catalyze GDP dissociation, allowing GTP to bind. The α-GTP complex in turn regulates the activity of specific effectors. The lifetime of the activated α-GTP complex is controlled by an intrinsic GTPase encoded in the α subunit that hydrolyzes the bound GTP to GDP, returning the α subunit to an inactive state (5).

Among the 20 or so G protein α subunits whose sequences have been defined, there are both common and unique functions for each encoded in the structure of the α chain polypeptide. The common features include the functions involved in regulation of the α subunit itself, 1) GDP/GTP binding domains, 2) intrinsic GTPase activity, and 3) binding sites for association with $\beta\gamma$ subunits. The unique functions for each α chain include 1) selectivity for regulating specific effectors and 2) coupling to specific receptors.

Two G protein members, G_s and G_i, regulate the common effector adenylyl cyclase but couple to different receptors. G_s activates and G_i inhibits adenylyl cyclase activity (6). G_s and G_{i2} α subunit polypeptides are approximately 65% identical in amino acid sequence (4, 7). For this reason the α_s and α_{i2} polypeptides have proven to be extremely valuable for characterizing both the common and unique functions of G protein α subunits by chimera and amino acid mutation analysis.

We previously showed that the chimera $\alpha_{i(54)/s}$, which has the amino-terminal 61 residues of α_s replaced by the corresponding amino-terminal 54 residues of α_{i2}, was a strong activator of adenylyl cyclase, compared with the wild-type α_s polypeptide (8–12). Properties of the $\alpha_{i(54)/s}$ chimera, compared with the wild-type α_s polypeptide, include 1) an accelerated rate of adenylyl cyclase activation in the presence of GTP, 2) altered regulation of GTP activation by $\beta\gamma$ subunit complexes, 3) normal GTPase activity, and 4) normal coupling to the β-adrenergic receptor (8–12). Consistent with the $\alpha_{i(54)/s}$ chimera having a change in the regulation of activation by guanine nucleotides was the finding that its activation characteristics

This work was supported by United States Public Health Service Grants GM30324 and DK37871 and Grant CA09313-01 from the National Cancer Institute.

ABBREVIATIONS: PCR, polymerase chain reaction; kb, kilobase(s); GTPγs, guanosine-5′-*O*-(3-thio)triphosphate.

were independent of the intrinsic α_s GTPase function. To more precisely define the amino-terminal region of the α_s polypeptide responsible for regulation of α_s activation by GTP, we have constructed a series of α_{i2}/α_s chimeras. These chimeras are similar to $\alpha_{i(54)/s}$ in that they each have portions of the amino-terminus of α_s replaced by the analogous region from α_{i2}. We examined the ability of these various α_{i2}/α_s chimeras to activate adenylyl cyclase, compared with the wild-type α_s polypeptide, when transiently expressed in COS-1 cells. Using this strategy regions of the α_s polypeptide that control activation by guanine nucleotides independently of the GTPase function have been defined.

Materials and Methods

Construction of α_{i2}/α_s chimeras. To construct the α_{i2}/α_s chimeras a previously described PCR strategy was used (11). Briefly, for $\alpha_{i(17)/s}$ a cDNA fragment was synthesized containing the 105-base pair 5′ noncoding sequence and the first 17 codons of α_{i2} using the oligonucleotides CCAAGCTTGAGAGCTTCCCGCAGAG (5′ primer) and TGCTTCTCGATCTTCTTAGAGCGCTCGG (3′ primer) and the rat α_{i2} cDNA as a template. The cDNA product encoding α_s residues 25–144 was synthesized by PCR using the oligonucleotides CCGAGCGCTCTA AGAAGATCGAGAAGCA (5′ primer) and ATAGAATTCAGGTGGGAA (3′ primer) and the rat α_s cDNA as a template. The internal complementarily of the α_{i2} 3′ primer and the α_s 5′ primer was used to anneal these two products into a chimeric cDNA with a second PCR reaction, using the α_{i2} 5′ primer and the α_s 3′ primer oligonucleotides. This product encodes a *Hind*III site at the 5′ end and an *Eco*RI site at the 3′ end. The chimeric cDNA was digested with *Hind*III and *Eco*RI and shuttled into pUC18 for sequence analysis. The *Hind*III-*Eco*RI fragment was then ligated with the *Eco*RI-*Hind*III fragment of α_s, which encodes amino acids 145–394 and the α_s 3′ noncoding sequence. The product was verified by restriction enzyme analysis and DNA sequencing.

Additional chimeras were constructed using this PCR strategy. The α_{i2} fragments for $\alpha_{i(27)/s}$, $\alpha_{i(34)/s}$, $\alpha_{i(79)/s}$, $\alpha_{i(94)/s}$, and $\alpha_{i(109)/s}$ were each synthesized using the α_{i2} 5′ primer oligonucleotide described above and the 3′ primers GCCCGGTAGACCTGGCCGTCCTCCCGCA, AGCAGCAGGCGCACCTCCCGTGCCG, ATGGTTTCAATGGCCTGGATGGTGTTGC, TTGGCCAGCTCCACGTCCATCTGCAGGT, and TTCATCACGCTCAGTGCGAACAGCTGCC, respectively. The α_s fragments for the chimeras $\alpha_{i(27)/s}$, $\alpha_{i(34)/s}$, $\alpha_{i(79)/s}$, $\alpha_{i(94)/s}$, and $\alpha_{i(109)/s}$ were synthesized using the α_s 3′ primer described above and the 5′ primers TGCGGGAGGACGGCCAGGTCTACCGGGC, CGGCACGGGAGGTGCGCCTGCTGCTGCT, GCAACACCATCCAGGCCATTGAAACCAT, ACCTGCAGATCGACGTGGAGCTGGCCAA, and GGCAGCTGTTCGCACTGAGCGTGATGAA, respectively. Each PCR product was verified by DNA sequencing.

The various α_{i2}/α_s chimeras were placed in combination with the point mutation α_{sQ227L} using a similar strategy as described above. The chimera cDNA was digested with *Hind*III and *Eco*RI and ligated in a three-piece ligation with the *Eco*RI-*Hind*III fragment from α_{sQ227L}, which encodes amino acids 145–394 (containing the Q227L mutation) and the α_s 3′ noncoding sequence, and *Hind*III-digested pCW1 expression plasmid. Ligations were verified by restriction analysis.

