Loss of the Effector Function in a Transducin-α Mutant Associated with Nougaret Night Blindness*

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A missense mutation, G38D, was found in the rod transducin α subunit (Gαt) in individuals with the Nougaret form of dominant stationary night blindness. To elucidate the mechanism of Nougaret night blindness, we have examined the key functional properties of the mutant transducin. Our data show that the G38D mutation does not alter the interaction between Gαt and Gβγ or activation of transducin by photoexcited rhodopsin (R*). The mutant Gαt has only a modestly (2.5-fold) reduced kat value for GTP hydrolysis. The GTPase activity of GαtG38D can be accelerated by photoreceptor regulator of G protein signaling, RGS9. Analysis of the GαtG38D interaction with cGMP phosphodiesterase revealed marked impairment of the mutant effector function. GαtG38D completely fails to bind the inhibitory PDE γ subunit and activate the enzyme. Altogether, our results demonstrate a novel molecular mechanism in dominant stationary night blindness. In contrast to known forms of the disease caused by constitutive activation of the visual cascade, the Nougaret form has its origin in attenuated visual signaling due to loss of effector function by transducin G38D mutant.

The visual transduction cascade in vertebrate rod photoreceptor cells is among the best understood G protein signaling systems. In the outer segments of rod photoreceptor cells (ROS),1 the visual G protein, transducin (Gt), couples the light receptor, rhodopsin (R), to the effector enzyme, cGMP phosphodiesterase (PDE). Photolyzed rhodopsin (R*) binds Gt and induces GDP/GTP exchange on the Gαt subunit, which upon dissociation from R* and Gβγ activates PDE. To activate the enzyme, Gαt-GTP interacts with the inhibitory PDE γ subunits (Pγ) and prevents them from blocking catalytic sites on the PDE αβ subunits. The following decrease in intracellular cGMP concentration leads to a closure of cGMP-gated channels in the ROS plasma membrane (1–3). An analogous visual cascade operates in cone photoreceptor cells. Unlike the cones that mediate high intensity daytime color vision, rod photoreceptors are designed for dim light vision during nighttime.

Defects in the genes encoding key components of the visual transduction cascade have been linked to a number of retinal diseases. Certain forms of retinitis pigmentosa, a progressive photoreceptor degeneration, are caused by mutations in rhodopsin (4), α- and β-subunits of PDE (5–7), or the α-subunit of the cGMP-gated channel (8). Mutations in rhodopsin (9, 10), PDEβ (11), or Gαt (12) have been identified in different forms of congenital stationary night blindness. Stationary night blindness is not associated with retinal degeneration and manifests itself in the inability to see in the dark, whereas daytime vision is largely unaffected. A missense Gαt mutation, G38D, was found in the Nougaret form of congenital stationary night blindness (12). Initially, it was thought that Nougaret night blindness resulted from a loss of rod function. However, a recent study using electroretinographic analysis of Nougaret patients has indicated the presence of a detectable albeit subnormal rod function coupled with slight impairment of cone function (13). A clear outcome of the disease is a significant loss of rod light sensitivity (13).

Functional consequences of the G38D mutation for transducin signaling have not been examined, although a Gly residue at the corresponding position in other GTP-binding proteins has been actively investigated. This Gly is located within the conserved P-loop with the consensus sequence GXXXX(X/T) that binds the α- and β-phosphate of GDP and GTP in the superfamily of G proteins (14). One of the most common transforming mutations in p21ras is a substitution of the corresponding residue Gly-12 by Val. This mutation essentially blocks the GTPase activity of p21ras and prevents its stimulation by GAPs leading to constitutive activation of p21ras-mediated pathways (15–17). Recently, a similar observation has been made for the analogous mutant of Gαs, G42V, which hydrolyzes GTP with a 30-fold lower rate than that of the parent protein (18). The ability of GαsG42V to inhibit the effector enzyme, adenyl cyclase, was not evaluated. Interestingly, Gαs, in which the Gly residue is replaced by Ser, has a characteristically low kat for GTP hydrolysis (19). The biochemical properties of p21rasG12V and GαsG42V seem to point to the constitutive activity of the GαsG38D mutant as a cause of Nougaret night blindness. However, the alternative possibility that the GαsG38D mutation leads to an inactive visual cascade remained. Such a possibility is supported by analysis of an analogous Gαt mutant, GαtG49V. The rate of GTP hydrolysis for GαsG49V is ~4-fold lower than that for Gαt and the GTP-bound mutant is capable of activation of adenylyl cyclase in vitro (20). However, GαsG49V was a very poor activator of adenylyl cyclase when expressed in a cyc-S49 cell line (21). The loss of light sensitivity of the Nougaret rod photoreceptors could potentially be caused either by the desensitization due to constitutive activity of Gαt or by reduced ability to transduce a visual signal due to impaired Gαt function.

