Rhodopsin Recognition by Mutant Gsα Containing C-terminal Residues of Transducin*

(Received for publication, July 21, 1999, and in revised form, September 28, 1999)

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The C-terminal regions of the heterotrimeric G protein α-subunits play key roles in selective activation of G proteins by their cognate receptors. In this study, mutant Gsα proteins with substitutions by C-terminal residues of transducin (Gtα) were analyzed for their interaction with light-activated rhodopsin (R*) to delineate the critical determinants of the Gtα/R* coupling. In contrast to Gtα, a chimeric Gsα/Gtα protein containing only 11 C-terminal residues from transducin was capable of binding to and being potently activated by R*. Our results suggest that Cys347 and Gly348 are absolutely essential, whereas Asp346 is more modestly involved in the Gtα activation by R*. In addition, the analysis of the intrinsic nucleotide exchange in mutant Gsα indicated an interaction between the C terminus and the switch II region in Gsα-GDP. Mutant Gsα containing the Gtα C terminus and substitutions of Asn339 and Asp340 (switch II) by the corresponding Gtα residues, Glu312 and Gly315, displayed significant reductions in spontaneous guanosine 5'-O-(3-thiotriphosphate)-binding rates to the levels approaching those in Gtα. Communication between the C terminus and switch II of Gtα does not appear essential for the activation coupling between Gtα and R*, but may represent one of the mechanisms by which Ga subunits control intrinsic nucleotide exchange.

In the visual transduction cascade, photolyzed rhodopsin (R*) activates the photoreceptor G protein, transducin (Gtαβγ), by catalyzing GDP/GTP exchange on the Gtα subunit. Transducin sites of interaction with R* have been extensively investigated (1, 2). Despite the fact that the Gtβγ subunit participates in the interaction with R* and enhances transducin affinity for R* (3, 4), the Gtα subunit contains principal sites for specific recognition of the activated receptor. Abundant evidence points to the C-terminal domain of Gtα as the major R* contact site. An ADP-ribosylation of Gtα Cys347 by pertussis toxin uncouples Gtα from R* (5, 6). A synthetic peptide corresponding to the 11 C-terminal amino acid residues of Gtα, Gtα(340–350), is capable of competing with Gtα for binding to R* and stabilizing the metarhodopsin II conformation (7). Mutational analysis of the Gtα C-terminal tail (8, 9) and screening of the combinatorial peptide library (10) have identified residues within Gtα(340–350) that are required for interaction with R*. All studies have consistently implicated conserved Leu344 and Leu349 as being absolutely essential for Gtα binding to R* (8–10). However, the receptor role of other C-terminal residues of Gtα has not been conclusively established. An NMR analysis of structural changes induced in the synthetic peptide Gtα(340–350) upon its binding to R* suggested a unique role for Gly348 in the transition from a disordered Gtα(340–350) to the R*-binding conformation with an open reverse turn and a helix-terminating C-capping motif (11). In agreement with the role of Gly348, activation of the Gtα mutant Gly348→Ala expressed in COS-7 cells was severely impaired (8). Furthermore, Cys347 and Gly348 were invariant in R* binding peptides obtained with a combinatorial library (10). In contrast, Cys347→Ala isolated from transfected COS-7 cells (8) or in vitro translated Gly348→Ala (9) showed no significant defects in interaction with R*. Thus, results of the “loss-of-function” experiments (Ala mutagenesis of Gtα) are not entirely consistent with a “gain-of-function” combinatorial selection of peptides.

To elucidate the role of individual C-terminal residues of Gtα in the specific interaction with R*, we initially generated a chimeric Gtα/Gsα protein containing 11 C-terminal residues of Gtα. The finding that, in contrast to Gtα, this chimera was capable of effective and specific interaction with R* allowed us to utilize Gtα as a background for introduction of selected C-terminal residues of Gsα. Analysis of the Gsα mutants identified minimal insertions of Gtα residues required for R* recognition and revealed a potential role of the C-terminal and switch II residues Glu212 and Gly213 in providing for a low intrinsic GDP/GTP exchange rate characteristic for Gtα.

EXPERIMENTAL PROCEDURES

Materials—[35S]GTPγS (1160 Ci/mol) was purchased from Amersham Pharmacia Biotech. Restriction enzymes were from New England Biolabs Inc. T4 DNA ligase was from Roche Molecular Biochemicals. Cloned Pfu DNA polymerase was from Stratagene. 1-1-2,2-Tosylamido-2-phenylthyl chloromethyl ketone-treated trypsin was from Worthington. All other chemicals were from Sigma or Fisher.

