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Roles of the Transducin α-Subunit α4-Helix/α4-β6 Loop in the Receptor and Effector Interactions*

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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 ($G_t\alpha$) 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*-$G_t\alpha$ interface and suggests that the $G_t\alpha$ residues Arg^{310} and Asp^{311} 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 $G_t\alpha$, our data indicate that none of the α4-β6 residues directly or significantly participate in the interaction with and activation of phosphodiesterase. However, Ile^{299}, Phe^{303}, and Leu^{306} form a network of interactions with the α3-helix of $G_t\alpha$, which is critical for the ability of $G_t\alpha$ to undergo an activational conformational change. Thereby, Ile^{299}, Phe^{303}, and Leu^{306} play only an indirect role in the effector function of $G_t\alpha$.

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

The two central interactions of $G_t\alpha$ with R* and Pγ during visual excitation have been extensively investigated, and the $G_t\alpha$ interaction sites have been localized. Evidence points to the C terminus of $G_t\alpha$ as the major R* contact site that is critical for $G_t\alpha$ activation (4–9). A second essential site of $G_t\alpha$ interaction with R* includes the α4/β6 loop (residues 305–315) (6, 10, 11). A peptide, $G_t\alpha$-311–328, competed for the G_t-R* interaction (6). The tryptic cleavage site at Arg^{310} of $G_t\alpha$ was protected upon $G_t\alpha\beta\gamma$ 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 $G_t\alpha$, $G_t\alpha$-293–314, that has been implicated in the transducin-effector interaction (12–16). A synthetic peptide, $G_t\alpha$-293–314, 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 $G_t\alpha$ 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 $G_t\alpha$ α4-β6 region in the effector interaction remains unclear. An insertion of the $G_t\alpha$-295–314 segment into $G_i\alpha_1$ 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 $G_t\alpha$ and $G_i\alpha$ folding may interfere with the ability of $G_t\alpha$-293–314 to assume the proper effector-binding conformation in the context of $G_i\alpha$. More importantly, the apparent ability of peptide $G_t\alpha$-293–314 to potently stimulate PDE (12, 16) is inconsistent with the mutational analysis of $G_t\alpha$ (18, 19). The latter indicates the requirement of the switch II and α3 regions for effector activation (18, 19).

In light of the importance of the $G_t\alpha$ α4-β6 region for the G_t-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 $G_t\alpha$. Our analysis of mutant $G_t\alpha$ 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 $G_t\alpha$ effector function via requirement of the α4/α3 coupling for the activational conformational change.

EXPERIMENTAL PROCEDURES

Preparation of ROS Membranes, $G_t\alpha$GDP, $G_t\beta\gamma$, 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). $G_t\beta\gamma$ was purified according to Kleuss *et al.* (22). Pγ labeled with the fluorescent probe, 3-(bromoacetyl)-7-diethyl aminocoumarin (PγBC), was obtained and purified as described previously (23).

Ala-scanning Mutagenesis of the α4-β6 Region of $G_t\alpha$—Substitutions of $G_t\alpha$ residues by Ala were introduced into $G_t\alpha/G_i\alpha_1$ chimeric protein, $G_t\alpha$*, which contains only 16 residues from $G_i\alpha$. $G_t\alpha$* was made based on another $G_t\alpha/G_i\alpha_1$ chimeric protein, Chi8, which is competent to interact with R* and $G_t\beta\gamma$ (17, 19). To generate $G_t\alpha$*, all the $G_i\alpha$ residues in the α3-helix and the α3-β5 loop of Chi8, except for Met^{247} (corresponding to Leu^{243} of $G_t\alpha$) were replaced by $G_t\alpha$ residues. The following $G_t\alpha$ residues were introduced into Chi8: His^{244}, Asn^{247}, His^{252}, Arg^{253}, Tyr^{254}, Ala^{256}, and Thr^{257}. The PCR-directed mutagenesis was

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[1] The abbreviations used are: R*, light-activated (bleached) rhodopsin; $G_t\alpha$, rod G-protein (transducin) α-subunit; PDE, rod outer segment cGMP phosphodiesterase; Pαβ and Pγ, α, β, and γ subunits of PDE; ROS, rod outer segment(s); uROS, urea-stripped ROS membranes; PγBC, Pγ labeled with 3-(bromoacetyl)-7-diethyl aminocoumarin; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PCR, polymerase chain reaction.

carried out essentially as described in Natochin *et al.* (19).