The plasmid $\alpha_{s/i(38)}$ described previously (13), which encodes α_s amino acids 1–356 and α_{i2} residues 320–355 (replacing the carboxyl terminus α_s residues 357–394), was digested with *Eco*RI and *Hind*III. The resulting fragment (0.95 kb), which encodes α_s residues 145–356 and α_{i2} residues 320–355, was isolated and used to construct the new chimeras listed in Fig. 5A. For example, $\alpha_{i(17)/s}$ was digested with *Eco*RI and *Hind*III and the fragment (0.54 kb) encoding α_{i2} residues 1–17 and α_s residues 25–144 was isolated and used in a three-piece ligation with the fragment described above (α_s residues 145–356 and α_{i2} residues 320–355) and pCW1 linearized with *Hind*III. Proper construction and orientation of each new chimera were verified by restriction enzyme analysis.

Site-directed mutagenesis. Point mutations in α_s were constructed using the Promega Altered Sites *in vitro* mutagenesis system. All the mutations synthesized were verified by DNA sequencing. The large form of α_s, encoding the 46.5-kDa α_s polypeptide, was used for all of the constructions.

Expression analysis of α chains. For immunoblots, 70 μg of membrane protein from transfected COS-1 cells were resolved on a sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide), transferred to nitrocellulose, and probed with an α_s-specific antibody. The blots were washed with ^{125}I-Protein A and autoradiographed as described previously (12, 13). The expressed α subunits were found to be membrane associated, with little or no soluble form detected for any of the constructs.

cAMP assay. The expression plasmid pCW1 (13) was used for transfection of cDNAs in COS-1 cells using DEAE-dextran (14). Cells were analyzed 65 hr after transfection. For cAMP measurements cells were incubated for 10 min at room temperature in the presence of 500 μM isobutylmethylxanthine. Cellular cAMP was extracted with 2 ml of ice-cold 65% ethanol and lyophilized. cAMP levels were measured using an Amersham radioimmunoassay kit, according to the manufacturer's instructions (15). We have extensively characterized this assay to demonstrate that cAMP measurements, in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine, are a reliable assay of the adenylyl cyclase activity in intact transfected COS-1 cells (11–13, 15). The measurement of cAMP levels and immunoblotting therefore provide a quantitative way to evaluate the functional activity of the expressed α_s polypeptides. The results shown in each figure are from a single experiment, but the measurements are representative of at least five independent transfections and cAMP measurements for each construct. The range of duplicate determinations is shown for each construct in the figures; the determinations generally differed by <5% of the mean.

Adenylyl cyclase assay. COS-1 cells were transfected as described previously, and 65 hr after transfection the cells were harvested and membranes were prepared. For measurement of adenylyl cyclase activity 20 μg of membrane protein were incubated at 30° in 100 μl of a reaction mixture that included 50 mM Tris·HCl, pH 8.0, 2.5 mM $MgCl_2$, 1 mM EDTA, 0.01% bovine serum albumin, 2 mM dithiothreitol, 1 mM isobutylmethylxanthine, and 0.4 mM Na_2ATP. The reaction was started by the addition of 100 μM GTPγS after a preincubation of 5 min and was stopped at specific time points by the addition of 1 ml of 65% ethanol. The samples were dried and cAMP was measured with an ^{125}I radioimmunoassay kit, according to the manufacturer's instructions (15). The results shown are from a single experiment but are representative of three independent transfections and adenylyl cyclase assays.

Results

We have constructed a series of α_{i2}/α_s chimeras (Figs. 1A and 2A) and defined their ability to stimulate cAMP synthesis. The chimeras have portions of the amino terminus of α_s replaced by the analogous residues from α_{i2}. For example, in $\alpha_{i(17)/s}$ the first 24 residues of α_s are replaced with the first 17 amino-terminal residues of α_{i2}. Figs. 1B and 2B show the ability of the various α_{i2}/α_s chimeras to stimulate cAMP synthesis, relative to the wild-type α_s polypeptide. Substitution of the first 24 residues of α_s with the corresponding 17 residues of α_{i2} (there are seven unique α_s amino acids at the amino terminus that are absent in α_{i2}) had no discernible influence on the ability of the chimeric α_s polypeptide to stimulate cAMP synthesis, relative to that observed with the wild-type α_s polypeptide (Fig. 1B). It is important to note that overexpression of the wild-type α_s polypeptide increases cAMP synthesis, relative to the control

