

# Obligatory role in GTP hydrolysis for the amide carbonyl oxygen of the $Mg^{2+}$ -coordinating Thr of regulatory GTPases

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**When G-protein  $\alpha$  subunits binds GTP and  $Mg^{2+}$ , they transition from their inactive to their active conformation. This transition is accompanied by completion of the coordination shell of  $Mg^{2+}$  with electrons from six oxygens: two water molecules, the  $\beta$  and  $\gamma$  phosphoryls of GTP, a helix- $\alpha$ 1 Ser, and a switch I domain (SWI) Thr, and the repositioning of SWI and SWII domains. SWII binds and regulates effector enzymes and facilitates GTP hydrolysis by repositioning the  $\gamma$ -carbonyl of a Gln. Mutating the Ser generates regulatory GTPases that cannot lock  $Mg^{2+}$  into its place and are locked in their inactive state with dominant negative properties. Curiously, mutating the Thr appears to reduce GTP hydrolysis. The reason for this difference is not known because it is also not known why removal of the Thr should affect the overall GTPase cycle differently than removal of the Ser. Working with recombinant  $G\alpha$ , we report that mutating its SWI-Thr to either Ala, Glu, Gln, or Asp results not only in diminished GTPase activity but also in spontaneous activation of the SWII domain. Upon close examination of existing  $\alpha$  subunit crystals, we noted the oxygen of the backbone carbonyl of SWI-Thr and of the  $\gamma$ -carbonyl of SWII Gln to be roughly equidistant from the oxygen of the hydrolytic  $H_2O$ . Our observations indicate that the Gln and Thr carbonyls play equihierarchical roles in the GTPase process and provide the mechanism that explains why mutating the Thr mimics mutating the Gln and not that of the Ser.**

enzymes/hydrolases | protein/metal ion interaction | catalytic water | heterotrimeric G proteins

The equilibrium dissociation constant of the interaction of  $Mg^{2+}$  with GTP is in the neighborhood of 60  $\mu M$  at the physiologic pH of 7.4 (1).<sup>†</sup> This value changes more than a 1,000-fold to 10–15 nM when Mg interacts with GTP bound to an  $\alpha$  subunit of a trimeric G protein (3). The increased affinity for  $Mg^{2+}$  is in good agreement with the apparent  $K_m$  determined for  $Mg^{2+}$  supporting the intrinsic GTPase of a  $G\alpha$  subunit (4). The reason for the increased affinity of  $Mg^{2+}$  for the GTP complexed to the protein is that the protein contributes with two coordination bonds that replace weaker coordinating water oxygens. As a result, whereas  $Mg^{2+}$  is held by free GTP with only two coordination bonds, it is held in place by four coordination bonds in the context of protein-bound GTP (cf. figure 1 in ref. 5). Thus, upon promoting the nucleotide exchange reaction, receptors not only promote binding of GTP but also the simultaneous creation of a high affinity binding site for  $Mg^{2+}$ . The combined binding of GTP and  $Mg^{2+}$  to G-protein  $\alpha$  subunits causes the transition of  $\alpha$  subunits from their inactive to their active conformation able to interact with effectors. In the case of the  $\alpha$  subunit of Gs, these effectors are primarily the different adenylyl cyclase isoforms whose catalytic activity is enhanced by their interaction with the  $G\alpha$ - $Mg^{2+}$ -GTP complex. In addition to leading to the switching into its effector-interacting conformation, occupancy of the  $\alpha$  subunit by  $Mg^{2+}$ -GTP also leads to activation of the subunit's second function, its intrinsic GTPase activity. The role, if any,

that  $Mg^{2+}$  plays in this conformational transition has, to our knowledge, not been addressed.

