THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 29, Issue of October 15, pp. 21889–21894, 1993
Printed in U.S.A.

Random Mutagenesis of G protein α Subunit $G_o\alpha$

MUTATIONS ALTERING NUCLEOTIDE BINDING*

(Received for publication, May 29, 1993, and in revised form, July 7, 1993)

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Nucleotide binding properties of the G protein α subunit $G_o\alpha$ were probed by mutational analysis in recombinant *Escherichia coli*. Thousands of random mutations generated by polymerase chain reaction were screened by *in situ* [^{35}S]GTPγS (guanosine 5′-(3-*O*-thio)-triphosphate) binding on the colony lifts following transformation of bacteria with modified $G_o\alpha$ cDNA. Clones that did not bind the nucleotide under these conditions were characterized by DNA sequence analysis, and the nucleotide binding properties were further studied in crude bacterial extracts. A number of novel mutations reducing the affinity of $G_o\alpha$ for GTPγS or Mg^{2+} were identified. Some of the mutations substitute amino acid residues homologous to those known to interact with guanine nucleotides in $p21^{ras}$ proteins. Other mutations show that previously unstudied residues also participate in the nucleotide binding. Several mutants lost GTPγS binding but retained the capacity to interact with the βγ subunit complex as determined by pertussis toxin-mediated ADP-ribosylation. One of these, mutant S47C, was functionally expressed in *Xenopus laevis* oocytes along with the G protein-coupled thyrotropin-releasing hormone (TRH) receptor. Whereas wild-type $G_o\alpha$ increased TRH-promoted chloride currents, S47C significantly decreased the hormone-induced Cl^- response, suggesting that this mutation resulted in a dominant negative phenotype.

Heterotrimeric GTP-binding proteins (G proteins)[1] link the excitation of cell-surface receptors to the activity of intracellular effector enzymes or ion channels. G protein specificity is thought to be determined at least in part by the α subunits that are most divergent and contain the GTP binding site. The protein-protein interactions of the α subunit with the βγ subunit complex, receptor, and effector depend upon a cycle of binding and hydrolysis of the nucleotide. In the GDP-bound form, the protein is essentially in a resting state primed to receive the incoming signal. In the GTP-bound active form, the G protein can interact with its effector starting a chain of downstream intracellular events. Activation of G protein is a complicated process that includes receptor-catalyzed dissociation of the pre-bound GDP and binding of GTP and Mg^{2+}, which triggers a strong change in α subunit conformation and dissociation of the αβγ heterotrimer (for reviews, see Refs. 1–4). Much of the understanding of the mechanisms of G protein function has come from "freezing" the G protein cycle at certain phases via ADP-ribosylation by bacterial toxins (5, 6) or by site-specific mutagenesis (7–12). However, most of the functional mutations introduced into mammalian G proteins were based upon analogies with the well-studied related GTP-binding $p21^{ras}$ proteins (13). We have developed a system for large scale systematic mutational analysis of the α subunit of the heterotrimeric G protein G_o expressed in *Escherichia coli* (14). We generated numerous random mutations in different parts of the α subunit by means of PCR and then screened the clones expressing recombinant mutant protein for changes in specific G protein functions. Mutants with altered functional properties were further characterized in a series of *in vitro* assays and by DNA sequence analysis. Using this approach we can 1) map mutations that affect specific functions, and 2) obtain functional mutants that can be used in general signal-transduction studies, like those that determine receptor and effector specificity. In the present study we have focused on guanosine triphosphate binding: the key event in G protein activation. A number of novel mutants with altered nucleotide binding properties have been identified and characterized. Some of the mutations abolished GTPγS binding while retaining specific interactions between the α subunit and βγ complex even in the presence of guanosine triphosphate. *In vivo*, such mutants may block certain phases of specific G protein-mediated pathways, by competing with the wild-type Gα for endogenous βγ (or receptors), and therefore produce a dominant negative phenotype. We provide evidence that one of the mutants behaves as a dominant negative when coexpressed with the TRH receptor in *Xenopus* oocytes.

EXPERIMENTAL PROCEDURES

Random Mutagenesis of the $G_o\alpha$ cDNA and Construction of the Mutant Libraries—The target $G_o\alpha$ (subtype A) cDNA modified to have five unique restriction sites was placed under T7 promoter control in the expressing plasmid $pG_o\alpha$ (14). The mutations were introduced by means of PCR, as described previously for the *Nco*I-*Bst*XI fragment (14); in the present study, the mutagenized regions cover the entire sequence. 20 cycles of PCR were performed; the reaction mixture contained 0.5 mM $MnCl_2$. To confine the mutations to certain regions of the $G_o\alpha$, the five fragments of mutated cDNA (about 200 base pairs each) were ligated with the $pG_o\alpha$ vector at the corresponding restriction sites to substitute the wild-type sequence leaving a specific fragment with the mutation. *E. coli* strain BL21DE3 was transformed by electroporation to produce five libraries of random mutations in recombinant $G_o\alpha$.

