Guanine nucleotide exchange in heterotrimeric G proteins catalyzed by G protein-coupled receptors (GPCRs) is a key event in many physiological processes. The crystal structures of the GPCR rhodopsin and two G proteins as well as binding sites on both catalytically interacting proteins are known, but the temporal sequence of events leading to nucleotide exchange remains to be elucidated. We employed time-resolved near infrared light scattering to study the order in which the Ga and Gγ C-terminal binding sites on the holo-G protein interact with the active state of the GPCR rhodopsin (R*) in native membranes. We investigated these key binding sites within mass-tagged peptides and G proteins and found that their binding to R* is mutually exclusive. The interaction of the holo-G protein with R* requires at least one of the lipid modifications of the G protein (i.e. myristoylation of the Ga N terminus and/or farnesylation of the Gγ C terminus). A holo-G protein with a high affinity Ga C terminus shows a specific change of the reaction rate in the GDP release and GTP uptake steps of catalysis. We interpret the data by a sequential fit model where (i) the initial encounter between R* and the G protein occurs with the Gβγ subunit, and (ii) the Ga C-terminal tail then interacts with R* to release bound GDP, thereby decreasing the affinity of R* for the Gβγ subunit. The mechanism limits the time in which both C-terminal binding sites of the G protein interact simultaneously with R* to a short lived transitory state.

In eukaryotes, signal transduction across cell membranes is in many cases based on the interplay between G protein-coupled receptors (GPCRs) and heterotrimeric guanine nucleotide-binding proteins (G proteins, Goβγ). Binding of extracellular signaling molecules like hormones, neurotransmitters, or odorants to GPCRs triggers structural rearrangements in the receptor, such that its intracellular domain becomes competent to catalyze nucleotide exchange in the heterotrimeric G protein (1).

Rhodopsin is the visual pigment in retinal rod photoreceptors, those cells responsible for seeing under dim light conditions, and is the prototypical GPCR of the large family of rhodopsin-like GPCRs. Rhodopsin’s ligand, the chromophore 11-cis-retinal, is covalently bound and recognizes a photon as an extracellular signal. Within 200 femtoseconds, the energy of the photon causes cis \rightarrow trans isomerization of the retinal, thereby triggering the conversion of inactive dark-adapted rhodopsin into the active receptor conformation (R*), which is reached after milliseconds and is capable of interacting with transducin, the G protein of the rod cell (2).

High resolution structures of transducin (Gt (3) and the closely related heterotrimeric G protein Gtαβγ (4)) and rhodopsin (in the dark-adapted 11-cis-retinal bound state (5)) are available (Fig. 1). However, static crystal structures alone cannot elucidate the dynamics of the receptor-G protein interaction. Previous studies have focused on identifying structural domains involved in catalysis. The key binding sites on transducin are the C-terminal tails (CT) of the Ga subunit and the farnesylated Gγ subunit of the Gtγ dimer (CTα and CTγ-far, respectively), which specifically recognize and bind to R* (6–8).

In current models of nucleotide exchange, it is assumed that from these sites act simultaneously on the distant nucleotide binding domain by a pull or lever mechanism (8–14).

In this work, we have investigated the unknown temporal sequence of interaction between the CTα and CTγ-far sites and R*. We used “mass-tagged” peptides (in which the key CTs are fused to functionally neutral maltose-binding protein (MBP)), modified G proteins (wild type and modified in their attached lipids or C-terminal Ga amino acid sequence), and a kinetic near infrared light scattering assay (15–17) to monitor protein-protein interactions in real time. This approach allowed us to investigate how individual receptor binding sites on the G protein are functionally linked with each other. Based on the experimental data, we propose that nucleotide exchange requires a sequential two-step interaction of the G protein with R*. An encounter of CTγ-far with R* initiates the interaction and thereby makes CTα available for binding to R*. In the second step, R* shifts interaction from CTγ-far toward CTα. Now the nucleotide binding site has low affinity for GDP and is prepared for the uptake of GTP.