G-protein  $\alpha$  subunits, as well as regulatory GTPases that share the GTPase fold discovered in EF-Tu and *ras* (6, 7), all bind the  $Mg^{2+}$  of the GTP- $Mg^{2+}$  complex in essentially the same fashion—i.e., with the participation of the OH groups of one Ser and one Thr: Ser17 and Thr35 in *ras* and Ser54 and Thr204 in  $G\alpha$ . In the realm of structure activity studies, the effect of mutating the Ser has been extensively studied, owing in part to the finding that *ras* and *ras*-like GTPases with a Ser to Asp mutation have dominant negative properties (cf. ref. 8). The effect of mutating Thr35 in *ras* and the cognate Thr in other regulatory GTPases, including  $\alpha$  subunits of heterotrimeric G proteins has, in contrast, received little attention. In small regulatory GTPases the only record we could find is an attempt to test the structural change of mutating Thr35 to Ser and Ala by analyzing crystals by X-ray diffraction and their structure in solution by NMR (9). No information could be collected from crystals because the Thr35 region, the so-called switch I domain (SWI) of *ras*, appears to be highly flexible and did not diffract constructively. In the same study, the flexibility of *ras*'s SWI domain was confirmed by NMR spectroscopy. In the heterotrimeric G-protein field, the sole data on record appear to be on the effect of mutating Thr182 to Ala in  $G\beta$  (T182A- $G\beta$ ) by Nishina et al. (10). The mutant protein displayed increased rates of GDP release, and a decreased GTP hydrolysis rate, without changes in its interaction with  $G\beta\gamma$  dimers or membrane-bound receptors. Whereas facilitated release of nucleotide might be an expected consequence of removing one of the coordination bonds that hold the Mg-nucleotide complexes in place, the mechanistic basis for a reduction in GTPase activity, which appears to be the opposite from that of mutating the Mg-coordinating Ser, was unexpected and not explored further.

Given the central role that the OH groups of the Mg-coordinating Ser and Thr play in locking Mg-GTP into place with attendant “activation” of the protein, we sought to learn more about the role of Thr204 in  $G\alpha$  by mutating it to Ala or Gln and studying the properties of the mutated  $\alpha$  subunit as well as comparing the changes to previously observed properties of T182A- $G\beta$ . We report that mutation of the Mg-coordinating Thr to Gln, Glu, or Ala results in a similar loss of their intrinsic GTPase activity and, remarkably, in activation of the effector regulating function without requiring the cooperation of a G-protein coupled receptor. The activation by endogenous nucleotide was observed both in adenylyl cyclase assays and in tryptic digestion studies in which the mutant proteins display trypsin resistance in

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<sup>†</sup>Although the affinity of  $Mg^{2+}$  for GTP $\gamma$ S has apparently not been reported, that of  $Mg^{2+}$  for 5'AMP $\alpha$ S has been reported to be the same as that for 5'AMP (2). On this basis we assume that the affinity of  $Mg^{2+}$  for GTP $\gamma$ S is not significantly different from that of GTP—i.e., 60  $\mu M$ .

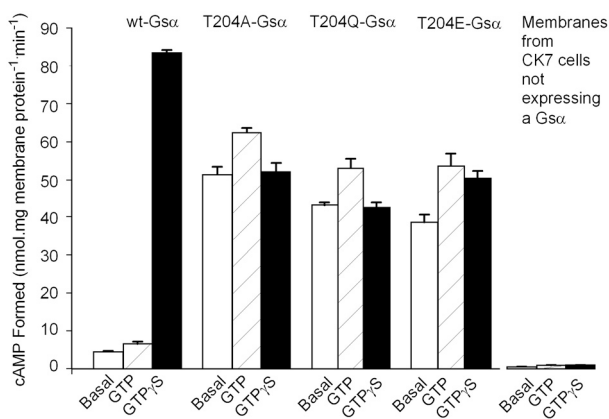
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the absence of exogenous addition of nonhydrolyzable GTP analogue. The loss of the Mg-coordinating Thr has thus quite different consequences than the loss of the Mg-coordinating Ser. The role of the amide carbonyl group of the Mg-coordinating Thr in activation of the GTPase activity is discussed as the likely mechanism by which intrinsic GTPase is diminished in Thr to Ala or Gln mutants, highlighting a previously unappreciated role for this Thr.

