The visual GTP-binding protein, transducin, couples light-activated rhodopsin (R*) with the effector enzyme, cGMP phosphodiesterase in vertebrate photoreceptor cells. The region corresponding to the α4-helix and α4-β6 loop of the transducin α-subunit (Gt α) has been implicated in interactions with the receptor and the effector. Ala-scanning mutagenesis of the α4-β6 region has been carried out to elucidate residues critical for the functions of transducin. The mutational analysis supports the role of the α4-β6 loop in the R*-Gt α interface and suggests that the Gt α residues Arg310 and Asp311 are involved in the interaction with R*. These residues are likely to contribute to the specificity of the R* recognition. Contrary to the evidence previously obtained with synthetic peptides of Gt α, our data indicate that none of the α4-β6 residues directly or significantly participate in the interaction with and activation of phosphodiesterase. However, Ile299, Phe303, and Leu306 form a network of interactions with the α3-helix of Gt α, which is critical for the ability of Gt α to undergo an activation conformational change. Thereby, Ile299, Phe303, and Leu306 play only an indirect role in the effector function of Gt α.

Upon transduction of the visual signal in vertebrate photoreceptor cells, photoexcited rhodopsin (R*) binds the retinal G protein, transducin (Gt), leading to Gt activation. The α-subunit of Gt (Gt α) complexed with GTP is then released to stimulate the effector enzyme, cGMP phosphodiesterase (PDE), by reversing the inhibitor imposed by two PDE γ subunits (Pγ) on the PDE catalytic dimer (Pβ). Activated PDE rapidly hydrolyzes cGMP resulting in closure of cGMP-gated channels in the photoreceptor plasma membrane (1–3).

The two central interactions of Gt α with R* and Pγ during visual excitation have been extensively investigated, and the Gt α interaction sites have been localized. Evidence points to the C terminus of Gt α as the major R* contact site that is critical for Gt α activation (4–9). A second essential site of Gt α interaction with R* includes the α4/β6 loop (residues 305–315) (6, 10, 11). A peptide, Gt α311–328, competed for the Gt α-R* interaction (6). The tryptic cleavage site at Arg310 of Gt α was protected upon Gt αβγ binding to R* (10). Several mutants with Ala substitutions of residues from the α4/β6 loop had impaired binding to R* and reduced degrees of activation (11). Interestingly, this R* binding site overlaps with a region of Gt α293–314, that has been implicated in the transducin-effector interaction (12–16). A synthetic peptide, Gt α293–314, corresponding to the α4-β6 region was shown to activate PDE in vitro and to bind to Pγ (12, 13). Sites of chemical cross-linking of the Pγ-subunit to Gt α were localized to within the α4-β6 loop (14, 15). A study using substituted peptides identified five nonconserved effector residues within this region (16). Despite the large body of evidence, the significance of the Gt α α4-β6 region in the effector interaction remains unclear. An insertion of the Gt α295–314 segment into Gt βα only marginally improved the latter’s ability to bind Pγ (17). This finding suggests that if the α4-β6 region is important for the interaction with PDE, then likely the conserved residues within α4-β6 are essential for the function of the effector. Alternatively, even small differences in Gt α and Gt β folding may interfere with the ability of Gt α293–314 to assume the proper effector-binding conformation in the context of Gt α. More importantly, the apparent ability of peptide Gt α293–314 to potently stimulate PDE (12, 16) is inconsistent with the mutational analysis of Gt α (18, 19). The latter indicates the requirement of the switching 2 and 3 regions for effector activation (18, 19).

In light of the importance of the Gt α α4-β6 region for the Gt α-R* interaction and substantial but conflicting evidence on its role for PDE activation, we carried out Ala-scanning mutational analysis of the α4-helix (residues 293–304) and the α4-β6 loop of Gt α. Our analysis of mutant Gt α interactions with R* and PDE has underscored the role of the α4-β6 loop for the receptor function but revealed only indirect involvement of the α4-helix in the Gt α effector function via requirement of the α4/α3 coupling for the activation conformational change.