Screening of the Mutant Libraries—The five mutant libraries were analyzed separately by *in situ* [^{35}S]GTPγS binding and staining with the carboxyl-terminal antibody, as described previously (14). Clones that produced the full-size protein but did not bind [^{35}S]GTPγS *in situ* ("G$^-$" mutants) were isolated and sequenced. The $G_o\alpha$ mutants were further studied in the soluble lysates (14) of these clones. Typically, 16 different mutant *E. coli* clones were grown simultaneously in 25-ml cultures and induced by isopropyl-1-thio-β-D-galactopyranoside at 30 °C.

Determination of Bound Nucleotide—Determination of GTPγS binding activity of the recombinant G protein α subunits was performed as described previously (14). 20 μl of each extract (concentration of total *E.*

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[1] The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; TRH, thyrotropin-releasing hormone; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; PCR, polymerase chain reaction.

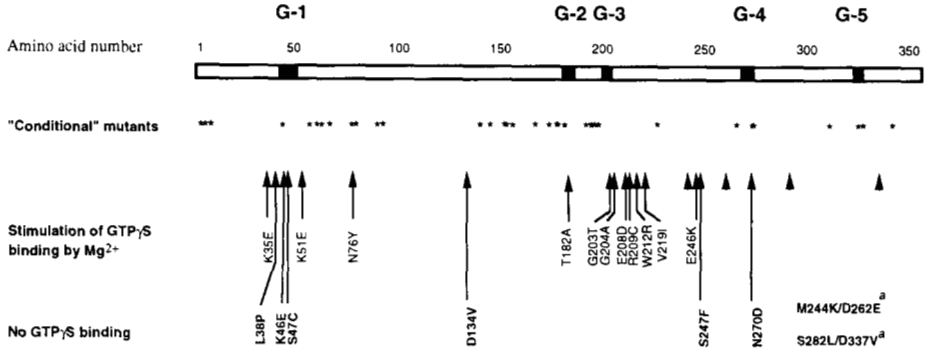

FIG. 1. **Distribution of mutations altering the guanine nucleotide in the $G_o\alpha$ protein sequence.** The *open bar* represents the linear sequence of $G_o\alpha$ with the number of the amino acid residue indicated above. The *shaded boxes* designate locations of the regions G-1 to G-5, which participate in binding and hydrolysis of GTP (13). Positions of the G⁻ mutations are indicated with *asterisks* or *arrows* with the phenotypes specified to the *left*. *Asterisks* indicate positions of conditional mutants: those that showed no [^{35}S]GTPγS binding in the *in situ* assay but were found to be similar to the wild-type $G_o\alpha$ in the crude extract preparation (see text for explanation). *Arrows* indicate positions of the mutations that result in a significant reduction in affinity for GTPγS in the presence of low concentrations of Mg^{2+}. *Longer arrows* indicate mutations causing a dramatic reduction or complete loss of affinity for the guanine nucleotide regardless to the magnesium ion concentration. *a* indicates two double mutants belonging to the class of mutants showing no GTPγS binding indicated with *arrowheads*.

TABLE I
Functional properties of $G_o\alpha$ mutants

The G⁻ mutants with one or two amino acid substitutions are included in the table. Mutants are divided into three groups, according to the degree of loss of affinity for GTPγS. ND, not determined; NA, data not available.

Mutation	Extent of GVTP-γS binding[a]	High affinity GTPγS binding in the presence of[b]		Reversibility of Mg^{2+} effect on GTPγS binding[e]		βγ-Subunit-dependent ADP-ribosylation by pertussis toxin[f]
		Mg^{2+}[c]	EDTA[d]	Mg^{2+}[c]	EDTA[d]	
Wild-type rGoá	Mg < EDTA	+	+	No	No	Inhibited by GTPγS
Conditional mutants						
S44T[g]	Mg < EDTA	+	+	No	No	Inhibited by GTPγS
S62P[h]	Mg > EDTA	+	+	No	No	Inhibited by GTPγS
Mg^{2+}-dependent mutants						
K35E	Mg = EDTA	+	–	NA		Very low in general (14)
K51E	Mg = EDTA	+	–	NA		Inhibited by GTPγS[i]
N76Y	Mg > EDTA	+	–	No	Yes	Inhibited by GTPγS[i]
T182A	Mg > EDTA	+	–	No	Yes	Inhibited by GTPγS[i]
G203T	Mg ≫ EDTA	–	–	Yes	Yes	Inhibited by GTPγS
G204A	Mg ≫ EDTA	–	–	Yes	Yes	Inhibited by GTPγS[i]
E208D	Mg ≫ EDTA	–	–	Yes	Yes	Inhibited by GTPγS[i]
R209C	Mg = EDTA	+	–	NA		ND
R209C/D238D	Mg ≫ EDTA	–	–	Yes	Yes	**Not inhibited by GTPγS**
W212R	Mg < EDTA	+	–	ND		Inhibited by GTPγS[i]
V219I	Mg = EDTA	+	–	NA		ND
V219I/N256S	Mg ≫ EDTA	–	–	Yes	Yes	Inhibited by GTPγS[i]
E246K	Mg = EDTA	+	–	NA		ND
E246K/F216S	Mg ≫ EDTA	ND	–	ND	Yes	ND
No GTPγS binding						
L38P/T4M	Not detected	NA		NA		ND
K46E	Not detected	NA		NA		Not detected
S47C	Not detected	NA		NA		**Not inhibited by GTPγS**
D134V	Very low, Mg ≫ EDTA	NA		NA		Not detected
M244K/D262E	Not detected	NA		NA		Not detected
S247F	Not detected	NA		NA		ND
N270D	Very low, Mg = EDTA	NA		NA		**Not inhibited by GTPγS**
S282L/D337V	Not detected	NA		NA		**Not inhibited by GTPγS**

