Communication between Switch II and Switch III of the Transducin α Subunit Is Essential for Target Activation*

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Qiubo Li and Richard A. Cerione‡
From the Department of Pharmacology,
Veterinary Medical Center, Cornell University,
Ithaca, New York 14853-6401

Comparisons of the tertiary structures of the GDP-bound and guanosine 5′-O-(thiotriphosphate) (GTPγS)-bound forms of the α subunit of transducin (αT) indicate that there are three regions that undergo changes in conformation upon αT activation. Two of these regions, Switch I and Switch II, were originally identified in Ras, while Switch III appears to be unique to trimeric GTP-binding proteins (G proteins). We find that replacement of the Switch III region (aspartic acid 227 through asparagine 237) with a single alanine residue yields an αT subunit that fully binds and hydrolyzes GTP but no longer stimulates the activity of the cyclic GMP phosphodiesterase (PDE), the physiological target for transducin. We also show that changing glutamic acid 232 of αT to a leucine (E232L) had no effect on rhodopsin-stimulated GTP-GDP exchange nor on the GTP hydrolytic activity of αT. However, the GTPγS-bound form of the αT E232L mutant was unable to stimulate the activity of the cyclic GMP PDE. The lack of stimulation was not due to an inability of the αT E232L mutant to bind to the target. Taken together, these results indicate that glutamic acid 232 mediates a conformational coupling between Switch II and Switch III, which is essential for converting GTP-dependent G protein-target interactions into a stimulation of target-effector activity.

GTP-binding proteins (G proteins) serve as molecular switches in a wide variety of biological response systems. Two large families, the Ras-related small G proteins and the trimeric G proteins, have received a great deal of attention because of their central role in signal transduction. The trimeric G proteins serve as intermediate signal transducers for seven-transmembrane-spanning (also called heptahelical or serpentine) receptors that are involved in responses to hormonal and neurotransmitter signals (1, 2). These G proteins consist of two functional units, the guanine nucleotide-binding α subunit (Gα) and the βγ complex (Gβγ). The molecular switch capability of a trimeric G protein is mediated through the Gα subunit, which cycles between the GDP-bound (inactive) and GTP-bound (active) states. A signal received from a receptor promotes the activation of a trimeric G protein by catalyzing the exchange of GTP for GDP on Gα. This results in the dissociation of the GTP-bound Gα from the Gβγ complex, thereby enabling these subunits to regulate the activities of downstream target/effectors. GTP hydrolysis on the Gα subunit promotes its re-association with Gβγ, thus terminating the signal.

The vertebrate phototransduction system represents one of the best characterized G protein-coupled signaling cascades. In this system, the photoreceptor rhodopsin activates a trimeric G protein, transducin, generating a GTP-bound α subunit (αT-GTP), which stimulates the target/effector enzyme, the cyclic GMP phosphodiesterase (PDE). The three-dimensional structures of the αT in different guanine nucleotide-bound states, and more recently for the αT-βγ complex, have been solved by x-ray crystallography (3–6), as have the corresponding structures for the inhibitory GTP-binding protein of the adenyl cyclase system, Gαi (7–9). This structural information now provides the foundation for understanding the molecular basis of many aspects of G protein-mediated signaling.

Three distinct regions on trimeric G protein α subunits have been shown to undergo conformational changes in response to GTP/GDP exchange (4, 5). Two of these regions, designated Switch I (Ser172 to Thr183 in αT) and Switch II (Phe195 to Thr215), are structurally analogous to the two conformationally-sensitive regions found in Ras (10) and EF-Tu (11), whereas the third region, designated Switch III (Arg227 to Arg238 in αT), is unique to the α subunits of trimeric G proteins. The various available x-ray crystallographic structures of G proteins show that the conformational changes in Switch I and II are the direct result of GTP binding to residues within these regions. Specifically, the structural changes in Switch I are induced by the interaction of the γ-phosphate of GTP with Thr177, while the changes in Switch II result from a hydrogen bond between Gly199 and the γ-phosphate (4). However, Switch III does not directly contact GTP. Rather, it was shown to respond to Switch II through a series of polar interactions that were mediated and/or promoted by GTP-induced conformational changes in Switch II (4). At present, the functional role of the Switch III domain or the importance of its conformational coupling to Switch II is not known. The fact that the residues proposed to be responsible for this conformational coupling are conserved in all trimeric G protein α subunits (4, 7) supports a critical role for Switch II-Switch III communication in some event associated with G protein activation, such as the GTP-mediated dissociation of rhodopsin and/or βγ from αT or the GTP-dependent interaction of αT with the cyclic GMP PDE. In the present work, we have examined the importance of Switch III in G protein function and find that a conserved glutamic acid residue within the Switch III domain of αT is essential for the regulation of target-effector activity.