**EXPERIMENTAL PROCEDURES**

Native and Modified Ga Subunits and Gγβγ Dimers—G proteins used in this study were either the G protein of the rod photoreceptor cell (transducin, purified from bovine eyes), purified recombinant Gtαγ, or a Gtα construct. Because of the known very low expression of the soluble transducin Ga subunit (Gtα) in Escherichia coli and S99 cells, single amino acid substitutions were introduced into Gtα to yield a Gtα/Gtα chimera that contained 18 residues of Gtα in a Gtα background (18). Gtαγ is not present in photoreceptor cells but belongs to the same Go subfamily as Gtα and for...
Recombinant G\(\alpha_{\text{1}}\) (which contains an internal His\(_{6}\) tag) but not for the G\(\alpha_{\text{0}}/G\(\alpha_{\text{a}}\) chimeric (which contains an N-terminal His tag). Most experiments were performed in parallel with G\(\alpha_{\text{a}}\) purified from bovine retinae, the G\(\alpha_{\text{a}}/G\(\alpha_{\text{a}}\) chimeric, and G\(\alpha_{\text{a}}\) with similar results (see figure legends). All G\(\beta\)\(\gamma\) dimers consisted of the \(\beta_{12}\), isoform, the G\(\beta\)\(\gamma\) dimer of transducin (G\(\beta\)\(\gamma\)), and were expressed in SF9 cells or purified from bovine retinae. Farnesyl-free G\(\beta\)\(\gamma\) was obtained by expression of the recombinant G\(\gamma_{17}\)C71S mutant concomitantly with G\(\alpha_{\text{1}}\) in SF9 cells or by proteolysis of G\(\beta\)\(\gamma\) obtained from bovine retinae. The results obtained with both preparations were similar.

Cloning, Expression, and Purification of Chimeric G\(\alpha_{\text{0}}/G\(\alpha_{\text{a}}\) and G\(\alpha_{\text{a}}\) Subunits—The expression vector pHias-G\(\alpha_{\text{a}}\)*, which was generously provided by M. Natochin and N. Artemyev, contains a chimeric bovine G\(\alpha_{\text{0}}/G\(\alpha_{\text{a}}\) coding sequence, preceded by a six-histidine sequence encoding six histidines as an affinity tag (18). The chimeric G\(\alpha_{\text{1}}\) protein contains only 16 residues from G\(\alpha_{\text{1}}\) and was shown to be similar to native G\(\alpha_{\text{1}}\) in receptor interaction and basal nucleotide exchange. For construction of the mutants, the SpeI-HindIII fragment of pHias-G\(\alpha_{\text{a}}\) was introduced into the pLmtmus 38 cloning vector (New England Biolabs), yielding the precursor pl-G\(\alpha_{\text{a}}\). An oligonucleotide duplex with the respective mutation was cloned into the Thh111 and SapI restriction sites of pl-G\(\alpha_{\text{a}}\), and the modified SpeI-HindIII fragment was subcloned into SpeI/HindIII-digested pHias-G\(\alpha_{\text{a}}\). Recombinant G\(\alpha_{\text{1}}\) was expressed and purified (19) using the plasmid pQE-60 (Qiagen) harboring the rat G\(\alpha_{\text{1}}\) coding sequence (20). This protein was designed to have an N-terminal His\(_{6}\) epitope (a modification of pGEX-HGGGMATA) after position 121, where the homologous G\(\alpha_{1}\) from yeast has a long insert compared with mammalian G\(\alpha_{\text{1}}\) subunits. For expression of myristoylated G\(\alpha_{\text{1}}\) and mutants thereof, the respective G protein-encoding plasmids were cotransfected with pBB131 (coding for yeast N-myristoyltransferase; a generous gift of Jeffrey Gordon (21)) into E. coli JM109. Cultures were grown at 30 °C, induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside at an A\text{600} of 0.8 and harvested 12–14 h later. Expression and purification of the G\(\alpha_{\text{0}}/G\(\alpha_{\text{a}}\) chimeras was performed as described (18, 19, 22).

Expression and Purification of G\(\beta\)\(\gamma\) Dimers—Nonfarnesylated G\(\beta\)\(\gamma\) complexes were expressed in and purified from SF9 cells infected with the respective baculoviruses. Baculoviruses encoding G\(\beta\)\(\gamma\), and G\(\gamma_{17}\)C71S were a generous gift from A. G. Gilman and P. Gierschik, respectively. G\(\beta\)\(\gamma\)/G\(\gamma_{17}\)C71S was recovered from the cytosolic fraction of SF9 lysates as described (23) and purified by Ni\(^{2+}\)-nitrilotriacetic acid co-chromatography with His-tagged G\(\alpha_{\text{1}}\) (20).