[a] [^{35}S]GTPγS binding was determined at saturation conditions (*i.e.* 35–45 min) in the crude bacterial extract. We found that the extent of the nucleotide binding to the wild-type recombinant $G_o\alpha$ was slightly (10–30%) inhibited when millimolar Mg^{2+} was present in the reaction (Fig. 2*A*). Since this effect decreased with dilution of the sample (data not shown), we consider the inhibition to be an artifact related to impurity of the lysates. At the same time, Mg^{2+} reproducibly stimulated (10–60%) nucleotide binding of the $G_o\alpha$ mutants belonging to the 2nd group of "conditional" mutants (see footnote *h*). This effect, however, was not as strong as with the "Mg^{2+}-dependent mutants," where it was very distinctive and apparently related to the nature of mutation and not to an artifact of the assays. We presume that all of these proteins were myristoylated (14).

[b] Pseudo-irreversible binding of ^{35}S determined by "chasing" with the unlabeled nucleotide, as shown in Fig. 2*B*.

[c] Typically, 4 mM free Mg^{2+}.

[d] 1 mM excess of EDTA over Mg^{2+}, *i.e.* micromolar Mg^{2+}.

[e] Reversibility of the Mg^{2+} effect upon the GTPγS binding was determined in an experiment as shown in Fig. 2 (*C* and *D*).

[f] In the present work, determination of the labeling has been performed qualitatively on the basis of radioautograms, without quantitation of the effect of βγ (14).

[g] Similar phenotype was also revealed by the following G⁻ mutants: G2E, C3R, S6N, I56N, N76I, L78T, M88V, L91S, C140R, R145W, K154G, L176F, V180D, F192L, K193E, N194Y, A227T, K272N, K311E, C325S, T327A, E43G/Q52I, and K211E/K272E. Mutants from this group with a single amino acid substitution are designated with asterisks in Fig. 1.

[h] Similar phenotype was also revealed by the following G⁻ mutants: E64G, D65G, D151N, G164V, E172G, L176I, N194I, K271E, I343N and K271E/K272R. Mutants from this group with a single amino acid substitution are designated with asterisks in Fig. 1.

[i] Inhibition of the labeling of this mutant by 100 μM GTPγS was less pronounced than for the wild-type $G_o\alpha$.

coli protein was 5 mg/ml) was placed in the wells of a microtiter dish. They were mixed at 22 °C with 220 µl of the TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol) containing 0.25 µM [^{35}S]GTPγS (40,000 cpm/pmol). For the time-course experiments, eight parallel aliquots (18 µl) were withdrawn from the reactions with an eight-channel pipette. They were diluted with 150 µl of ice-cold TED buffer in the wells of matching eight-well filter strip (Costar, product 8523, 0.45 µm nitrocellulose), filtered, and washed four times with the same buffer under vacuum. The filter strips were dried, the wells separated, and the amount of bound radioactivity determined on each filter by scintillation counting. By using this modification of the assay we could process about 200 (usually 256 (64 × 4) or 192 (96 × 2)) samples within 3–4 h.

ADP-ribosylation of Recombinant $G_o\alpha$—Pertussis toxin-catalyzed ADP-ribosylation was performed in crude extracts as described previously (14).

In Vitro Transcription—The cRNA of $G_o\alpha$ mutants and TRH receptor (15) was synthesized using T7 polymerase with *in vitro* transcription kit (Ambion) according to the manufacturer's protocol.

Injection and Treatment of Oocytes—*Xenopus* oocyte maintenance, cRNA injections, and electrophysiological methods are described in detail elsewhere (16). Briefly, oocytes were defolliculated and maintained at 18 °C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4), 2.5 mM sodium pyruvate, 50 µg/ml gentamicin, and 5% horse serum (17). The cRNAs were diluted to appropriate concentrations in water, injected into the oocytes in 50-nl volumes, and allowed to translate for 2–4 days. Approximately 3 h prior to measurement, the oocytes were transferred to incubation medium without horse serum. During the experiment, the oocytes were superfused continuously with Ca^{2+}-free ND96, and TRH was applied in this solution. Whole cell currents were measured at room temperature using a Dagan 8500 amplifier in a standard two-electrode voltage-clamp configuration. The oocytes were voltage-clamped at a holding potential of −80 mV, and changes in current were monitored by oscilloscope and chart recorder.

