A Switch 3 Point Mutation in the $\alpha$ Subunit of Transducin Yields a Unique Dominant-negative Inhibitor*

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The rhodopsin/transducin-coupled vertebrate vision system has served as a paradigm for G protein-coupled signaling. We have taken advantage of this system to identify new types of constitutively active, transducin-$\alpha$ ($\alpha T$) subunits. Here we have described a novel dominant-negative mutation, made in the background of a chimera consisting of $\alpha T$ and the $\alpha$ subunit of G$_{11}$ (designated $\alpha T^*$), which involves the substitution of a conserved arginine residue in the conformationally sensitive Switch 3 region. Changing Arg-238 to either lysine or alanine had little or no effect on the ability of $\alpha T^*$ to undergo rhodopsin-stimulated GDP-GTP exchange, whereas substituting glutamic acid for arginine at this position yielded an $\alpha T^*$ subunit ($\alpha T^*$(R238E)) that was incapable of undergoing rhodopsin-dependent nucleotide exchange and was unable to bind or stimulate the target/effect enzyme (cyclic GMP phosphodiesterase). Moreover, unlike the GDP-bound forms of $\alpha T^*$, $\alpha T^*$(R238A) and $\alpha T^*$(R238K), the $\alpha T^*$R238E) mutant did not respond to membrane fluoride (AlF$_3^-$), as read out by changes in Trp-207 fluorescence. However, surprisingly, we found that $\alpha T^*$(R238E) effectively blocked rhodopsin-catalyzed GDP-GTP exchange on $\alpha T^*$, as well as rhodopsin-stimulated phosphodiesterase activity. Analysis by high pressure liquid chromatography indicated that the $\alpha T^*$(R238E) mutant exists in a nucleotide-free state. Nucleotide-free forms of $\gamma$ subunits were typically very sensitive to proteolytic degradation, but $\alpha T^*$(R238E) exhibited a resistance to trypsin-proteolysis similar to that observed with activated forms of $\alpha T^*$. Overall, these findings indicated that by mutating a single residue in Switch 3, it is possible to generate a unique type of dominant-negative G protein subunit that can effectively block signaling by G protein-coupled receptors.

The vertebrate phototransduction system operating in retinal rods has served as a prototype for G protein-coupled receptor (GPCR)-dependent signal transduction (1). The photoreceptor, rhodopsin, a seven-membrane-spanning GPCR, is the initial component in this pathway and is capable of responding to a single photon of light. Light-activated rhodopsin engages the heterotrimeric GTP-binding protein (G protein), transducin, and catalyzes the exchange of GDP for GTP on its $\alpha$ subunit ($\alpha T$). The activated, GTP-bound $\alpha T$ subunit targets are a significant distance from the guanine nucleotide-binding domain (5–7). It is generally agreed that the rate-limiting step for G protein activation is the dissociation of GDP from the $\alpha$ subunit. The retinal G protein transducin exhibits an especially slow rate of intrinsic GDP dissociation and basal GDP-GTP exchange (8) and therefore is dependent on light-activated rhodopsin to catalyze these events. Unlike their small G protein counterparts, the $\alpha$ subunits of the large G protein family contain a helical domain in addition to their guanine nucleotide-binding (Ras-like) domain (9–14). Thus, it has been suspected that the role of GPCRs (e.g. rhodopsin) in stimulating GDP-GTP exchange involves changing the juxtaposition of the helical domain relative to the Ras-like domain, as well as perturbing the interactions that normally hold GDP tightly in place (via residues that stabilize the guanine ring and phosphate moieties). Recently, we have tested the importance of altering the relative positions of the helical and Ras-like domains (15).

Substitutions were made for the conserved residues that comprise the linker regions connecting these two major domains. Indeed, we found that mutations in the linker regions of $\alpha T$ yielded molecules capable of constitutive GTP-GTP exchange in the absence of rhodopsin.