To identify the molecular mechanism of Nougaret night blindness, we have introduced the G38D substitution into Gαt-
like, effector-competent, Ga\(\alpha\)/Ga\(\alpha\) chimeric \(-\alpha\)-subunit. An extensive examination of the functional properties of this mutant has revealed a complete loss of the effector function.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(^{35}\)S]GTP-S (1160 Ci/mmol) and [\(^{32}\)P]NAD (1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Restriction enzymes were from New England Biolabs. T4 DNA ligase was from Roche Molecular Biochemicals. Cloned Pfu DNA polymerase was from Stratagene. TFE-treated trypsin was from Worthington. All other chemicals were from Sigma or Fisher. Urea-washed ROS membranes were prepared as described previously (22). Bovine ROS membranes were prepared as described previously (22). Urea-washed ROS membranes (uROS) prepared from the trypsin-protected assay. When the trypsin digestion was performed with 25 \(\mu\)M trypsin/ml for 15 min at 25 \(^\circ\)C and stopped with simultaneous addition of SDS sample buffer and heat treatment (100 \(^\circ\)C, 5 min).

**GTP-S Binding Assay**—gloN prostas (1 \(\mu\)M) alone, mixed with 2 \(\mu\)M Ga\(\alpha\) or 2 \(\mu\)M Ga\(\alpha\)* (0.5 \(\mu\)M rhodopsin) were incubated for 3 min at 25 \(^\circ\)C. Binding reactions were started by addition of 5 \(\mu\)M [\(^{35}\)S]GTP-S (0.2 Ci). Aliquots of 50 \(\mu\)l were withdrawn at the indicated times, mixed with 1 ml of ice-cold 20 \(\mu\)M Tris-\(\mathrm{HCl}\) (pH 8.0) buffer containing 130 mM NaCl and 10 mM MgSO\(_4\), and passed through Whatman cellulose nitrate filters (0.45 \(\mu\)m). The filters were then washed with 30 \(\mu\)l with the same buffer (2 ml, ice-cold) and counted in a liquid scintillation counter. After the filter was dried, 3 \(\mu\)l peroxidase (preactivated with 100 mM dithiothreitol and 0.25% SDS for 10 min at 30 \(^\circ\)C). The reaction was started by addition of 5 \(\mu\)M [\(^{32}\)P]NAD and allowed to proceed for 1 h at 25 \(^\circ\)C. Afterward, reaction mixtures were diluted with 1 ml of ice-cold 20 \(\mu\)M Tris-\(\mathrm{HCl}\) (pH 8.0) buffer containing 100 mM NaCl and filtered through Whatman cellulose-nitrate filters. The filters were washed four times with the same buffer and counted in a liquid scintillation counter. In some experiments, 10-\(\mu\)l aliquots were withdrawn from reaction mixtures, mixed with sample buffer for SDS-PAGE, and analyzed by electrophoresis in 12% gels.

**Single-turnover GTPase Assay**—Single-turnover GTPase activity measurements were carried out in suspensions of uROS membranes (5 \(\mu\)M rhodopsin) reconstituted with Ga\(\alpha\)* or Ga\(\alpha\)*/Ga\(\delta\) (2 \(\mu\)M) and Ga\(\beta\)* (12 \(\mu\)M) as described for the trypsin-removal assay. When the trypsin digestion was performed with 25 \(\mu\)M trypsin/ml for 15 min at 25 \(^\circ\)C and stopped with simultaneous addition of SDS sample buffer and heat treatment (100 \(^\circ\)C, 5 min).