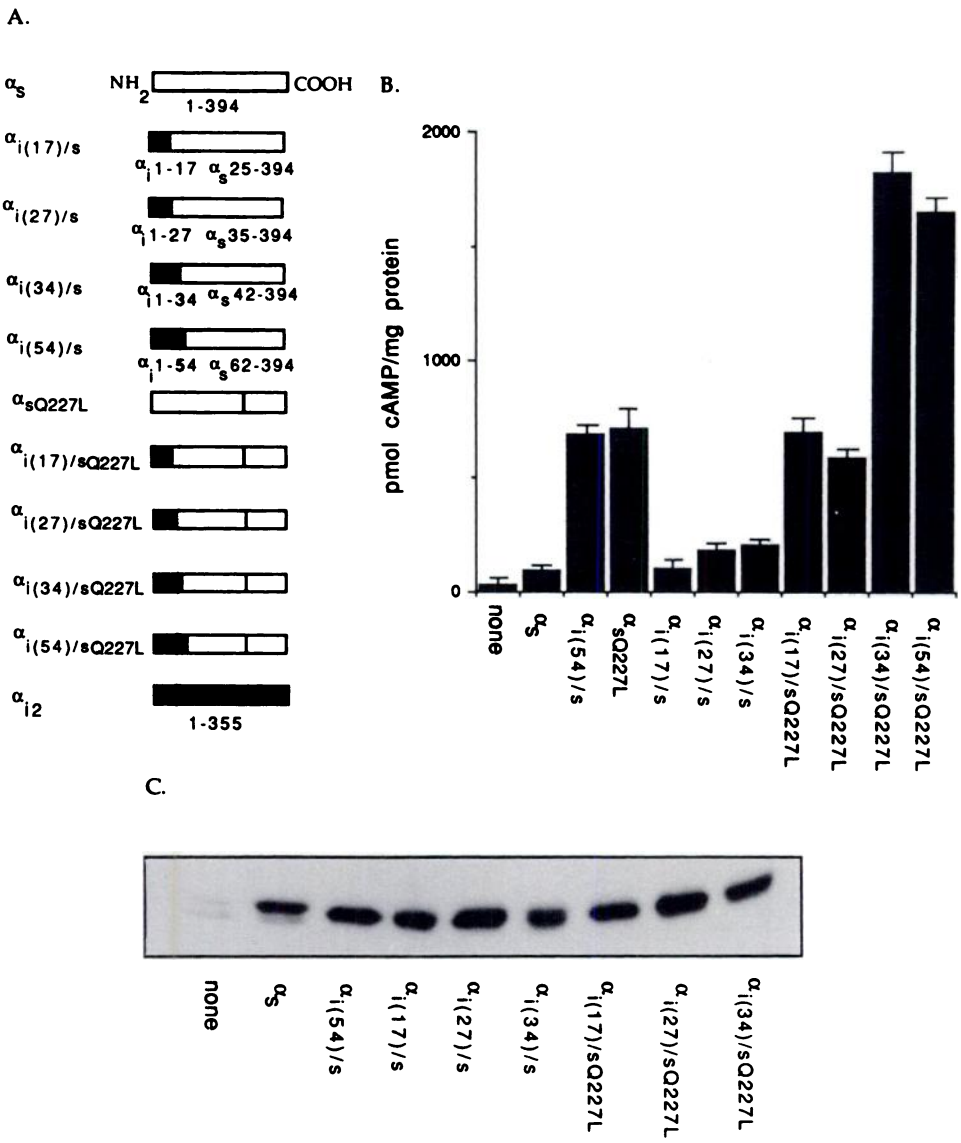

Fig. 1. A, The relative contribution of α_s (□) and α_{i2} (■) is shown for each α_{i2}/α_s chimera. Each chimera has portions of the amino terminus of α_s replaced by corresponding residues of α_{i2}. For example, $\alpha_{i(17)/s}$ is a chimera that contains the amino-terminal 17 amino acids of α_{i2} (replacing the amino-terminal 24 residues of α_s) linked to α_s residues 25–394. B, Chimeras were transiently expressed in COS cells and cAMP levels were measured as an indication of adenylyl cyclase activation in the transfected cells. Sixty-five hours after transfection the cells were incubated at room temperature for 10 min in the presence of 500 μM isobutylmethylxanthine, a cAMP phosphodiesterase inhibitor, and then fixed in ethanol and cAMP levels were measured by radioimmunoassay as described in Materials and Methods. C, Immunoblots, using anti-α_s antisera, of COS-1 cells transfected with wild-type α_s or chimeras showed an overexpression of each of the chimeras. *none*, COS-1 cells transfected in the absence of α subunit cDNA.

mock-transfected cells. In the presence of a phosphodiesterase inhibitor to prevent cAMP degradation, we previously showed that this accurately reflects the synthesis of cAMP by adenylyl cyclase. All of the α_{i2}/α_s chimeras we have expressed are functional α_s polypeptides and have intrinsic cAMP synthesis properties similar to or greater than those of the wild-type α_s polypeptide. Comparing the α_s residues 1–24 with the substituted α_{i2} sequence 1–17 found in $\alpha_{i(17)/s}$, there are six nonconserved residues and seven unique α_s amino acids whose substitution or loss had no dramatic influence on the activation of cAMP synthesis. The $\alpha_{i(27)/s}$ sequence has four additional nonconserved amino acids, relative to the $\alpha_{i(17)/s}$ sequence, when both are compared with α_s. The $\alpha_{i(27)/s}$ chimera had modest effects on cAMP synthesis, relative to expression of the α_s polypeptide. Additional mutation of the α_s amino terminus found in $\alpha_{i(34)/s}$ also produced only a modest increase in cAMP synthesis, relative to wild-type α_s or $\alpha_{i(17)/s}$, when similar levels of expression were obtained (Fig. 1B). Within the α_s residues 1–41 and the corresponding α_{i2} sequence 1–34 there are a total of 16 nonconserved residues, six more than are present in the $\alpha_{i(27)/s}$ chimera. The modest effects observed with the $\alpha_{i(27)/s}$ and $\alpha_{i(34)/s}$ chimeras, relative to wild-type α_s or $\alpha_{i(17)/s}$ suggest that the mutations introduced have little effect by themselves on the regulation of cAMP synthesis.

Remarkably, expression of the $\alpha_{i(54)/s}$ chimera dramatically stimulated cAMP synthesis, compared with the $\alpha_{i(34)/s}$ polypeptide (Figs. 1B and 2B). The stimulation of cAMP synthesis resulting from expression of the $\alpha_{i(54)/s}$ chimera resulted from an increased rate of GTP activation, resulting from enhanced GDP dissociation from the mutant α subunit polypeptide (11, 12). The only amino acid differences between $\alpha_{i(34)/s}$ and $\alpha_{i(54)/s}$ are two conserved arginine to lysine substitutions between α_s and α_{i2} at residues 42 and 61 in the α_s sequence. The $\alpha_{i(54)/s}$ chimera was reproducibly 3–6 times more effective, depending on the experiment, in its ability to stimulate cAMP synthesis, compared with similar expression of the wild-type α_s polypeptide. The previously characterized $\alpha_{i(64)/s}$ polypeptide was the only other chimera with activation potential similar to that of $\alpha_{i(54)/s}$ (11).

Larger α_i/α_s chimeras such as $\alpha_{i(94)/s}$ and $\alpha_{i(109)/s}$ were functionally similar to wild-type α_s in their ability to stimulate cAMP synthesis (Fig. 2B). The $\alpha_{i(94)/s}$ and $\alpha_{i(109)/s}$ chimeras were