We have tried to identify mutations that yield constitutively active $\gamma$ subunits that fully mimic the functional outcome of GPCR-mediated interactions, just as we have done for the small G protein Cdc42 (16, 17). Along these lines, we have examined several different regions on $\alpha T$, with one of these being the conformationally sensitive Switch 3 domain. The Switch 3 domain has been shown to play a critical role in G protein activation by linking the binding of target/effectors at Switch 2 to the stimulation of effector activity (18, 19) and to be important for regulator of G protein signaling-stimulated GTP hydrolysis (20). Switch 3 might also help to ensure that $\alpha T$ reaches the appropriate activated conformation to engage downstream target/effectors and, in doing so, perhaps to influence nucleotide binding and the nucleotide exchange reaction.

To examine such possibilities, we have changed conserved residues in Switch 3 in the background of an $\alpha T$/ar1 chimera ($\alpha T^*$, see “Experimental Procedures”) and determined the consequences of these substitu-
tions on αT* activation and αT*-target/effecter interactions. Here we have reported the surprising finding that specific substitutions for one of these residues, Arg-238, can influence the ability of αT* to reach the activated (GTP-bound) state and to ultimately dissociate from the receptor (rhodopsin). Although certain changes at position 238 showed no apparent effect on the ability of αT* to functionally couple to rhodopsin, we found that when glutamic acid was substituted for the conserved arginine residue, an αT* subunit was generated that was capable of blocking the functional coupling between rhodopsin and transducin. The αT*(R238E) mutant was isolated in a nucleotide-free state but exhibited a reduced sensitivity to trypsin proteolysis, when compared with GDP-bound αT*. Thus, we believe that the R238E substitution yields a Gα mutant that remains stable in the absence of bound nucleotides and therefore can serve as a dominant-negative inhibitor of the interactions of a GPCR with its G protein substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—GTP, GDP, GTPγS, cGMP, tetrabutylammonium bromide, trypsin, and other reagents (HEPES, MgCl₂, dodecyl maltoside, NaF, AlCl₃) were purchased from Sigma. BA85 nitrocellulose filters were from Schleicher & Schuell. [35S]GTP was obtained from PerkinElmer Life Sciences. Frozen, dark-adapted bovine retinas were obtained from W. L. Lawson Co. (Lincoln, NE).

**Mutagenesis**—Degenerate primers from Invitrogen were used to generate different mutants of αT*(Arg-238). A plasmid containing the αT* gene, which codes for an αT-α1 chimera in which the corresponding region from α1 was inserted between residues 215 and 294 of αT, was prepared as described previously (15). This served as a template to perform site-directed mutagenesis by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). The parental DNA template was digested using DpnI endonuclease, and the nicked vector DNA with the desired mutation was transformed into Escherichia coli XL-1 Blue competent cells. DNA was purified from single colonies using the Qiagen plasmid miniprep kit and sequenced at the BioResource Center of Cornell University.

**Protein Expression and Purification**—The recombinant αT* and the αT*(Arg-238) mutants were expressed and prepared using procedures similar to those described by Skiba et al. (21). Briefly, the αT* subunits were expressed in BL21(DE3) competent cells and purified in the presence of 50 μM GDP. The proteins were eluted from a nickel-nitrilotriacetic acid column with 200 mM imidazole and then eluted from a Q column with a NaCl gradient. The αT* subunits were further purified by gel filtration chromatography on a HiLoad Superdex G75 HR 26/60 column equilibrated with 20 mM HEPES, pH 7.5, and 10% glycerol (Buffer G). The samples were aliquoted, snap-frozen, and stored at −80 °C. The final yield of αT*, αT*(R238A), and αT*(R238K) ranged from 1 to 1.5 mg of pure protein/liter of bacterial culture. Some aggregation was noticed during the purification of the αT*(R238E) mutant, and the final yield of this mutant ranged from 0.1 to 0.2 mg of pure protein/liter of culture. However, gel filtration chromatography of purified αT* and αT*(R238E) yielded similar profiles with roughly 50% of the recombinant αT* subunits eluting at the expected size of ~40 kDa and ~50% of the total protein eluting at the size of ~80 kDa due to dimerization.