**EXPERIMENTAL PROCEDURES**

**Purification of Retinal Proteins**—Rod outer segments (ROS) were prepared as described previously (12, 13). Transducin and the cyclic GMP PDE were purified by exposing ROS to room light and repeated washings with 10 mM Hepes, pH 7.5, 6 mM MgCl₂, 1 mM dithiothreitol (DTT), and 100 mM NaCl (isotonic buffer) and then with 10 mM Hepes, pH 7.5, 6 mM MgCl₂, and 1 mM DTT (hypotonic buffer). The cyclic GMP

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‡ To whom correspondence should be addressed. Tel.: 607-253-3888; Fax: 607-253-3659.

1 The abbreviations used are: PDE, phosphodiesterase; ROS, rod outer segment(s); DTT, dithiothreitol; GTPγS, guanosine 5′-O(thiotriphosphate).
PDE is released into the hypotonic wash and is further purified by hydroxyapatite chromatography (13). Transducin is released from ROS by washing with hypotonic buffer in the presence of 0.1 mM GTP or 0.1 mM GTPyS. The αT and βγT subunit complexes are then resolved by Blue Sepharose chromatography (14).

Measurement of GMP PDE Activity—Dark-adapted ROS membranes containing hydroxyapatite-purified cyclic GMP PDE were assayed for cyclic GMP hydrolysis by measuring proton release, as originally described by Yee and Liebman (15). The assays are performed at room temperature in a buffer containing 10 mM Hepes, pH 8.0, 60 mM KCl, 30 mM NaCl, 1 mM DTT, and 5 mM MgCl₂, together with the protein components described in the legend to Fig. 3. The assays were initiated by the addition of cyclic GMP (5 mM), with the pH being recorded for 1–2 min at one determination/s. The PDE activity (nanomoles) was calculated as the ratio of the slope of the pH change (millivolts) and the buffering capacity of the medium (millivolt/nmol) (16).

Expression of Recombinant αT Subunits—The coding region of the bovine αT was amplified by the polymerase chain reaction using primers that create a 5'-end NdeI site and a 3'-end BamHI site. The polymerase chain reaction product was digested with NdeI and BamHI and ligated into pET15b (Novagen). The resultant vector was digested with NcoI and PstI to release the coding region of αT, with its 5'-end fused in-frame to the hexa-His tag present in the vector pET15b. This released fragment was then blunt-ended using T4 DNA polymerase and ligated into pVL1393. To generate the αTΔ1 deletion mutant (Asp227 through Asn237 replaced by a single Ala), and the αTΔ232L point mutant (Glu232 replaced by Leu), we employed a single-stranded DNA-based mutagenesis strategy (17), using synthetic oligonucleotide primers containing the indicated deletion or point mutation (αTΔ1: 5'-CAGGCTCTCCTGATTCATTGCCGAGCTCAG-3' and αTΔ232L: 5'-CACCTGCTGCTTGAGCACCAGGC-3'). The pVL1393 vector carrying either the wild type, αTΔ1, or αTΔ232L gene was introduced into Sf9 insect cells using the Baculogold transfection kit (PharMingen). The recombinant extracellular virus (rECV) was purified by a limiting dilution procedure (18). For production of the recombinant proteins, Sf9 insect cells were infected at 80% confluence with the purified rECVs at a multiplicity of infection of 5 and harvested typically 60 h post-infection. The His-tagged αT proteins were purified through Ni²⁺-nitrilotriacetic acid affinity chromatography following a protocol provided by Qiagen. The purified proteins were then dialyzed against HMDN buffer (20 mM Hepes, pH 7.4, 5 mM MgCl₂, 150 mM NaCl, and 1 mM DTT) containing 40% of glycerol and stored at −20 °C.