Site-directed Mutagenesis of Gsα—The short splice form of bovine Gsα was subcloned into the vector pHIs-Gsα for bacterial expression using PCR-directed cloning and the NcoI and HindIII restriction sites (12). The long splice form Gsα numbering is used throughout the text for clarity. pHIs-Gsα was used as a template for PCR-based mutagenesis. GsαGtα(340–350) was generated by replacing the C terminus of Gsα with the 11 C-terminal residues of Gtα. The PCR included a forward primer carrying a BglII site overlapping codons for Gsα 294QDL296 and a reverse primer coding for the C terminus of Gsα and carrying a HindIII site overlapping the stop codon. The PCR product (~300 base pairs) was digested with BglII and HindIII and subcloned into the pHIs6 vector. Similarly, all other mutations within the Gtα C terminus were introduced using reverse primers carrying the desired mutation(s). To substitute Asn339→Asp340 in Gtα for the corresponding Gsα residues, Gtu312Glu213, a forward 5’-phosphorylated primer carrying these substitutions was paired with the appropriate reverse C-terminal primers and PCR-amplified using pHIs6-Gsα or mutant pHIs6-Gtα as a template.
The products were ligated to the upstream \( \alpha \) cDNA fragment, which was obtained by PCR amplification with the N-terminal primer and the reverse 5'-phosphorylated primer coding for the sequence adjacent to \( \alpha \) Asn240-Asp240. After the ligation, DNA fragments of the appropriate size (~1200 base pairs) were separated on an agarose gel, excised, and analyzed as templates for PCR amplification with primers flanking the \( \alpha \) open reading frame. Amplified mutant cDNAs were subcloned into the phIGE vector as described above. Sequences of all \( \alpha \) mutants were confirmed by automated DNA sequencing at the University of Iowa DNA Core Facility. Typically, for protein expression, 500 ml of 2× TY medium (16 g liter tryptone, 10 g liter yeast extract, 5 g liter NaCl) was inoculated with 15 ml of overnight culture of BL21(DE3) cells transformed with the mutant \( \alpha \) cDNAs. At \( A_{600} = 0.5 \), expression was induced by 30 mM isopropyl-\( \beta \)-thiogalactopyranoside for 18 h at 25–30 °C. Cells were pelleted and kept frozen at ~70 °C. Purification of \( \alpha \) and its mutants was carried out as described (12, 13). Yields for 80–90% pure mutant \( \alpha \) were normally 3–5 mg.

Trypsin Protection Assay—\( \alpha \) mutants (20 mM) were incubated for 30 min at 25 °C in 20 mM HEPES (pH 8.0) containing 130 mM NaCl, 50 mM GDP, and 5 mM MgSO\(_4\). Where indicated, 50 mM GTP-S or 10 mM NaF and 30 mM AlCl\(_3\) were included in the buffer. Trypsin digestions were performed with 50 \( \mu \)g/ml trypsin for 20 min at 25 °C and stopped by simultaneous SDS sample buffer addition and heat treatment (100 °C, 5 min). The time courses of GTP-S binding in the trypsin protection assay were followed by trypsinolysis of mutant \( \alpha \) with 100 \( \mu \)g/ml trypsin for 10 min at 25 °C.

Binding of \( \alpha \), \( \alpha \), \( \alpha \), and \( \alpha \) (Gα340–350) Reconstituted with Gβ/γ to R* in uROS Membranes—\( \alpha \), \( \alpha \), and \( \alpha \) were like Gα340–350 chimera (Gα340–350) complexed with Gβ/γ (14), Gα340–350 (1 μM each), Gβ/γ (1 μM), and uROS membranes (10 μM rhodopsin) were mixed in 100 μl of 20 mM HEPES (pH 7.6) containing 150 mM NaCl and 4 mM MgCl\(_2\). The mixture was then illuminated with a white light transilluminator lamp (Fisher) for 5 min at 25 °C. Rod outer segment membranes were centrifuged for 10 min at 20,000 \( \times \)g, and the pellets were washed twice with 1 ml of the buffer. Bound G proteins were then extracted using 50 μl of 10 mM GTP-S in hypotonic buffer, and the supernatants (150,000 \( \times \)g, 15 min) were concentrated and analyzed by SDS-polyacrylamide gel electrophoresis.

GTP-S Binding Assay—\( \alpha \) or its mutants (1 μM) alone or mixed with 2 μM Gβ/γ or with 2 μM Gβ/γ and uROS membranes (300 nm rhodopsin if not otherwise indicated) were incubated for 3 min at 25 °C. Binding reactions were started by addition of 5 μM [35S]GTP-S (1 Ci/mmol). Aliquots of 20 μl were withdrawn at the indicated times and passed through Whatman cellulose nitrate filters (0.45 μm), which were then washed three times with 1 ml of ice-cold 20 mM Tris-HCl (pH 8.0) containing 130 mM NaCl and 10 mM MgSO\(_4\). Upon complete dissolution in a 3a7b8 mixture, the filters were counted in a liquid scintillation counter. The \( k_{app} \) values for the binding reactions were calculated by fitting the data to the following equation: \% GTP-S bound = 100(1 – e\(^{-k_{app}(t)}\)). A linear regression fit was used when <25% of total mutant \( \alpha \) bound GTP-S during the time course of the binding reactions.