**Purification of Retinal Proteins**—Rod outer segment membranes were isolated using a sucrose gradient as described in Papermaster and Dreyer (22). Holotransducin and PDE6 were purified from rod outer segment membranes using previously described procedures (23). The PDE was further purified by gel filtration chromatography on a HiLoad Superdex G200 HR26/60 column equilibrated with 20 mM HEPES, pH 7.5, and 10% glycerol. Urea-washed rod outer segment membranes, prepared as described (24), provided the rhodopsin used in all experiments.

The αT and Gβγ subunit components of holotransducin were separated on a Blue-Sepharose column equilibrated with 10 mM HEPES, pH 7.5, 6 mM MgCl₂, 1 mM dithiothreitol, and 25% glycerol. The Gβγ complex was eluted from the Blue-Sepharose column with 250 mL of low salt buffer (i.e., the equilibration buffer containing 100 mM KCl) and further purified by gel filtration chromatography on a HiLoad Superdex G75 HR 26/60 column equilibrated with buffer G. The final yield of Gβγ ranged from 3 to 5 mg of pure protein/300 retina. The purified subunit complex was aliquoted, snap-frozen, and stored at −80 °C.

**HPLC Analysis**—A Sunfire C-18 reversed phase column (0.46 × 25 cm) filled with 5 μm silica was obtained from Waters. The system consisted of a Waters 1525 binary HPLC pump machine and a Waters 2487 dual absorbance UV detector. Chromatography was performed in phosphate buffer (100 mM, pH 6.5) containing 10 mM tetrabutylammonium bromide, 7.5% (v/v) acetonitrile, and 0.2 mM NaF (HPLC buffer) at ambient temperature with a flow rate of 1 mL/min. In this system, the order of elution of guanine nucleotides is GMP, GDP, and GTP (retention times 4.8, 8, and 14 min, respectively). The amount and identity of the guanine nucleotide bound to αT* was determined by adding ~400 μg of protein sample to HPLC buffer and then centrifuging for 10 min at 12,000 × g to remove denatured protein. The supernatant was added directly to the column, and guanine nucleotides were chromatographed using the same buffer. The column was calibrated with solutions of the different guanine nucleotides.

**Trypsin Proteolysis Assay**—The patterns of tryptic proteolysis were determined as described previously (25). Briefly, 20-μL reactions containing 5 μg of either αT* or αT*(R238E) were performed for 10 min at 25 °C in 20 mM HEPES (pH 7.5) containing 100 μM GDP. Where indicated, 20 mM NaF and 600 μM AlCl₃ were added to the buffer to form the aluminum fluoride (AlF₄⁻) complex and incubated at 25 °C for 10 min. Trypsin digestions were performed by adding 2 μL of trypsin (100 μg of trypsin/ml) and incubating for 10 min at 25 °C. The reactions were stopped with the addition of SDS sample buffer and heat treatment (95 °C, 5 min). Proteolytic fragments were resolved on 15% SDS–gel electrophoresis and stained with Coomassie Blue.

**Fluorescence Measurements**—Fluorescence measurements were made on a Varian Eclipse fluorescence spectrophotometer in 1 mL of HMM buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, and 100 mM NaCl) at 25 °C. αT*-GDP (400 nM) was preincubated in HMM buffer for 5 min at 25 °C. The AlF₄⁻ complex was formed by adding 10 mM NaF and 50 μM AlCl₃. The binding of AlF₄⁻ was measured by monitoring the enhancement of the intrinsic tryptophan fluorescence of αT* upon excitation at 300 nm and emission at 345 nm (15).

**[35S]GTPγS Binding Assays**—Rhodopsin (4 nM), αT*-GDP (500 nM), and Gβγ (500 nM) were incubated in a total volume of 100 μl of buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, and 0.01% dodecyl maltoside) for 20 min at room temperature, in the presence of light. Binding reactions were started by the addition of 100 μl of 10 μM [35S]GTPγS (1 Ci/mmol). Aliquots (20 μl) were removed at the times indicated and applied directly to prewetted nitrocellulose filters (pore size = 0.45 μm) on a suction manifold. The filters were washed twice with HMM buffer and counted in a scintillation counter (LS6500 Multipurpose Scintillation counter) after the addition of 3 ml of scintillation liquid (Scinbase 30% cocktail). The kₐ values for the binding reactions were obtained by fitting the data to the equation Y = Bmax(1 − exp(−kt)) using GraphPad Prism software.