RESULTS AND DISCUSSION

The original finding that a third region on heterotrimeric G protein α subunits undergoes structural changes upon GTP-GDP exchange (i.e., in addition to the Switch I and Switch II regions originally identified in Ras and EF-Tu (10, 11)) suggests that it may play a critical role in a GTP-dependent G protein function. To obtain experimental support for this suggestion, we examined the properties of an αT deletion mutant in which the entire Switch III domain (residues Asp⁹⁷⁷ through Asn³⁷⁷) was replaced by a single alanine residue. The deletion mutant, designated αTΔ₁₂, was expressed in Spodoptera frugiperda (Sf9) cells as a hexahistidine (His)-tagged fusion protein and purified by Ni²⁺ affinity chromatography. This results in a rapid and highly effective purification of the recombinant αT subunit, as shown in Fig. 1. The first three lanes in A show the Coomassie Blue-stained profiles for the αT subunit purified from bovine retina, the recombinant wild type His-tagged αT purified from Sf9 cells (which has a slightly slower mobility on SDS gels because of the His-tag), and the His-tagged αTΔ₁₂ mutant purified from Sf9 cells. B shows the corresponding Western blots that were obtained using a specific antibody raised against the carboxy-terminal 10 amino acids of αT (16).

We first examined whether the deletion of the Switch III domain from αT affected rhodopsin- and βγT-p promoting [³²P]GTPyS/GDP exchange. Fig. 2A shows that as has been documented previously (19, 20), when αT purified from bovine retina was added to urea-stripped ROS containing light-activated rhodopsin, there was a marked increase in [³²P]GTPyS binding that was strongly stimulated by the addition of purified retinal βγT. Virtually identical results were obtained with the Sf9-expressed, His-tagged wild type αT and the His-tagged αTΔ₁₂ deletion mutant. Likewise, the αTΔ₁ site mutant was able to fully hydrolyze [γ⁻³²P]GTP (Fig. 2B). Taken together, the results presented in Fig. 2, A and B, indicated that the deletion of the Switch III domain did not impair the ability of αT to interact with rhodopsin nor with the βγT subunit complex and that removal of Switch III did not interfere with the GTP-binding/GTP hydrolytic cycle of the G protein.

We then examined the ability of the αTΔ₁₂ mutant to functionally couple to the cyclic GMP PDE, by first loading the αTΔ₁₂ mutant with GTPyS (by incubation with ROS and bovine retinal βγT) and then assaying cyclic GMP PDE activity, by measuring the H⁺ release that accompanies cyclic GMP hydrolysis. The results presented in Fig. 3A illustrate that the bovine retinal αT subunit and the recombinant wild type αT were essentially equivalent in their abilities to stimulate cyclic GMP hydrolysis. However, the αTΔ₁₂ deletion mutant was unable to stimulate PDE activity (relative to the basal activity measured in the absence of added αT). Thus, these results suggested that the integrity of the Switch III domain was essential for αT-mediated regulation of its target/effecter enzyme.

An interesting possibility that was originally proposed following an examination of the x-ray crystallographic structure of the αT-GTPyS complex (3) was that the acidic amino acid residues, Asp³⁷⁷, Asp³⁸⁴, and Glu³⁸⁵ formed a potential binding site for a basic stretch of amino acids on the γ subunit. If this were the case, it would then explain why the deletion of the Switch III domain yields an αT subunit that is unable to stimulate effector activity. However, we have expressed and purified an αT mutant from Sf9 cells in which the three acidic amino acids were replaced by alanine residues and found that this triple mutant was fully active, not only in its ability to bind and hydrolyze GTP, but also in its ability to stimulate cyclic GMP PDE activity (data not shown). This then led us to examine another possibility, namely that a conserved glutamic acid residue in the Switch III region, Glu³⁸⁵ is responsible for mediating the conformational communication between the Switch I and Switch III domains (4). To test this, we generated a mutant of αT in which a leucine residue was substituted for Glu³⁸⁵ (αTΔE232L) and expressed it.
in S. frugiperda (S19) insect cells as a hexahistidine (His)-tagged protein (see lane 4 in Fig. 1, A and B). Like the α2Δ12 deletion mutant, we found that the α2E232L mutant was able to functionally couple to rhodopsin and/ or βγt, as read-out by its ability to undergo [35S]GTPγS/GDP exchange and GTP hydrolysis in a rhodopsin- and βγt-dependent manner (Fig. 2, A and B). Moreover, just as was the case for the Switch III deletion mutant, the α2E232L mutant was unable to stimulate target/effectector (PDE) activity (Fig. 3A), even when using amounts of the mutant that were in 10-fold excess relative to the retinal or recombinant wild type α2 proteins (data not shown). Thus, the mutation of the single conserved Glu232 residue appeared to fully mimic the effects obtained upon the removal of the entire Switch III domain.