**RESULTS**

**Expression and Trypsin Sensitivity of the Ga\(\alpha\)*/Ga\(\delta\) Mutant**—A mutation, G38D, was introduced into the Ga\(\alpha\)*-like Ga\(\alpha\)/Ga\(\delta\) chimeric protein (25), which contained –94% of Ga\(\alpha\) residues including the two key Ga\(\alpha\) effector residues His-244 and His-247 and carried a Ga\(\alpha\)-like GTPase activity (26). Ga\(\alpha\)* was chosen as a template for mutagenesis because it has efficient expression, which allows complete examination and comparison of functional properties of Ga\(\alpha\)* and Ga\(\alpha\)*/Ga\(\delta\). Expression levels in E. coli of soluble mutant Ga\(\alpha\)*/Ga\(\delta\) were analogous to those of Ga\(\alpha\)* (–4–5 mg/liter of culture). Activation of Ga\(\alpha\) subunits induces a conformational change that protects the Ga switch II region from proteolysis with trypsin. Fig. 1 demonstrates the

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The provided text is too large to be fully transcribed here. It contains detailed experimental procedures and results related to the study of Ga\(\alpha\), Ga\(\alpha\), and Ga\(\alpha\)*/Ga\(\delta\) chimeric proteins. The text is technical and scientific in nature, focusing on methods for preparing and analyzing proteins, as well as detailed results from experiments performed on these proteins. The text includes methods for protein expression, purification, and functional assays, along with results indicating the absence of effector function in Ga\(\alpha\)*/Ga\(\delta\) mutants.
results of the trypsin-protection assay for Gαt, and GαtG38D. Both proteins were capable of undergoing an activation-dependent conformational change upon R* induction of GTPγS (Fig. 1). However, the GDP-AlF4-induced conformation of GαtG38D is different from that of Gαt, as indicated by the lack of its resistance to trypsin in the presence of AlF4. Such a trypsin resistance pattern of GαtG38D is consistent with similar results reported for the G42V mutant of Gαt (18).

**Kinetics of R* dependent GTPγS binding to Gαt and GαtG38D—**The ability of R* to activate Gαt and GαtG38D was examined by measuring their GTPγS-binding kinetics in the presence of Gβγ. The rate of GTPγS-binding is controlled by a rate-limiting GDP release of Ga subunits. Similarly to native Gαt (25), GαtG38D and GαtG38D in the absence (data not shown) or in the presence of Gβγ (Fig. 2) displayed very slow intrinsic rates of GDP release as measured by GTPγS binding. This observation, that the GαtG38D mutation does not significantly affect a high affinity of Gαt for GDP, is in agreement with the GDP binding properties of GαtG42V (18). In the presence of uROS membranes (100 nM R* and Gβγ (2 μM), the rates of GTPγS binding were markedly enhanced (Fig. 2). The comparable R* induced GTPγS binding rates by GαtG38D (k_{app} = 0.17 min⁻¹) and GαtG38D (k_{app} = 0.11 min⁻¹) indicate that rhodopsin recognition in the mutant GαtG38D is generally not impaired.

**Pertussis Toxin-catalyzed ADP-ribosylation of Gαt and GαtG38D—**Pertussis toxin effectively ADP-ribosylates Gαt-GDP at Cys-347 (36, 37). The heterotrimeric complex, GαtGβγγ is a notably better substrate than Gαt alone (38), making ADP-ribosylation a useful tool to assess Gαt-Gβγγ interaction with Gβγγ. The GTPγS-binding properties of GαtG38D in the presence of R* and Gβγγ indicated that the mutation did not grossly alter the GαtG38D binding to Gβγ. However, an excess of Gβγγ was used in the binding assay to avoid the influence of potential defects in the GαtG38D/Gβγ coupling on activation of GαtG38D by R*.

To further examine the interaction of GαtG38D with Gβγγ, we carried out a pertussis toxin-catalyzed ADP-ribosylation of Gαt and GαtG38D in the presence of increasing concentrations of Gβγγ. The dose dependences of Gβγγ-supported ADP-ribosylation of Gαt and GαtG38D were similar, suggesting that GαtG38D retains intact interaction with Gβγγ (Fig. 3).

**GTPase Activity of GαtG38D and Effects of RGS9—**Unaltered interaction of GαtG38D with Gβγγ and activation by R* allowed examination of mutant GTPase activity under single turnover conditions. The GTPase activities of Gαt and GαtG38D were measured in the reconstituted system with Gβγγ and uROS membranes. uROS membranes lack the activity of a photoreceptor GAP, RGS9 (27). The calculated k_{cat} for GTP hydrolysis by GαtG38D was 0.020 ± 0.003 s⁻¹ (Fig. 4A), GαtG38D hydrolyzed GTP with a notably lower rate (k_{cat} of 0.008 ± 0.0004 s⁻¹) (Fig. 4B). The reduction in the k_{cat} value for GTP hydrolysis caused by the GαtG38D mutation (−2.5-fold) is proportional to that observed in the G42V mutant of Gαt (20) but considerably smaller than a 30-fold decrease in the k_{cat} of the GαtG42V mutant (18). The GTPase activity of the p21ras/G12V mutant is insensitive to the p21ras GAP (17). We tested the effects of a truncated RGS9 protein (amino acids 284−461) containing the RGS domain on rates of GTP hydrolysis for Gαt and GαtG38D. Addition of 5 μM RGS9−284−461 resulted in a 10-fold decrease in the GTPase activity of Gαt by 6-fold (k_{cat} = 0.12 ± 0.01 s⁻¹) (Fig. 4A) and of GαtG38D by 3-fold (k_{cat} = 0.023 ± 0.002 s⁻¹).

