Rapid Activation of Transducin by Mutations Distant from the Nucleotide-binding Site

EVIDENCE FOR A MECHANISTIC MODEL OF RECEPTOR-CATALYZED NUCLEOTIDE EXCHANGE BY G PROTEINS*

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G proteins act as molecular switches in which information flow depends on whether the bound nucleotide is GDP (“off”) or GTP (“on”). We studied the basal and receptor-catalyzed nucleotide exchange rates of site-directed mutants of the α subunit of transducin. We identified three amino acid residues (Thr-325, Val-328, and Phe-332) in which mutation resulted in dramatic increases (up to 165-fold) in basal nucleotide exchange rates in addition to enhanced receptor-catalyzed nucleotide exchange rates. These three residues are located on the inward facing surface of the α5 helix, which lies between the carboxy-terminal tail and a loop contacting the nucleotide-binding pocket. Mutation of amino acid residues on the outward facing surface of the same α5 helix caused a decrease in receptor-catalyzed nucleotide exchange. We propose that the α5 helix comprises a functional microdomain in G proteins that affects basal nucleotide release rates and mediates receptor-catalyzed nucleotide exchange at a distance from the nucleotide-binding pocket.

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§ The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; Go, α subunit of transducin; Gβγ, βγ subunits of transducin; GPCR, G protein-coupled receptor; R*, signaling active conformation of rhodopsin; GTPγS, guanosine 5′-O-(thiotriphosphate).

G protein1-coupled receptors (GPCRs) activate G proteins by catalyzing the release of GDP bound to the G protein α subunit (Go) (1–3). GDP-bound Go proteins regulate a variety of cellular effector enzymes or ion channels. The active Go is turned off kinetically by intrinsic GTPase activity, which is highly regulated by bound effector and regulator of G protein signaling proteins (4, 5). High resolution structural analysis of several nucleotide-bound forms of Go (6–9) and of complexes of Go subunits with regulatory proteins (10, 11) have provided insights into key conformational changes required for G protein function. In addition, the crystal structure of rhodopsin (12), a prototypical GPCR, provides a basis for understanding several key features of the molecular physiology of rhodopsin (13). However, structural information is lacking for the receptor-bound nucleotide-free conformation of Go, and the molecular details of how GPCRs switch G proteins from their inactive to active states remains unknown. Interestingly, the receptor must act at a distance because extensive evidence from biochemical and mutagenesis work suggests that the cytoplasmic loops of the active receptor (R*) do not directly contact the nucleotide-binding pocket of Go subunits (14).

To address the question of how GPCRs catalyze the switching of GDP for GTP by G proteins, we studied the mechanism of nucleotide exchange in transducin (Go). Because it exhibits an extremely low basal GDP release rate (15) and a very rapid R*-catalyzed nucleotide exchange rate (16) consistent with its role in rod cell visual phototransduction where low biochemical noise levels are required. We tested the hypothesis that the α5 helix of Go, one of the structural features that resolves the action at a distance problem and explains how R* induces nucleotide switching by G proteins (17, 18). The α5 helix of Go (amino acid residues 325–339) connects the carboxy-terminal tail of Go to the β6/α5 loop. The carboxy tail of Go, especially amino acid residues 340–350, is a well characterized binding domain for R* (19–23), and the β6/α5 loop directly contacts the guanine ring of the bound nucleotide. Consequently, R* might induce nucleotide exchange in Go, by using the α5 helix to communicate between the carboxyl terminus and the β6/α5 loop.

We report that mutation of several specific residues on the buried surface of α5 caused dramatic increases in the basal nucleotide exchange rates in the resulting Go mutants. The evidence suggests that the mechanism of R*-catalyzed nucleotide exchange by Go involves conformational changes in these α5 residues. R* may induce changes in α5 either directly by binding to α5 or indirectly by binding to the adjacent carboxyl terminus. We propose that the α5 helix controls the basal nucleotide exchange rate and mechanistically couples R* binding to the guanine nucleotide in Go.

EXPERIMENTAL PROCEDURES

Most of the methods used in this paper have been described previously (24). Brief descriptions are given below.