RESULTS

Expression and Characterization of the G\( \alpha \)/G\( \alpha \) Chimera Containing the G\( \alpha \) C Terminus—To assess the suitability of G\( \alpha \)/G\( \alpha \) chimera and G\( \alpha \) mutants as tools for mapping the G\( \alpha \)cR* interface, we first expressed G\( \alpha \) and G\( \alpha \)/G\( \alpha \) (340–350) chimera as His-tagged proteins in Escherichia coli and examined their interactions with R*. G\( \alpha \) and G\( \alpha \)/G\( \alpha \) (340–350) were fully capable of undergoing an activating conformational change upon the binding of GTP-S or GDP/AlF\(_3\), as tested in the trypsin protection assay (Fig. 1A). Direct binding experiments of G\( \alpha \) and G\( \alpha \)/G\( \alpha \) (340–350) to R* were carried out in suspensions of uROS reconstituted with G\( \beta \)/γ (15). For the purpose of comparison with native G\( \alpha \), recombinant His-tagged G\( \alpha \) and the transducin-like chimeric G\( \alpha \)/G\( \alpha \) protein G\( \alpha \) (14) were included (Fig. 1B). Predictably, the very myristoylated G\( \alpha \) showed the strongest binding to R*, followed by recombinant non-myristoylated G\( \alpha \) and G\( \alpha \). Relatively potent binding of G\( \alpha \) to R* is not surprising. G\( \alpha \) has a C-terminal sequence almost identical to that of G\( \alpha \), and activation of G\( \alpha \) by R* has been reported (13). Under the conditions of this assay, G\( \alpha \) showed no detectable binding to R*. However, the incorporation of the G\( \alpha \)C terminus into G\( \alpha \) resulted in notable binding to R* (Fig. 1B). G\( \alpha \)/G\( \alpha \) (340–350) bound to R* significantly more weakly compared with G\( \alpha \), in part likely due to the lack of the N-terminal myristoylation of the chimeric protein. Somewhat weaker binding of G\( \alpha \)/G\( \alpha \) (340–350) to R* in comparison with G\( \alpha \) probably reflects additional R*-interacting sites in these proteins, although a lower affinity of G\( \alpha \)/G\( \alpha \) (340–350) for G\( \beta \)/γ may have contributed as well.

Further functional evaluation of G\( \alpha \), G\( \alpha \)/G\( \alpha \) (340–350) interactions with R* was performed using a GTP-S binding assay. In control experiments, the very slow GTP-S binding by G\( \alpha \) to intrinsic nucleotide exchange (\( k_{app} = 0.002 \) min\(^{-1}\)) was greatly accelerated in the presence of R* and G\( \beta \)/γ (\( k_{app} = 0.35 \) min\(^{-1}\)) (Fig. 2A). As expected, binding of GTP-S to G\( \alpha \) due to spontaneous nucleotide exchange (\( k_{app} = 0.030 \) min\(^{-1}\)) was significantly faster than that to G\( \alpha \) (Fig. 2B). Addition of G\( \beta \) inhibited the GTP-S binding to G\( \alpha \) (\( k_{app} = 0.003 \) min\(^{-1}\)) (Fig. 2B). This effect is consistent with the inhibition of the G\( \alpha \) GDP/GTP-S exchange by G\( \beta \) demonstrated earlier (16). The presence of uROS membranes only slightly enhanced GTP-S binding to G\( \beta \) complexed G\( \alpha \) (\( k_{app} = 0.006 \) min\(^{-1}\)) (Fig. 2B). It is not clear whether this small increase is due to very weak affinity of G\( \alpha \) for R* or perhaps just to reduction of effective G\( \beta \) concentration caused by its partitioning to rod outer segment membranes. We favor the latter explanation because no interaction between G\( \alpha \) and R* was observed in the direct binding experiments shown in Fig. 1B. Nonetheless, the rate of GTP-S binding to G\( \alpha \) in the presence of both uROS and G\( \beta \) was still considerably slower than the intrinsic rate. Similarly to G\( \alpha \), G\( \alpha \)/G\( \alpha \) (340–350) had a high intrinsic GTP-S binding rate (0.031 min\(^{-1}\)), which was inhibited by G\( \beta \) (\( k_{app} = 0.002 \) min\(^{-1}\)) (Fig. 2C); yet addition of uROS to G\( \alpha \)/G\( \alpha \) (340–350) in the presence of G\( \beta \) had a profound effect. The GTP-S binding rate was accelerated to levels (\( k_{app} = 0.27 \) min\(^{-1}\)) far exceeding the G\( \alpha \)/G\( \alpha \) (340–350) intrinsic GTP-S binding rate (Fig. 2C). Overall, analysis of the interaction of G\( \alpha \) and G\( \alpha \)/G\( \alpha \) (340–350) with R* supports previous conclusions that the C terminus of G\( \alpha \) is the major determinant of the G\( \alpha \) interaction with R*.