**EXPERIMENTAL PROCEDURES**

**Preparation of ROS Membranes, Gt αGDP, Gt βγ, and PγBC**—Bovine ROS membranes were prepared as described previously (20). Urea-washed ROS membranes (uROS) were prepared according to protocol described by Yamanaka et al. (21). Gt βγ was purified as described by Kleuss et al. (22). Pγ labeled with the fluorescent probe, 3-(bromoacetyl)-7-diethylaminocoumarin (PγBC), was obtained and purified as described previously (23).

**Ala-scanning Mutagenesis of the α4-β6 Region of Gt α**—Substitutions of Gt α residues by Ala were introduced into Gt αα/Gt α, chimeric protein, Gt α*, which contains only 16 residues from Gt α. Gt α* was made based on another Gt αα/Gt β, chimeric protein, Chib, which is competent to interact with R* and Gt βγ (17, 19). To generate Gt α*, all the Gt α residues in the α3-helix and the α3-β5 loop of Chib, except for Met247 (corresponding to Leu243 of Gt α) were replaced by Ala residues. The following Gt α residues were introduced into Chib: His284, Asn287, His252, Arg253, Tyr254, Ala266, and Thr257. The PCR-directed mutagenesis was...
carried out essentially as described in Natochin et al. (19).

Single substitutions of Gαi residues at positions 293, 294, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 308, 309, 310, 311, 312, 313, and 314 were introduced by PCR-directed mutagenesis. In the PCR reactions, forward mutant primers were paired with a reverse primer carrying a BamHI site and corresponding to a sequence 50 base pairs downstream of the stop codon. The pHis6-Gαi plasmid was used as a template. The PCR products (~200 base pairs) were purified on agarose gel and used for a second round PCR amplification as reverse primers combined with a forward primer containing the unique Gαi BamHI site. The 500-base pair PCR products were digested with BamHI and HindIII and ligated into pHis6-Gαi cut with the same enzymes. The sequences of all mutants were verified by automated DNA sequencing at the University of Iowa DNA Core Facility. Gαi and all mutants were expressed and purified as described previously (19).

**GTPγS Binding Assay—** Gαα4 or mutants (0.4 μM each) were pre-mixed with Gβγ (2 μM) in 0.5 ml of 20 mM HEPES buffer (pH 8.0) containing 100 mM NaCl and 8 mM MgSO4. The binding of GTPγS to Gαi or mutants was initiated by addition of 5 μM [35S]GTPγS (0.2 μCi) and uROS membranes (100 nM rhodopsin). Aliquots (100 μl) were withdrawn at the indicated times, passed through the Whatman cellulose nitrate filters (0.45 μm), washed three times with an ice-cold binding buffer and counted. The kapp values for the binding reactions were calculated by fitting the data to the equation, GTPγS binding (%) = 100(1 - e-kapp*t).

**Fluorescence Assays—** Fluorescence assays of interaction between Gαα4 and PγBC were performed on a F-2000 fluorescence spectrophotometer (Hitachi) in 1 ml of 20 mM HEPES buffer (pH 7.6), 100 mM NaCl, 5 mM dithiothreitol, and 4 mM MgCl2, essentially as described in (19, 23). Where indicated, the buffer contained 30 mM AlCl3 and 10 mM NaF. Fluorescence of PγBC was measured with excitation at 445 nm and emission at 495 nm. Concentration of PγBC was determined using εAmax = 53,000. The AIFγ-induced increases in the tryptophan fluorescence of Gαα4 GDP and its mutants were recorded on an AB2 fluorescence spectrophotometer (Electronic Instruments) in a stirred 1-ml cuvette with excitation at 280 nm and emission at 340 nm as described previously (19).

**PDE Activation Assay—** HoloPDE was extracted from ROS membranes and purified as described earlier (24). PDE (0.2 mM) was reconstituted with 2 μM Gαα4 GDP or the GDP-bound Gαα4 mutants and 2 μM Gβγ in suspensions of uROS membranes containing 10 μM rhodopsin. GTPγS (10 μM) was added to the reaction mixture, and PDE activity was measured using [3H]GMP similarly as described previously (19).