Protolytic Defarnesylation of G\(\beta\)\(\gamma\)—The GGC-farnesyl fragment was removed from purified native G\(\beta\)\(\gamma\) by proteolysis as described earlier (24). Digests with Endo-Lys-C (sequencing grade; Roche Applied Science) and chymotrypsin were performed as described previously (28). The amino acid sequences of the peptides are given in Fig. 2A. The amino termini of the peptides were unmodified, and carboxyl termini of the G\(\gamma\)-derived peptides were amidated. The peptide CDKNPFKELKGGCFarnesy1 was synthesized as C-terminal amide on a Rink resin (loading 0.25 mg/mol; Rapp Polymere, Tübingen, Germany) with a Pioneer Synthesizer (Applied Biosystems) using an Fmoc (N-9-fluorenylmethoxycarbonyl) strategy. The C-terminal Cys-sulfur was protected by C-terminal Cys-sulfanylation. For intein-mediated ligation, was protected by an S-(tert-butylsulfenyl) group. All other side chain functions were protected with trifluoroacetic acid groups. The peptide was cleaved from the resin with 95% trifluoroacetic acid using triisopropylsilane as a scavenger. The crude peptide was precipitated twice with ether and lyophilized from acetonitrile/water (1:3). For farnesylation, 100 mg of the crude peptide (0.065 mmol), dissolved in 3 ml of N,N-dimethylformamide, was treated with 1.4 eq of farnesyl bromide (0.09 mmol) and an equal amount of disopropyl ethylamine (0.16 mmol). The formation of oxidation products was suppressed by degrading with NaH\(_{2}\). After 1 h, the crude farnesylation mixture was purified by preparative HPLC. For deprotection of the S-(tert-butylsulfenyl) group from cysteine, 20 eq of triis(2-carboxyethyl)-phosphine hydrochloride were added to a solution of the peptide in 50% acetonitrile/water. The mixture was adjusted to pH 5 with NH\(_{4}\) and stirred at room temperature for 5 h and then separated by HPLC. The farnesylated peptide obtained was characterized by mass spectrometry and analytical HPLC.

Disc sequence and Transducin Preparation—Preparations were performed as described previously (17).

Measurement of G Protein Conformational Changes by Fluorescence—The basal GDP/GTP exchange of Gs subunits (2 μM) in the absence of G\(\beta\)\(\gamma\) and rhodopsin was monitored by detection of fluorescence emission at 340 nm (excitation at 300 nm, measured at 20 °C with constant stirring) using a SPECTRO fluorometer (see Ref. 16 and references therein). Activation of Gs was started by adding GTP-S (10 μM final concentration). Activation of the whole Gs pool was completed by adding NaF and then AlCl\(_{3}\) (yielding 100 μM AlF\(_{3}\) final concentration). Traces were normalized to show the same total increase of intensity of fluorescence emission induced by GTP-S and AlF\(_{3}\).

Kinetic Light Scattering—Changes in intensities of scattered near infrared light were measured on the same spectrophotometer. The sensitivity of the light scattering assay is given by the measuring conditions and the experimental setup (15–17). As employed in the present study, the light scattering change is proportional to the generation binding of “mass-tagged” peptides (MBP-epitope fusion proteins) to R* but not binding of short synthetic peptides to R* because of the small peptide mass. All measurements were performed in 10-mm path cuvettes at pH 7.4 (20 mM bis-tris propane, 130 mM NaCl, 1 mM MgCl\(_{2}\), pH 7.5) and 25 ± 1 °C. Reactions were triggered by flash photolysis of rhodopsin (3 μM) using flashes of green light (500 ± 20 nm). Binding signals were recorded with 32% flash-activated rhodopsin. For data evaluation, see “Appendix.”

**RESULTS**

Monitoring the Interaction between R* and C-terminal Binding Sites of the G Protein by Kinetic Light Scattering—To monitor in real time binding between R* in disk membranes isolated from the rod outer segment and interacting CTs of the heterotrimeric G protein (namely CTs and CTy-far; Figs. 1 and 2A), the kinetic near infrared light scattering assay was employed (see “Experimental Procedures” and Refs. 15 and 16).

“Mass-tagged” peptides, which can be obtained by expression and purification of MBP-epitope fusion proteins, allow convenient measurement of the binding to R* (Fig. 2B).

A typical binding signal triggered by flash activation of rhodopsin is shown in Fig. 2B for MBP-CT\(\alpha_{1}\)HA1 (top trace). This MBP fusion protein contains the well known G\(\alpha_{1}\) high affinity analog CT sequence (Fig. 2A, CT\(\alpha_{1}\)HA1), which was identified by Hamm and co-workers (26). Since both the small synthetic and MBP “mass-tagged” CT\(\alpha_{1}\)HA1 peptides bind to R*, binding of MBP-CT\(\alpha_{1}\)HA1 to R* can be inhibited by an excess of the competing small synthetic CT\(\alpha_{1}\)HA1 peptide (Fig. 2B, middle trace). A further control showing that the assay monitors the
interaction between R* and MBP-CTαHA1 is provided by the effect of hydroxylamine, which affects this interaction by hydrolyzing the retinal Schiff base in a competing reaction (20 mM), which was farnesylated at Cys-71.