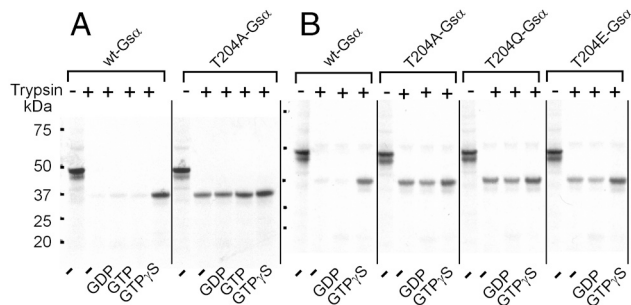
## Results

To study functional consequences resulting from mutating the Mg<sup>2+</sup>-coordinating Thr in G $\alpha$  (Thr204), we used purified membrane from S49 cyc- kin- (CK7) cells (deficient in G $\alpha$  activity) mixed with wild-type and mutant G $\alpha$  subunits synthesized in vitro by using reticulocyte lysates (11), in order to analyze the Gs-regulated adenylyl cyclase system. As shown in Fig. 1, under the conditions of the assay that used 10  $\mu$ g CK7 cell membrane proteins, the presence of wild-type G $\alpha$  allows measurement of low levels of adenylyl cyclase in the absence of exogenous guanosine nucleotide, followed by a 35% and 17-fold G $\alpha$ -stimulated activity increased when GTP or GTP $\gamma$ S are added at 100  $\mu$ M. In contrast, G $\alpha$  with Thr204 (T204) mutated to Ala, Glu, or Gln led to much larger adenylyl cyclase activity that was increased neither by GTP nor by GTP $\gamma$ S and in the presence of GTP were between 63% and 74% of the activity seen with wild-type G $\alpha$  in the presence of GTP $\gamma$ S. GTP increased their activities between 20% and 35%, whereas GTP $\gamma$ S either did not affect activity (T204A-G $\alpha$  and T204Q-G $\alpha$ ) or increased it to the same level as seen with GTP (T204E-G $\alpha$ ). These findings indicated that the mutant proteins are constitutively active.

To explore the effect of mutating G $\alpha$  Thr204 on structural changes, we synthesized wild-type and mutant G $\alpha$  subunits in vitro in the presence of [<sup>35</sup>S]methionine, incubated the products in the presence of nucleotides and Mg, and followed this by partial tryptic digestion. The products were resolved by SDS-PAGE and visualized by autoradiography. Under these conditions, wild-type G-protein  $\alpha$  subunits in their inactive conformation are fully digested but after activation with a nonhydrolyzable GTP analogue are partially resistant to tryptic digestion owing to the conformational change that occurs in their switch II domain (12). This conformational change diminishes access of trypsin to



**Fig. 1.** Reconstitution of Gs-regulated adenylyl cyclase activities by wt-G $\alpha$ , T204A-G $\alpha$ , T204E-G $\alpha$ , and T204Q-G $\alpha$  in S49 cyc- kin- (CK7) cell membranes shows high basal activities for the mutant G $\alpha$  subunits in the absence of added nucleotides. Membranes of CK7 cells (10  $\mu$ L, 5–10  $\mu$ g protein) were mixed with 10  $\mu$ L in vitro synthesized G $\alpha$  subunits prepared as described in *Materials and Methods* and allowed to stand on ice for 10 min. Adenylyl cyclase assay reagents were then added to determine basal activities (no nucleotide addition, *Open Bars*) and activities in the presence of 100  $\mu$ M GTP (*Stippled Bars*) or 100  $\mu$ M GTP $\gamma$ S (*Black Bars*). Incubations were for 20 min at 32 °C. Conversion of  $\alpha$ -[<sup>32</sup>P]ATP to [<sup>32</sup>P]cAMP was determined as described in *Materials and Methods*.



**Fig. 2.** Incubation with trypsin reveals that G $\alpha$  with Thr204 mutated to Ala or Gln displays partial resistance to tryptic digestion in the absence of added guanine nucleotide or in the presence of GDP or GTP, as is seen in the wild-type G $\alpha$  only in the presence of nonhydrolyzable GTP analogues, in this instance GTP $\gamma$ S. Wild-type and Thr mutants of G $\alpha$  were synthesized in vitro in the presence of [<sup>35</sup>S]methionine and incubated for 15 min at 25 °C in 20  $\mu$ L of 20 mM Hepes-NaOH, pH 7.5 and 5 mM MgCl<sub>2</sub> with 10  $\mu$ L/mL trypsin in the absence or presence of 200  $\mu$ M GDP or GTP, followed by addition of 2  $\mu$ L of 100  $\mu$ g/mL trypsin and a second incubation for 15 min at 25 °C. The reactions were stopped by addition of 20  $\mu$ L of Laemmli's sample buffer with 1% SDS. The samples were subjected to SDS-PAGE through a 4–12% polyacrylamide gel and visualized by autoradiography.