RESULTS AND DISCUSSION

Screening of the Mutant Libraries—The libraries of $G_o\alpha$ mutants expressed in *E. coli* were subjected to two rounds of screening. First, [^{35}S]GTPγS binding was studied on colony lifts from agar plates (14). This method allowed rapid analysis of about 6000 transformants. The average mutagenesis rate determined by sequencing of randomly chosen colonies was about 30% (*i.e.* approximately 2000 mutants were screened for GTPγS binding). A total of 147 clones that expressed the full-size recombinant protein but did not bind the radionucleotide *in situ* (G^- mutants) were identified. Sequencing allowed us to eliminate the clones bearing more than two mutations and to avoid repetitions (*i.e.* clones with the same mutation), reducing the number to 52. The amino acid changes in the mutants were distributed nearly evenly throughout the entire sequence (Fig. 1, Table I), and their number seemed high in light of the rather short length of the homology regions that have been suggested to contain the residues involved in nucleotide binding. These clones were tested in a secondary screen by measuring [^{35}S]GTPγS binding in the soluble fraction of bacterial lysates (Fig. 2). Under these conditions we identified only 16 mutants (see Fig. 1 and Table I) with dramatically reduced nucleotide binding. Most of the G^- mutants did not reveal distinct changes in phenotype (*i.e.* 23 of the clones could not be distinguished from the wild-type $G_o\alpha$) (Table I, footnote g), while 12 mutants (Table I, footnote h) were only slightly inhibited in the extent of binding by excess EDTA. We classified such mutants as "conditional," since it is most likely that the secondary screen could not detect them due to differences in the assay procedures. The *in situ* binding protocol includes exposure of the expressed $G_o\alpha$ to chloroform vapor upon cell lysis and immobilization on the nitrocellulose filter prior to the binding with the nucleotide. Some of the G^- mutations could result in a slight conformational change that reduces stability of the recombinant G protein and promotes its denaturation. Under the relatively mild conditions of the assay performed in the bacterial extract this

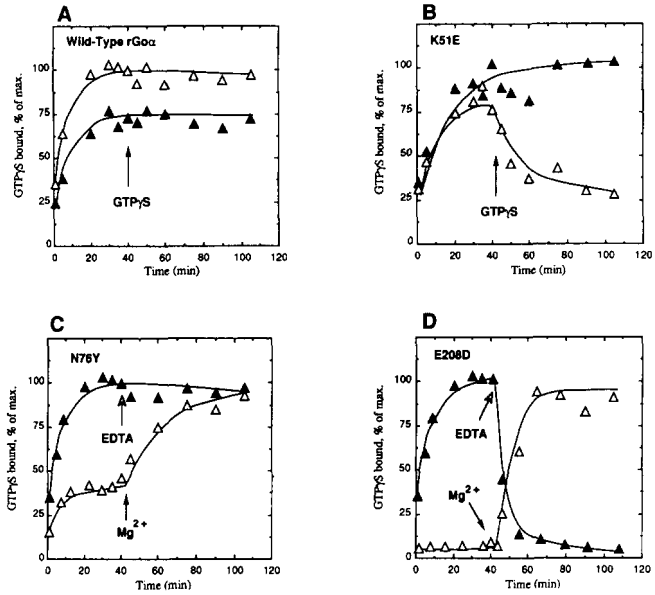

FIG. 2. **GTPγS binding to wild-type and mutant recombinant $G_o\alpha$.** 20 µl of the *E. coli* extract containing wild-type (*A*) or mutant (*B–D*) G_oa was diluted 12-fold with TED buffer with 0.25 µM [^{35}S]GTPγS (40,000 cpm/pmol) and incubated at 22 °C. At the indicated times, 18-µl aliquots were withdrawn and assayed for the bound nucleotide. [^{35}S]GTPγS was allowed to bind in the presence of 5 mM $MgCl_2$ (*black triangles*) or 0.1 mM $MgCl_2$ plus 1.0 mM EDTA (*open triangles*). *A* and *B*, after 40 min, 100 mM unlabeled GTPγS was introduced. *B*, the data shown are a single experiment representative of three experiments (n = 3) performed with K51E extract. Similar binding characteristics were obtained for the mutants K35E (14), R209C, W212R, V219I, and E246K. *C* and *D*, after 40 min, either EDTA or $MgCl_2$ was added to final concentrations of 7.5 and 5 mM, respectively. *C*, representative experiment with mutant N76Y is shown; a similar phenotype was observed with mutant T182A. *D*, representative experiment with mutant E208D is shown; a similar phenotype was observed with mutants R209C/D238E, V219I/N256S, and F216S/E246K, as well as mutants G203T and G204A (14).

phenotype might not be detected. We found that several G^- mutants (for example mutants N76I,[2] N194I, and C325S) were distinctively less stable to freeze-thawing in the crude bacterial extract than the wild-type recombinant $G_o\alpha$. Another reason for the lower selectivity of *in situ* GTPγS binding could be that in this assay the filters were washed for a long time (30–60 min) at room temperature following [^{35}S]GTPγS exposure, whereas in the regular binding assay (in solution), washing the unbound radioligand from the filters by vacuum filtration occurs in less than 1 min in ice-cold buffer.