By testing peptides with modified C-terminal sequences, we were able to identify a single amino acid exchange (K341L in Gtα) that increases the affinity for R* of the corresponding CTα peptide (termed CTαHA2) to a level similar to CTαHA1 (details to be published). "Mass-tagging" of CT peptides with MBP was extended to CTα and CTαHA2 and the farnesylated CTγ-far. All of these MBP-CT fusion proteins showed binding to R*, were sensitive to competition by the corresponding synthetic CT peptide, and were sensitive to the effect of hydroxylamine (Fig. 3C and data not shown). MBP lacking a fused G protein CT peptide tail showed no binding to R* (data not shown).

Only One of the C-terminal Peptides, CTα or CTγ-far, Can Bind to R* at One Time—To investigate whether CTα and CTγ-far interact simultaneously with R* (see Fig. 1), competition of the two CTs for binding to R* was investigated with the kinetic light scattering assay. The interaction between the MBP-CTαHA1 fusion protein and R* could be inhibited in a concentration-dependent manner by the presence of a synthetic peptide corresponding to CTγ-far (Fig. 3A). Analogously, binding of MBP-CTαHA2 and MBP-CTα to R* could be suppressed by excess CTγ-far peptide (data not shown). The competition between the MBP-CTαHA1 fusion protein and the CTγ-far peptide for R* could be supported by a centrifugation assay (Fig. 3B). In this biochemical binding assay, rhodopsin in disk membranes is pelleted in the presence of MBP-CT fusion pro-
teins in the dark or light, followed by subsequent analysis of the pellet and supernatant by SDS-PAGE and protein staining. The centrifugation assay also showed that the MBP-CTa peptide reduced the binding signal of MBP-CTaHA1 (0.45 μM; Kᵢ = 0.2 μM) and R⁺ (1 μM) measured by light scattering in a concentration-dependent manner (concentrations as indicated). Affinity and competition results are similar for MBP-CTaHA2. MBP-CTa peptide can also effectively inhibit MBP-CTot (data not shown). B, biochemical competition measurement. Rhodopsin in disk membranes (10 μM) was incubated with MBP-CTaHA1 (0.5 μM) and pelleted in the dark or light in the absence or presence of CT-far peptide (concentrations as indicated). Supernatants (s) and pellets (p) were analyzed by SDS-PAGE and proteins were visualized by Coomassie Blue staining. C, competition experiment under reversed conditions. MBP-CTa-far was produced semisynthetically by intein-mediated protein ligation (27). The binding signal of 60 μM MBP-CT-far (1 μM R⁺) is inhibited in a concentration-dependent manner by the presence of CTaHA1 peptide (concentrations as indicated). Also CTaHA2 and CTot peptides can inhibit binding of MBP-CT-far to R⁺ (data not shown).

The G protein’s lipid modification is mandatory for interaction with activated rhodopsin. Interaction of light-activated rhodopsin (1 μM R⁺; the arrow indicates time of activation) with 0.6 μM Goαβγ (see “Experimental Procedures”) was measured by kinetic light scattering as described in Fig. 2. The amount of G protein bound to disk membranes (3 μM rhodopsin) in the dark (D) and light (L) was determined by pelleting the disk membranes, subsequent SDS-PAGE analysis of the pellet, and protein staining with Coomassie Blue (G protein bands are boxed; SDS-PAGE data are part of an extensive study on G protein lipid modifications (M. Heck, O. P. Ernst, R. Herrmann, K. P. Hofmann, and C. Kleuss, manuscript in preparation). Samples contained lipid modifications as indicated (myristoylation of Goα, or farnesylation (far) of Gy). Under the experimental conditions the Gal/ Gβγ combinations shown formed holoproteins (data not shown), which were not bound to the disk membrane when at least one lipid modification was lacking. The amplitude of the binding signal of the +myr/+far combination is reduced, because this holoprotein shows like native transducin considerable binding to the disk membrane in the dark, an effect, which is more pronounced for the holoprotein containing Goα. Consequently, the concentration of soluble G protein, which is available to contribute to changes of the intensity of scattered light (17), is reduced. Similar results were obtained for transducin when heterogeneously fatty acylated native Goα, the nonmyristoylated GdαGtα, chimeras and Gβγ with and without farnesyl moiety were used (data not shown). No difference in behavior was observed when enzymatically defarnesylated Gβγ or the recombinant Gβγ(C71S) mutant was used. The increase of intensity of scattered light, the intensity change is smaller for MBP-CT-far.