**Miscellaneous Procedures—** Protein concentrations were determined by the method of Bradford (25) using IgG as a standard or using calculation of extinction coefficients at 280 nm. Rhodopsin concentrations were calculated extinction coefficients at 280 nm. Rhodopsin concentrations were determined using the filter binding assay. The calculated kapp values are 0.004 s⁻¹ (■) and 0.079 s⁻¹ (▲).

**Fig. 1. The time course of GTPγS binding to Gαα4.** The binding of GTPγS to Gαα4 (0.4 μM) was initiated by addition of 5 μM of [35S]GTPγS (0.2 μCi) ▲. When the effect of R* was measured, Gαα4 was premixed with Gβγ (2 μM), and the binding was initiated by addition of 5 μM of [35S]GTPγS and uROS (100 nM rhodopsin) (▲). Aliquots were withdrawn at the indicated times, and GTPγS bound to Gαα4 was determined using the filter binding assay. The calculated kapp values are 0.004 s⁻¹ (■) and 0.079 s⁻¹ (▲).

**Results**

**Expression and Characterization of the Receptor and Effector Competent Chimeric Gαα4—** We have previously found that residue Leu243 of Gαi is mainly responsible for the low level expression of Gαi/Gαi chimeras containing the Gαi-237–270 (α4-β5) segment (19). Gαα4 was obtained based on the Gαα4/Gαi chimera, Chii, which contains the αβ-β5 region of Gαi (17). The nonconserved Gαi residues from the α3-helix and the α3-β5 loop of Chii were replaced by the corresponding Gαi residues (except for Leu243) (19). Two of the introduced Gαi residues, His244 and Asn247, are important for the Gαi-PDE interaction (19). The resulting chimeric Gαi was not only efficiently expressed in Escherichia coli (yields of soluble protein of 3–5 mg/liter culture) but was also fully competent for interaction with Gβγ and R* and capable of high affinity effector binding. The ability of Gαα4 to interact with R* in the presence of Gβγ was evaluated using the GTPγS binding assay. The very slow GTPγS binding rate to Gαα4 (kapp = -0.004 s⁻¹), which is limited by the rate of GDP dissociation (26), was significantly accelerated in the presence of R* and Gβγ (kapp = -0.079 s⁻¹) (Fig. 1). A fluorescence read-out assay was utilized to monitor the interaction between Gαi and the Pγ subunit (23). Using this assay, Gαα4 GDP bound fluorescently labeled Pγ, PγBC, with a Kd value of 28 nM (Fig. 2A and Table I). When Gαα4 GDP was activated in the presence of AIFγ, it bound to PγBC with an almost 6-fold higher affinity (Kd of 5.1 nM) (Fig. 2B and Table I). Thus, Gαα4, which contains only 16 Gαi residues, represents a well suited tool for mutational analysis to identify residues that are essential for both receptor and effector interactions of transducin.

**Expression of Gαα4 Mutants with Ala Substitutions of the α4-β6 Residues—** Residues at positions 293, 294, 297, 298, 300, 301, 302, 304, 305, 306, 308, 309, 310, 311, 312, 313, and 314 within the α4-helix and the α4-β6 loop of Gαi are surface exposed (27) and were substituted with Ala residues. In addition to a modestly solvent-exposed Leu243, two buried residues, Ile299 and Phe303, are involved in coupling the α4-helix with the α3-helix (27). This linkage might be important for stabilization of the receptor and/or effector-competent conformations of Gαi. Substitutions of Ile299 and Phe303 were made to test this possibility. Expression of all but three of the Gαα4 mutants in E. coli have yielded similar amounts of soluble proteins (~3–5 mg/liter of culture). Mutants Y298A, I299A, and F303A had notably reduced expression levels (~0.5–1 mg/liter). The crystal structure of Gαi GTPγS shows that the Tyr299 side chain makes contact with Tyr303, whereas Ile299 and Phe303 interact with the α3-helix (27). Perhaps, the reduction in mutant expression reflects lower rates of proper protein folding due to the lack of stabilizing contacts between α4 and the α3-α4 loop or the α3-helix.