$G_o\alpha$ Mutations That Affect High Affinity Binding of GTPγS and Mg^{2+}—The key event in G protein-mediated signaling is activation of the G protein promoted by the binding of guanosine triphosphate. The active conformation of the α subunit is characterized by an increased intrinsic fluorescence and resistance of the protein to proteolysis (18–20). This conformation requires the high affinity binding of Mg^{2+} and represents the complex $G\alpha\cdot GTP\cdot Mg^{2+}$. Both ligands, the cation and the nucleotide, are bound very tightly ("pseudoirreversibly") and can dissociate only after hydrolysis of GTP (18, 19, 21, 22). Therefore, reduction of affinity to either Mg^{2+} or GTP can result in the inability of the G protein to assume the active conformation.

Many of the mutants with reduced affinity for GTPγS showed a stimulatory effect of Mg^{2+} on nucleotide binding. The

[2] Mutant proteins are designated by the wild-type amino acid residue (single-letter code), the position of this residue, and the residue used for the displacement.

extent of the stimulation varied with different mutants, apparently reflecting the difference in their affinity for Mg^{2+} (Fig. 2). Micromolar concentrations of free Mg^{2+} promoted by an excess of EDTA over the cation are sufficient to support the high affinity binding of GTPγS to the wild-type recombinant $G_o\alpha$ (Fig. 2*A*). The tightly bound [^{35}S]GTPγS cannot dissociate and be replaced by unlabeled nucleotide subsequently added to the reaction mixture. In contrast, several mutants (mutant K51E is shown as an example in Fig. 2*B*) could bind GTPγS pseudoirreversibly only in the presence of millimolar Mg^{2+}. For these mutants, the extent of [^{35}S]GTPγS binding in the presence of excess EDTA was approximately the same as in the presence of millimolar Mg^{2+}, but the bound [^{35}S]GTPγS could be readily exchanged for subsequently added unlabeled ligand. Interestingly, this phenotype (*i.e.* loss of the pseudoirreversible nucleotide binding at low Mg^{2+} concentrations) was characteristic for a number of mutations located in distant parts of the polypeptide chain: K35E (14), N76Y, T182A, R209C, W212R, V219I, and E246K.

Another set of mutants was characterized by a stronger effect of Mg^{2+} on the extent of nucleotide binding. Millimolar Mg^{2+} increased the level of [^{35}S]GTPγS binding 3–5-fold for the mutants N76Y and T182A, compared to binding in excess EDTA. In the presence of EDTA, the nucleotide could be exchanged for unlabeled ligand. However, addition of 7.5 mM EDTA to the assay in which 5 mM Mg^{2+} was present initially did not reverse the binding, suggesting that once Mg^{2+} is bound, affinity of the Gα·Mg^{2+}·GTPγS complex is high (Fig. 2*C*). Reversal of [^{35}S]GTPγS binding by excess EDTA was characteristic of the mutants E208D, R209C/D238E, V219I/N256S, and F216S/E246K; EDTA promoted a rapid dissociation of nucleotide from the mutant α subunit. In the reciprocal experiment, when excess Mg^{2+} was added to these mutants after preincubation with EDTA, nucleotide binding reached the level observed when Mg^{2+} was present initially (Fig. 2*D*). Even in the presence of 5 mM Mg^{2+}, GTPγS binding to these mutants was not pseudoirreversible.

The nucleotide binding to the mutants S47C, D134V, and N270D was hardly detectable above background. Addition of unlabeled GTPγS to the assay with or without Mg^{2+} instantly reduced [^{35}S]GTPγS binding to zero (data not shown). With mutants T4M/L38P, K46E, M244K/D262E, S247F, and S282L/D337V, specific nucleotide binding in the extracts was not detected, although Western analysis indicated that mutants were present in the extracts at least at the same level as the wild-type recombinant protein (data not shown). These mutants could reveal the residues that are directly involved in nucleotide binding or that may be critical for maintaining the overall tertiary structure (folding) of the protein.