When we performed the competition experiment with the MBP-CT-far fusion protein and CTa peptides, an analogous inhibition by the CTa peptide in a concentration-dependent manner was seen in the light scattering assay (shown for CTaHA1 in Fig. 3C). At higher concentrations of the competing CTa peptide (>1 μM), a transient small binding signal becomes visible, which sits on the normal light scattering change with competitively reduced amplitude. It may reflect a delayed action of the CTa peptide, in agreement with the data presented below. The binding of MBP-CT-far to the disk membrane in the dark causes a high background in the centrifugation assay, thereby limiting the use of this assay in competition experiments involving MBP-CT-far (data not shown). Taken together, the competition experiments show that R⁺ can interact with only one C-terminal binding domain of the G protein at one time, either CTa or CTγ-far.

The First Interaction Step Requires Lipid-modified G Protein—The competition experiments described above suggest that in the holo-G protein, the two CT binding domains interact sequentially and not simultaneously with R⁺. Therefore, the question arose which CT binding domain of the holo-G protein is involved in the initial encounter step with R⁺. For interaction of a synthetic CTγ-far peptide with R⁺, it is known that the farnesyl moiety is indispensable (7, 8). However, for the transducin holoprotein, farnesylation is controversially discussed (29, 30). Thus, we prepared the Gβγ dimer with and without farnesylation. Furthermore, we prepared the Ga subunit with and without myristoylation at the N terminus in order to investigate whether the hydrophobic modification on the N terminus of the Ga subunit (Fig. 1) is involved in the interaction with R⁺ as suggested by its close proximity to the farnesyl moiety (31).
Light scattering and centrifugation experiments were performed with native transducin subunits (G\(_{\alpha}\alpha\), G\(_{\beta}\gamma\), G\(_{\alpha}\alpha\) with its physiological myristoyl moiety, and G\(_{\beta}\gamma\) lacking its farnesyl moiety. Recombinant nonmyristoylated Go subunits were obtained by expression in E. coli, either a G\(_{\alpha}\alpha\) construct or G\(_{\alpha}\alpha\), which can couple like transducin to R\(^*\) (see “Experimental Procedures”). By light scattering, rapid and strong binding to R\(^*\) was seen with G proteins that are missing one of the two lipid modifications, either myristoylation or farnesylation (Fig. 4). This was supported by the centrifugation assay, as seen by the intensity of the Ga and G\(_{\beta}\gamma\) protein bands, which reflect the amount of G protein bound to the disk membrane in dark and light, respectively (Fig. 4, protein bands are boxed). Native holo-G protein, which carries both lipid modifications, is like MBP-CT\(_{\gamma}\)-far. Partially membrane bound and therefore yields only a smaller binding signal. No interaction with rhodopsin or R\(^*\) is observed when both lipid modifications are lacking. This cannot be explained by a lack of holo-G protein formation, since the Go/\(G_{\beta}\gamma\) combinations tested formed heterotrimers in solution as determined by analytical gel filtration. The data presented therefore imply that one lipid modification in the holo-G protein is required for interaction with R\(^*\). In the absence of Go farnesylation, myristoylation of Go can replace CT\(_{\gamma}\)-far.

We have shown above that the MBP-CT\(_{\alpha}\) fusion protein interacts with R\(^*\). Therefore, CT\(_{\alpha}\) as part of the non-lipid-modified holo-G protein should be able to confer affinity to the G protein to bind to R\(^*\). However, this was not observed in the holo-G protein lacking both lipid modifications (Fig. 4), suggesting that this binding domain of Go is not available for interaction with R\(^*\) in the holo-G protein. Taken together, this argues for an initial encounter of R\(^*\) with the hydrophobic farnesyl/myristoyl pair, which starts nucleotide exchange catalysis and subsequently enables CT\(_{\alpha}\) to interact with R\(^*\).

Sequence of Interactions—Another line of evidence for the CT\(_{\alpha}\) binding site being the second in the interaction of the holo-G protein with R\(^*\) is shown in Fig. 5. We investigated the influence of the CT\(_{\alpha}\) sequence on the reaction. By a spectroscopic binding assay (“Extra-MII” assay; see Ref. 16), a CT\(_{\alpha}\) peptide (CT\(_{\alpha}\)HA2; Fig. 2A) was determined to develop an affinity for R\(^*\) 2 orders of magnitude higher than the native CT\(_{\alpha}\) peptide (CT\(_{\alpha}\) or CT\(_{\alpha}\)I), similar to an already described high affinity CT\(_{\alpha}\) peptide (CT\(_{\alpha}\)HA1) (26). Nonmyristoylated Go either with the native or the high affinity CT\(_{\alpha}\) (CT\(_{\alpha}\)HA2) was tested for R\(^*\) binding in combination with farnesylated G\(_{\beta}\gamma\). In the absence of additional nucleotides (only the endogenous GDP was present in the nucleotide binding site), both combinations showed identical binding signals. The same rates of R\(^*\) binding support the notion that CT\(_{\alpha}\) is not involved in the initial encounter step.