Single substitutions of $G_t\alpha$ 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 *Hin*dIII site and corresponding to a sequence 50 base pairs downstream of the stop codon. The pHis6-$G_t\alpha$* 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 $G_t\alpha$ *Bam*HI site. The 500-base pair PCR products were digested with *Bam*HI and *Hin*dIII and ligated into pHis6-$G_t\alpha$* cut with the same enzymes. The sequences of all mutants were verified by automated DNA sequencing at the University of Iowa DNA Core Facility. $G_t\alpha$* and all mutants were expressed and purified as described previously (19).

GTPγS Binding Assay—$G_t\alpha$* or mutants (0.4 μM each) were premixed with $G_t\beta\gamma$ (2 μM) in 0.5 ml of 20 mM HEPES buffer (pH 8.0) containing 100 mM NaCl and 8 mM $MgSO_4$. The binding of GTPγS to $G_t\alpha$* or mutants was initiated by addition of 5 μM [^{35}S]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 k_{app} values for the binding reactions were calculated by fitting the data to the equation, GTPγS bound (%) = $100(1 - e^{-kt})$.

Fluorescence Assays—Fluorescence assays of interaction between $G_t\alpha$* 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 $MgCl_2$ essentially as described in (19, 23). Where indicated, the buffer contained 30 μM $AlCl_3$ and 10 mM NaF. Fluorescence of PγBC was monitored with excitation at 445 nm and emission at 495 nm. Concentration of PγBC was determined using ϵ_{445} = 53,000. The AlF_4^--induced increases in the tryptophan fluorescence of $G_t\alpha$*GDP and its mutants were recorded on an AB2 fluorescence spectrophotometer (Spectronic 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 nM) was reconstituted with 2 μM $G_t\alpha$*GDP or the GDP-bound $G_t\alpha$* mutants and 2 μM $G_t\beta\gamma$ in suspensions of uROS membranes containing 10 μM rhodopsin. GTPγS (10 μM) was added to the reaction mixture, and PDE activity was measured using [^{3}H]cGMP similarly as described previously (19).

Miscellaneous Procedures—Protein concentrations were determined by the method of Bradford (25) using IgG as a standard or using calculated extinction coefficients at 280 nm. Rhodopsin concentrations were measured using the difference in absorbance at 500 nm between "dark" and bleached ROS preparations. Fitting of the experimental data was performed with nonlinear least squares criteria using GraphPad Prizm (v.2) software. The results are expressed as the means ± S.E. of triplicate measurements. Examination of the crystal structure of $G_t\alpha$ was performed using RasMol (v.2.6) software.

RESULTS

Expression and Characterization of the Receptor and Effector Competent Chimeric $G_t\alpha$—We have previously found that residue Leu^{243} of $G_t\alpha$ is mainly responsible for the low level expression of $G_t\alpha/G_i\alpha$ chimeras containing the $G_t\alpha$-237–270 (α3-β5) segment (19). $G_t\alpha$* was obtained based on the $G_t\alpha/G_i\alpha$ chimera, Chi8, which contains the α3-β5 region of $G_i\alpha$ (17). The nonconserved $G_i\alpha$ residues from the α3-helix and the α3-β5 loop of Chi8 were replaced by the corresponding $G_t\alpha$ residues (except for Leu^{243}). Two of the introduced $G_t\alpha$ residues, His^{244} and Asn^{247}, are important for the $G_t\alpha$-PDE interaction (19). The resulting chimeric $G_t\alpha$ 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 $G_t\beta\gamma$ and R* and capable of high affinity effector binding. The ability of $G_t\alpha$* to interact with R* in the presence of $G_t\beta\gamma$ was evaluated using the GTPγS binding assay. The very slow GTPγS binding rate to $G_t\alpha$* (k_{app} = ~0.004 s^{-1}), which is limited by the rate of GDP dissociation (26), was significantly accelerated in the presence of R* and $G_t\beta\gamma$ (k_{app} = ~0.079 s^{-1}) (Fig. 1). A fluorescence read-out assay was utilized to monitor the interaction between $G_t\alpha$ and the Pγ subunit (23). Using this assay, $G_t\alpha$*GDP bound fluorescently labeled Pγ, PγBC, with a K_d value of 28 nM (Fig. 2*A* and Table I). When $G_t\alpha$*GDP was activated in the presence of AlF_4^- it bound to PγBC with an almost 6-fold higher affinity (K_d of 5.1 nM) (Fig. 2*B* and Table I). Thus, $G_t\alpha$*, which contains only 16 $G_i\alpha$ residues, represents a well suited tool for mutational analysis to identify residues that are essential for both receptor and effector interactions of transducin.