We used the α2E232L mutant to further examine the importance of Switch II domain-Switch III domain coupling in the stimulation of target/effectector activity. We found that the inability of the α2E232L mutant to stimulate PDE activity cannot be attributed to its inability to bind to its PDE target. This was determined through competition experiments. Fig. 3B shows that like the GDP-bound wild type α2 subunit (open bar in column 2), the GDP-bound form of the α2E232L mutant (open bars in columns 3 and 4) did not competitively inhibit the PDE stimulatory activity of the GTPγS-bound retinal α2 subunit (shown as the solid bar in column 1). This was as expected, since the GDP-bound form of α2 has only a weak affinity for the γPDE subunit. However, the GTPγS-bound form of α2E232L showed a dose-dependent inhibition (hatched bars in columns 3 and 4 in Fig. 3B), thus indicating that the α2E232L mutant can bind to γPDE in a GTPγS-dependent manner. The fact that the GTPγS-bound wild type α2 subunit did not competitively inhibit the stimulatory activity of the retinal GTPγS-bound α2 (column 2), 20 nM recombinant α2E232L mutant (column 3), or 40 nM recombinant α2E232L mutant (column 4) in either the GDP-bound (open bars) or GTPγS-bound state (hatched bars). The PDE activity stimulated by the bovine retinal α2 in the absence of any competitor (column 1, solid bar) is set as 100% (800–1000 nmol of cGMP hydrolyzed per s and per nmol of PDE).
bound wild type αT yields two stable fragments, an ∼23-kDa fragment (shown in lane 2 under αTwt in Fig. 4) and an ∼9-kDa fragment (not shown), whereas trypsin treatment of the GTPγS-bound wild type αT yields a stable 32-kDa (precursor) fragment (lane 3 under αTtet in Fig. 4). Based on the information provided from the tertiary structures for the different nucleotide forms of αT (4, 5), it is now clear that the protection afforded by GTPγS directly reflects a GTPγS-dependent conformational change that occurs within the Switch II domain and effectively moves the trypsin-sensitive Arg204 residue from a solvent-exposed environment to a less accessible position (by virtue of its interaction with Glu232). Thus, the protection against trypsin proteolysis afforded by GTPγS serves as a highly sensitive read-out for GTPγS-induced conformational changes within the Switch II domain and has frequently been used as a monitor for αT activation (23). The results presented in Fig. 4 (lanes 2 and 3 under αTE232L) show that GTPγS binding to the αTE232L mutant provides a similar protection against trypsin proteolysis, as observed with the wild type αT subunit. Therefore, the mutation of Glu232 neither perturbs GTPγS binding nor the GTPγS-induced conformational alteration of Switch II. However, mutation of Glu232, while preserving the GTP-dependent binding of the αT subunit to the cyclic GMP PDE, completely uncouples this binding from target/effecter stimulation.

The location of Glu232 in the loop connecting the β4 strand and α3 helix of αT places it in a prime position to couple conformational transitions between Switch II and Switch III. In particular, x-ray crystallographic analysis shows that upon GTPγS binding, Glu232 is engaged in direct interactions with Arg201 and Arg204 of Switch II and in a water-mediated interaction with Gly199 of Switch II (5). Given our findings, we conclude that the conformational coupling between Switch II and Switch III is responsible for converting a primary binding interaction between activated αT and its target (γPDE), perhaps involving residues in Switch II (24) or in other regions of αT (25–27), into a secondary stimulatory interaction between the γPDE subunit and the αCβ7C residues 305–314 of αT (28). Moreover, these results indicate that Glu232 plays an essential role in mediating this conformational coupling, thereby translating αT-target (PDE) interactions into a specific regulatory event. The fact that this glutamic acid residue is conserved in all trimeric Gα subunits further suggests that it plays a fundamental role in converting target binding into target/effecter regulation in a wide variety of G protein-coupled signaling pathways.

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