It also indicates that G\( \alpha \) provides an appropriate background for introduction of specific G\( \alpha \)C-terminal residues to test their role for G\( \alpha \) activation by R*.
The binding of GTP

\[ \text{FIG. 2.} \]

(a) Time courses of GTP\( ^\alpha \)-binding, % maximal for Gt\( ^\alpha \* \)

(b) Time courses of GTP\( ^\alpha \)-binding, % maximal for Gs

(c) Time courses of GTP\( ^\alpha \)-binding, % maximal for Gs/Gt\( ^\alpha \)-340–350

Tyr-Glu/Asp-Cys-Gly). To limit the number of potential combinations of substituted residues, we decided to introduce mutations into mutant G\( ^\alpha \) with Glu\( ^{-3} \) replaced by Gly. The Gly residue is highly conserved in the G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \) family and is crucial to the ability of the G\( ^\alpha \) C terminus to adopt the R*\(^k\)-binding conformation (11). Furthermore, Gly\( ^{348} \) is invariant in peptides selected by binding to R* (10), and its replacement with Ala leads to a severe impairment of G\( ^\alpha \) activation by R* (8).

Surprisingly, a point mutation of G\( ^\alpha \), Glu\( ^{-3} \) \(\rightarrow\) Gly, led to a strong reduction in the expression levels and functional activity as judged by the lack of trypsin protection in the presence of AIF\( _1 \) and severely impaired GTP\( ^\alpha \)-binding (data not shown). Thus, the conformational changes of the G\( ^\alpha \) C terminus due to insertion of Gly\( ^{-3} \) appear to influence the overall structural integrity of G\( ^\alpha \). Interestingly, additional substitutions of G\( ^\alpha \) residues by corresponding G\( ^\alpha \) residues in the two triple G\( ^\alpha \) mutants, Met\(^{-9} \) \(\rightarrow\) Glu/His\(^{-8} \) \(\rightarrow\) Asn/Glu\(^{-3} \) \(\rightarrow\) Gly (EN-G mutant) (Table I) and Gln\(^{-5} \) \(\rightarrow\) Asp/Tyr\(^{-4} \) \(\rightarrow\) Cys/Glu\(^{-3} \) \(\rightarrow\) Gly (DCG mutant), rescued expression levels and the ability of mutant G\( ^\alpha \) to undergo an activational conformational change. Both mutants EN-G and DCG had expression levels comparable to that of G\( ^\alpha \) and were analogous to G\( ^\alpha \) in the trypsin protection assay (data not shown). EN-G and DCG had similar intrinsic GTP\( ^\alpha \)-binding rates, 0.033 and 0.029 min\(^{-1} \), respectively (Fig. 3). Binding of G\( ^\beta \gamma \) strongly inhibited these rates \((k_{\text{app}} = 0.003 \text{ min}^{-1})\), as seen for G\( ^\alpha \). The key difference between EN-G and DCG was revealed in the presence of R*. Whereas R* in the presence of G\( ^\beta \gamma \) did not meaningfully affect the GTP\( ^\alpha \)-binding \((k_{\text{app}} = 0.005 \text{ min}^{-1})\), it significantly stimulated the rate of GTP\( ^\alpha \)-binding by DCG \((k_{\text{app}} = 0.073 \text{ min}^{-1})\) (Fig. 3). To more accurately assess the relative abilities of G\( ^\alpha \), G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350, and DCG to interact with R*, the kinetics of R*\(^k\)-induced GTP\( ^\alpha \)-binding were analyzed at varying concentrations of uROS membranes. Fig. 4 shows the apparent rates of GTP\( ^\alpha \)-binding to G\( ^\alpha \), G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350, and DCG plotted as functions of the photoreceptor membrane (rhodopsin) concentration. The apparent activation constants \(k_{\text{app}}\) for G\( ^\alpha \), G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350, and DCG from the slopes in Fig. 4 are 1.33 \(\times\) \(10^6\), 0.66 \(\times\) \(10^6\), and 0.23 \(\times\) \(10^6\) M\(^{-1}\) \(\text{min}^{-1}\), respectively. This analysis underscores the role of the G\( ^\alpha \) C terminus and the residues Asp\(^{346} \), Cys\(^{347} \), and Gly\(^{348} \) in particular, in the interaction with R*.

| Mutant     | Position from the C terminus\(^a\) |
|------------|-----------------------------------|
| G\( ^\alpha \)/G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350 | 11QRMHLRQYELL\(^{-1}\) |
| G\( ^\alpha \)/G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350 | 11QRMHLRQYELL\(^{-1}\) |
| G\( ^\alpha \)/G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350 | 11QRMHLRQYELL\(^{-1}\) |
| G\( ^\alpha \)/G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350 | 11QRMHLRQYELL\(^{-1}\) |

\(^a\) Mutated residues are underlined.

\(^b\) G\( ^\alpha \)/G\( ^\alpha \)/G\( ^\beta \)/G\( ^\gamma \)-340–350, and the DCG mutant were also made with substitutions Asn\(^{239} \) \(\rightarrow\) Glu and Asp\(^{346} \) \(\rightarrow\) Gly to produce EG-G\( ^\alpha \), EG-G\( ^\alpha \)/G\( ^\alpha \)-340–350, and EG-DCG, respectively.