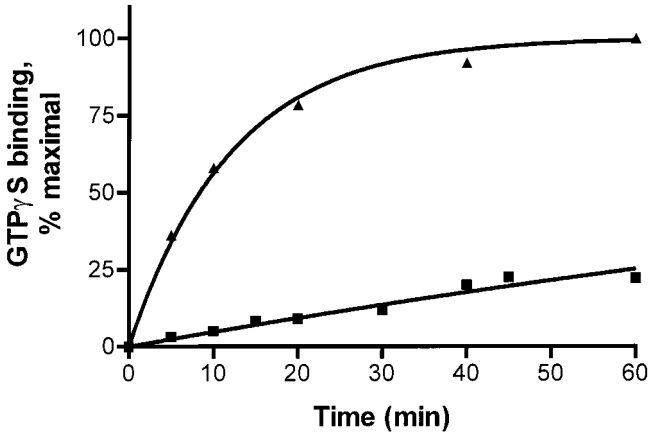

FIG. 1. **The time course of GTPγS binding to $G_t\alpha$*.** The binding of GTPγS to $G_t\alpha$* (0.4 μM) was initiated by addition of 5 μM [^{35}S]GTPγS (0.2 μCi) (■). When the effect of R* was measured, $G_t\alpha$* was premixed with $G_t\beta\gamma$ (2 μM), and the binding was initiated by addition of 5 μM of [^{35}S]GTPγS and uROS (100 nM rhodopsin) (▲). Aliquots were withdrawn at the indicated times, and GTPγS bound to $G_t\alpha$* was determined using the filter binding assay. The calculated k_{app} values are 0.004 s^{-1} (■) and 0.079 s^{-1} (▲).

Expression of $G_t\alpha$ 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 $G_t\alpha$ are surface exposed (27) and were substituted with Ala residues. In addition to a modestly solvent-exposed Leu^{306}, two buried residues, Ile^{299} and Phe^{303}, 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 $G_t\alpha$. Substitutions of Ile^{299} and Phe^{303} were made to test this possibility. Expression of all but three of the $G_t\alpha$* 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 $G_t\alpha$GTPγS shows that the Tyr^{298} side chain makes contact with Tyr^{286} from the αG-α4 loop, whereas Ile^{299} and Phe^{303} 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 αG-α4 loop or the α3-helix.

The ability of $G_t\alpha$ mutants to undergo a conformational change upon addition of AlF_4^- 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 AlF_4^-, whereas the fluorescence change for L306A was intermediate to that for $G_t\alpha$* (not shown).

R-induced GTPγS Binding to $G_t\alpha$* Mutants*—The ability of R* to interact with $G_t\alpha$* mutants and cause them to release GDP was examined by measuring the rates of GTPγS binding to these mutants in the presence of R* and $G_t\beta\gamma$. 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 $G_t\alpha$* mutants, Y298A, I299A, and F303A, did not appreciably bind GTPγS. A correlation between the low expression