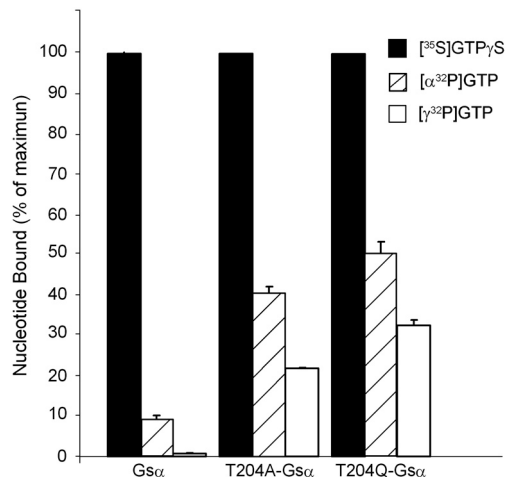
its triple basic amino acid sequence (RRK in G $\alpha$  and RKK in  $\alpha$  subunits of the Gi/o and transducin family). As seen in Fig. 2A, activation of wild-type G $\alpha$  with GTP $\gamma$ S and Mg caused the protein to become resistant to partial tryptic digestion and accumulation of a fragment that migrated with an apparent molecular mass of ca. 37 kDa. This fragment is likely to be G $\alpha$ (1–231), cleaved between the two Arg of the switch II RRRK motif (12). This fragment did not accumulate if no nucleotide was added, or if GTP or GDP were added, conditions known not to allow for accumulation of an activated form of G $\alpha$  (Fig. 1). The right panel of Fig. 2A shows that, in contrast to wild-type G $\alpha$ , T204A-G $\alpha$  adopts the “activated” conformation even in the presence of GDP. We refer to this conformation as constitutively active. T204Q-G $\alpha$  and T204E-G $\alpha$  behaved essentially the same as T204A-G $\alpha$  (Fig. 2B). Thus, the appearance of the structural change in the T204 mutants of G $\alpha$  responsible for adenylyl cyclase activation was independent of the presence of the effector. The remainder of the experiments were performed with the T204A and T204Q mutants of G $\alpha$ .

Which are the functional changes responsible for the constitutive activity of T204 mutants? To answer this question, we prepared highly purified recombinant G-protein  $\alpha$  subunits and studied their intrinsic GTPase activities and the rates of association and dissociation of GTP $\gamma$ S and analyzed the GTP/GDP ratio of the guanine nucleotide after exposing the purified proteins to GTP. His-tagged G-protein  $\alpha$  subunits were expressed in *Escherichia coli* (13) and purified as follows: (i) chromatography over a nitrilotriacetate-Ni column, eluting with buffer containing EDTA—i.e., removing the Ni from the column and thereby avoiding the deleterious effect that imidazole has on the stability of G $\alpha$  activity. This was followed by (ii) size-exclusion chromatography over a Superdex 200 GL column (Fig. 3 *Inset*). The G $\alpha$  proteins obtained in this manner were more than 95% pure. The ca. 22–23 kDa contaminant remaining in the preparations was determined by mass spectrometry to be a fragment of G $\alpha$  formed after the Ni-affinity purification step. Analysis of the intrinsic GTPase activity of wild-type and mutant G $\alpha$  subunits showed the GTPase activities of the T204A and T204Q mutants of G $\alpha$  to be 1/20 and 1/10 of that of the wild-type G $\alpha$  (Table 1 and Fig. 3A).

To establish whether the effect of mutating the Mg<sup>2+</sup>-coordinating Thr of G $\alpha$  was peculiar to G $\alpha$ , we sought to extend our observations to another G-protein  $\alpha$  subunit in which the Mg<sup>2+</sup>-coordinating Thr of the GTPase domain was mutated to