**Measurement of cGMP PDE Activity**—A pH microelectrode was used to measure the change in pH (in millivolts) that results from the release...
of one proton for each molecule of cGMP hydrolyzed (26). In a typical assay, purified αT*-GDP (1 μM) was preincubated with 500 nM Gβγ, 20 nM rhodopsin, and 100 μM GTPyS in HMN buffer for 20 min at 25 °C, in the presence of light. PDE (100 nM) was added, and the reaction was incubated for an additional 10 min at 25 °C. The substrate, cGMP (5 mM), was added to the reaction mix, and the relative cGMP hydrolysis rate was calculated based on the change in pH over time.

Additional Procedures—Protein concentrations were measured as described by Bradford (27) using bovine serum albumin as a standard. SDS-PAGE was performed by the method of Laemmli (28) in 12 or 15% acrylamide gels. GraphPad Prizm (version 4) software was used to fit experimental data.

RESULTS

The Conformationally Sensitive Switch 3 Region of αT Influences Rhodopsin-stimulated Activation of Transducin—Comparisons of the x-ray crystal structures for the GDP-bound (signaling-inactive) and GTPyS-bound (signaling-competent) forms of αT (12) indicated that there were three regions that undergo conformational changes in response to GDP-GTP exchange. Two of these regions correspond to the conformational sensitive segments initially identified in Ras (29) and E-Tu (30), designated as Switch 1 and Switch 2. The third region, designated as Switch 3, undergoes changes in αT and other Gx subunits but not in Ras nor in related small G proteins. Switch 3 was previously shown to play a key role in linking the binding of Switch 2 to target/effectors proteins, with the stimulation of effector activity (19). Thus, removal of the entire Switch 3 loop, as well as mutation of the conserved Glu-232 in this segment of αT, yields an αT subunit that is capable of binding to the PDE in a GTP-dependent manner but is unable to stimulate PDE activity (cyclic GMP hydrolysis). These findings raised the possibility that Switch 3 may be communicating with another region of the αT subunit that was directly responsible for effector regulation.

Fig. 1a shows the x-ray crystal structure for the GDP-bound form of retinal αT (10), with the Switch 3 segment highlighted in magenta. Arginine 238 is a conserved residue within Switch 3. In GDP-bound αT, Arg-238 contacts Glu-39 (Fig. 1b), which is in the phosphate-binding P-loop lying just upstream from the beginning of the large helical domain, whereas in the activated (GTPyS-bound) αT subunit, Arg-238 contacts both Glu-39 and Gln-143 of the helical domain. It has been suggested that the helical domain of Gx subunits plays a role in mediating the regulation of target/effectors, and in the particular case of αT, may be essential for the stimulation of PDE activity (31–33). Thus, we wanted to see whether disruption of the apparent links between the conserved Arg-238 in Switch 3 and Glu-39 and/or Gln-143 might compromise αT activation and/or its ability to bind and regulate the PDE. To facilitate the expression of different αT mutants in E. coli, point mutations were prepared in the background of an αT/oit chimera referred to as αT* (see “Experimental Procedures”). Unlike wild-type αT, the αT* subunit is expressed in a soluble form in E. coli and (like retinal αT) is fully capable of functionally coupling to rhodopsin (15).