In the presence of additional GDP (~800-fold excess), the amount of R\(^*\)G complex formed was reduced as seen in the lower final level of the binding signals at completion (Fig. 5B). The effect was less pronounced in the case of the modified holo-G protein, containing the high affinity CT\(_{\alpha}\). Kinetic anal-

\(^{2}\) M. Heck, O. P. Ernst, R. Herrmann, K. P. Hofmann, and C. Kleuss, manuscript in preparation.

\(^{3}\) R. Herrmann, P. Henklein, K. P. Hofmann, and O. P. Ernst, manuscript in preparation.
ysis according to the reaction model (Fig. 5A) revealed that the high affinity CTα has no influence on the initial encounter step but specifically affects the second step (GDP release). It favors the formation of the nucleotide-free Rα-G complex, which is seen in a shift of equilibrium 2 by a factor of 7 (shown for GtHA2 in Fig. 5, B and C, Table I). The presence of the high affinity CTα on the G protein accelerates significantly GTP uptake and Rα-G complex dissociation by a factor of 3 (Table I). This suggests that upon GDP release, CTα is the site that remains engaged with Rα, thereby determining the G protein conformation in which GTP can be taken up. 

**Gβγ-dependent Binding of CTα**—The single Lys → Leu substitution in CTot (K341L in Gtα) not only elevated the affinity of CTα for Rα but also revealed that CTα is apparently not available for interaction with Rα in the isolated Go subunit. Nonmyristoylated Go with wild type CTα is incapable of interacting with Rα in the presence of farnesyl-lacking Gβγ (Fig. 4, lowest trace) or absence of farnesylated Gβγ (Fig. 6A, lowest trace), whereas the corresponding MBP-CTα fusion protein binds readily to Rα (data not shown). However, the mutant Go subunit containing the high affinity Lys → Leu substitution (Go-CTαHA2) showed slow Gβγ-independent binding to Rα (Fig. 6A). Interestingly, this mutant Go subunit does not interact with rhodopsin in the dark but was detected in rhodopsin-containing membranes after illumination (Fig. 6B). Light-induced binding of the mutant Go-CTαHA2 subunit to Rα was also observed in the presence of GTP and when this mutant Go was preactivated with AIF2α (Fig. 6A). The mutation had very little effect on the basal nucleotide exchange rate of Go and the conformational change induced by AIF2α uptake as measured with a fluorescence activation assay (Fig. 6C). The mutant Go-CTαHA2 subunit showed binding to Rα similar to the MBP-CTα fusion proteins, indicating that in this mutant Go, the high affinity CTα is now available for interaction with Rα in any conformational state of Go. However, the capability of this mutant Go to bind to Rα is lost when a holo-G protein is formed with Gβγ lacking its farnesyl moiety (Fig. 6A). In contrast, farnesylated Gβγ in the mutant holoprotein containing Go-CTαHA2 accelerated binding to Rα and enabled Rα-catalyzed GDP/GTP exchange, corroborating that the initial interaction of Rα with Gβγ makes CTα available for interaction with Rα (Fig. 6A).

**DISCUSSION**

**The Interaction Model**—We performed kinetic experiments to elucidate how in the rhodopsin/transducin model system the G protein heterotrimer is presented to its receptor at the membrane surface, to illuminate the sequence of interactions of key binding sites of the G protein with the activated GPCR (Rα) and how this sequence is associated with nucleotide exchange. We performed the experiments with rhodopsin embedded in disk membranes of rod cells and two G proteins, consisting of Gtα or Gtα1 and Gtβγ, obtaining comparable results. We have provided four lines of experimental results, namely (i) the competition of isolated C-terminal sequences of Go (CTα) and Gγ (CTγ-far) for binding to Rα, (ii) the necessity of a hydrophobic lipid modification on either the Gγ C terminus or Go N terminus (Fig. 1) to initiate the interaction with Rα, (iii) the influence of the CTα sequence on GTP-dependent dissociation of the Rα-G complex, and (iv) shielding of a constitutively available C terminus of a mutant Go subunit by holoprotein formation with Gβγ. Our results are consistent with a model in which the interaction of the G protein with Rα is initiated by the encounter of Rα with the Gβγ subunit. Either the membrane interaction of the G protein or, more likely, the contact of the G protein with Rα is required to make CTα available for interaction with Rα. GDP may be released when both CTα and CTγ-far are transiently engaged in the interaction with Rα. Eventually, however, the affinity of the receptor for the Gβγ subunit decreases, relocating the main interaction of Rα to CTα and resulting in a nucleotide-free Rα-G complex into which GTP can be taken up. It is important to note that such a mechanism does not exclude simultaneous interaction of both sites with Rα but embeds it into the sequence of events as a short lived transitory state (Fig. 5A). This may provide a general scheme according to which GPCRs activate their G proteins.