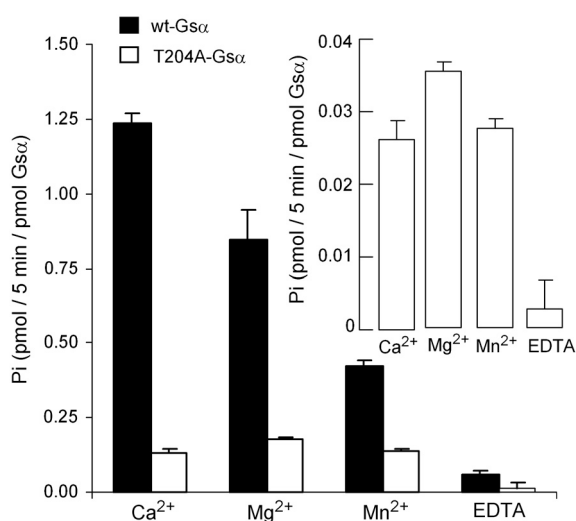






**Fig. 5.** Guanine nucleotide bound at equilibrium after exposure to GTP shows a higher proportion of the resident guanine nucleotide in the GTP as opposed to GDP from mutant Gs<sub>α</sub> subunits. Wild-type Gs<sub>α</sub>, T204A-Gs<sub>α</sub>, and T204Q-Gs<sub>α</sub> (5 pmol GTP<sub>γ</sub>S binding sites) were incubated at 32 °C with 1,000 nM [<sup>35</sup>S]GTP<sub>γ</sub>S (specific activity 17,000 cpm/pmol), 1,000 nM [α-<sup>32</sup>P]GTP (specific activity 16,000 cpm/pmol), or 1,000 nM [γ-<sup>32</sup>P]GTP (specific activity 15,000 cpm/pmol). After 15 min, triplicate aliquots were removed and bound nucleotides analyzed by liquid scintillation counting as described in *Materials and Methods*. Radioactive nucleotide recovered from Gs<sub>α</sub> exposed to [γ-<sup>32</sup>P]GTP (Open Bars) is taken as the GTP bound; that recovered from Gs<sub>α</sub>s exposed to [α-<sup>32</sup>P]GTP (Hatch Bars) is the sum of GTP plus GDP. The difference between GTP<sub>γ</sub>S (Black Bars) binding sites and [α-<sup>32</sup>P]GTP is assumed to be nucleotide-free Gs<sub>α</sub>.

chimera, it appears that the tethering of the Thr to Mg<sup>2+</sup> is a general requirement for activation of the GTPase activity. The “facilitation” of activation by GTP seen in the mutants compared to wild-type Gs<sub>α</sub> is likely because of the fact that exit of GDP from the wild-type protein is rate limiting and slower than the intrinsic GTPase activity, so that the GTP form cannot accumulate unless the release of GDP is facilitated, which is what G-protein coupled receptors do. As shown in Fig. 4, GTP<sub>γ</sub>S dissociation is faster in Gs<sub>α</sub> subunits with Thr204 mutated to Ala or Gln, and GDP release is likely to be faster as well.



**Fig. 6.** The GTPase activity of Gs<sub>α</sub> varies inversely with the atomic radius of the divalent cation. The hydrolysis of GTP by 20 pmol each of wild-type Gs<sub>α</sub> (Black Bars) and T204A-Gs<sub>α</sub> (Open Bars and Inset) was assessed as described in *Materials and Methods* in the absence and presence of 10 mM of the indicated divalent cations. The data are representative of three different experiments with similar results.

Which critical interaction(s) of Thr needed for GTPase activation are disrupted in the mutant Gs<sub>α</sub> subunits? The mechanism by which GTP is hydrolyzed by regulatory GTPases has been extensively studied. The current consensus appears to be that it is of the dissociative type (18). That is, the β-γ phosphate bond is weakened by a nucleophilic attack of H<sub>2</sub>O from the distal side, opposite to the diester bond. This nucleophilic attack subtracts charge from the β-γ ester bond so that its oxygen remains with the β phosphoryl and the γ phosphoryl leaves the reaction as a phosphate carrying the oxygen of the attacking water. It has been shown previously that the hydrolytic attack is facilitated by a Gln—227 in Gs<sub>α</sub>, 204 in Gi1<sub>α</sub>, and 200 in T<sub>α</sub>—located at the beginning of the so-called switch II domain (SWII), which positions the attacking water, and by an Arg—201 in Gs<sub>α</sub>, 178 in Gi1<sub>α</sub>, and 174 in T<sub>α</sub>—which further withdraws electron density from the β-γ ester bond. As is visible in crystals of both transducin α (e.g., 1GOT, 1TAG) and Gi1<sub>α</sub> (e.g., 1GP2, 1BOF) in their GDP and GDP-Mg<sup>2+</sup> liganded, GTPase-inactive conformations, the catalytic Arg points “away” from the β-γ phosphodiester bond as does the Mg<sup>2+</sup>-coordinating Thr. Close inspection of the difference between the crystals with GDP and crystals with GTP<sub>γ</sub>S or GMP-P(NH)P reveals that the attacking water is also positioned in front of the γ phosphoryl by the backbone carbonyl of a Thr on SWI. Thus, the coordinating Mg bond serves the function of pulling the backbone carbonyl of the Thr into position to, in turn, stabilize the attacking H<sub>2</sub>O allowing for better catalysis of the diester bond between the β and γ phosphates of the GTP. Thus, the process by which the Mg-GTP complex is locked into place causes not only the activation of the protein's effector regulating function but also through stabilization of a H<sub>2</sub>O molecule at *ca.* 3 Å from the γ phosphoryl that is to be removed from GTP. The importance of the Thr carbonyl oxygen in positioning the hydrolytic H<sub>2</sub>O for the hydrolytic reaction to proceed is further strengthened by the fact that in the crystal of the Mg<sup>2+</sup>-GDP-Al<sub>4</sub>F<sup>-</sup> complex the carbonyl oxygen of the Thr has moved to just 2.86 Å (1FOK, Fig. 7B), significantly closer than it is found when the nonhydrolyzable GTP<sub>γ</sub>S is bound to the protein (3.40 Å—1TND, Fig. 7A).