Site-directed Mutagenesis of Go and Expression in Vitro—Site-directed mutations were created using the QuickChange system (Stratagene). The parent for all Go constructs was pGEM2/Ta, the synthetic bovine Go gene cloned into the pGEM2 plasmid under the control of a SP6 promoter (25). Go and Go mutant proteins were prepared in vitro using the TNT Quick Coupled transcription/translation system (Promega). The translated products were passed over BioSpin 6 gel filtration spin columns (Bio-Rad) twice consecutively to remove excess nucleotides and [35S]methionine. The volume of each sample was then adjusted to 100 μl in Buffer A (5 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.01% (w/v) n-dodecyl-β-D-maltoside). If the sample was to be studied in a R*-catalyzed assay, Gβγ was added to a final concentration of 30 nM. Every experiment was performed using freshly translated material.
The α5 Helix of Transducin

Nucleotide Exchange Rate Assays—Samples (70 μl each) of translated Goα or mutant Go, kept on ice in Buffer A were quickly warmed to room temperature in a water bath. For basal exchange rate assays (Fig. 1), the experiment was initiated by the addition of GTP-γS to a final concentration of 100 μM. Five aliquots (8 μl) were withdrawn during a period ranging from 2 to 6 h and digested. For R5-catalyzed assays (Fig. 2), the experiment was initiated by the addition of a mixture of R5 and GTP-γS (4 μl) to a final concentration of 30 nM R5 and 14 μM GTP-γS. Immediately before addition to the reaction, the rhodopsin was photolyzed by illumination for 15 s with a fiber optic cable connected to a Dolan Jenner lamp equipped with a >495-nm long pass filter. The samples were incubated at room temperature under illumination. Aliquots (8 μl) were withdrawn and digested at 1, 2, 3, 5, 10, and 20 min. The digestion procedure was adapted from Garcia et al. (20). Aliquots were mixed with 1.5 μl of digest buffer (5% Lubrol, 2 mM GDP, 1 mg/ml l-1-tosylamide-2-phenylthyl chloromethyl ketone-treated trypsin) and incubated on ice for 30 min. Digestion was terminated by the addition of 2.5 μl of termination solution (10 mg/ml aprotinin, 10 μM phenylmethylsulfonyl fluoride), followed by 6 μl of 34% SDS sample buffer (New England Biolabs). The proteolytic fragments were resolved by SDS-polyacrylamide gel electrophoresis using precast 15% gels (Bio-Rad). The intensities of the bands were quantitated using a Storm Imager and ImageQuant software (Molecular Dynamics). Goα, in the GDP-bound inactive conformation yielded an ~23-kDa fragment, whereas, in the active conformation (GDP-γS- or GDP-AlF4-bound) yielded an ~34-kDa fragment following trypsin digestion. The fraction of Goα activated in a given sample was determined from the relative intensity of the two bands. The ~23-kDa bands were generally absent from digests of the F185A and F192A mutants of Goα (not shown), although they could bind GDP as indicated by activation by AlF4- and the rate of GTP uptake. Instead, the mutations seemed to affect the folding or stability of the proteins. The rates of nucleotide exchange in F185A and F192A could be estimated from the intensities of the ~34-kDa bands.

RESULTS AND DISCUSSION

We carried out alanine-scanning mutagenesis of the 14 non-alanine amino acid residues in the α5 helix of Goα. Proteins were expressed in vitro in rabbit reticulocyte lysate to overcome the well documented problems with the heterologous expression of Goα (26, 27). Nucleotide exchange rates were determined using a quantitative trypsin digest assay specifically adapted for kinetic measurements. The assay is directly sensitive to the nucleotide-bound status of Goα (28) and does not rely on potentially confounding second messenger readouts. The sensitivity and fidelity of this assay has been described in detail (24).

Three of the fourteen mutants (T325A, V328A, and F332A) exhibited tremendous increases in their rates of basal nucleotide exchange (Fig. 1, A and B; Table I). The basal exchange rates of V328A, F332A, and T325A were accelerated by 77-,

and 100-fold, respectively, over the basal exchange rate of wild-type Goα, measured under the identical conditions. These rates are comparable with or in excess of the basal exchange rate of mutant A322S (63-fold increase) (Fig. 1, A and B; Table I). Ala-322 is known to reside in the β6/α5 loop and to be in contact with GDP. The A322S mutant is also analogous to mutations in Goα and Goβ that have been reported to greatly increase the basal nucleotide exchange rates (29, 30). These results suggest that alteration of the α5 helix, which is distant from the nucleotide, can accelerate GDP release to an even greater degree than replacement of amino acids known to be in direct contact with the nucleotide.