The initial encounter of the G protein with Rα is likely to be associated with a conformational change in Gβγ. A conformational switch in Gβγ was previously proposed on the basis of a lack of accessibility of CTγ to carboxypeptidase Y in the solubilized Gβγ or holotransducin, suggesting that CTγ is masked at least in the non-membrane-bound state of the G protein (32). In a recent NMR study of a CTγ-far peptide, it was reported that CTγ is unstructured in the presence of an inactive receptor but forms an amphipathic helix upon rhodopsin activation (33). In the initial encounter complex, the contact of Rα with the lipid modifications of the holo-G protein appears to trigger the process of making the second CT binding site, CTα, available for interaction. Interestingly, myristoylation at the N terminus of Go can functionally replace farnesylation of Gβγ (Fig. 4). The precise role of the myristoylated N terminus of Go in the physiological process with farnesylated Gβγ remains to be studied. Also unresolved is how dissociation of the Rα-G complex occurs after GTP uptake. We described a mutant Go subunit, containing a high affinity CTα, which binds to Rα independent of its GDP- or GTP-bound conformational state but dissociates like native transducin from Rα when Gβγ is present in the complex with Rα (Fig. 6; see below). This suggests that the GTP-induced Rα-G complex dissociation passes through a similar but reversed sequence of interaction steps as established for Rα-G complex formation. The subsequent disso-
started by the addition of NaF and AlCl3 from separate stock solutions to disk membranes (10 μM). Experimentally, the interaction with R* is accelerated in the presence of Gβγ (0.6 μM Gβγ plus 0.6 μM Gαi-CTa/H9252/H9253). When an excess of GTP (50 μM) is present, the R*-Gαi-CTa/H9252/H9253 complex goes through nucleotide exchange and subsequent complex dissociation and therefore does not accumulate. R*-Gαi-CTa/H9252/H9253-Gβγ complex formation does not occur when deamidated Gβγ is used (2 μM Gβγ (C71S mutant) plus 2 μM Gαi-CTa/H9251). In contrast to nonamidated Gαi-CTa/H9252/H9253, the nonamidated Gαi-CTa/H9251 alone (10 μM) does not bind to R* (lowest trace). R*, rhodopsin in disk membranes (10 μM) was incubated with nonamidated Gαi-CTa/H9251-CTa/H9252 (0.6 μM) in complex with Gβγ (0.6 μM, upper panel) or as isolated subunit (10 μM, lower panel) in the presence or absence of GTP (50 μM) and pelleted. Supernatants (s) and pellets (p) were analyzed by SDS-PAGE and proteins were visualized by Coomassie Blue staining. Rhodopsin (R) was not completely removed during the SDS sample preparation and a small fraction is observed in the pellet lanes (17). Similar results in both assays (light scattering and centrifugation) were obtained when recombinant Gαi with CTa/H9252 was used. C, basal nucleotide exchange of nonamidated Gαi with high or low affinity Gα C terminus (CTa/H9252 or native CTa/H9251, respectively) was monitored by measuring the increase in fluorescence emission. Traces of Gαi-CTa/H9252 and Gαi-CTa/H9251 are superimposed. At the time indicated by the arrow, the reaction was started by the addition of GTPγS. Activation of remaining GαGDP was started by the addition of NaF and AlCl3 from separate stock solutions to form AlF4− at the times indicated by the arrows.

The Physiological Necessity of a Sequential Interaction—We have described that nucleotide exchange catalysis in Go can be improved by increasing the affinity of CTA for R* (Fig. 5, Table I). An intriguing question that arises from this finding is why the native system does not employ a Go subunit with a high affinity CTA. An important consequence of the high affinity mutation is that nonamidated Go subunits can bind independent of Gβγ to R*. Binding to R* is possible and strong in both the GDP-bound and the activated GDP-AlF4− conformation, suggesting that this mutation in the Go monomer uncouples the nucleotide binding site from CTA and exposes CTA for interaction with R*. It takes the complex with Gβγ to make this high affinity CTA unavailable for direct binding to R* (shown by the farnesylation-dependent binding to R*) (Fig. 6A). Any high affinity Go subunit that is not bound to Gβγ would, through its more accessible CTA, represent a severe threat to signal transduction in the cell, since it would block the active GPCR and thereby poison the catalyst. Both efficient shielding of CTA in Go and holo-G proteins and a secure mechanism for making CTA accessible upon catalytic interaction with R* are therefore essential for maximal effectiveness of signal transduction. One would hope to learn the structural basis for the inaccessibility of CTA in monomeric inactive Go-GDP and active Go-GTP subunits from the crystal structure of Gαi3 (34). Unfortunately, for the holo-G proteins the crystal structures are not informative, because most of CTA is not resolved (3, 4), leaving molecular details open for further studies.