The same, roughly equal distances of the Thr amide carbonyl and Gln γ carbonyl oxygens to the hydrolytic water (3.03 and 2.93 Å, respectively) are also seen in the transition state crystal of rasGAP bound to H-ras in complex with Mg<sup>2+</sup>-GDP-Al<sub>4</sub>F<sup>-</sup> (1WQ1). This suggests that the observations made here, assigning an equihierarchical role for the two oxygens in GTP hydrolysis in heterotrimeric GTPase α subunits, is likely to be a general feature of all regulatory GTPases.

### Material and Methods

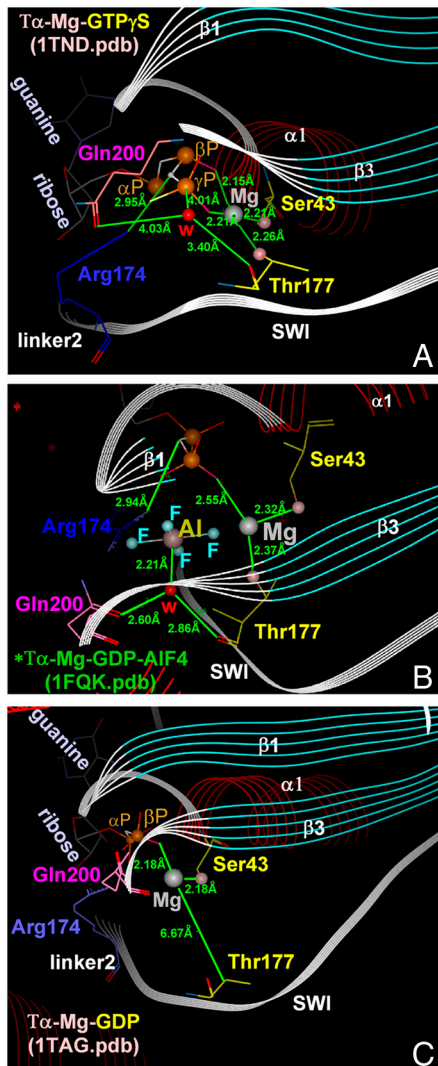
Radioisotopes α-<sup>32</sup>P]ATP, [<sup>3</sup>H]cAMP, [<sup>35</sup>S]GTP<sub>γ</sub>S, [γ-<sup>32</sup>P]GTP, and [<sup>35</sup>S]methionine were purchased from NEN-PerkinElmer.

Trypsin and GTP were from Sigma-Aldrich; and GTP<sub>γ</sub>S was from Roche. All other chemicals and biochemicals were of the highest purity commercially available and used without further purification.

CK7 cells, derivatives of S49 thymoma cells that are *cyc*<sup>-</sup> and *kin*<sup>-</sup>, i.e., deficient in Gs<sub>α</sub> activity and in cAMP-dependent protein kinase activity, were obtained from the Cell Culture Facility at University of California at San Francisco.

Standard recombinant DNA techniques were used throughout (19). The mutants of Gs<sub>α</sub> were generated in the cDNA coding for the 394-aa splice variant of the human Gs<sub>α</sub> (20). Single codon mutations were made by using the Quikchange II XL Site-Directed Mutagenesis kit from Stratagene.

**In Vitro Synthesis of Gs<sub>α</sub> Subunits.** Wild-type Gs<sub>α</sub> and the indicated T204 mutants of Gs<sub>α</sub> were synthesized by using the TNT Quick Coupled Transcription/Translation System kit from Promega following the manufacturer instructions by using 1 μg of plasmid