Distribution of G^- Mutations in the $G_o\alpha$ Polypeptide Chain: Homology with $p21^{ras}$ Proteins—Analysis of the crystallized complexes of $p21^{ras}$ with GTP and GDP (23) provided important information about these proteins and identified amino acid residues that interact with the nucleotides. G protein α subunits share distinctive sequence homology with $p21^{ras}$ proteins in certain conserved regions that are considered to be involved in the nucleotide binding (13). Most of the mutations that we found in our screens were located within or in close proximity to these regions (Fig. 1). In many cases the affected amino acid residues are homologous to those involved in nucleotide binding with $p21^{ras}$. For example, mutation K46E involves the residue corresponding to Lys-16 in $p21^{ras}$, which forms bonds with α- and β-phosphates of either GDP or GTP. Residues Ser-47 and Thr-182 of $G_o\alpha$ are homologous to Ser-17 and Thr-35 of $p21^{ras}$ that were found to coordinate Mg^{2+} ion. The side chain of the latter residue interacts directly with the γ-phosphate of GTP and points away from GDP bound to $p21^{ras}$ (13). In $p21^{ras}$ the entire region downstream of threonine 35 (corresponding to Thr-182 in $G_o\alpha$) undergoes a dramatic conformational change upon shifting from the GDP- to the GTP-bound form. In the α subunits of heterotrimeric G proteins, the sequence ^{201}DVGGQR is conserved through the entire family. The glycine residues in positions 203 and 204 are reported to be important for high affinity binding of GTP, while residue Gln-205 is critical for hydrolysis of the nucleotide (10–14). The nucleotide binding characteristics of mutants E208D, R209C/D238E, and V219I/N256S were similar to those of G203T and G204A mutants. Therefore, not only the 2 glycine residues 203 and 204 but the entire stretch of 15–20 downstream amino acids may be important for the movements of the polypeptide chain during GTP-promoted activation.

Interestingly, homology with $p21^{ras}$ proteins can also be found for the amino acid residues that are not directly involved in nucleotide binding. The amide protons of the Asn-116 of $p21^{ras}$ stabilize the nucleotide binding allosterically due to hydrogen bonds with residues Lys-13 and Ser-14 that are directly involved in coordination of the ligand. In $G_o\alpha$, mutation of the homologous residue Asn-270 to aspartate dramatically reduces the protein affinity for GTPγS, perhaps by a similar mechanism.

There are also certain differences in the molecular mechanism of guanine nucleotide binding between G proteins and $p21^{ras}$. For example, substitution of residue Lys-271 of $G_o\alpha$ by the "opposite" residue (*i.e.* negatively charged glutamate) had no effect, whereas the homologous residue Lys-117 in $p21^{ras}$ was found to be important for the binding of the nucleotide. Substitution of other amino acid residues that are conserved in all Gα proteins (K272E, C325S, T327A, etc.; see Table I legend) had little or no effect on the activities examined. On the other hand, mutations like D134V and E246K that reduced GTPγS binding do not lie near any of the conserved sequences previously known to be related to nucleotide binding. These mutations may function through a mechanism that is specific to the heterotrimeric GTP-binding proteins.

Potential Dominant Negative Mutants—The expression of a dominant negative mutant provides a useful way to specifically block a G protein-mediated signaling pathway. However, information about such mutants in mammalian G proteins is quite limited; essentially, only one putative dominant negative mutation (G226T in G_s) has been tested *in vivo*. Expression of $G_s\alpha$ mutant G226T in COS-1 cells has been shown to inhibit β-adrenergic receptor stimulation of cAMP synthesis (10), and the homologous mutation in $G_i\alpha$ (G204A) has been reported to slow the growth of NIH 3T3 fibroblasts (9), suggesting involvement of G_i in the control of the cell proliferation. However, these mutants had only partial inhibitory effects, mutants with a stronger dominant negative phenotype that would completely block the pathways could provide more efficient tools for the assessment of the roles of various G proteins *in vivo*.

To find mutants with reduced nucleotide binding that retain the capacity to interact with βγ subunits and thus act as dominant negative mutants, we screened by ADP-ribosylation with pertussis toxin which requires interaction with βγ to modify the Gα subunits (14, 24). Some of the mutants, namely L38P, K46E, D134V, and M244K/D262E, did not serve as substrates for pertussis toxin, suggesting that these mutations may prevent the proper folding of the protein or cause its denaturation. In contrast, mutants S47C, N270D, R209C/D238E, and S282L/D337V, which also could not bind GTPγS, retained the ability to be ADP-ribosylated by the toxin in the presence of exogenous βγ in a manner similar to the wild-type recombinant $G_o\alpha$. 100 mM GTPγS had no effect upon modification of these mutants, whereas it completely blocked the labeling of the wild-type $G_o\alpha$, presumably by causing dissociation of α from βγ subunits (Fig.

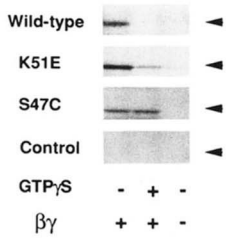

FIG. 3. **Influence of GTPγS on pertussis toxin-mediated ADP-ribosylation of wild-type and mutant $rG_o\alpha$.** G proteins were ADP-ribosylated in the crude extracts with pertussis toxin in the presence of [^{32}P]NAD (14). The reactions contained: *E. coli* extract (10–20 μg of protein), 100 ng of retinal βγ subunit complex, and 100 μM GTPγS. After 45 min at 22 °C, the reaction was stopped by heating for 3 min at 90 °C in SDS-polyacrylamide gel electrophoresis sample buffer. The samples were resolved on a 12% SDS gel, which was subjected to autoradiography. The *arrows* indicate the position of recombinant $G_o\alpha$ (39 kDa) on the gel. *Control*, *E. coli* extract without expressed G protein.