Receptor Dimerization—Another open question is whether R* is represented by a receptor monomer or dimer. The mechanism of receptor-G protein interaction given above is compatible with an interaction between the G protein and a receptor monomer as well as a receptor dimer and does not require receptor dimerization for activation of the G protein. Recently, atomic force microscopy showed that rhodopsin can form dimers in disk membrane preparations (35). Future studies will have to address this point and determine the molecular identity of R*, which may consist of one or two activated receptor molecules or a dimer between one activated and one inactive receptor molecule. It even appears possible that the G protein induces receptor dimerization during the interaction process. It is anticipated to be that a dimer between two light-activated rhodopsin molecules is not required for G protein activation. Such dimers do not exist in the single photon working range (36) of the rod photoreceptor cell. Furthermore, it remains to be studied how the sequence of catalytic steps in R*-G protein coupling identified here relate to different conformations of the activated receptor, which are separated by protonation changes in rhodopsin (2, 37–41). The G protein may use different receptor conformations, which can bind either CTA or CTγ-far, to allosterically control the affinity of the two binding sites on the receptor for efficient nucleotide exchange catalysis.

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APPENDIX

Kinetic Analysis of Receptor-G Protein Coupling—Data analysis of the binding signals of Goβγ (Fig. 5, B and C) was performed on the basis of the reaction scheme depicted in Fig. 5A. The time dependence of the concentrations of each reactant (the first derivative over time) can be described by the following differential equations.

\[ \frac{d[G\text{-GDP}]}{dt} = -k_{1,\text{on}}[R^*][G\text{-GDP}] + k_{1,\text{off}}[R^*G\text{-GDP}] + k_{2}[G\text{-GTP}] \]  
\[ \text{(Eq. 1)} \]

\[ \frac{d[R^*G\text{-GDP}]}{dt} = k_{1,\text{on}}[R^*][G\text{-GDP}] - k_{1,\text{off}}[R^*G\text{-GDP}] - k_{2,\text{on}}[R^*G\text{-GDP}] + k_{2,\text{off}}[R^*G\text{-GDP}] \]  
\[ \text{(Eq. 2)} \]

\[ \frac{d[R^*]}{dt} = -k_{1,\text{on}}[R^*][G\text{-GDP}] + k_{1,\text{off}}[R^*G\text{-GDP}] + k_{2,\text{on}}[R^*G\text{-GDP}] + k_{2,\text{off}}[R^*G\text{-GDP}] \]  
\[ \text{(Eq. 3)} \]

\[ \frac{d[G\text{-GTP}]}{dt} = k_{2}[R^*G\text{-GDP}] - k_{2}[G\text{-GTP}] \]  
\[ \text{(Eq. 4)} \]
The light scattering change $\Delta I/I$ is proportional to the sum of the concentrations of the formed $R^*$-G protein complexes, $[R^*G]$ and $[R^*-G^*]$. Therefore, the experimental values for $\Delta I/I$ could be fitted using a scaling factor $f$ (for further details, see Ref. 17).

The set of three binding signals of the G protein (with either native or high affinity CTx in the presence of different nucleotides (no nucleotide, GDP added, or GTP added; Fig. 5, B and C) was fitted simultaneously using the differential equations above and a numerical multiple least square fit procedure (Scientist software; MicroMath Scientific Software, Salt Lake City, UT). The initial concentrations of G protein, R*, GDP, and GTP were fixed for each individual experiment, and the other parameters ($k_{1,\text{on}}, k_{1,\text{off}}, k_{2,\text{on}}, k_{2,\text{off}}, k_3, k_4, f$) were allowed to vary. The initial conditions were as follows: $[R^*] = 1 \mu M$; $[G-GDPC] = 0.6 \mu M$; $[GDP] = 0$ and 500 $\mu M$, respectively; $[GTP] = 0$ and 0.6 $\mu M$, respectively; $[R^*G^*G] = 0$; $[R^*G] = 0$; $\Delta I/I = 0$.

A first fit yielded kinetic parameters for $G_{i\alpha}\gamma/G_{i\beta}\gamma$. Data fitting of $G_{i\alpha}\gamma\alpha_i\text{CTxHA2}/G_{i\beta}\gamma\beta$ was performed with fixed rate constants for equilibrium 1 (Fig. 5A, $k_{1,\text{on}}$ and $k_{1,\text{off}}$) using the values of $G_{i\alpha}\gamma/G_{i\beta}\gamma$. Parameters obtained are given in Table I.
