Interaction of Transducin with Uncoordinated 119 Protein (UNC119)

**IMPLICATIONS FOR THE MODEL OF TRANSDUCIN TRAFFICKING IN ROD PHOTORECEPTORS** *‡*

The key visual G protein, transducin undergoes bi-directional translocations between the outer segment (OS) and inner compartments of rod photoreceptors in a light-dependent manner thereby contributing to adaptation and neuroprotection of rods. A mammalian uncoordinated 119 protein (UNC119), also known as Retina Gene 4 protein (RG4), has been recently implicated in transducin transport to the OS in the dark through its interaction with the N-acylated GTP-bound transducin-α subunit ($\text{G}_{\alpha_1}$). Here, we demonstrate that the interaction of human UNC119 (HRG4) with transducin is dependent on the N-acylation, but does not require the GTP-bound form of $\text{G}_{\alpha_1}$. The lipid specificity of UNC119 is unique: UNC119 bound the myristoylated N terminus of $\text{G}_{\alpha_1}$ with much higher affinity than a prenylated substrate, whereas the homologous prenyl-binding protein PrBP/δ did not interact with the myristoylated peptide. UNC119 was capable of interacting with $\text{G}_{\alpha_1}$GDP as well as with heterotrimeric transducin ($\text{G}_t$). This interaction of UNC119 with $\text{G}_t$ led to displacement of $\text{G}_{\beta_1}\gamma_1$ from the heterotrimer. Furthermore, UNC119 facilitated solubilization of $\text{G}_t$ from dark-adapted rod OS membranes. Consistent with these observations, UNC119 inhibited rhodopsin-dependent activation of $\text{G}_t$ but had no effect on the GTP-hydrolysis by $\text{G}_{\alpha_1}$. A model for the role of UNC119 in the IS→OS translocation of $\text{G}_t$ is proposed based on the UNC119 ability to dissociate $\text{G}_t$ subunits from each other and the membrane. We also found that UNC119 inhibited activation of $\text{G}_t$ by D2 dopamine receptor in cultured cells. Thus, UNC119 may play conserved inhibitory role in regulation of GPCR-G protein signaling in non-vertical tissues.

In rod photoreceptors, exposure to bright light causes translocation of the visual G protein, transducin from the photosensitive outer segments (OS)² to the inner compartments of the cells (reviewed in Refs. 1–3). The light-dependent translocation of transducin is thought to play an important role in light-adaptation and neuroprotection (4, 5). Significant advances have been made in understanding the mechanism of this phenomenon. The current evidence supports a simple diffusion model, whereby the activation of transducin by photoexcited rhodopsin (R*) causes dissociation of transducin-α ($\text{G}_{\alpha_1}$) and $\text{G}_{\beta_1}\gamma_1$ subunits allowing them to diffuse into the inner segment (1–9). However, translocated transducin must return to the OS during dark adaptation to restore rod sensitivity. This retrograde translocation occurs on a relatively slow time scale with a half-life of 2.5 h (4). The precise mechanism of transducin return to the OS in the dark is not known. Formation of heterotrimeric $\text{G}_t$ in the inner segment (IS) appears to be a prerequisite for correct transport of transducin to the OS. Heterotrimeric $\text{G}_t$ forms in the IS in the absence of R* following hydrolysis of $\text{G}_{\alpha_1}$-bound GTP. GTP and GTPγS both caused light-dependent transducin redistribution from the OS in permeabilized retinas, but only GTP-translocated $\text{G}_t$ returned to the OS in the dark (8). Likewise, the time course of the IS→OS transport in the dark was exceedingly slow for the GTPase deficient $\text{G}_{\alpha_1}$, Q200L mutant (6). The kinetics of $\text{G}_{\alpha_1}$ and $\text{G}_{\beta_1}\gamma_1$ return to the OS in the dark is identical, supporting the transport of $\text{G}_t$ as the heterotrimer (4). In dark-adapted $\text{G}_{\alpha_1}$ knock-out mice $\text{G}_{\beta_1}\gamma_1$ is mislocalized and spread throughout the photoreceptor cells (10). Furthermore, $\text{G}_{\alpha_1}$ is severely down-regulated and mislocalized in $\gamma_2$ knock-out mice (11). Thus, $\text{G}_{\alpha_1}$ and $\text{G}_{\beta_1}\gamma_1$ depend on each other for proper targeting.