The mutants T327A and I339A also exhibited statistically significant gain of function phenotypes (Table I). Together, the five mutants that displayed an increase in basal nucleotide exchange rate are found to be clustered along the buried surface of the α5 helix (Fig. 3). Thr-325 is located in the first turn of the α5 helix, immediately adjacent to the β6/α5 loop. Asn-327 is also located on the first turn, a structure that appears to be stabilized by a network of hydrogen bonds. Substitution of the amino acid side chains in this region might be expected to disrupt some of these hydrogen bonds and alter the structure of the first turn and possibly the β6/α5 loop. Two other residues, Val-328 and Phe-332, point in toward the center of Goα and contribute to its hydrophobic core. Val-328 and Phe-332 make extensive contacts with residues from the α1 helix, as well as the β2 and β3 sheets. Loops emanating from these structural elements contribute to the canonical nucleotide-binding surfaces of Goα and other G proteins (31). Replacement of Val-328 or Phe-332 with alanine is likely to perturb the packing of the β2 and β3 sheets and the α1 helix in a manner that affects the structure of the nucleotide-binding pocket.

All of the alanine replacement mutants, including V328A,
Lys-188, which extends from a loop between domain, hydrogen bonds with the main chain carbonyl of Thr-33. NMR structural studies of Ras F156L revealed alterations in secondary structures.

A equivalent of Phe-332 was replaced with leucine in Ras, the and to accelerate greatly the rate of nucleotide exchange appears to form ionic interactions with two aspartic acid residues. The gain of function phenotypes of the T325A, V328A, and F332A were capable of binding GDP and GTPγS and could be activated by GDP/AlF$_4$ (Fig. 1A and data not shown). In addition, the typical band patterns of trypsin digestion were observed with all of the mutants (Fig. 1A and data not shown). These results indicate that the mutations did not disrupt the overall folding of the mutant proteins.

The equivalents of residues Val-328 and Phe-332 are highly conserved not only in Go subunits, but also among small monomeric G proteins of the Ras superfamily (32). When the equivalent of Phe-328 was replaced with leucine in Ras, the F156L mutant was found to have transforming properties in vivo and to accelerate greatly the rate of nucleotide exchange in vitro (33). NMR structural studies of Ras F156L revealed alterations in secondary structures α1, α5, and β1–β3 near position 156. The mutation did not appear to reduce the stability of the protein, perhaps due to induction of new intramolecular contacts not present in the wild-type protein. This observation suggests that Go$_{α5}$, F332A might exhibit higher stability than wild-type Go$_{α5}$ in the absence of bound nucleotide.

Because the mutagenesis studies indicate that perturbation of residues in the α5 helix in Go$_{α5}$ causes a dramatic increase in the nucleotide exchange rate, one would predict the existence of intramolecular contacts that maintain the α5 helix in a specific rigid conformation to avoid inappropriate release of GDP. In fact, mutation of residues in the protein core that interact with the α5 helix also resulted in moderate increases in the basal nucleotide exchange rate (Fig. 3C).

### Table I

| Mutant     | $k_{app}^a$ | $\text{Fold increase}^b$ |
|------------|-------------|--------------------------|
| Wild-type  | 8.6 ± 0.7   | 1.0 ± 0.1                |
| Q168A      | 26.4 ± 5.2  | 3.1 ± 0.7                |
| F187A      | 4.8 ± 0.8   | 0.6 ± 0.1                |
| K188A      | 48.5 ± 13.1 | 5.7 ± 1.6                |
| A322S      | 53.6 ± 10.9 | 62.6 ± 13.7              |
| T325A      | 1410 ± 47.4 | 165 ± 57                 |
| Q326A      | 6.6 ± 1.3   | 0.8 ± 0.2                |
| N327A      | 198 ± 60    | 23 ± 7.2                 |
| V328A      | 661 ± 185   | 77 ± 23                  |
| K329A      | 9.0 ± 0.9   | 1.0 ± 0.1                |
| F330A      | 5.3 ± 1.2   | 0.6 ± 0.1                |
| V331A      | 9.6 ± 2.0   | 1.1 ± 0.3                |
| F332A      | 1300 ± 256  | 151 ± 32                 |
| D333A      | 6.4 ± 0.3   | 0.7 ± 0.1                |
| V335A      | 17.7 ± 2.2  | 2.1 ± 0.3                |
| T336A      | 7.4 ± 2.0   | 0.9 ± 0.2                |
| D337A      | 17.3 ± 4.2  | 2.0 ± 0.5                |
| I338A      | 9.3 ± 1.1   | 1.1 ± 0.2                |
| I339A      | 35.4 ± 23   | 4.1 ± 2.7                |

$^a$ The apparent rate constants for basal nucleotide exchange were derived from fits of each data set to the exponential rise equation, $y = c + 100(1 - \exp(-k_{app}t))$. Each mutant protein was assayed at least 3 times (wild-type Go$_{α5}$ protein was assayed 26 times), and an independent fit was made to each data set. The values reported are the mean $k_{app} \times 10^4$ min$^{-1} \pm 2 \times$ S.E.