The ability of Gαi mutants to undergo a conformational change upon addition of AIFγ was analyzed by measuring their intrinsic tryptophan fluorescence (18). Mutants Y298A, I299A, and F303A failed to display an increase in tryptophan fluorescence upon addition of AIFγ, whereas the fluorescence change for I299A was intermediate to that for Gαα4 (not shown).

**R*-induced GTPγS Binding to Gαα4 Mutants—** The ability of R* to interact with Gαi mutants and cause their GDP release was examined by measuring the rates of GTPγS binding to these mutants in the presence of R* and Gβγ. The release of GDP is a rate-limiting step in activation of G protein α subunits, and thus it controls the rate of GTPγS binding (26). Three Gαα4 mutants, Y298A, I299A, and F303A, did not appreciably bind GTPγS. A correlation between the low expression...
levels of these mutants and the lack of GTP-$\gamma$S binding indicates that defects in the overall folding might be responsible for the loss of the R*-dependent activation. However, the finding that these mutants were able to specifically interact with the effector (see below) rules out gross misfolding. Alternatively, the $\alpha$4-$\alpha$3 coupling could represent an important element in maintaining proper conformation of the R*-binding regions, or it is essential for the ability of Gt$\alpha$ to undergo a conformational change upon binding of GTP-$\gamma$S. The latter possibility is supported by the lack of the tryptophan fluorescence enhancement with addition of AlF$_4^-$ to Y298A, I299A, and F303A. The GTP-$\gamma$S binding properties of the L306A mutant, in which another residue that contacts $\alpha$3 was substituted, were seriously compromised but not abolished. Fitting of the GTP-$\gamma$S binding data for L306A yielded a value for maximal binding at $\sim$35% of that for Gt$\alpha^*$ with an $\sim$4-fold lower rate ($k_{app} = 0.019$ s$^{-1}$) (Fig. 3 and Table I). Gt$\alpha^*$L306A was expressed in E. coli comparably to Gt$\alpha^*$ but showed diminished ability for the conformational change in the presence of AlF$_4^-$. This suggests that L306A has a similar but more mildly expressed phenotype than mutants Y298A, I299A, and F303A.

A substantial loss of the receptor function was observed when Asp$^{311}$ was replaced by Ala. The D311A mutant in comparison with Gt$\alpha^*$ maximally bound only $\sim$50% GTP-$\gamma$S with a reduced rate of 0.023 s$^{-1}$ (Fig. 3 and Table I). A relatively mild alteration in R$^*$ activation was found for the R310A mutant. Gt$\alpha^*$R310A had a saturating level of GTP-$\gamma$S binding similar to that of Gt$\alpha^*$, but the rate of binding was decreased by $\sim$2-fold (Fig. 3 and Table I). Previously, Arg$^{309}$, Val$^{312}$, and Lys$^{313}$ were implicated in the Gt$\alpha$-R$^*$ interaction using an assay of Gt$\alpha$ activation in microsomes of COS7 cells expressing rhodopsin and mutant Gt (11). We observed no significant changes in the kinetics of Gt$\alpha$ activation caused by these three or other remaining mutations under our experimental conditions (Table I).

**FIG. 2.** Binding of Gt$\alpha^*$ to P$_{2\beta}$C. The relative increase in fluorescence ($F/F_0$) of P$_{2\beta}$C (10 nM) (excitation at 445 nm; emission at 495 nm) was determined after addition of increasing concentrations of Gt$\alpha^*$-GDP in the absence (A) or in the presence (B) of AlF$_4^-$. The D311A mutant in comparison with Gt$\alpha^*$ showed a similar but more mildly expressed phenotype than mutants Y298A, I299A, and F303A.