3). Mutants S47C, N270D, S247F, R209C/D238E, and S282L/D337V are candidates for proteins that may possess strong dominant negative phenotypes, because the inactive αβγ complex of these mutants does not dissociate in the presence of guanosine triphosphate. These mutations may have a stronger effect than the previously described G204A, because, in the same type of experiment, the ADP-ribosylation of the latter mutant was inhibited by GTPγS (14). Several other mutants, such as K51E (Fig. 3), may also be potential dominant negatives because the effect of GTPγS on the ADP-ribosylation was noticeably reduced.

We have begun to assess the functional properties of these $G_o\alpha$ mutants by examining their effect on G protein-linked, receptor-mediated responses in *Xenopus* oocytes. The advantage of the oocyte system for screening $G_o\alpha$ mutants is that endogenous G_o in the oocytes apparently acts on phospholipase C to mediate a Ca^{2+}-activated Cl^- conductance that can be measured electrophysiologically (25, 26). The effects of one $G_o\alpha$ mutant, S47C, on TRH receptor-mediated Cl^- currents is shown in Fig. 4. Application of TRH to oocytes injected with cRNA for the TRH receptor results in the generation of a transient inward current. This inward current is reduced in oocytes that have been coinjected with cRNA for the mutant S47C (Fig. 4*A*). In all four oocyte batches tested, coexpression of TRH receptor and S47C resulted in an approximately 50% reduction in Cl^- current when compared to oocytes injected with receptor alone. This is in contrast to oocytes coinjected with TRH receptor and wild-type $G_o\alpha$, which results in an approximately 2-fold increase in hormone-induced current. When the TRH receptor is coexpressed with equal amounts of wild-type and mutant G protein α subunit cRNAs, an intermediate response is obtained (Fig. 4*B*), as though the wild-type and mutant G protein α subunits are competing for receptor binding, for βγ binding, or both. The dominant negative phenotype of mutant S47C is further supported by the evidence presented in Fig. 4*C*, in which the extent of TRH-induced Cl^- current reduction was correlated with increasing amounts of injected S47C. These data may reflect competition between the S47C mutant and the endogenous $G_o\alpha$. Further experiments are necessary to understand the molecular mechanism of the inhibition. Interestingly, the S47C mutant found in our screen is homologous to the S17N mutant of $p21^{ras}$ that has been reported to inhibit growth of NIH 3T3 fibroblasts (27) and attenuate nerve growth factor-induced neuronal differentiation of PC12 cells (28). It is currently not known what phenotypes will be produced by homologous S47C mutation in other G protein α subunits or in other *in vivo* systems. This study suggests, however, that further analysis of the other Gα mutants described here in signaling pathways of oocytes and transfected mammalian cells will be of interest.