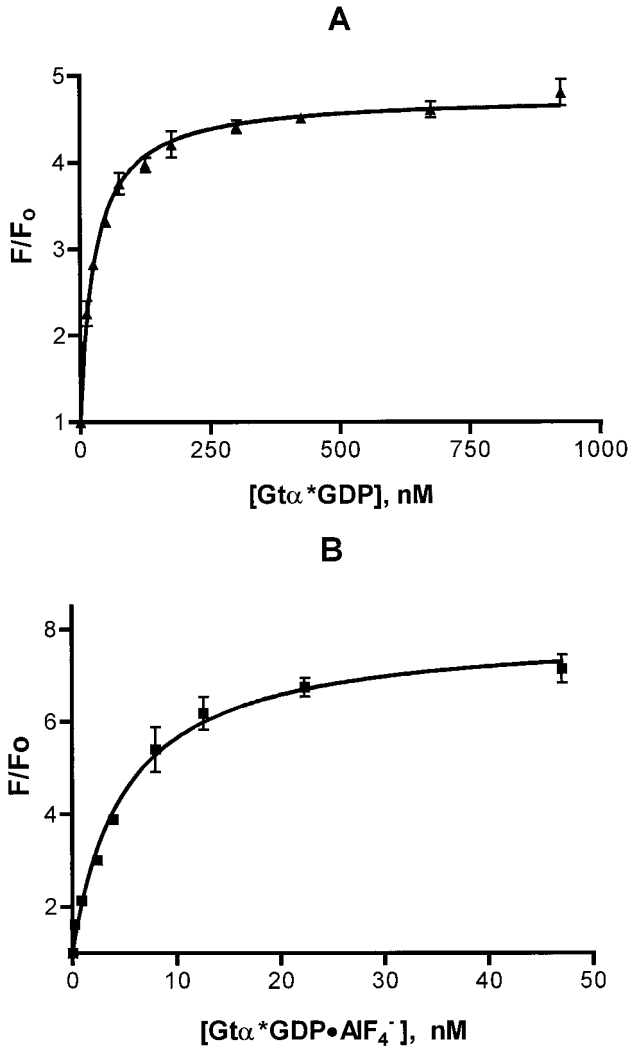

FIG. 2. **Binding of $G_t\alpha$* to PγBC.** The relative increase in fluorescence (F/F_o) of PγBC (10 nM) (excitation at 445 nm; emission at 495 nm) was determined after addition of increasing concentrations of $G_t\alpha$*GDP in the absence (*A*) or in the presence (*B*) of AlF_4^-.

TABLE I
Interaction of $G_t\alpha$ mutants with PγBC and activation by R**

	Binding to PγBC (K_d)		GTPγS binding (k_{app})
	$G_t\alpha$*GDP·AlF_4^-	$G_t\alpha$*GDP	
	nM		s^{-1}
$G_t\alpha$*	5.1 ± 0.5	28 ± 2	0.079 ± 0.010
E293A	5.0 ± 0.8	20 ± 1	0.057 ± 0.012
E294A	4.4 ± 0.2	34 ± 2	0.059 ± 0.009
N297A	6.6 ± 0.7	49 ± 3	0.081 ± 0.003
Y298A	54 ± 7	49 ± 4	
I299A	35 ± 3	37 ± 2	
K300A	7.9 ± 0.7	42 ± 2	0.084 ± 0.009
V301A	7.5 ± 0.8	79 ± 6	0.077 ± 0.004
Q302A	6.9 ± 0.7	27 ± 2	0.073 ± 0.010
F303A	60 ± 8	50 ± 4	
L304A	3.0 ± 0.4	28 ± 2	0.053 ± 0.003
E305A	3.5 ± 0.1	22 ± 3	0.065 ± 0.002
L306A	15 ± 1	34 ± 4	0.019 ± 0.001
M308A	6.5 ± 0.3	27 ± 1	0.079 ± 0.006
R309A	8.7 ± 1.2	33 ± 2	0.065 ± 0.003
R310A	4.8 ± 0.7	37 ± 3	0.034 ± 0.004
D311A	8.5 ± 0.9	34 ± 4	0.023 ± 0.001
V312A	4.2 ± 0.4	52 ± 2	0.095 ± 0.004
K313A	6.1 ± 0.4	73 ± 9	0.092 ± 0.005
E314A	7.9 ± 0.6	49 ± 2	0.107 ± 0.009

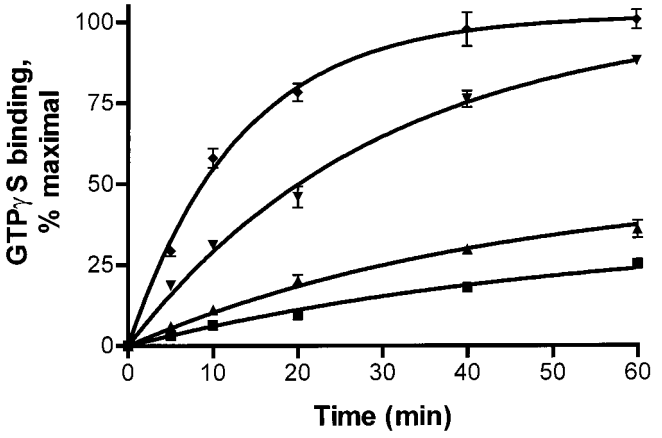

FIG. 3. **GTPγS binding to $G_t\alpha$* mutants.** The binding of GTPγS to $G_t\alpha$* mutants (0.4 μM mutant $G_t\alpha$*; 2 μM $G_t\beta\gamma$) was initiated by addition of 5 μM [^{35}S]GTPγS (0.2 μCi) and uROS membranes (100 nM rhodopsin). Protein-bound GTPγS was determined using the filter binding assay. The V301A mutant (♦) is representative of $G_t\alpha$* mutants with intact kinetics of GTPγS binding. The L306A (■), R310A (▼) and D311A (▲) mutants had impaired GTPγS binding characteristics.