Transducin may return to the OS in dark by a motor-driven mechanism, diffusion or a combination of the two transport modes. The apparent energy-independence of the $\text{G}_t$ return transport suggests diffusion as a primary mode (12). $\text{G}_t$ is modified with two lipid anchors, fatty acyl at the N-terminal Gly of $\text{G}_{\alpha_1}$ and thioether-linked farnesyl attached to the C terminus of $\gamma_2$ (13–15). Two lipid anchors are generally thought to be sufficient for stable membrane attachment of a protein that precludes protein dissociation and diffusion in the cytosol (16–19). Intriguingly, $\text{G}_t$ is capable of significant interdisc transfer in...
the OS (20, 21). We hypothesized that the longitudinal diffusion of Gt1β1γ1, which may allow to “refill” apical discs with Gt during dark adaptation, is facilitated by sequestration of one or the other subunit via interactions with a binding partner (21). The same mechanism may underlie the entire IS → OS route of Gt in the dark. Potential partners include phosducin and prenyl-binding protein PrBP/δ also known as PDEδ (22–25). PrBP/δ, in particular, was shown to be critical for the IS → OS transport of a number of prenylated proteins (26).

UNC119, a mammalian ortholog of Caenorhabditis elegans unc-119 (27), also known as Retina Gene 4 protein (RG4), has recently emerged as the protein essential for transducin transport in darkness (28). Human UNC119 (HRG4) was originally identified in a screen for candidate retinal degeneration genes (29). The C-terminal domain of UNC119 shares significant sequence and structural homology with PrBP/δ (28, 30). UNC119 is relatively abundant in the photoreceptor synapses and the IS (31), but also is expressed in a number of other tissues (32, 33). Truncation mutation in UNC119 that deletes the PrBP/δ homology domain has been linked to cone-rod dystrophy in human patients (34). A transgenic mouse model of the human mutation revealed severe synaptic degeneration (34). In contrast, a knock-out mouse model of UNC119 (MRG4) revealed a different dysfunction at distal IS/OS regions that may allow to “refill” apical discs with Gt during darkness based on diffusion of the stable UNC119-Gt complex (28). Here, we examined the interaction of human UNC119 with transducin and the lipid specificity of UNC119 in comparison to that of PrBP/δ in order to gain insights into the mechanism of IS → OS transport of Gt in the dark.

**EXPERIMENTAL PROCEDURES**

**Materials—**Mystioylated peptides, [MYR]-GAGASAEK, and [MYR]-GCGASAEK were synthesized by Promimmune Ltd. BC (3-(bromo acetyl)-7-dithyl amino coumarine) fluorophore was purchased from Molecular Probes, Inc. 6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester (AMCA-X, SE) was purchased from Invitrogen. S-Farnesyl-cysteine methyl ester was purchased from Enzo Life Sciences. All other chemicals are acquired from Sigma.

**Preparation of ROS Membranes, Gt1,GDP, Gt1 γ1, and Chimeric Gt1**—Bovine ROS membranes were prepared as previously described (37). Urea-washed ROS membranes (uROS) were prepared according to protocol in Yamanaka et al. (38). Gt1,GDP was prepared and purified according to published protocol (39). Recombinant Gt1,G was expressed using the baculovirus/sf9 cell system. The Gt1 baculoviral stock was obtained from Dr. S. Chen (University of Iowa). To generate Gt1 bacuviroles, the Gt1 cDNA was PCR amplified from bovine retinal library with the introduction of the coding sequence for the N-terminal His6 tag, and cloned into the pFast HTB vector using Rsrl/NheI sites. Generation of the recombinant bacmids, transfection of Sf9 cells, and viral amplifications were carried out according to the manufacturer’s recommendations (Invitrogen). For expression of the Gt1β1γ1 heterodimer, Sf9 cell cultures (2 × 10⁶ cells/ml) were co-infected with Gt1 and Gt1 γ1 baculoviruses at MOI of 4–6. The Gt1β1γ1 heterodimer was purified using affinity chromatography on Ni-NTA resin (Novagen) as previously described (40).

The Gt1 chimera (Gt1*) with the His6 sequence inserted between Met15 and Pro116 of the helical domain of Gt1 (41) was generated to allow the N-terminal myristoylation of the protein. The Gt1–115 sequence was PCR-amplified from the Chi8 template using a 5’-primer with an Ncol site and a 3’-primer coding the His6 sequence added to Gt1 specific sequence. The Chi8 116–350 sequence was PCR-amplified using a 5’-primer with the His6 sequence added to the Gt1-specific sequence, and a 3’-primer containing an Xhol site. The two resulting PCR products were used in the PCR reaction with the flanking primers, and the PCR product was subcloned into the pET15b vector using the Ncol/Xhol sites. Gt1* was expressed and purified as previously described (41). To obtain myristoylated Gt1* (myrGt1*), BL21-codon plus Escherichia coli cells were co-transformed with kanamycin-resistant plasmid pbb131 expressing yeast N-myristoyl transferase (42).