G\( ^\beta \gamma \) and uROS membranes (300 \(\text{nm}\) rhodopsin) \((\Uparrow)\) was initiated by addition of 5 \(\mu\text{M}\) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\). Gs-bound GTP\( ^\alpha \)-S was counted by withdrawing aliquots at the indicated times and passing them through Whatman cellulose nitrate filters (0.45 \(\mu\text{m}\)). GTP\( ^\alpha \)-S binding is expressed as percent of maximal binding, calculated based on protein concentration. The calculated \(k_{\text{app}}\) values are as follows: 0.005 min\(^{-1} \) \((\bullet)\) and 0.35 \(\pm\) 0.02 min\(^{-1} \) \((\Uparrow)\); B: 0.030 \(\pm\) 0.002 min\(^{-1} \) \((\Uparrow)\), 0.005 min\(^{-1} \) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\); A, and 0.006 \(\pm\) 0.0005 min\(^{-1} \) \((\Uparrow)\); and C, 0.031 \(\pm\) 0.001 min\(^{-1} \) \((\Uparrow)\), 0.002 min\(^{-1} \) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\), and 0.27 \(\pm\) 0.02 min\(^{-1} \) \((\Uparrow)\).

G\( ^\beta \gamma \) and uROS membranes (300 \(\text{nm}\) rhodopsin) \((\Uparrow)\) was initiated by addition of 5 \(\mu\text{M}\) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\). Gs-bound GTP\( ^\alpha \)-S was counted by withdrawing aliquots at the indicated times and passing them through Whatman cellulose nitrate filters (0.45 \(\mu\text{m}\)). GTP\( ^\alpha \)-S binding is expressed as percent of maximal binding, calculated based on protein concentration. The calculated \(k_{\text{app}}\) values are as follows: 0.005 min\(^{-1} \) \((\bullet)\) and 0.35 \(\pm\) 0.02 min\(^{-1} \) \((\Uparrow)\); B: 0.030 \(\pm\) 0.002 min\(^{-1} \) \((\Uparrow)\), 0.005 min\(^{-1} \) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\); A, and 0.006 \(\pm\) 0.0005 min\(^{-1} \) \((\Uparrow)\); and C, 0.031 \(\pm\) 0.001 min\(^{-1} \) \((\Uparrow)\), 0.002 min\(^{-1} \) \((\text{[5}\text{S}\text{]}\text{GTP})\text{S}\), and 0.27 \(\pm\) 0.02 min\(^{-1} \) \((\Uparrow)\).
Fig. 3. GTPγS binding to the EN-G and DCG mutants of Gα. The binding of GTPγS to the EN-G (A) and DCG (B) mutants of Gα (1 μM each) alone (■) or mixed with 2 μM Gβγ (△) or with 2 μM Gbγ and uROS membranes (300 nM rhodopsin) (▲) was initiated by addition of 5 μM [35S]GTPγS. The calculated kapp values are as follows: A, 0.033 ± 0.002 min⁻¹ (●), 0.003 ± 0.0003 min⁻¹ (△), and 0.005 ± 0.001 min⁻¹ (▲); and B, 0.029 ± 0.001 min⁻¹ (●), 0.003 ± 0.0002 min⁻¹ (△), and 0.073 ± 0.010 min⁻¹ (▲).

The above results suggest that one or more of these Gα residues, Asp240, Cys347, and Gly348, are crucial for Rα recognition. Based on these findings, three double mutants of Gα, Gin⁻→Asp/Tyr⁻→Cys (DC), Tyr⁻→Cys/Glu⁻→Gly (CG), and Gin⁻→Asp/Glu⁻→Gly (D-G), were generated and analyzed (Table I). The DC mutant had reduced levels of expression and trypsin protection, and the GTPγS binding by DC was not notably affected by Rα (data not shown). The CG and D-G mutants were expressed and trypsin-protected in the presence of AlF3 similarly to Gα (Fig. 5A). Unexpectedly, the CG mutant demonstrated a lowered trypsin protection after incubation with GTPγS, but not with ATP (Fig. 5A). Examination of the rates of intrinsic nucleotide exchange confirmed a relatively slow basal GTPγS-binding rate by CG (kapp = 0.007 min⁻¹) as compared with Gα or other Gα mutants (Fig. 5B). In contrast to the D-G mutant, which showed no significant enhancement of the GTPγS-binding rate by Rα in relation to Gα (Fig. 5C), the GTPγS-binding rate of CG was accelerated by the activated receptor (kapp = 0.047 min⁻¹; best fit with Bmax = 45%) (Fig. 5B). Although the Rα-induced activation of CG was somewhat lower than that of DCG, the data seem to indicate the central role of Gα Cys347 and Gly348.