levels of these mutants and the lack of GTPγS 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 α4-α3 coupling could represent an important element in maintaining proper conformation of the R*-binding regions, or it is essential for the ability of $G_t\alpha$ to undergo a conformational change upon binding of GTPγS. The latter possibility is supported by the lack of the tryptophan fluorescence enhancement with addition of AlF_4^- to Y298A, I299A, and F303A. The GTPγS binding properties of the L306A mutant, in which another residue that contacts α3 was substituted, were seriously compromised but not abolished. Fitting of the GTPγS binding data for L306A yielded a value for maximal binding at ~35% of that for $G_t\alpha$* with an ~4-fold lower rate (k_{app} = ~0.019 s^{-1}) (Fig. 3 and Table I). $G_t\alpha$*L306A was expressed in *E. coli* comparably to $G_t\alpha$* 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 $G_t\alpha$* maximally bound only ~50% GTPγS 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. $G_t\alpha$*R310A had a saturating level of GTPγS binding similar to that of $G_t\alpha$*, but the rate of binding was decreased by ~2-fold (Fig. 3 and Table I). Previously, Arg^{309}, Val^{312}, and Lys^{313} were implicated in the $G_t\alpha$-R* interaction using an assay of $G_t\alpha$ activation in microsomes of COS7 cells expressing rhodopsin and mutant G_t (11). We observed no significant changes in the kinetics of $G_t\alpha$* activation caused by these three or other remaining mutations under our experimental conditions (Table I).

Binding of $G_t\alpha$ Mutants to PγBC*—To delineate potential effector residues within the α4-β6 region, the $G_t\alpha$* mutants in the GDP-bound or active AlF_4^--induced conformations were tested for binding to PγBC. Interestingly, mutants Y298A, I299A, and F303A, which had low expression levels and lacked R*-induced GTPγS binding, in the GDP-bound conformations displayed affinities for PγBC comparable with $G_t\alpha$*GDP (Table I). This result indicates that in the inactive conformation their effector interface is not significantly affected. Predictably, these three $G_t\alpha$* mutants had significant defects in binding to Pγ in the presence of AlF_4^-. Addition of AlF_4^- produced no

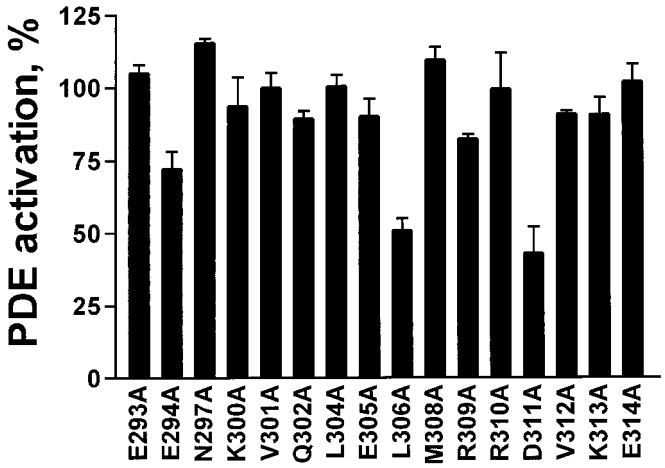

FIG. 4. **Activation of PDE by $G_t\alpha$* mutants.** The cGMP hydrolytic activity of rod holoPDE (0.2 nM) was measured in suspensions of uROS membranes (10 μM rhodopsin) reconstituted with 2 μM $G_t\beta\gamma$ and 10 μM GTPγS in the presence of 2 μM $G_t\alpha$* mutants. The PDE activation is expressed as a percentage of that elicited by $G_t\alpha$* (the basal and $G_t\alpha$*-stimulated PDE activities were 14 and 210 mol cGMP/s mol PDE, respectively).

enhancement in the mutant interaction with PγBC, evidently due to the inability of these mutants to assume an active conformation. In addition, the interaction of the L306A mutant with PγBC was less sensitive than that of $G_t\alpha$* to AlF_4^-. In the presence of AlF_4^-, L306A bound to PγBC with a K_d only 2-fold lower than when AlF_4^- was absent (Table I). This is consistent with the limited competency of L306A to assume an active conformation. The $G_t\alpha$* mutants, V301A and K313A, had mild defects in effector binding. These mutants retained a high affinity for PγBC in the AlF_4^--bound conformations but revealed a somewhat reduced interaction with the effector in the absence of AlF_4^- (Table I). All other $G_t\alpha$* mutants demonstrated affinities for PγBC comparable with that of $G_t\alpha$ (Table I).