**TABLE I**

| Binding to P$_{2\beta}$C ($K_d$) | Gt$\alpha^*$-GDP-AlF$_4^-$ | Gt$\alpha^*$-GDP | GTP-$\gamma$S binding ($k_{app}$) |
|-----------------------------|-----------------|-----------------|------------------------|
| Gt$\alpha^*$               | $5.1 \pm 0.5$   | $28 \pm 2$     | 0.079 $\pm 0.010$     |
| E293A                      | $5.0 \pm 0.8$   | $20 \pm 1$     | 0.057 $\pm 0.012$     |
| E294A                      | $4.4 \pm 0.2$   | $34 \pm 2$     | 0.059 $\pm 0.009$     |
| N297A                      | $6.6 \pm 0.7$   | $49 \pm 3$     | 0.081 $\pm 0.003$     |
| Y298A                      | $54 \pm 7$      | $49 \pm 4$     | 0.077 $\pm 0.000$     |
| I299A                      | $35 \pm 3$      | $37 \pm 2$     | 0.054 $\pm 0.000$     |
| R300A                      | $7.9 \pm 0.7$   | $42 \pm 2$     | 0.084 $\pm 0.009$     |
| V301A                      | $7.5 \pm 0.8$   | $79 \pm 6$     | 0.077 $\pm 0.004$     |
| Q302A                      | $6.9 \pm 0.7$   | $27 \pm 2$     | 0.073 $\pm 0.010$     |
| F303A                      | $60 \pm 8$      | $50 \pm 4$     | 0.072 $\pm 0.004$     |
| L304A                      | $3.0 \pm 0.4$   | $28 \pm 2$     | 0.053 $\pm 0.003$     |
| E305A                      | $3.5 \pm 0.1$   | $22 \pm 3$     | 0.068 $\pm 0.002$     |
| L306A                      | $15 \pm 1$      | $34 \pm 4$     | 0.019 $\pm 0.001$     |
| M308A                      | $6.5 \pm 0.3$   | $27 \pm 1$     | 0.079 $\pm 0.006$     |
| R309A                      | $8.7 \pm 1.2$   | $33 \pm 2$     | 0.065 $\pm 0.003$     |
| R310A                      | $4.8 \pm 0.7$   | $37 \pm 3$     | 0.034 $\pm 0.004$     |
| D311A                      | $8.5 \pm 0.9$   | $34 \pm 4$     | 0.023 $\pm 0.001$     |
| V312A                      | $4.2 \pm 0.4$   | $52 \pm 2$     | 0.095 $\pm 0.004$     |
| K313A                      | $6.1 \pm 0.4$   | $73 \pm 9$     | 0.092 $\pm 0.005$     |
| E314A                      | $7.9 \pm 0.6$   | $49 \pm 2$     | 0.107 $\pm 0.009$     |

**FIG. 3.** GTP-$\gamma$S binding to Gt$\alpha^*$ mutants. The binding of GTP-$\gamma$S to Gt$\alpha^*$ mutants (0.4 $\mu$M mutant Gt$\alpha^*$, 2 $\mu$M Gt$\gamma$P) was initiated by addition of 5 $\mu$M [$^{[35]S}$GTP/$\gamma$S (0.2 $\mu$Ci) and uROS membranes (100 nM rhodopsin). Protein-bound GTP-$\gamma$S was determined using the filter binding assay. The V301A mutant (○) is representative of Gt$\alpha^*$ mutants with intact kinetics of GTP-$\gamma$S binding. The L306A (■), R310A (▲) and D311A (▲) mutants had impaired GTP-$\gamma$S binding characteristics.