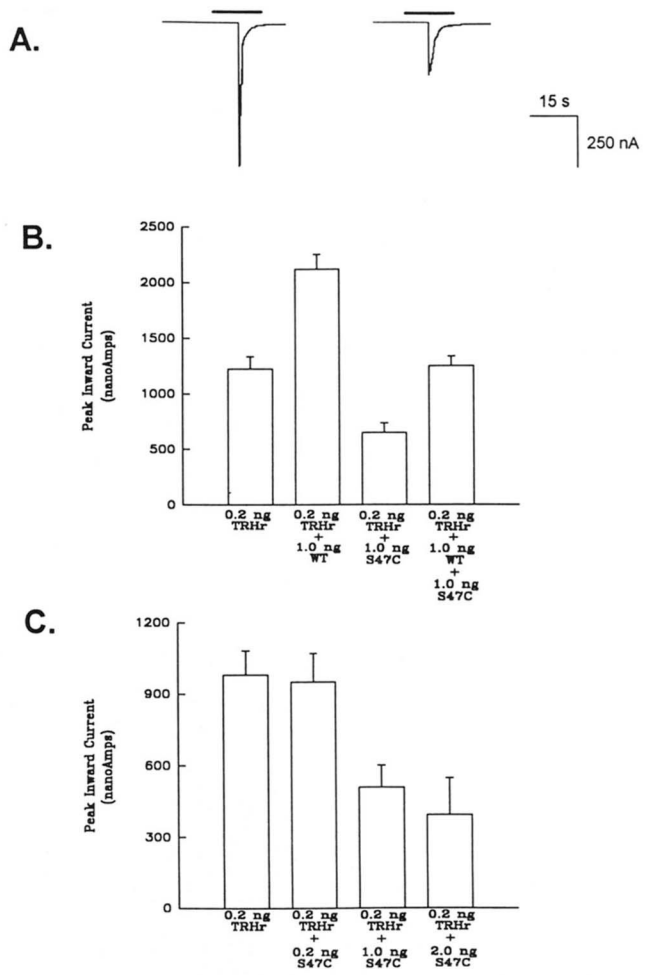

FIG. 4. **Effect of wild-type $G_o\alpha$ and mutant S47C expression on TRH-induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes.** Oocytes were injected with cRNAs encoding the TRH receptor alone or the TRH receptor in combination with wild type $G_o\alpha$, the S47C mutant, or both. Functional expression of these proteins was monitored by examining the oocyte's endogenous Ca^{2+}-activated Cl^- conductance generated in response to application of 1 mM TRH. *A*, representative raw data traces for two oocytes injected with either 0.2 ng of TRH receptor cRNA (*left trace*) or 0.2 ng of TRH receptor cRNA and 1.0 ng of S47C cRNA (*right trace*). The *downward traces* reflect inward current generated by TRH applied for the time course depicted by the *bars above* each *trace*. *B*, differences in TRH-induced inward currents in oocytes coexpressing either wild type $G_o\alpha$, S47C, or both. *C*, the effect of increasing the amount of coinjected S47C cRNA on peak inward currents induced by application of TRH. In *B* and *C*, the data are plotted as the peak inward current response (mean ± S.E.) as a function of different cRNAs and concentrations injected. At least five oocytes were measured for each RNA variant. The data shown are representative of four independent experiments on different oocyte batches.

REFERENCES

1. Birnbaumer, L., Abramowitz, J., and Brown, A. M. (1990) *Biochim. Biophys. Acta* **1031,** 163–224
2. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) *Annu. Rev. Biochem.* **60,** 349–400
3. Simon, M. I., Strathman, M. P., and Gautam, N. (1991) *Science* **252,** 802–808
4. Gilman, A. G. (1987) *Annu Rev. Biochem.* **56,** 615–649
5. Cassel, D., and Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2669–2673
6. Katada, T., and Ui, M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 3129–3133
7. Masters, S. B., Miller, R. T., Chi, M.-H., Chang, F.-H., Beiderman, B., Lopez, N. G., and Bourne, H. R. (1989) *J. Biol. Chem.* **264,** 15467–15474
8. Graziano, M. P., and Gilman, A. G (1989) *J. Biol. Chem. 264,* 15475–15482
9. Hermouet, S., Merendino, J. J., Gutkind, J. S., and Spiegel, A. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10455–10459
10. Osawa, S., and Johnson, G. L. (1991) *J. Biol. Chem.* **266,** 4673–4676
11. Lee, E., Taussig, R., and Gilman, A. G. (1992) *J. Biol. Chem.* **267,** 1212–1218
12. Berlot, C. H., and Bourne, H. R. (1992) *Cell* **68,** 911–922

13. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature* **349,** 117–126
14. Slepak, V. Z., Wilkie, T., M., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 1414–1423
15. Aragay, A. M., Katz, A. and Simon, M. I. (1992) *J. Biol. Chem.* **267,** 24983–24988
16. Quick, M. W. and Lester, H. A. (1993) in *Methods in Neuroscience: Ion Channels of Excitable Cells* (Conn, P. M., ed) Academic Press, Orlando, FL, in press
17. Quick, M. W, Naeve, J, Davidson N, and Lester H. A (1992) *Bio/Techniques* **13,** 358–362
18. Higashijima, T., Ferguson, K. M., Sternweis, P. C., Ross, E. M., Smigel, M. D., and Gilman, A. G. (1987) *J. Biol. Chem.* **262,** 752–756
19. Higashijima, T., Ferguson, K. M., Smigel, M. D., and Gilman, A. G. (1987) *J. Biol. Chem.* **262,** 757–761
20. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem.* **259,** 13806–13813
21. Higashijima, T., Ferguson, K. M., Sternweis, P. C., Smigel, M. D., and Gilman, A. G. (1987) *J. Biol. Chem.* **262,** 762–766
22. Higashijima, T., Burnier, J., and Ross, E. M. (1990) *J. Biol. Chem.* **265,** 14176–14186
23. De Vos, A. M., Tong, L., Milburn, M. V., Maniatis, P. M., Jancarik, J., Noguchi., S., Nishimura, S., Muira, K., Ohtsuka, E., and Kim, S.-H. (1988) *Science* **239,** 888–893
24. Casey, P. J., Graziano, M. P., and Gilman, A. G (1989) *Biochemistry* **28,** 611–616
25. Padrell, E., Carty, D. J., Moriarty, T. M., Hildebrandt, J. D., Landau, E. M., and Iyengar, R. (1991) *J. Biol. Chem.* **266,** 9771–9777
26. Blitzer, R. D., Omrri, G., Devivo, M., Carty, D. J., Premont, R. T., Codina, J., Birnbaumer, L., Cotecchia, S., Caron, M. G., Lefkowitz, R. J., and Iengar, R. (1993) *J. Biol. Chem.* **268,** 7532–7537
27. Szeberenyl, J., Cai, H., and Cooper, G. M. (1990) *Mol. Cell. Biol.* **10,** 5324–5332
28. Feig, L. A, and Cooper, G. M. (1988) *Mol. Cell. Biol. 10,* 3235–3243