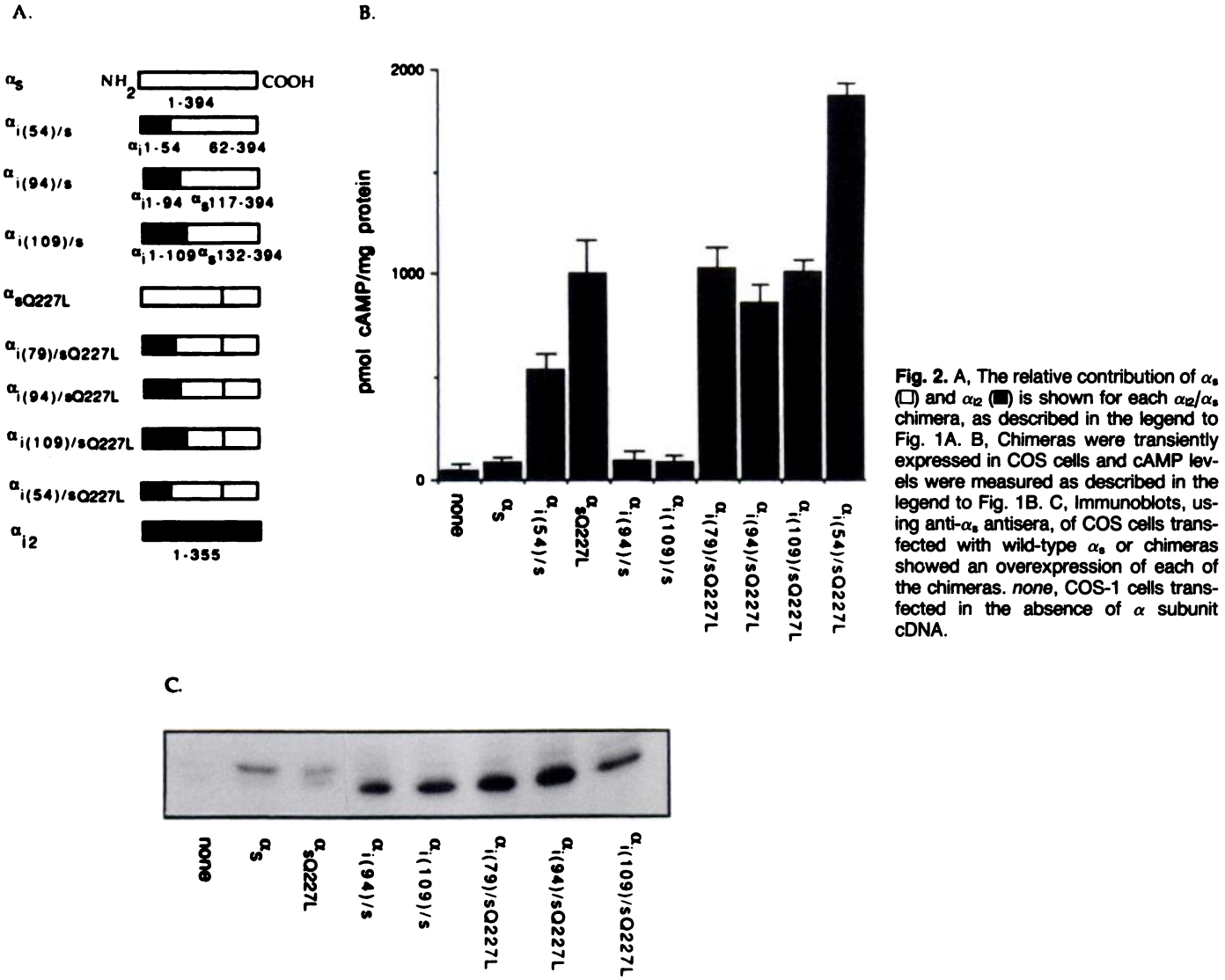

Fig. 2. A, The relative contribution of α_s (□) and α_{i2} (■) is shown for each α_{i2}/α_s chimera, as described in the legend to Fig. 1A. B, Chimeras were transiently expressed in COS cells and cAMP levels were measured as described in the legend to Fig. 1B. C, Immunoblots, using anti-α_s antisera, of COS cells transfected with wild-type α_s or chimeras showed an overexpression of each of the chimeras. *none*, COS-1 cells transfected in the absence of α subunit cDNA.

expressed at levels similar to those of $\alpha_{i(54)/s}$ (compare Figs. 1C and 2C) and encode an even larger number of nonconserved amino acid substitutions. However, they were not constitutively activated. Cumulatively, the findings suggest that the functional domain controlling α_s activation by GTP resides within a boundary beginning at α_s residue Lys-25 and ending before Pro-116. Disruption of this regulatory domain by mutation, similarly to the $\alpha_{i(54)/s}$ chimera, results in release of an inhibitory function, resulting in activation of the chimeric polypeptide.

To further characterize the boundaries within the amino-terminal regulatory domain controlling GTP activation of α_s, the chimeras were placed in combination with the mutation of Gln-227 to leucine (Q227L). The α_{sQ227L} mutation inhibits the intrinsic GTPase activity by >95% (5, 16, 17). The $\alpha_{i(54)/s}$ chimera has normal GTPase activity (8–12). Placing the Q227L mutation within the α_i/α_s chimeras allows an amplification of the activation state of mutations that enhance GDP dissociation, because the GTPase turn-off mechanism is inhibited (5, 16, 17). For example, the mutant α_s polypeptide encoding both $\alpha_{i(54)/s}$ and α_{sQ227L} mutations is generally additive or more than additive in the ability to activate adenylyl cyclase, relative to the mutant $\alpha_{i(54)/s}$ and α_{sQ227L} polypeptides alone. The greater activity is related to the fact that $\alpha_{i(54)/s}$ and α_{sQ227L} mutations influence the rate of GTP activation and GTPase turn-off of the α_sGTP complex, respectively, the two rate-limiting determinants in G protein α subunit regulation of effector enzymes.

Figs. 1B and 2B show that the combined mutations $\alpha_{i(17)/sQ227L}$, $\alpha_{i(27)/sQ227L}$, $\alpha_{i(79)/sQ227L}$, $\alpha_{i(94)/sQ227L}$, and $\alpha_{i(109)/sQ227L}$ were all similar in their ability to stimulate adenylyl cyclase, relative to α_{sQ227L} alone. This finding confirms that these amino-terminal α_i/α_s chimeras do not significantly influence regulation of α_s activation by GTP. These results contrast with those for the $\alpha_{i(54)/sQ227L}$ polypeptide, which strongly activated adenylyl cyclase, relative to either $\alpha_{i(54)/s}$ or α_{sQ227L} alone. A dramatic result was the finding that $\alpha_{i(34)/sQ227L}$ activated adenylyl cyclase to similar levels as did $\alpha_{i(54)/sQ227L}$, even though $\alpha_{i(34)/s}$ was similar to α_s in its activation of cAMP synthesis. This result indicates that inhibition of the intrinsic α_s GTPase activity amplifies the phenotype of the $\alpha_{i(34)/s}$ chimera because it allows accumulation of the GTP-liganded complex, which is required for stimulation of cAMP synthesis. The $\alpha_{i(79)/sQ227L}$ construct further narrows the boundaries for the amino-terminal domain, relative to

$\alpha_{i(94)/s}$. For unknown reasons, the $\alpha_{i(79)/sQ227L}$ polypeptide was efficiently expressed (Fig. 2C), whereas $\alpha_{i(79)/s}$ polypeptide expression was not detected. Thus, the amino-terminal regulatory domain is between Lys-25 and Glu-101.