The results presented in Fig. 2 show that changing the arginine at position 238 to either alanine or lysine, within the αT background, had only modest effects on the ability of αT* to respond to rhodopsin and Gβγ and to undergo GDP-[35S]GTPyS exchange. The same appears to be true when Arg-238 is changed to glutamine (20). However, when Arg-238 was changed to glutamic acid, the resulting αT* mutant was completely ineffective in undergoing rhodopsin-stimulated activation. The αT*(R238E) Mutant Is Unable to Activate the Cyclic GMP PDE—We also examined the abilities of different position 238 mutations within an αT background to stimulate PDE activity. Both the αT*(R238A) and the αT*(R238K) mutants, upon binding to GTPyS in a rhodopsin-dependent manner, were compromised in their ability to stimulate PDE activity (Fig. 3a). Their partial ability to stimulate the target/effectors may reflect the importance in maintaining the linkage between Arg-238 and Glu-143 of the helical domain for proper effector regulation. However, the αT*(R238E) mutant was completely incapable of stimulating PDE activity, consistent with its inability to become activated in response to rhodopsin. Still, we wondered whether the αT*(R238E) mutant might be able to bind to PDE and prevent wild-type αT* from binding and stimulating the target/effectors. However, as
shown in Fig. 3b, this was not the case, as there was no detectable inhibition of the αT*-mediated stimulation of PDE activity when excess αT*(Arg-238) was preincubated with PDE prior to the addition of activated (GTPγS-bound) αT* to the assay.

The αT*(R238E) Mutant Is Unresponsive to Aluminum Fluoride Treatment—We also examined whether substitutions for the conserved Arg 238 in Switch 3 had any effect on the ability of αT* to respond to aluminum fluoride (AlF4−) and undergoing conformational changes (34, 35). Substituting either alanine or lysine for arginine at position 238 had no effect on the ability of αT* to respond to AlF4− when monitoring Trp-207 fluorescence (Fig. 4). However, the αT*(R238E) mutant was unresponsive to AlF4−, showing no detectable change in its intrinsic tryptophan fluorescence.

The αT*(R238E) Mutant Is Nucleotide-depleted—The inability of αT*(R238E) to respond to AlF4− together with the fact that AlF4− only binds to the GDP-bound form of Gα subunits, prompted us to examine the nucleotide-bound state of R238E mutant by HPLC. Fig. 5, a and b, show the HPLC profiles obtained following treatment with acetonitrile to release any prebound GDP or GTP from αT* and αT*(R238E), respectively. Fig. 5c shows the control elution profiles for GDP and GTP. It was clear that although αT* was purified in the GDP-bound state, thus accounting for its ability to respond to AlF4−, αT*(R238E) was isolated in a nucleotide-free state. This explained the inability of the R238E mutant to bind AlF4−. However, although it has been generally assumed that nucleotide-depleted Gα subunits are highly susceptible to proteolysis and degradation, somewhat surprisingly, the αT*(R238E) mutant did not show an enhanced sensitivity to trypsin, and in fact, showed a greater degree of resistance than did the GDP-bound form of retinal αT or the recombinant αT* protein. For example, it has been well established that when the GDP-bound form of retinal αT is treated with trypsin, a sequence of proteolytic events occurs that culminates in the cleavage of arginine 204 in Switch 2, yielding fragments of ~23 and ~10 kDa (36, 37). When activated forms of αT* (either bound to AlF4− or bound to GTPγS) are exposed to trypsin, the proteolytic site in Switch 2 is protected, as an outcome of the activating conformational change, thus resulting in the production of a stable fragment of ~34 kDa. The same was true when the recombinant αT* subunit was treated with trypsin. Fig. 6 shows that the 23-kDa fragment was generated when proteolysis of αT* was performed in the absence of AlF4−, whereas complete proteolysis was blocked when αT* was treated with the activating agent, yielding first an ~38-kDa fragment and then the 34-kDa fragment. When the same experiments were performed with the αT*(R238E) mutant, there was a slight shift in the mobility of the two core fragments of αT*(R238E), perhaps because of the point mutation; however, there was little or no generation of a fragment in the range of 20–23 kDa. These results suggested that the conformation of the Switch 2 domain of αT*(Arg-238) at least partially mimics that of the activated Gα subunits are highly susceptible to proteolysis and degradation, somewhat surprisingly, the αT*(R238E) mutant did not show an enhanced sensitivity to trypsin, and in fact, showed a greater degree of resistance than did the GDP-bound form of retinal αT or the recombinant αT* protein. 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state, thus protecting the trypsin-sensitive Arg-204 residue and enabling this nucleotide-free mutant to exhibit increased resistance to proteolytic degradation.