Intrinsic and Rα-induced GTPγS Binding of Gα Mutants containing Gα Gly212 and Gly213—The lower rate of intrinsic GTPγS binding for the CG mutant prompted us to further investigate this phenomenon. In one of the three molecules in the asymmetric unit of the Gα-GTPγS crystal, the C-terminal residues 343–349 are seen to make contacts with residues 212–215 from the αβ loop (switch II) of transducin (17). This interaction may contribute to the slow spontaneous nucleotide exchange of Gα and possibly provide a plausible explanation for the property of the CG mutant. The residues Glu212 and Gly213 of Gα are substituted in Gα by the non-homologous residues Asn239 and Asp240. The latter residues may interfere significantly with the coupling between switch II and the C terminus in Gα-GTPγS (340–350) or the DCG mutant, but perhaps to a lesser extent in the CG mutant. To test this hypothesis, we introduced the Asn239 → Glu and Asp240 → Gly substitutions into Gα (EG-Gα) (EG-Gα/Gα-(340–350)) and the DCG mutant (EG-DCG). These mutants were fully functional as assessed by the levels of expression and trypsin protection in the presence of AlF3 (Fig. 6, A and B). EG-Gα was functionally indistinguishable from Gα (data not shown). The nucleotide-binding rates for EG-Gα/Gα-(340–350) and EG-DCG were considerably slower than that for Gα, as shown by the trypsin protection test performed after time courses of GTPγS binding (Fig. 6, A and B). Corroborating these observations, the rates of intrinsic GTPγS binding by EG-Gα/Gα-(340–350) and EG-DCG were 0.004 and 0.009 min⁻¹, respectively (Fig. 6, C and D). In fact, these rates were sufficiently slow to make the inhibition of nucleotide exchange by Gβγ appear insignificant. Rα-induced stimulations of GTPγS binding to EG-Gα/Gα-(340–350) (kapp = 0.20 min⁻¹) and EG-DCG (kapp = 0.064 min⁻¹) were comparable to those for Gα-Gα/Gα-(340–350) and DCG, respectively (Fig. 6, C and D).

DISCUSSION

Seven-transmembrane domain receptors transduce extracellular signals across the plasma membrane to the intracellular effectors by stimulating GDP/GTP exchange on the α-subunits of heterotrimeric GTP-binding proteins. Following the activation interaction with receptors, Go/Gβ and Gβγ are released to activate their targets. Although G protein-coupled receptors may activate more than one related G protein, typically G protein/receptor interactions are rather specific, and a particular receptor recognizes G proteins from one family (2). The
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Gβγ subunits contribute to the selectivity of G protein/receptor interactions, which apparently resides to a large extent in Gα subunits. Evidence suggests that 10 or fewer C-terminal residues of Gα subunits compose a major receptor-binding and specificity domain (7–11). The C termini are generally conserved within each of the four families of G proteins (Gs, Gi, Gq, and G12,13), but are diverse between members of different families, except for the two absolutely invariant Leu residues at positions −2 and −7. The C-terminal diversity is essential for the G protein/receptor selectivity. An elegant study demonstrated that replacement of just three C-terminal residues of G,α at positions −1, −3, and −4 by corresponding G,α residues allowed G, to switch specificity to a new, G,α-coupled receptor (18). Similar findings were later reported using Gα130, Gα99, and Gα340 C-terminal chimeras (19). Conservation of the C-terminal residues within one family requires participation of additional regions of Gα to achieve fine receptor tuning. For example, Glu104 and Glu308 in the α helix of G,α1 are important to the selectivity of the 5-hydroxytryptamine receptor for G,α1 over G,α2 (20). Chimeric Gα,Gα proteins have been very instrumental in gaining insight into the G,α/Gα,5-hydroxytryptamine receptor selectivity (20), but would not be helpful in elucidating the C-terminal determinants of the G,αR* interaction since the C termini of Gα and Gα are almost identical, and R* potently stimulates Gα in vitro (13). In fact, the “receptor switch” or “gain-of-recognition” type of experiment for R* has not until now been reported. Instead, in addition to Ala mutagenesis of the G,α C terminus, which identifies impairments of function (8, 9), a positive selection for R* binding was carried out using a com-

Fig. 5. Trypsin protection test and GTPγS binding of the G,α mutants CG and D-G. A, the CG and D-G mutants of G,α (20 μM each) were treated with trypsin (50 μg/ml) for 30 min at 25 °C in the absence or presence of 50 μM GDP, 50 μM GTP, 50 μM AlF4, or 50 μM GDP plus 50 μM AlF4. B and C, the binding of GTPγS to the CG and D-G mutants, respectively, of G,α (1 μM each) alone (●) or mixed with 2 μM G,βγ (▲) or with 2 μM G,βγ and uROS membranes (300 μM rhodopsin) (▼) was initiated by addition of 5 μM [35S]GTPγS. The calculated kapp values are as follows: B, 0.007 ± 0.001 min⁻¹ (●), 0.002 min⁻¹ (▲), and 0.047 ± 0.002 min⁻¹ (▼); and C, 0.029 ± 0.001 min⁻¹ (●), 0.003 min⁻¹ (▲), and 0.007 ± 0.001 min⁻¹ (▼).