To further elucidate important residues in the α_s amino-terminal regulatory region, point mutations were made to substitute several α_s amino acids with their counterparts in α_{i2}. Fig. 3 defines these mutations, which included switching of α_s Arg-38 to alanine (R38A), double mutation of Lys-32 and -34 to glutamate and glycine, respectively (KK32,34EG), mutation of Arg-42 to lysine (R42K), and mutation of Arg-61 to lysine (R61K). Residues α_s Arg-42 and Arg-61 are the only two amino acid differences between $\alpha_{i(34)/s}$ and $\alpha_{i(54)/s}$; these two residues are lysine in $\alpha_{i(54)/s}$, corresponding to the α_{i2} sequence (Fig. 3A). Expression of the four mutant α_s polypeptides indicated that only α_{sR42K} was possibly weakly activating in terms of enhanced cAMP synthesis, relative to expression of the wild-type α_s polypeptide (Fig. 3B). All of the chimeric polypeptides were expressed at similar levels (Fig. 3C). The α_{sQ227L} polypeptide was frequently found to be expressed at slightly lower levels. This did not appear to be due to its presence in the cytoplasm, as determined by immunoblotting (data not shown).

Placement of the α_{sR42K} mutation in combination with the α_{sQ227L} mutation in the same polypeptide ($\alpha_{sR42K+Q227L}$) yielded an α_s that was able to stimulate adenylyl cyclase activity to levels similar to those observed with $\alpha_{i(54)/sQ227L}$ (Fig. 4B). This finding indicated that mutation of Arg-42 to lysine was sufficient to alter the regulation of GTP activation if the mutation was placed in an α_s polypeptide whose GTPase activity was inhibited. Arg-42 is just upstream of the consensus G-1 binding domain (GAGESGKS) that is involved in binding the α- and β-phosphoryl groups of GDP and GTP (1, 18). Interestingly, a

A.

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                                      32 34 38  42
αs    MCGLGNSKTEDQRNEEKAQREANKKIEKQLQKDKQVYRATHRLLLLGAGESGKS
αi2   MGC---TVS----AEDKAAAERSKMIDKNLREDGEKAAREVKLLLLGAGESGKS

          61
αs    TIVKQMRILHVNGFNGEGGEEDPQAARSNSDGEKATKVQDIKNNLKEAIETIVA.....
αi2   TIVKQMKIIHEDGYSEE---------------ECRQYRAVVYSNTIQSIMAIVK.....
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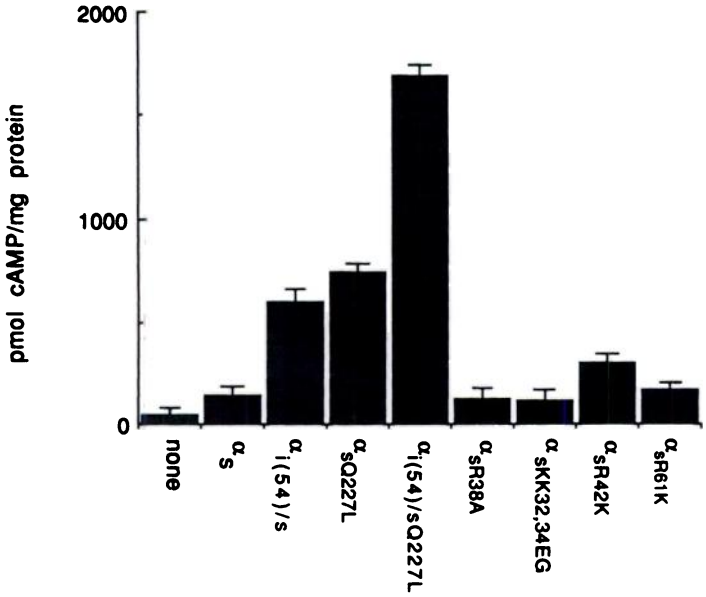
B.



C.

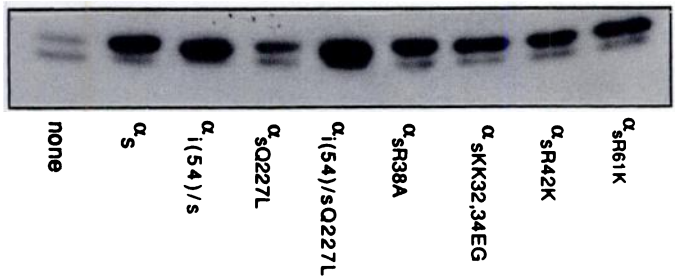


Fig. 3. A, Alignment of amino acid sequences of the amino-terminal portions of α_s and α_{i2}, with point mutations made in α_s *underlined*. B, Mutant α_s polypeptides were transiently expressed in COS cells and cAMP levels were measured as described in the legend to Fig. 1B. C, Immunoblots, using anti-α_s antisera, of COS cells transfected with wild-type α_s or mutant α_s showed similar levels of expression of each of the mutant α_s polypeptides. *none*, COS-1 cells transfected in the absence of α subunit cDNA.

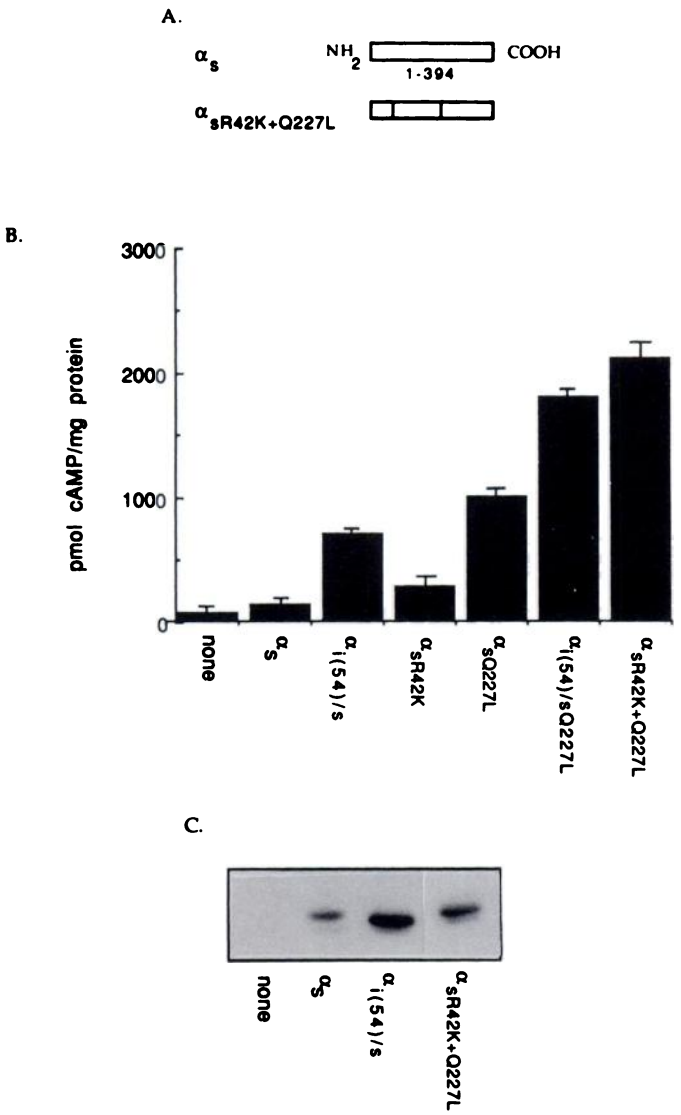


Fig. 4. A, Diagram of the double-point mutant $\alpha_{sR42K+Q227L}$. B, Mutant α_s polypeptides were transiently expressed in COS cells and cAMP levels were measured as an indication of adenylyl cyclase activation in the transfected cells, as described in the legend to Fig. 1*B*. C, Immunoblots, using anti-α_s antisera, of COS cells transfected with wild-type α_s or mutant α_s showed similar levels of expression of each of the mutant α_s polypeptides. *none*, COS-1 cells transfected in the absence of α subunit cDNA. Immunoblots for other constructs are shown in Fig. 3.