**Effectors Properties of GαtG38D—**A fluorescence read-out assay was used to study the interaction between GαtG38D and the Pγ subunit. It utilizes the Pγ subunit labeled at Cys-68 with the fluorescent probe, 3-(bromoacetyl)-7-diethyl aminocoumarin (Pγ-BC) (31). Binding of Gαt to Pγ-BC causes a large
increased in the probe fluorescence. Using this assay, affinities of Gaα*GTPγS (Kd 1.7 ± 0.3 nM) or Gaα*AlF4 (Kd 3.2 ± 0.3 nM) for PγBC were similar (Fig. 5). Remarkably, Gaα*G38D in both the GTPγS- and AlF4-activated conformations showed no detectable interaction with PγBC (Fig. 5). To test the possibility that Gaα*G38D binds PγBC without causing a fluorescence increase, we investigated the binding of Gaα* to PγBC in the presence of high concentrations of Gaα*G38D. No competition between Gaα* and Gaα*G38D for binding to PγBC was detected as the Kd value for the Gaα*/PγBC interaction was essentially unchanged in the presence of 100 nM Gaα*G38D (Fig. 5A). This result suggests the Gaα* mutation leads to a loss of the effector function. To confirm this conclusion, we evaluated the ability of Gaα*G38D to stimulate activity of holoPDE reconstituted with uROS membranes and Gβγ in the presence of GTPγS. For comparison, Gaα* (2 μM) was capable under these conditions of stimulating basal PDE activity by ~18-fold (Fig. 6). Gaα*G38D failed to activate cGMP hydrolysis (Fig. 6). Moreover, excess of the GTPγS-bound Gaα*G38D did not interfere with activation of holoPDE by Gaα*, further supporting the lack of competition between Gaα* and Gaα*G38D for the effector molecule (Fig. 6).

**DISCUSSION**

Heterotrimeric G proteins transduce a variety of extracellular signals (neurotransmitters, hormones, light, olfactory and taste signals) from specific cell surface receptors to intracellular effectors. Mutations in Ga subunits that lead to abnormal signaling are known to cause a large number of diseases (40). Abnormal G protein signaling might result from either attenuated or elevated signal transduction. Mutations of Ga subunits leading to lower protein stability, reduced ability for interaction with and activation by cognate receptors, inability to dissociate Gβγ, or diminished capacity to activate effectors would attenuate G protein transduction. Mutations causing an increase in spontaneous Gaα GDP/GTP exchange, a lower GTPase activity, or impairment of interaction with GAP proteins are often a source of excessive G protein signaling.

Recently, a mutation in the visual G protein, Gaα38D, was found in patients with the Nougaret form of dominant stationary night blindness (12). Although psychophysical and electrophysiological consequences of the Nougaret night blindness enhance the loss of photoreceptor function.
have been thoroughly investigated (13), the molecular mechanism of the disease remained unclear. Despite a lack of biochemical characterization of Goa38D, a wealth of information has been accumulated on mutations of an analogous Gly residue in small and heterotrimeric G proteins. The G12V mutation in p21ras produces constitutively active signaling by inhibiting the p21ras-GTPase activity and abolishing its stimulation by GAP proteins (15-17). Likewise, the Goa42V mutant has a 30-fold lower k_cat for GTP hydrolysis in comparison to the wild-type Goa (18). By analogy with the p21ras and Goa42V mutants, constitutive activity of Goa38D becomes the most appealing model for abnormal function of rod photoreceptors in the Nougaret pedigree. Persistent activation of PDE by the GTP-bound Goa38D would also provide a simple explanation to the dominant inheritance of the disease (12). Moreover, such a hypothesis would be consistent with existing biochemical evidence on dominant night blindness caused by two mutations in rhodopsin, G90D and A292E (9,10). Both rhodopsin mutants are constitutively active and capable of activating transducin even in the absence of the retinal chromophore (9,10). The PDE β-subunit gene, a third gene implicated in dominant stationary night blindness, carries a missense mutation H258N (11). Although properties of the PDE mutant are not known, it has been suggested that it might be constitutively active due to impaired interaction with the Pγ subunit (11). Finally, an Oguchi disease, a recessive form of stationary night blindness, was found to be associated with null mutations in the rhodopsin kinase gene (41). Rhodopsin kinase is intimately involved in inactivation of R*. Transgenic mice lacking rhodopsin kinase displayed larger and longer than normal single-photon responses (42). The excessive signaling is thought to saturate rod responses at abnormally low light-intensities in Oguchi disease (42). Similarly to light adaptation, constitutive activation of the visual cascade would cause desensitization of rod photoreceptors by lowering cGMP levels, keeping the cGMP-gated channels closed, the plasma membrane hyperpolarized, and lowering intracellular Ca^2+ concentration. Supporting the hypothesis for constitutive activity of Goa38D, some of the abnormalities seen in the rod and cone functions of Nougaret patients have been simulated by light adaptation of the normal retina (13). However attractive, constitutive activity toward effectors of Goa mutants with substitution of the Gly residue has not been firmly established. Examination of Goa42V revealed that it can normally stimulate adenylyl cyclase in the reconstituted system (20), but only poorly in cyc^-S49 cells expressing the mutant (21).