Fig. 6. Trypsin protection test and the time courses of GTPγS binding and kinetics of GTPγS binding of G,α-G,α-(340–350), EG-G,α-G,α-(340–350), DCG, and EG-DCG. A and B, G,α mutants (20 μM each) were incubated for the indicated times at 25 °C in 20 mM HEPES (pH 8.0) containing 130 mM NaCl, 5 mM MgSO4, and 50 μM GTPγS. The time courses of GTPγS binding were followed by trypsinolysis of mutants with 100 μg/ml trypsin for 10 min at 25 °C. In the lanes marked AlF4, the samples were incubated for 30 min in the buffer containing 10 mM NaF and 30 μM AlCl3 instead of GTPγS. C and D, the binding of GTPγS to EG-G,α-G,α-(340–350) and EG-DCG (1 μM each), respectively, alone (●) or mixed with 2 μM G,βγ (▲) or with 2 μM G,βγ and uROS membranes (300 μM rhodopsin) (▼) was initiated by addition of 5 μM [35S]GTPγS. The calculated kapp values are as follows: C, 0.004 ± 0.0005 min⁻¹ (●), 0.003 ± 0.0003 min⁻¹ (▲), and 0.20 ± 0.02 (▼); and D, 0.009 ± 0.0005 min⁻¹ (●), 0.005 ± 0.0002 min⁻¹ (▲), and 0.064 ± 0.004 min⁻¹ (▼).
roles of C-terminal residues of G\(_{\alpha}\)

Our approach to delineation of the C-terminal contacts of G\(_{\alpha}\) with R\(^*\) involved using G\(_{\alpha}\) for introduction of nonconserved G\(_{\alpha}\) residues and examining activation of mutant G\(_{\alpha}\) by the receptor. The choice of G\(_{\alpha}\) was based on its apparent inability to bind and be activated by R\(^*\). In contrast, chimeric G\(_{\alpha}\)/G\(_{\alpha}\) containing only 11 C-terminal residues from transducin was capable of efficient coupling to R\(^*\). The analysis of R\(^*\)-induced rates of GTP\(_{\gamma}\)S binding by mutant G\(_{\alpha}\) reconstituted with G\(_{\beta}\gamma\) demonstrated the key role of transducin Cys\(^{347}\) and Gly\(^{348}\) in the G\(_{\alpha}\) interaction with the activated receptor. In addition, Asp\(^{346}\) appears to be important, as the DCG mutant containing all three residues was stimulated by R\(^*\) to a greater extent than the mutant with the Cys and Gly residues only. This result is in excellent agreement with the study on sequence selection based on R\(^*\)/peptide interaction, which demonstrated an invariant presence of Cys and Gly residues at positions \(-4\) and \(-3\) in peptides with high affinity for R\(^*\) (10). Interestingly, the Asp residue at position \(-5\) was also highly conserved in R\(^*\)-binding peptides (10). Our study extends the previous findings by demonstrating the correlation of the ability of C-terminal peptides containing the key residues to bind R\(^*\) (10) with the activation of G\(_{\alpha}\) subunits containing the same residues. The model of the mutant G\(_{\alpha}\) C-terminal sequence with Asp, Cys, and Gly at positions \(-5, -4,\) and \(-3\), respectively, is shown in Fig. 7B. This model was generated using the R\(^*\)-induced structure of G\(_{\alpha}\)(340–350) as a template (Fig. 7A) (11). It indicates that the presence of the key G\(_{\alpha}\) residues would enable the C terminus of G\(_{\alpha}\), upon binding to R\(^*\), to adopt an overall conformation very similar to that of G\(_{\alpha}\)(340–350).

Our data are also consistent with the study showing the critical role of the Cys\(^{-4}\) and Gly\(^{-3}\) residues of G\(_{\alpha}\) in the selectivity of several G\(_i\)-coupled receptors (19). Cys\(^{-4}\) and Gly\(^{-3}\) are conserved in the G\(_i\) family (G\(_i\), G\(_i\), G\(_{\alpha}\), and G\(_{\alpha}\)), but other G protein families have non-homologous substitutions at these positions. Therefore, residues at positions \(-3\) and \(-4\) are likely to serve as major determinants that discriminate G\(_i\)-coupled receptors from receptors signaling through other G proteins.

In the course of this study, we made an interesting observation that the CG mutant of G\(_{\alpha}\) had notably reduced intrinsic nucleotide exchange in comparison with G\(_{\alpha}\), DCG, and G\(_{\alpha}/G\(_{\alpha}\)(340–350). We then hypothesized that this effect may have resulted from the interaction of the C-terminal residues in the CG mutant with the \(\alpha_{\beta_4}\) loop from the switch II region. Such an interaction is seen between residues 343–349 and 212–215 in one of the G\(_{\alpha}/G\(_{\alpha}\)(340–350) crystal structures (17) and is supported by analysis of the conformational changes using fluorescently labeled G\(_{\alpha}\) (22). The results of our study suggest that a similar interaction is present in the GDP-bound conformation of G\(_{\alpha}\). The residues Cys\(^{212}\) and Gly\(^{213}\) of G\(_{\alpha}\) are substituted by Asn\(^{219}\) and Asp\(^{240}\) in G\(_{\alpha}\), and we speculated that because of these differences, coupling between the \(\alpha_{\beta_4}\) loop and the C terminus may not be possible in DCG and G\(_{\alpha}/G\(_{\alpha}\)(340–350). This hypothesis has been confirmed by significant decreases in the rates of intrinsic GTP\(_{\gamma}\)S binding in DCG and G\(_{\alpha}/G\(_{\alpha}\)(340–350) upon substitution of Asn\(^{219}\) and Asp\(^{240}\) by the Glu and Gly residues, respectively. We have modeled the substitutions Cys\(^{212}\) → Asn and Gly\(^{213}\) → Asp in G\(_{\alpha}\) using the