**Fig. 7.** The crystal structures of transducin  $\alpha$  show that in the MgGTP activated conformation (1TND), the amide carbonyl group of the Mg-coordinating Thr177 and the  $\gamma$ -carbonyl group of Gln200 are equidistant from the catalytic H2O located in “front” of the  $\gamma$ -phosphoryl of GTP. (A) In the GTP $\gamma$ S-Mg liganded form of the protein, SWI, SWII (to which Gln200 belongs), and Arg174 are oriented in such a way that the catalytic H<sub>2</sub>O that will capture the  $\gamma$ PO3 split off the GTP and leave the active site as part of the newly formed P<sub>i</sub>. The Mg-coordinating Ser43 and Thr177 are shown in gray, with the O of the OH groups shown as pink balls, and Mg is shown with four of the six coordinating oxygens: one from the brown  $\gamma$ P, one from the brown  $\beta$ P, and one each from the OH groups of Ser and Thr. The coordination shell is completed by the electrons from two additional water oxygens [not shown here, but seen in figure 1 of Zurita and Birnbaumer (17)]. Mg-O distances are shown as green lines with corresponding lengths in Å. Distances between the catalytic water oxygen (w) and the Thr177 amide carbonyl O, the  $\gamma$  carbonyl O of Gln200, and the  $\gamma$ -phosphoryl O are also shown as green lines with corresponding lengths in Å. The distance between the  $\beta$ - $\gamma$  ester bond (white) and Arg174, referred to as the “Arg finger” by Wittinghofer and colleagues (24) when GTPase activating G proteins (GAPs) are available to interact with *ras*-like regulatory GTPases, is also shown as a green line with the corresponding length in Å. (B) In the Mg-GDP-AlF<sub>4</sub> liganded conformation of \*T $\alpha$  stabilized by RGS4 (1FQK)—which is presumed to be equivalent to the transition form of the GTPase—one sees that the backbone carbonyl of Thr177 as well as the  $\gamma$  carbonyl of the Gln have both moved closer to the catalytic H<sub>2</sub>O oxygen. (C) In the GDP-Mg liganded conformation of T $\alpha$  (1TAG) none of the critical amino acids are close enough to establish meaningful contacts; especially Thr177 cannot be pulled in to confer an effector regulating conformation to SWII. Crystal structures of Gi1 $\alpha$  in the MgGTP conformation [1GIA with GTP $\gamma$ S or 1CIP with GMP-P(NH)P] and GDP conformation (1BOF, 1GP2) and of Gs $\alpha$  in the MgGTP $\gamma$ S conformation (e.g., 1AZT, 1AZS) are in agreement with structural features highlighted in this figure.

pSP72 (Promega) carrying the cDNA template. After the in vitro translation, the samples (50  $\mu$ L) were diluted into 4 mL of the desired buffer and concentrated to  $\sim$ 50  $\mu$ L by using a 30,000 NMWL Amicon Ultra filter from Millipore centrifuging at 3,500  $\times$  *g* for 35 min. <sup>35</sup>S-labeled G-protein  $\alpha$  subunits were synthesized as above by supplementing the in vitro translation step with 10  $\mu$ Ci of [<sup>35</sup>S]methionine (1,000 Ci/mmol).

**Bacterial Expression and Purification G $\alpha$  Proteins.** Two-liter overnight cultures of competent BL21-codon plus (DE3) RIPL cells (Stratagene), transformed with the vector pHis6 carrying the cDNAs of wild-type or mutants Gs $\alpha$ , were induced with 100  $\mu$ g/mL IPTG for 14 h at 25 °C in order to induce expression of the desired protein. The cells were harvested by centrifugation at 4,000 rpm at 4 °C for 20 min in a Sorvall SH-3000 rotor. Cells in the pellet were resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol (lysis buffer) and lysed by a single decompression cycle in a French Press (Thermo) pressurized with N<sub>2</sub> to 2,250 psi. The lysate was centrifuged at 43,000 rpm in a Beckman 45Ti rotor at 4 °C for 60 min. The supernatant was adjusted to 60 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% Lubrol PX (buffer A), and 20 mM imidazole and applied to a 3-mL nickel-agarose column (Qiagen). The column was washed with 10 volumes of buffer A with 20 mM imidazole and eluted with buffer A plus 50 mM EDTA. The eluted fractions containing the recombinant protein of interest (located either by activity analysis or by SDS-PAGE followed by Coomassie blue staining) were pooled, concentrated to 0.5 mL by ultrafiltration (Amicon Ultracel-10K; Millipore) and applied to a 10  $\times$  300 mm Superdex 200 GL column (GE Healthcare) equilibrated in buffer A. Fractions with wild-type and mutant Gs $\alpha$  subunits were concentrated by ultrafiltration and either stored at -70 °C for up to 1 yr or used immediately in the experiments reported below. Recombinant Gs $\alpha$  proteins prepared in this manner had a specific [<sup>35</sup>S]GTP $\gamma$ S binding activity between 0.8 and 0.92 pmol GTP $\gamma$ S per pmol protein using BSA as standard.