The αT*(R238E) Mutant Blocks Rhodopsin-stimulated Nucleotide Exchange on αT*—Given that nucleotide-binding-defective mutants of Ras and related small G proteins tightly associate with their upstream activators (guanine nucleotide exchange factors or GEFs) and act as dominant-negative inhibitors (38, 39), we then asked whether the αT*(R238E) mutant might block the functional coupling of rhodopsin to transducin. Indeed, we found that the αT*(R238E) was a potent inhibitor of rhodopsin-stimulated GDP-[35S]GTPγS exchange on αT*. Fig. 7a shows that when αT*(R238E) was added together with αT* to an assay incubation containing rhodopsin and Gβγ, the presence of the R238E mutant caused a significant reduction in the rhodopsin-dependent binding of [35S]GTPγS by αT*. The inhibitory effects of αT*(R238E) were enhanced when the mutant was preincubated with rhodopsin and Gβγ, prior to adding αT* (Fig. 7a). Likewise, preincubation of the αT*(R238E) mutant with rhodopsin and Gβγ potentially inhibited the rhodopsin-dependent stimulation of PDE activity by αT* (Fig. 7b). However, consistent with our previous findings (Fig. 3b), when αT* was first activated by rhodopsin in the absence of αT*(R238E), the (R238E) mutant was no longer able to inhibit PDE activity.

Fig. 8a shows that the inhibition of the rhodopsin-dependent activation of αT* by αT*(R238E) is dose-dependent and requires that αT*(R238E) is present in excess over αT*. However, the ability of αT*(R238E) to inhibit the rhodopsin-stimulated activation of αT* was not dependent on Gβγ. In fact, when the αT*(R238E) mutant was preincubated with rhodopsin in the absence of Gβγ, it was still able to effect an essentially complete inhibition of the rhodopsin-dependent GDP-[35S]GTPγS exchange activity of αT* (i.e. as assayed upon the addition of αT* together with excess Gβγ, Fig. 8b). Overall, these results indicated that although αT*(R238E) was incapable of undergoing rhodopsin-stimulated GDP-GTPγS exchange, it nonetheless was able to block rhodopsin-catalyzed G protein activation.

**DISCUSSION**

G proteins act as molecular switches in a variety of signaling pathways by linking the initial activation of cell surface GPCRs to the regulation of effector enzymes or ion channels. The two most fundamentally important steps in the actions of G proteins are the GTP binding event, which occurs as an outcome of receptor-stimulated GDP-GTP exchange, and GTP hydrolysis, which serves to terminate the signal and return the G protein to its basal, inactive state. The activation step needs to be tightly regulated, in the cases of both large and small G proteins, as the loss of regulation of GDP-GTP exchange results in constitutively active G proteins that have been implicated in a number of disease states (5, 40). A good deal of information is now available regarding the fundamental mechanisms underlying the activation of small G proteins by their upstream activators (GEFs) (41, 42). Based on x-ray crystal structures for different small G protein-GEF complexes, as well as various lines of biochemical study, it appears that the principle actions of GEFs are the perturbation of Mg\(^{2+}\) binding, as Mg\(^{2+}\) strongly influences the affinity of the G protein for GDP and the destabilization of the binding of the guanine ring moiety and the phosphate residues (43, 44). In the case of the G proteins, the underlying mechanism of activation by GPCRs is less well understood, owing to a lack of structural information for GPCR-G protein complexes.
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The Gα subunits hold a special challenge for the activation event, given that they are comprised of a conserved guanine nucleotide-binding domain, which is shared by Ras and other small G proteins and accordingly referred to as the GTPase domain, and a second large helical domain that has been described to fit over the GTPase domain like a "clam shell" (10–14). This implies that as a first step in the activation of large G proteins, GPCRs need to alter the juxtaposition of the helical domain relative to the GTPase domain. We have shown that mutations of conserved residues in αT* that are part of the linker regions connecting the helical and GTPase domains result in constitutive GDP-GTP exchange (15). However, the rate of nucleotide exchange measured for the αT* linker mutants is still slow relative to the rate of rhodopsin-stimulated nucleotide exchange. Thus, we have examined additional types of mutations in attempting to understand more about the regions on αT involved in regulating the activation event, with the ultimate aim being to identify a constitutively active Gα mutant that fully mimics the functional capability of a GPCR-activated G protein. This led us to examining the possible interactions between the conformationally sensitive Switch 3 loop and the helical domain.