$^b$ The fold increase in the nucleotide exchange rate measured for the mutant protein relative to that of uncatalyzed wild-type Go$_{α5}$ was calculated as the $k_{app}$(mutant)/$k_{app}$(wild-type).

F332A, and T325A, were capable of binding GDP and GTPγS and could be activated by GDP/AlF$_4$ (Fig. 1A and data not shown). In addition, the typical band patterns of trypsin digestion were observed with all of the mutants (Fig. 1A and data not shown). These results indicate that the mutations did not disrupt the overall folding of the mutant proteins.

The gain of function phenotypes of the T325A, V328A, and F332A are located on α2 and β3, respectively, are part of a phenylala-nine cluster that lies adjacent to Phe-332. These contacts may serve to stabilize the local tertiary structure near the α5 helix, to sense alterations in the position of Phe-332, and to communicate structural changes onto the nucleotide-binding pocket. This latter role is suggested by the structural changes observed in β2 and β3 in NMR studies of the F156L mutant in Ras (33).

It has been proposed previously that interactions between the carboxyl-terminal region of the α5 helix and the rest of the protein were involved in controlling the rate of basal (and possibly R$^*$-catalyzed) nucleotide exchange (34–36). We suggest that these contacts, like those discussed above, stabilize the position of residues in the amino terminus of α5 relative to the rest of Go$_{α5}$. Weakening these contacts, as in the I339A mutant, moderately accelerated nucleotide exchange (Table I), in agreement with previous results (36). However, the magnitude of increases in GDP release rates resulting from mutations of residues in the carboxy-terminal region of α5 were very small (~4-fold) compared with the effect of mutations closer to the amino terminus of α5.

The gain of function phenotypes of the T325A, V328A, and F332A mutants appear to mimic the effect of R$^*$ binding on Go$_{α5}$ in several key respects: (i) the basal rate of nucleotide exchange
is tremendously increased, (ii) the primary perturbation is at a distance from the nucleotide-binding site, and (iii) the nucleotide binding properties of the protein are preserved. In principle, perturbation of these residues by R* would be sufficient to catalyze nucleotide exchange.

To explore whether R* actually does induce conformational changes in this region, we measured the R*-catalyzed nucleotide exchange rates in each of the mutants of the α5 helix (Fig. 2). The Gαt mutants that showed the most drastic acceleration of basal nucleotide exchange rates (T325A, V328A, and F332A) could be further stimulated by R* and Gβγ1 (compare Fig. 2A with Fig. 1B). These mutants displayed faster R*-catalyzed rates than that of wild-type Gαt (Fig. 2A), as did N327A and I339A (not shown). These data demonstrate that the T325A, V328A, and F332A mutants could interact with R*. Furthermore, the conformations of the mutant proteins appear to be consistent with or possibly similar to the conformation of R*-bound Gαt.

Five mutations caused little or no change in the R*-catalyzed activation rate (Fig. 2B). These mutations, Q326A, K329A, D333A, V335A, and T336A, also did not affect the basal nucleotide exchange rate (Table I). Four of these mutations map to the same surface of α5 (green line). These residues communicate with the nucleotide-binding pocket. The perturbation may result from direct binding of R* to the exposed hydrophobic surface of α5 (red line).
indicated by the crystal structure and the basal nucleotide exchange rates of the corresponding mutants (Fig. 3C, Table I). In particular, Phe-330 is more than 4 Å from any other residue. Thus, the formation of important contacts involving Phe-330, Val-331, Asp-337, and Ile-338 or a subset

tions are needed to conclusively determine whether the inter-

structure of the G protein heterotrimeric G proteins (31, 42).

Go, a highly specialized molecular switch that exhibits an extremely low rate of basal GDP release and a very rapid rate of R*-catalyzed GTP uptake compared with other G proteins. However, considering the conserved nature of the α5 helix and its orientation to the nucleotide-binding pocket, it is likely that amino acid residues corresponding to Thr-325, Val-328, and Phe-332 on the inside surface of Go, serve a primary role in regulating nucleotide exchange rate in other Go subunits.

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