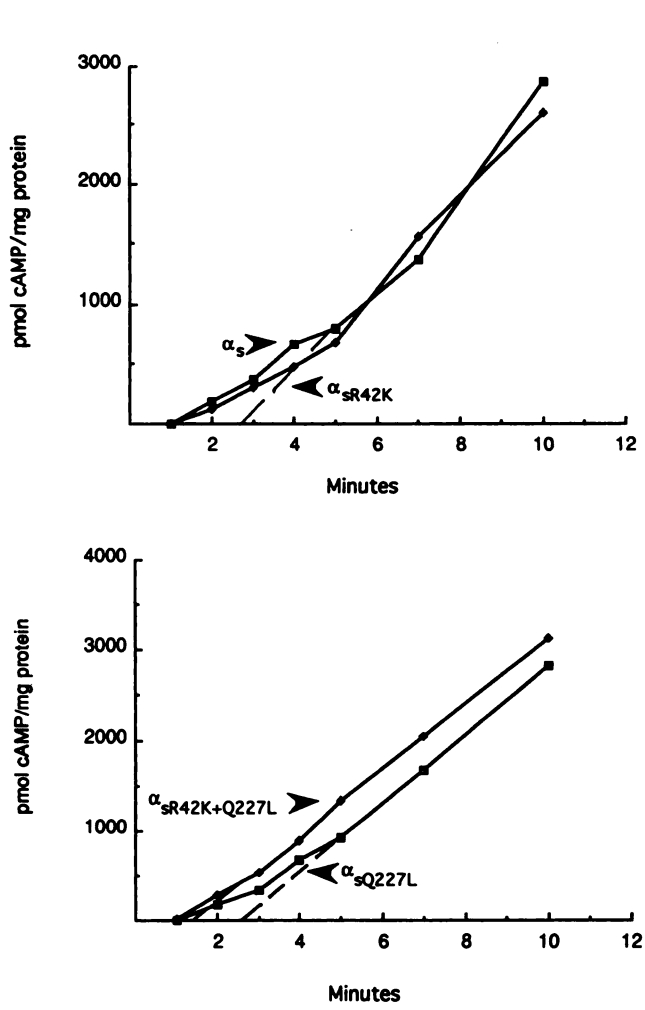


Fig. 5. A, The time-dependent activation of adenylyl cyclase by GTPγS with α_s (⊡) versus α_{sR42K} (♦) was measured as described in Materials and Methods. B, The time-dependent activation of adenylyl cyclase by GTPγS with α_{sQ227L} (⊡) versus $\alpha_{sR42K+Q227L}$ (♦) was measured as described in Materials and Methods. The time required to reach V_{max} was estimated by extrapolation of the maximal rate to the time line (– – –). For α_s, α_{sQ227L} and α_{sR42K} the time to reach V_{max} was approximately 2.5–2.75 min. The time to reach V_{max} for the $\alpha_{sR42K+Q227L}$-expressing membranes was approximately 1.5 min.

similar phenotype was observed with $\alpha_{i(34)/s}$ and the $\alpha_{i(34)/sQ227L}$ chimeras.

To demonstrate the influence of the α_{sR42K} mutation on adenylyl cyclase regulation, membranes were prepared from transfected COS cells. The time-dependent activation of adenylyl cyclase in the presence of GTPγS was then determined (Fig. 5). The α_{sR42K} mutation in the presence of the α_{sQ227L} mutation decreased the time required to reach maximal adenylyl cyclase activation, compared with α_{sQ227L} alone. Thus, introduction of the R42K mutation in the same polypeptide as the Q227L mutation altered the rate of α_s activation, similar to what was previously observed with the $\alpha_{i(54)/s}$ chimera (8, 9, 11, 12). However, the R42K mutation alone was not sufficient to activate α_s in the absence of GTPase inhibition. This contrasts with the $\alpha_{i(54)/s}$ chimera, which strongly activated cAMP synthesis in the absence of the Q227L mutation. Cumulatively, the findings indicate that Arg-42 is important but is not the only amino acid residue in the extreme amino terminus that influences the regulation of α_s activation.

We additionally constructed a series of chimeras that placed the previously described α_{i2}/α_s chimeras in combination with an α_{i2} substitution at the carboxyl terminus, which replaces α_s residues 357–394 with the analogous α_{i2} residues 320–355 (Fig. 6A). The chimera $\alpha_{i(17)/s/i(38)}$ was similar to wild-type α_s in its ability to stimulate cAMP synthesis, whereas $\alpha_{i(27)/s/i(38)}$ was slightly activating (Fig. 6B). The chimera $\alpha_{i(34)/s/i(38)}$ had an activated phenotype (Fig. 6B), and the previously described $\alpha_{i(54)/s/i(38)}$ (9) was an even stronger activator of adenylyl cyclase. An interesting result was found with the larger amino-terminal chimeras, which alone behaved essentially as wild-type α_s but in combination with the carboxyl-terminal α_{i2} substitution become very strongly constitutively activated. The chimeras $\alpha_{i(79)/s/i(38)}$, $\alpha_{i(94)/s/i(38)}$, $\alpha_{i(109)/s/i(38)}$, and previously described $\alpha_{i/s(Bam)/i(38)}$ (9) each activated adenylyl cyclase to levels similar