**Binding of Gt$\alpha^*$ Mutants to P$_{2\beta}$C—To delineate potential effector residues within the $\alpha$4-$\beta$6 region, the Gt$\alpha^*$ mutants in the GDP-bound or active AlF$_4^-$-induced conformations were tested for binding to P$_{2\beta}$C. Interestingly, mutants Y298A, I299A, and F303A, which had low expression levels and lacked R$^*$-induced GTP-$\gamma$S binding, in the GDP-bound conformations displayed affinities for P$_{2\beta}$C comparable with Gt$\alpha^*$-GDP (Table I). This result indicates that in the inactive conformation their effector interface is not significantly affected. Predictably, these three Gt$\alpha^*$ mutants had significant defects in binding to P$_{2\beta}$C (Fig. 3 and Table I). A relatively mild alteration in R$^*$ activation was found for the R310A mutant. Gt$\alpha^*$R310A had a saturating level of GTP-$\gamma$S binding similar to that of Gt$\alpha^*$, but the rate of binding was decreased by $\sim$2-fold (Fig. 3 and Table I). Previously, Arg$^{309}$, Val$^{312}$, and Lys$^{313}$ were implicated in the Gt$\alpha$-R$^*$ interaction using an assay of Gt$\alpha$ activation in microsomes of COS7 cells expressing rhodopsin and mutant Gt (11). We observed no significant changes in the kinetics of Gt$\alpha$ activation caused by these three or other remaining mutations under our experimental conditions (Table I).
enhancement in the mutant interaction with PyBC, evidently
due to the inability of these mutants to assume an active
conformation. In addition, the interaction of the L306A mutant
with PyBC was less sensitive than that of Gαα* to AlF4−. In
the presence of AlF4−, L306A bound to PyBC with a Kd only 2-fold
lower than when AlF4− was absent (Table I). This is consistent
with the limited competency of L306A to assume an active
conformation. The Gαα* mutants, V301A and K313A, had mild
defects in effector binding. These mutants retained a high
affinity for PyBC in the AlF4−-bound conformations but re-
vealed a somewhat reduced interaction with the effector in
the absence of AlF4− (Table I). All other Gαα* mutants demonstrated
affinities for PyBC comparable with that of Gαα* (Table I).

Activation of Rod PDE by Gαα* Mutants—The ability of Gαα*
mutants to stimulate activity of holoPDE (Pyβγγ) was tested in
the reconstituted system with additions of uROS membranes
and purified Gβγ in the presence of GTPγS. Gαα* as well as the
majority of its mutants activated holoPDE under these condi-
tions by ~12–18-fold. Not surprisingly, mutants Y298A, I299A,
and F303A were incapable of stimulating PDE (not shown).
Mutants L306A and D311A were notably less effective in the
PDE activation assay (Fig. 4). This reduction in the effector
function seems to correlate well with the decreased capacity
of these mutants to bind GTPγS in the presence of R*. Therefore,
residues Leu260 and Asp271 are unlikely to be directly involved
in interaction with and activation of PDE.

DISCUSSION

The α4-β6 region of Gα is an essential contributor to the
Gαα-rhodopsin interface (6, 11). The R* binding sites of Gα, the
α4-β6 loop (amino acids 305–315) and Gαα-340–350, are posi-
tioned on the same “receptor” face of Gαβγ as the N terminus
of Gα and the C terminus of Gγ (28). The β6-sheet and the
α5-helix project inward from the α4-β6 loop and Gαα-340–350
on the Gα surface to form the β6/α5 loop. The latter contains a
cluster of residues, Cys231, Ala232, and Thr233, intimately in-
volved in binding of the guanine ring (27). Mutations of the
residues within the β6/α5 loop promote dissociation of GDP
and GTP-GDP exchange on several Go subunits (29–31). Thus,
the Gα activation mechanism is likely to involve interaction of R*
with the α4-β6 loop and Gαα-340–350, leading to confor-
mational changes of the β6/α5 loop and dissociation of GDP.