The Switch 3 loop undergoes changes upon GDP-GTP exchange exclusively in the Gα subunits of large G proteins, thereby distinguishing it from the conformationally sensitive Switch 1 and 2 regions that are found in both small G proteins as well as in the α subunits of large G proteins. We had originally found that the Switch 3 loop plays a key role in linking the binding of PDE by activated αT (via its Switch 2 domain) to the stimulation of PDE activity (19). Given that Switch 3 can make contacts with the helical domain and because it had been proposed that the helical domain may be involved in the stimulation of PDE activity

Dose-dependent inhibition by αT*(R238E) of rhodopsin-stimulated activation of αT*. a, αT* (500 nM), αT*(R238E) (500 nM) ( ), or control, i.e. no αT* ( ), was incubated with rhodopsin (4 nM) and Gβγ (500 nM) at 25 °C for 20 min in the presence of light before [35S]GTPγS was added to start the reaction. In some cases, varying amounts of αT* (R238E), 500 nM, 1.5 μM ( ), or 3 μM ( ) were first preincubated with rhodopsin and Gβγ for 20 min at 25 °C, and then αT* (500 nM) was added and incubated for an additional 20 min at 25 °C before initiating the assay. Aliquots were withdrawn at the times indicated, filtered, and counted. Binding of GTPγS to proteins is expressed as the amount of GTPγS bound (in picomoles) as a function of time (minutes). b, the αT*(Arg-238) mutant (3 μM) was preincubated with rhodopsin (4 nM), in the presence ( ) or absence ( ) of Gβγ (2 μM), prior to the addition of αT* (500 nM), and if necessary, Gβγ (i.e., when Gβγ was absent from the preincubation). In other cases, αT* was assayed in the presence of rhodopsin and Gβγ ( ) or in the absence of rhodopsin ( ), and αT*(R238E) was assayed in the presence of rhodopsin and Gβγ ( ). The results are representative of three experiments.
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FIGURE 9. A model depicting the effects of the \( \alpha^T( R238E) \) mutant on the rhodopsin-regulated GTP binding/hydrolytic cycle of transducin. Mutating the conserved Arg-238 residue in Switch 3 of \( \alpha^T \) yields a mutant protein that can form a complex with rhodopsin even in the absence of GTP and blocks the binding of GDP-bound \( \alpha^T \). Thus, the R238E mutant can act as a dominant-negative inhibitor of GPCR-coupled signal transduction.

(31–33), it was attractive to consider that interactions between Switch 3 and the helical domain might be essential for effector regulation. Since we already had shown that changing the position of the helical domain relative to the GT-Pase domain can influence GDP-GTP exchange, we also wondered whether disrupting interactions between Switch 3 and the helical domain might impact \( \alpha^T \) activation. In the present study, we have looked at this in some detail by mutating a conserved residue in Switch 3, Arg-238, which, based on the available x-ray structures for \( \alpha^T \), appeared to make contacts with Glu-39 from the P-loop within the amino-terminal portion of the \( \alpha^T \) subunit, as well as with Gln-143 (the latter contact occurring when \( \alpha^T \) is in an activated conformation).