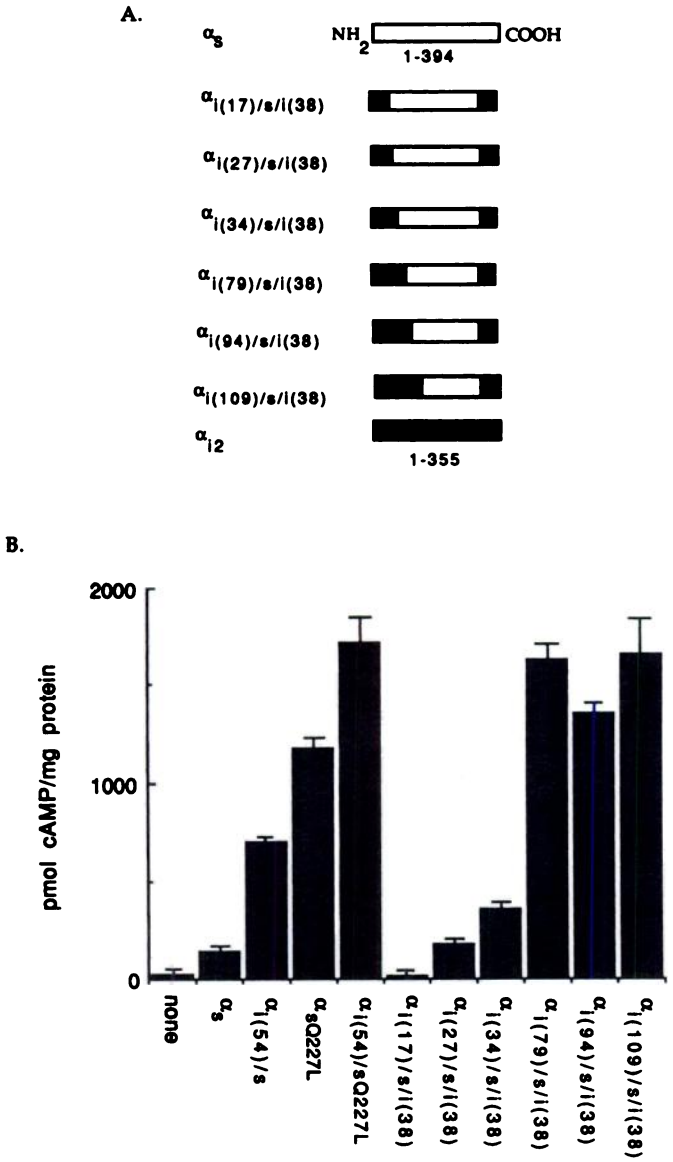


Fig. 6. A, The relative contribution of α_s (□) and α_{i2} (■) is shown for each chimera, as described in the legend to Fig. 1A. B, Chimeras were transiently expressed in COS cells and cAMP levels were measured as described in the legend to Fig. 1B.

to those seen with the strongly activating $\alpha_{i(54)/sQ227L}$ chimera (Fig. 6B). Thus, mutation at both the amino- and carboxyl termini within the same polypeptide confers a strongly constitutively activated α_s activity.

Discussion

The rate-limiting steps that control G protein regulation of effectors are 1) GDP dissociation from the α subunit, allowing subsequent GTP binding and activation, and 2) hydrolysis of the bound GTP by the intrinsic GTPase activity encoded in the α subunit, returning the α subunit to an inactive α-GTP complex. The consensus sequences among G protein α subunits involved in controlling GTPase activity have been defined (1, 18). Much less is known about the sequences involved in regulating GDP dissociation, a function that is regulated by G protein-coupled receptors and the $\beta\gamma$ subunit complex (4).

Substantial evidence suggests that the extreme carboxyl terminus of G protein α subunits functions as a major regulatory site for receptor-catalyzed GDP dissociation (2, 19). Appropriate mutation or truncation of the carboxyl terminus may lead to receptor uncoupling or enhanced GTP binding and activation (2, 15). Additionally, pertussis toxin-catalyzed ADP-ribosylation of α_i-like proteins at a cysteine four residues from the carboxyl terminus results in receptor uncoupling (20, 21).

The α subunit amino terminus appears to be a region involved in regulating interactions with the $\beta\gamma$ subunit complex (11, 22). Our work with the α_{i2}/α_s chimeras and α_s point mutations confirms that specific sequences in the α_s amino-terminal region control activation of the α_s polypeptide (9, 11, 12). We have now defined this α_s region as surrounding the G-1 sequence involved in forming the GDP/GTP binding site (1, 18, 23). Fig. 7 shows the regulatory sequence surrounding the GAGESGKS sequence comprising the G-1 region. The G-1 sequence is involved in binding the α- and β-phosphoryl groups of GDP and GTP (23). With the $\alpha_{i(54)/s}$ chimera we have shown that the ability of $\beta\gamma$ subunit complexes to inhibit the rate of GTPγS activation of adenylyl cyclase is significantly diminished, relative to that of the wild-type α_s polypeptide (24). This finding indicates that appropriate mutation in this regulatory region does indeed influence the ability of $\beta\gamma$ subunits to regulate α_s activation, without influencing the GTPase function of the α_s polypeptide.

The amino-terminal α subunit sequence defined within α_s residues Lys-25 to Glu-101 (corresponding to α_{i2} residues Met-18 to Gln-79) may be functionally switched between α_s and α_{i2} with maintenance of a wild-type-like α_s polypeptide. However, mutation within this region results in the loss of normal regulation. This is particularly apparent with the $\alpha_{i(34)/s}$, $\alpha_{i(54)/s}$, and $\alpha_{i(64)/s}$ chimeras (Fig. 7) (11). Short stretches of α_{i2} sequence up to approximately 17 amino acids or large amino-terminal α_{i2} sequences of 79 or more amino acids result in a wild-type-like α_s chimeric polypeptide, because the regulatory region is left intact and is able to properly control regulation of α_s by guanine nucleotides.

A critical residue appears to be α_s Arg-42. Mutation of α_s Arg-42 to lysine, a conserved substitution present in α_{i2}, has very modest influences on the activity of the mutant α_s, relative to the wild-type α_s polypeptide. When placed in a background of inhibited GTPase activity (α_{sQ227L}) the R42K mutant activates adenylyl cyclase as effectively as does the $\alpha_{i(54)/sQ227L}$ construct. This indicates that the R42K mutation alone has only modest effects but when the intrinsic GTPase activity is inhibited the mutant α_s has an increased ability to accumulate in an activated GTP-liganded state. The difference in basic charge distribution between the arginine and lysine side chains at α_s residue 42 is sufficient to induce this change in regulation of the α_s polypeptide by guanine nucleotides. Recently it was shown that mutation of Lys-35 to glutamate in the α_o polypeptide reduced the affinity of the mutant α_o for guanine nucleotides (25). This finding is consistent with our results showing that amino acids flanking the G-1 domain influence the binding of GDP and GTP. Our results demonstrate that a more subtle mutation in α_s, Arg-42 to lysine, can have dramatic effects on the regulation of effector enzymes by the mutant α subunit, but only in a background of inhibited GTPase activity.