**Tryptic Digestions.** G $\alpha$  subunits, synthesized in vitro in the presence of [<sup>35</sup>S]methionine and subjected to two rounds of 100-fold dilution with 20 mM Hepes-NaOH, pH 8.0, 5 mM MgCl<sub>2</sub> (dilution buffer) followed by concentration by ultrafiltration, were incubated for 15 min at room temperature in a final volume of 20  $\mu$ L of dilution buffer with or without 200  $\mu$ M GDP, GTP, or GTP $\gamma$ S, followed by tryptic digestion initiated by addition of 2  $\mu$ L 100  $\mu$ g/mL trypsin (Sigma-Aldrich). The reactions were terminated after 15 min at room temperature by addition of 20  $\mu$ L of 2 $\times$  Laemmli’s sample buffer (1 $\times$  Laemmli’s; buffer 62,5 Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5%  $\beta$ -mercaptoethanol). The tryptic fragments were resolved by 4–12% SDS-PAGE. The gel slabs were dried under vacuum and subjected to autoradiography at room temperature for 15–30 min by using a Kodak BioMax MR film.

**Adenylyl Cyclase Assays.** Activity was measured as described by Iyengar et al. (21) with modifications. Briefly, unless specified otherwise, the reactions were performed by incubating CK7 membranes (10  $\mu$ g protein) resuspended in 20 mM Hepes-NaOH (pH. 8.0) with 50 mM NaCl, 1 mM DTT, and 1 mM EDTA for 20 min at 32 °C in a final volume of 50  $\mu$ L containing 25 mM Tris-HCl, pH 8.0, 1 mM [3H]cAMP (10,000 cpm), 1 mM EDTA, 6 mM MgCl<sub>2</sub>, 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (5  $\times$  10<sup>6</sup> cpm), and a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/mL of creatine phosphokinase (282 units/mg), and 0.02 mg/mL of myokinase (1,500 units/mg). When present, GTP and GTP $\gamma$ S were 100  $\mu$ M. The reactions were stopped by addition of 100  $\mu$ L of 40 mM ATP, 10 mM cAMP, and 1% SDS, and the [32P]cAMP formed was isolated by the method of Salomon et al. (22) as modified by Bockaert et al. (23).

**GTPase Assays.** GTP hydrolysis by wild-type and mutant G-protein  $\alpha$  subunits was determined as described by Sunyer et al. (4) with modifications. Briefly, 1–5 pmol purified proteins were incubated in the presence of 500 nM [ $\gamma$ - $^{32}$ P]GTP (specific activity 40,000 cpm/pmol) in a final volume of 100  $\mu$ L containing 20 mM Tris-bis-propane (pH 6.5, or as stated), 100 mM NaCl, 1 mM EDTA, 4 mM  $\beta$ -mercaptoethanol, and 10 mM MgCl<sub>2</sub>. The reactions were incubated at 32 °C and stopped at the desired times by addition 0.7 mL of an ice-cold 5% (wt/vol) suspension of charcoal (Sigma) in 20 mM phosphoric acid (pH 2.1). The mixtures were allowed to stand on ice for 15 min followed by centrifugation for 15 min at 10,000  $\times$  g at 4 °C. The  $^{32}$ P<sub>i</sub> released from the  $^{32}$ P-labeled GTP was estimated in the supernatant by scintillation counting by using 2.8 mL of Ecolume liquid scintillation cocktail from MP Biomedical in a Tri-Carb 2800 TR liquid scintillation counter from PerkinElmer.

**Guanine Nucleotide Binding.** Binding of guanine nucleotides to wild-type and mutant purified G $\alpha$  was determined as described

in Graf et al. (5) with modifications. Briefly, purified G $\alpha$  proteins were incubated in the presence of 1,000 nM of [ $^{35}$ S]GTP $\gamma$ S, 1,000 nM [ $\alpha$ - $^{32}$ P]GTP, or 1,000 nM [ $\gamma$ - $^{32}$ P]GTP (specific activity 17,000 cpm/pmol), 20 mM Hepes-NaOH, pH. 8.0, 100 mM NaCl, 0.05% (wt/vol) BSA, 1 mM EDTA, 4 mM  $\beta$ -mercaptoethanol, and 2 mM MgCl<sub>2</sub>. Incubations were for 15 min, or as stated in the figures, at 32 °C and were stopped by addition of 1 mL ice-cold 20 mM Hepes-NaOH, pH. 8.0, 100 mM NaCl, 1 mM EDTA, 4 mM  $\beta$ -mercaptoethanol, and 2 mM MgCl<sub>2</sub>, followed by filtration through MF-Millipore membrane with 0.45- $\mu$ m pore size. After filtration, the filters were washed two times with 7.5 mL of the same buffer, and the guanine nucleotide bound estimated by liquid scintillation counting as above.

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