Fig. 7. A, the “backbone” and “sticks” representations of the R\(^*\)-induced structure of G\(_{\alpha}\)(340–350) (11); B, the model structure of the C-terminal sequence of DCG (mutant G\(_{\alpha}\) with Asp, Cys, and Gly at positions \(-5, -4,\) and \(-3\), respectively). The model was generated using the Swiss-PDB Viewer (21) and coordinates of the R\(^*\)-induced structure of G\(_{\alpha}\)(340–350) as a template (11). The images in A and B were obtained using RasMol Version 2.6.

Fig. 8. A, a Connolly diagram of the \(\alpha_{\beta_4}\) loop (in red) and the C terminus (in green) from the structure of G\(_{\alpha}\)-GTP\(_{\gamma}\)S (17). The linkage is formed primarily by van der Waals contacts. B, the substitutions Cys\(^{212}\) → Asn and Gly\(^{213}\) → Asp modeled into the G\(_{\alpha}\)-GTP\(_{\gamma}\)S structure. The mutation Gly\(^{213}\) → Asp leads to steric hindrances between the Asp side chain and Gly\(^{214}\) and Leu\(^{219}\). The model and the images were generated using SYBYL Version 6.5.3 (Tripos Associates, St. Louis, MO).
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\( \Gamma \alpha \)-GTP\( \gamma \)S structure as a template (17). Fig. 8 demonstrates that introduction of Asp instead of \( \Gamma \alpha \) Gly\(^{213} \) creates multiple steric hindrances between the Asp side chain and Gly\(^{349} \) and Leu\(^{349} \), thus providing a structural basis for the lack of interaction between the \( \alpha_b \beta_b \) loop and the C terminus in DCG and \( \Gamma \alpha / \Gamma \alpha -(340–350) \). Although this model does not reveal an explanation for the functional properties of the CG mutant, it is possible that the steric hindrances are reduced when Gln is present at position –5 instead of Asp.

Altogether, our results suggest that the interaction between the C terminus and the switch II region in \( \Gamma \alpha \) contributes to the low intrinsic rate of nucleotide exchange of transducin. Furthermore, this interaction must be present not only in the active conformations of \( \Gamma \alpha \) (17, 22), but in the GDP-bound conformation as well because GDP release is the rate-limiting step. It is unlikely that an analogous interaction controls the GDP affinity in \( \Gamma \alpha \) as it has an Asp residue, similarly to \( \Gamma \alpha \), instead of Gly\(^{213} \) in \( \Gamma \alpha \). Contacts of the Ile-Ile residues at positions –12 and –11 from the C terminus with Val\(^{34} \) and Leu\(^{195} \) appear to be involved in regulating \( \Gamma \alpha \) affinity for GDP (23). Lack of the C terminus switch II contacts in \( \Gamma \alpha \) and \( \Gamma \alpha \) may in part account for the relatively high spontaneous GDP/GTP exchange rate. The \( \Gamma \beta \gamma \) subunit binding is required to lower the basal exchange rate of these \( \Gamma \) subunits. The C terminus switch II coupling is likely to be just one of the mechanisms that control the rate of spontaneous nucleotide exchange. \( \Gamma \alpha \) has a relatively high intrinsic GDP/GTP exchange rate despite the fact that the sequences corresponding to \( \Gamma \alpha \) residues 343–349 and 212–215 are identical in \( \Gamma \alpha \) and \( \Gamma \alpha \). The lack of discrete interdomain contacts between the Ras-like and the \( \alpha \)-helical domains in \( \Gamma \alpha \)-GDP may account for the rapid dissociation of GDP (24).

Both groups of mutant \( \Gamma \) subunits with high (\( \Gamma \alpha / \Gamma \alpha -(340–350) \) and DCG) and low (EG-\( \Gamma \alpha / \Gamma \alpha -(340–350) \) and EG-DCG) intrinsic GTP\( \gamma \)S-binding rates were comparably activated by \( \Gamma \) in the presence of \( \Gamma \beta \gamma \). This indicates either that binding of \( \Gamma \beta \gamma \) blocks the contacts between the \( \Gamma \alpha \) C terminus and the switch II region or that these contacts do not hinder or enhance the coupling of the \( \Gamma \alpha \)-GTP C terminus to light-activated rhodopsin. Consequently, potential communication between the C terminus and the switch II region of \( \Gamma \alpha \) in \( \Gamma \alpha \beta \gamma \) is not an essential component of \( \Gamma \)-induced GDP release. The latter process likely involves transmission of the conformational change from the C terminus via the \( \alpha_c \) helix to the \( \beta_b \alpha_c \) loop that contains the guanine ring-binding residues (25).

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