Certain changes at position 238, when made in the background of the recombinant \( \alpha^T \) subunit, appeared to be largely without effect when assaying rhodopsin-stimulated nucleotide exchange; these included a conserved substitution (i.e. lysine for arginine) or when changing the arginine to alanine or glutamine (20). Changes at position 238 did impact the ability of the recombinant \( \alpha^T \) subunit to stimulate PDE activity to varying extents. The R238A and R238K mutants reduced the extent of activation by about 50%, whereas an R238Q mutant showed an even greater impairment (20). These findings may reflect the importance of maintaining proper Switch 3 domain-helical domain interactions for full effector regulation. However, when we changed Arg-238 to glutamic acid, we completely eliminated the ability of the \( \alpha^T \) subunit to undergo rhodopsin-stimulated GDP-GTP exchange. Consequently, this mutant \( \alpha^T \) subunit was also unable to stimulate PDE activity.

At the moment, we do not fully understand why this single point mutation has such a dramatic effect on rhodopsin-dependent nucleotide exchange. It may have something to do with disrupting the normal interaction of Arg-238 with Glu-39, which lies in the P-loop. The x-ray crystal structures for \( \alpha^T \) also suggested that Arg-238 comes into proximity of the conserved NKXD motif, which participates in binding the guanine ring moiety. Thus, perhaps reversing the side-chain charge at position 238 (i.e. Arg to Glu) caused some destabilization of the guanine ring. Still, what was particularly striking and unexpected was the finding that the \( \alpha^T( R238E) \) mutant inhibited the ability of rhodopsin to activate wild-type \( \alpha^T \) subunits. Thus, as depicted in Fig. 9, the R238E mutant has the ability to act as a dominant-negative inhibitor. This carries a number of interesting implications. One concerns the traditional difficulty in generating nucleotide-depleted \( \alpha^T \) mutants. Although the nucleotide-depleted forms of Ras-like G proteins work extremely well as dominant-negative inhibitors by forming stable complexes with their respective GEFs and blocking GEF-stimulated activation of the endogenous G protein counterparts (38), nucleotide-depleted forms of \( \alpha^T \) subunits can be extremely unstable. This then raises the question of how \( \alpha^T( R238E) \) was able to remain sufficiently stable so as to effectively act as a dominant-negative inhibitor. The answer may lie in its ability to assume what appears to be a partially activated conformation, as judged from its relative insensitivity to trypsin treatment. Specifically, the \( \alpha^T( R238E) \) mutant yielded a stable core fragment after exposure to trypsin, similar to what is observed with activated forms of both retinal \( \alpha^T \) and recombinant \( \alpha^T \) and distinct from what one sees when treating GDP-bound forms of the \( \alpha^T \) subunits with the protease (resulting in further degradation and the production of a 23-kDa fragment). Thus, although nucleotide-free forms of \( \alpha^T \) subunits are thought to be especially sensitive to proteolytic degradation, the \( \alpha^T( R238E) \) mutant, apparently by virtue of disrupting Switch 3, adopted a conformation that exhibits resistance to proteolysis and therefore represents a relatively stable \( \alpha^T \) subunit.

In conclusion, we have shown that a single point mutation of a conserved residue in the Switch 3 domain of a \( \alpha^T \) subunit significantly impacts its ability to bind guanine nucleotides and yields a unique type of dominant-negative inhibitor. Recently, a mutation in the \( \alpha^T \) subunit of the \( \alpha^T \) protein (\( \alpha^T(s( S54N)) \)), which corresponds to the well known Asn-17 mutations in Ras (38) and related small G proteins, has been shown to act as a dominant-negative inhibitor of signaling via the thyroid-stimulating hormone receptor (45). However, it seems unlikely that the \( \alpha^T(s( S54N)) \) mutant exists as a nucleotide-depleted protein because it behaves as a conditional dominant-negative inhibitor, showing some stimulation of basal adenyl cyclase activity (46). A dominant-negative mutant of \( \alpha^T \) that contains three sets of distinct mutations, influencing different functions of the G protein, has also been reported (47). However, to our knowledge, the R238E mutant represents the first demonstration that a single point-mutated, nucleotide-binding-defective \( \alpha^T \) subunit can be generated and used in a manner analogous to the Asn-17 Ras mutants as an inhibitor of GPCR-coupled signaling.

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