Activation of Rod PDE by $G_t\alpha$ Mutants*—The ability of $G_t\alpha$* mutants to stimulate activity of holoPDE ($P\alpha\beta\gamma_2$) was tested in the reconstituted system with additions of uROS membranes and purified $G_t\beta\gamma$ in the presence of GTPγS. $G_t\alpha$* as well as the majority of its mutants activated holoPDE under these conditions 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 Leu^{306} and Asp^{311} are unlikely to be directly involved in interaction with and activation of PDE.

DISCUSSION

The α4-β6 region of $G_t\alpha$ is an essential contributor to the $G_t\alpha$-rhodopsin interface (6, 11). The R* binding sites of $G_t\alpha$, the α4-β6 loop (amino acids 305–315) and $G_t\alpha$-340–350, are positioned on the same "receptor" face of $G_t\alpha\beta\gamma$ as the N terminus of $G_t\alpha$ and the C terminus of $G_t\gamma$ (28). The β6-sheet and the α5-helix project inward from the α4-β6 loop and $G_t\alpha$-340–350 on the $G_t\alpha$ surface to form the β6/α5 loop. The latter contains a cluster of residues, Cys^{321}, Ala^{322}, and Thr^{323}, intimately involved 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 Gα subunits (29–31). Thus, the $G_t\alpha$ activation mechanism is likely to involve interaction of R* with the α4-β6 loop and $G_t\alpha$-340–350, leading to conformational changes of the β6/α5 loop and dissociation of GDP.

The critical R*-binding region, $G_t\alpha$-340–350, has been investigated in great detail (6–9, 32). However, the role of the $G_t\alpha$ α4-β6 loop and its individual residues in binding to R* and $G_t\alpha$ activation is not well understood. Recently, mutants of $G_t\alpha$ with Ala substitutions of residues in the α4-β6 loop have been translated *in vitro* and expressed in COS-7 cells (11). Mutational analysis revealed that substitutions of four residues in the α4-β6 loop, Arg^{309}, Asp^{311}, Val^{312}, and Lys^{313} impaired $G_t\alpha$ interaction with R* (11). The Ala-scanning mutagenesis of the $G_t\alpha$ α4-β6 region in the context of $G_t\alpha$* readily expressed in *E. coli* has provided us with an opportunity for in depth investigation of the roles of individual α4-β6 residues in the receptor interaction. Reconstitution of the purified mutant $G_t\alpha$* with $G_t\beta\gamma$ and uROS membranes has enabled the examination of the effects of mutations on the kinetics of $G_t\alpha$* activation by R*. Our results confirm the role of Asp^{311} in the R*-dependent activation of $G_t\alpha$. A substitution of this residue led to a substantial decrease in both the rate of and total R*-induced GTPγS binding. A moderate alteration of the kinetics of R*-induced GTPγS binding was caused by the substitution of Arg^{310}. The R310A mutant bound GTPγS with an ~2-fold slower rate. Supporting the involvement of $G_t\alpha$ Asp^{311}, and probably Arg^{310}, in the interaction with R* is the fact that the trypsin cleavage site Arg^{310}-Asp^{311} is protected upon binding of $G_t\alpha$ to R* (10). In addition to effective activation of $G_t\alpha$, R* is capable of activating $G_i\alpha$ (17) but has no detectable interaction with $G_s\alpha$.[2] Arg^{310} and Asp^{311} of $G_t\alpha$ align with the Lys-Asp and Ser-Gly pairs in $G_i\alpha$ and $G_s\alpha$, respectively. Therefore, Arg^{310} and Asp^{311} along with $G_t\alpha$-340–350 may contribute to the specificity of the $G_t\alpha$-R* interaction.