At the carboxyl-terminal side of the highly conserved G-1 region is a second arginine to lysine substitution between α_s and α_{i2} polypeptides (α_s Arg-61 and α_{i2} Lys-54). Mutation of α_s

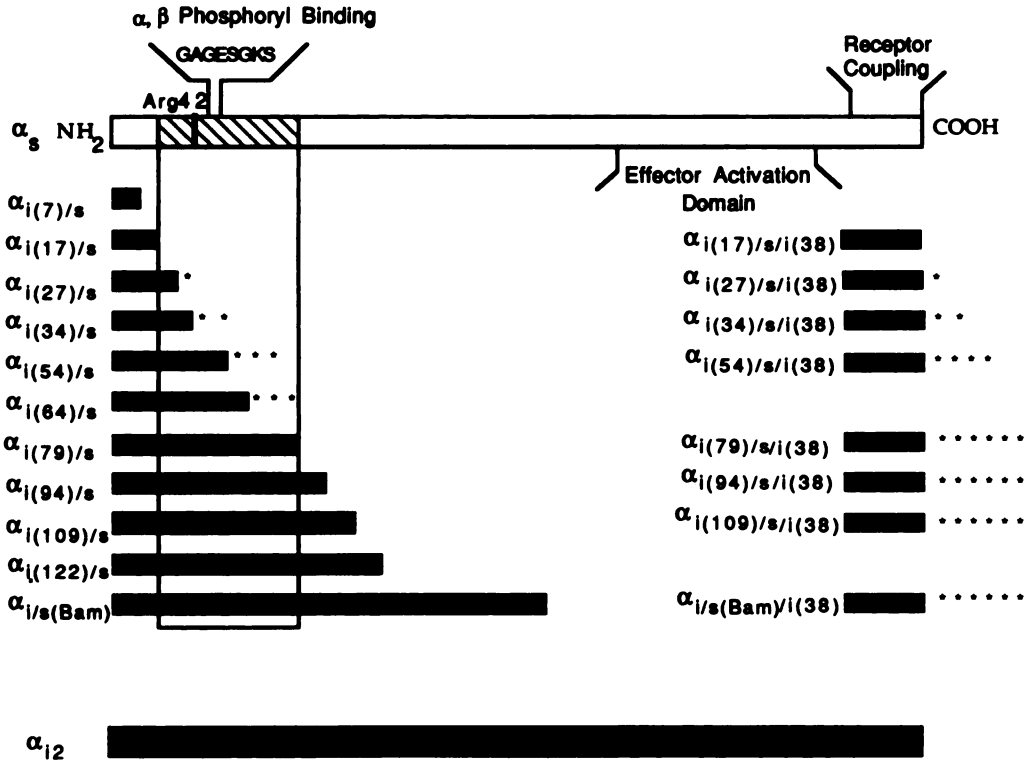


Fig. 7. Diagram of the α_s polypeptide. Highlighted by *diagonal lines* is the regulatory region, which contains Arg-42 and the G-1 binding domain, which is involved in α/β-phosphoryl binding. The portions of α_s that are replaced by the analogous α_{i2} sequence in various chimeras are shown by *black boxes*. *, Increased adenylyl cyclase activity of the chimeras, relative to wild-type α_s (*, slightly activating; ******, strongly activating). Also shown are the effector activation domain and the region involved in receptor coupling.

Arg-61 to lysine did not enhance the ability of the mutant α_s polypeptide to stimulate adenylyl cyclase activity. An important test would be to characterize the $\alpha_{sR42K,R61K}$ double-mutant polypeptide to see whether it mimicked the $\alpha_{i(54)/s}$ chimera. For reasons unknown at this time, we have been unable to express this α_s construct.

It is very important to note that mutation of the α subunit in other regions of the polypeptide generally has not produced a phenotype similar to that of the amino-terminal attenuator domain mutations. This includes mutations near or within additional sequences forming the GDP/GTP binding site (11, 26). The exception is appropriate mutation or truncation of the extreme carboxyl terminus; enhanced rates of GTPγS activation and GTP binding have been observed with these mutations (2, 13). This is the region of the α subunit that appears to interact with G protein-coupled receptors, which catalyze GDP dissociation, allowing GTP to bind. Thus, mutations in the extreme amino terminus implicated in regulation of $\beta\gamma$ interactions and in the extreme carboxyl terminus implicated in interaction with receptors yield similar phenotypes. The similarity of amino-terminal disruption and extreme carboxyl terminal mutation in enhancing GTP activation of α_s is consistent with the models predicting that the amino and carboxyl termini are in close proximity to one another (23, 26). Both the amino and carboxyl termini are predicted to be involved in the regulation of GDP dissociation and GTP activation (10, 23, 26). The activated nature of the chimeras containing different amino-terminal α_{i2} substitutions and the carboxyl-terminal 38 residues of α_{i2} supports the proposal that both the amino terminus and the carboxyl terminus are important in the regulation of α_s activation. The role of the extreme amino and carboxyl-termini in regulating α_s activation is clearly independent of the GTPase turn-off function.

Receptors that activate G_s do so by catalyzing GDP dissociation (4). This reaction must involve changes in the conformation of the α_s polypeptide that influence the affinity of α_s for GDP. The mutations we have defined that lead to enhanced GTP activation of α_s in several ways mimic the receptor activation of G_s. Both receptor activation and appropriate mutations are predicted to alter the interaction of amino acids, within the G-1 sequence, with the phosphoryl groups of GDP. This could effectively enhance GDP dissociation, allowing an accelerated rate of GTP activation. No mutations within or surrounding the G-2, G-3, or G-4 sequences of the GDP/GTP binding domain, except for mutations that inhibit GTPase, have yielded activated phenotypes similar to those observed with the amino- and carboxyl-terminal α_s mutations (10). This fact indicates that the activated nature of the amino- and carboxyl-terminal α_s mutation is due to specific changes in the α_s structure and cannot be created by generalized disruptions of other regions of the GDP/GTP binding domain.

It is interesting that the G_q α subunit (α_q) is quite divergent from the α_i and α_s sequences at the amino- and carboxyl-terminal regions (20). G_q also has a very slow GDP dissociation rate, relative to that of G_i or G_s in the absence of receptor (27). It will be of interest to determine whether mutations of the

extreme amino and carboxyl termini of α_q will alter this characteristic of high GDP affinity, relative to α_i or α_s. If so, it will substantiate the hypothesis that the amino- and carboxyl-terminal regions of G protein α subunits regulate GDP dissociation.

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