To elucidate the biochemical mechanism of Nougaret night blindness, we investigated the key functional properties of Goa38D, such as interaction with R* and Gγγγ, GTPase activity, interaction with RGS9, binding the Pγ subunit, and the ability to stimulate PDE. Goa42V interaction with Gβγ and activation by R* was found largely intact. In comparison with Goa*, the mutant had only a very modest ~2.5-fold reduction in the k_cat value for GTP hydrolysis. The decrease in Goa38D GTPase activity was significantly smaller than that seen in the G12V G42V or G42S mutants (18). In addition, unlike Goa42V (18), Goa38D retained reduced ability to interact with RGS proteins, particularly, with a photoreceptor-specific RGS9. In contrast, the effector function of Goa38D is markedly impaired. Goa38D fails to bind Pγ and activate PDE. The inability of the G38D mutant, in a background of wild-type Goa, to interact with the effector molecule was demonstrated using in vitro translated Goa and Goa38D. Lack of trypsin protection of Goa38D (or Goa42V) in the presence of AlF4^- indicates that the active conformation of the switch II region of the mutant Goa may differ from that in wild-type transducin. The switch II region of Goa is an essential effector binding domain (26,43), and alterations in this region provide a plausible rationale for the loss of effector function. Moreover, similar patterns of sensitivity to trypsin between active conformations of Goa42V (18) and Goa38D allow us to speculate that these mutations may have caused analogous conformational changes. A crystal structure of the GTPγS bound Goa42V shows that the Val-42 side chain forces the peptide planes of the switch II residues 203-206 to rotate leading to disruption of ionic contacts between Arg-205 and the switch III/α3-helix residues Asp-237 and Glu-245 (18). If a similar conformational change is caused by the Goa38D mutation, it would break the linkage between the Goa switch II Arg-201 and the switch III/α3-helix residues Glu-232 and Glu-241 (44). This linkage is central to the ability of Goa to assume the effector-competent conformation (26,45). All mutations of the Goa switch II region residues that are involved in the linkage with switch III/α3-helix have resulted in a severe impairment of Goa effector function (26).

An absolute inability of Goa38D to activate PDE may only in part account for desensitization of rod photoreceptors in the Nougaret form of dominant night blindness. Heterozygous Nougaret patients, who presumably have about 50% functional transducin, display more than 99% reduction in rod sensitivity (13). The observed rod sensitivity loss implies that Goa38D somehow interferes with expression of the wild-type allele, or the mutant has dominant negative properties. We demonstrated that Goa*38D is unable to prevent PDE activation by Goa*. Despite the lack of evidence to support the dominant negative nature of the G38D mutant, such a possibility cannot be ruled out. Goa*38D is fully capable of interaction with R*, and under dim light conditions and very low concentrations of R*, the mutant Goa may potentially compete with wild-type Goa for R*. Such a competition would slow the rate of formation of Goa*GTP and, consequently, attenuate PDE activation.

Overall, our results demonstrate a novel molecular mechanism in dominant stationary night blindness. In contrast to stationary night blindness caused by constitutive activation of the visual cascade by rhodopsin mutants (9,10), the Nougaret form has its basis in decreased visual signaling due to loss of transducin effector function.

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