Mutations Y298A, I299A, and F303A caused the loss of $G_t\alpha$* activation by R* or AlF_4^-, whereas the L306A mutation resulted in a less severe phenotype. This loss of function apparently resulted from the inability of the mutants to undergo the activational conformational change. Residues Ile^{299}, Phe^{303}, and Leu^{306} interact with Met^{239}, Leu^{243}, and Phe^{246}, respectively (27). This interaction network between the α4 and α3 helices may secure the proper positioning of Glu^{241}, Leu^{245}, Ile^{249}, and Phe^{255}. The latter residues, upon activation of $G_t\alpha$, engage the switch II residues Arg^{201}, Arg^{204}, and Trp^{207} to form another network of interactions, which is critical for the $G_t\alpha$ progression to the active conformation (33). Therefore, our results suggest that the coupling between helices α3 and α4 is critical for the transition of $G_t\alpha$ to the active state.

The key event in photoactivation of PDE is a direct interaction between the GTP-bound $G_t\alpha$ and Pγ. Both $G_t\alpha$GTP and $G_t\alpha$GDP are capable of binding Pγ. However, $G_t\alpha$GDP binds Pγ with ~10–30-fold lower affinity and is incapable of efficient activation of PDE (23, 34). The $G_t\alpha$ binding sites on Pγ have been firmly established (13, 35–37). The Pγ-binding surface on $G_t\alpha$ appears to be significantly more complex and less understood. Initially, the putative effector region of $G_t\alpha$ corresponding to the α4-helix and the α4-β6 loop was identified using synthetic $G_t\alpha$ peptides. A synthetic peptide, $G_t\alpha$-293–314, potently (K_a of 8 μM) stimulated activity of rod holoPDE (12) via binding to Pγ (13). Substitutions within the $G_t\alpha$-293–314 peptide have been made, and five nonconserved residues, Asn^{297}, Val^{301}, Glu^{305}, Met^{308}, and Arg^{310}, were found to contribute to the activational effects (16). However, evidence contradicting the role of α4-β6 as a major effector domain of $G_t\alpha$ has emerged from analysis of chimeric $G_t\alpha/G_i\alpha$ proteins and mutagenesis of $G_t\alpha$ (17–19). Two other effector-interacting domains of $G_t\alpha$, the switch II region and the α3-helix-α3/β5 loop, have been identified (17–19). These findings led to the apparent discrepancy between the ability of peptide $G_t\alpha$-293–314 to activate PDE and

[2] M. Natochin and N. O. Artemyev, unpublished observations.

the prerequisite of the switch II and α3-β5 regions of $G_t\alpha$ 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 $G_t\alpha$GDP. However, such a model is not supported by the crystal structures of $G_t\alpha$ (27, 33). Moreover, at least three residues, Ile^{208} (switch II), His^{244}, and Asn^{247} (α3) are likely to interact directly with Pγ in the GTP-bound $G_t\alpha$ 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 $G_t\alpha$/Pγ binding. Even substitutions of the residues Tyr^{298}, Ile^{299}, Phe^{303}, and Leu^{306}, which disabled the activation of $G_t\alpha$*, had no notable impact on the binding of the GDP-bound mutants to PγBC. Results on activation of PDE by the $G_t\alpha$ mutants correlated well with the Pγ binding experiments. All mutants with unimpaired capacity for R*-induced GTPγS binding were competent to stimulate cGMP hydrolysis by holoPDE. The studies on cross-linking of Pγ to $G_t\alpha$ attest to a close proximity of Pγ to the α4-β6 region in the $G_t\alpha$-Pγ complex (14, 15). Although our analysis seems to rule out strong major interactions between Pγ and $G_t\alpha$-293–314, a relatively weak van der Waals' contact(s) at this site cannot be entirely excluded. Rather, the role of the α4-β6 residues, Ile^{299}, Phe^{303}, and Leu^{306}, 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 $G_t\alpha$.

The most surprising finding in this work is that none of the mutations of five $G_t\alpha$ residues identified using synthetic peptides (16) meaningfully affected the $G_t\alpha$*-PDE interaction. A greater sensitivity of the peptide structure than that of $G_t\alpha$* 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 $G_t\alpha$-293–314 and $G_t\alpha$ 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 $G_t\alpha$-293–314 likely represents a major effector-activating domain of $G_t\alpha$ was reached based on the ability of the peptide to "mimic" $G_t\alpha$ 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 $G_t\alpha$ peptide does not appear to reflect the role of the corresponding region in $G_t\alpha$.

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Roles of the Transducin α-Subunit α4-Helix/α4-β6 Loop in the Receptor and Effector Interactions

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