The critical R*-binding region, Gαα-340–350, has been inves-
tigated in great detail (6–9, 32). However, the role of the Gα
α4-β6 loop and its individual residues in binding to R* and Gα
activation is not well understood. Recently, mutants of Gα
with Ala substitutions of residues in the α4-β6 loop have been
translated in vitro and expressed in COS-7 cells (11). Muta-
tional analysis revealed that substitutions of four residues in
the α4-β6 loop, Arg298, Asp311, Val312, and Lys313 impaired Gα
interaction with R* (11). The Ala-scanning mutagenesis of the
Gαα-α4-β6 region in the context of Gαα* readily expressed in E.
coli has provided us with an opportunity for in depth investi-
gation of the roles of individual α4-β6 residues in the recepto-
interaction. Reconstitution of the purified mutant Gαα* with
Gβγ and uROS membranes has enabled the examination of the
effects of mutations on the kinetics of Gαα* activation by R*.
Our results confirm the role of Asp311 in the R*-dependent
activation of Gα. A substitution of this residue led to a sub-
stantial decrease in both the rate of and total R*-induced
GTPγS binding. A moderate alteration of the kinetics of R*-in-
duced GTPγS binding was caused by the substitution of Arg310.
The R310A mutant bound GTPγS with an ~2-fold slower rate. Supporting the involvement of Gαα Asp311, and
probably Arg310, in the interaction with R* is the fact that the
tryptsin cleavage site Arg310 Asp311 is protected upon binding
of Gα to R* (10). In addition to effective activation of Gαα, R*

M2. Natochin and N. O. Artemyev, unpublished observations.
the prerequisite of the switch II and α3-β5 regions of Gα for the effector stimulation. Hypothetically, the discrepancy is nonexistent if the role of switch II and α3-β5 is only to obscure the α4-β6 region in GαGDP. However, such a model is not supported by the crystal structures of Gα (27, 33). Moreover, at least three residues, Ile<sup>208</sup> (switch II), His<sup>244</sup>, and Asn<sup>247</sup> (α3) are likely to interact directly with Py in the GTP-bound Gα conformation (19).

The Ala-scanning mutational analysis performed in this study demonstrated that none of the α4-β6 residues appear to participate directly and significantly in the Gα-Pγ binding. Even substitutions of the residues Tyr<sup>298</sup>, Ile<sup>299</sup>, Phe<sup>303</sup>, and Leu<sup>306</sup>, which disabled the activation of Gα<sup>α</sup>*, had no notable impact on the binding of the GDP-bound mutants to Py<sub>B</sub>C. Results on activation of PDE by the Gα<sup>α</sup> correlates well with the Py binding experiments. All mutants with unimpaired capacity for R<sup>α</sup>-induced GTPγS binding were competent to stimulate cGMP hydrolysis by holoPDE. The studies on cross-linking of Py to Gα attest to a close proximity of Py to the α4-β6 region in the Gα-Pγ complex (14, 15). Although our analysis seems to rule out strong major interactions between Py and Gα<sup>α</sup>-293–314, a relatively weak van der Waals’ contact<sup>α</sup>(s) at this site cannot be entirely excluded. Rather, the role of the α4-β6 residues, Ile<sup>299</sup>, Phe<sup>303</sup>, and Leu<sup>306</sup> is that they are critical for the activational conformational change via the interaction with the α3-helix and thus indirectly are important for the effector function of Gα.<br><br>The most surprising finding in this work is that none of the mutations of five Gα residues identified using synthetic peptides (16) meaningfully affected the Gα<sup>α</sup>-PDE interaction. A greater sensitivity of the peptide structure than that of Gα<sup>α</sup> to mutations may explain the different results. Although the NMR analysis of substituted peptides ruled out gross misfolding, inactivation of mutant peptides due to a conformational change remains a possibility (16). However, a more plausible explanation is that the peptide Gα<sup>α</sup>-293–314 and Gα activate PDE via different mechanisms. This raises a general concern regarding potential problems with interpretation of effects that might be observed using synthetic peptides as probes of protein-protein interactions. The conclusion that Gα<sup>α</sup>-293–314 likely represents a major effector-activating domain of Gα was reached based on the ability of the peptide to “mimic” Gα in PDE activation (12, 16) and provided the best explanation of the data in the absence of an alternative approach. Yet, the puzzling mimicking effect of the Gα peptide does not appear to reflect the role of the corresponding region in Gα<sup>α</sup>.