**Cloning, Expression, and Purification of Human UNC119 and PrBP/δ**—For expression of the His6-tagged UNC119, UNC119 (289–290) and PrBP/δ, corresponding cDNAs were PCR amplified from a human retina cDNA library and subcloned into the pET15b vector using the Ncol/Xhol sites (UNC119) or Ndel/BamHI sites (PrBP/δ). The N-terminally StrepII-tagged UNC119 (289–290) was amplified from a human retina cDNA library with a 5’ primer coding an Ncol site and the tag sequence WSHPQFEK with the SGG linker and a 3’ primer containing a Xhol site. The PCR product was subcloned into the pET15b vector using the Ncol/Xhol sites. Protein expression in BL21-codon plus E. coli cells was induced with the addition of 30 μM IPTG. UNC119 and StrepII-UNC119 (289–290) were expressed overnight at 16 °C, whereas UNC119 (289–290) and PrBP/δ were expressed for 5 h at 30 °C. The His6-tagged proteins were purified on Ni-NTA resin (Novagen). StrepII-UNC119 (289–290) was purified using StrepTactin Superflow Agarose (Novagen). UNC119 (289–290) and PrBP/δ were additionally purified by gel-filtration on a Superdex 75 Sepharose column, and UNC119 was purified by an ion-exchange chromatography on a Uno-Q1 column (Bio-Rad).

**Preparation of Hypotonic, GTP, and GTPγS Extracts of ROS Membranes**—To extract native transducin without exposure to photobleached R* and dissociation-reassociation of Gt1 and Gt1 γ1 (43), ROS membranes (200 μl, 160 μM R) were washed two times with 2 ml of isotonic buffer A (20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF and 120 mM KCl) under dim red light. The pellet after centrifugation was resuspended in 1 ml of hypotonic buffer B (5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and homogenized in a 2 ml tube using a disposable pestle under dim red light. To obtain GTP or GTPγS extracts of Gt, ROS membranes (200 μl, 160 μM R) were photobleached under fluorescent light.
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for 30 min on ice and resuspended in 1 ml of buffer A containing either 100 µM GTP or GTPγS and homogenized in a 2-ml microcentrifuge tube using a disposable pestle. The resulting mixtures were centrifuged at 125,000 × g for 30 min at 4 °C. Supernatants of hypotonic, GTTP, and GTPγS extracts were collected and dialyzed against buffer A at 4 °C for 4 h. DiaZyzed extracts were centrifuged at 125,000 × g for 30 min at 4 °C, aliquoted, and stored at −20 °C.

ROS Extraction with UNC119—ROS membranes washed twice with buffer A were resuspended in 1 ml of buffer A containing 5 µM purified UNC119 and incubated on ice for 30 min in the dark. The mixture was homogenized using a disposable pestle and centrifuged at 125,000 × g for 30 min at 4 °C. Cleared supernatant was dialyzed against buffer A at 4 °C for 4 h. DiaZyzed UNC119 extract of ROS was cleared by centrifugation, aliquoted, and stored at −20 °C.

Pull-down Assays—StrepII-UNC11955-240 was expressed as described above. Cell pellet was resuspended in Strep-Tactin binding buffer C (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and then disrupted by ultrasonication. The cleared supernatant after centrifugation (100,000 × g, 60 min) was incubated with 200 µl of StrepTactin Superflow Agarose resin at 1 h at 4 °C with gentle shaking. StrepII-UNC11955-240-bound resin was washed thoroughly with buffer C to remove unbound proteins. For pull-down assay, 15 µl of StrepII-UNC11955-240-bound resin was incubated with 5 µg each of purified Ga1tGDP, Ga1t*GTP, and myrGa1t*GTP with hypotonic, GTP, and GTPγS ROS extracts (10 µg protein each) for 30 min at room temperature in 1.5-ml microcentrifuge tubes. Resin was washed three times with buffer A to remove unbound proteins and 30 µl of SDS-PAGE sample buffer was added to the resin. The samples were separated on 4–12% gradient Bis-tris NuPage gels (Invitrogen).

Native PAGE—Samples were prepared by incubating hypotonic, GTTP, and GTPγS ROS extracts (typically 10 µg proteins each) with 5 µg His6-tagged UNC119 for 30 min at room temperature. Samples were run in 7.5% Mini-PROTEAN TGX precast acrylamide gels (Bio-Rad) at 25 °C with constant voltage of 150 V for 45 min.

Western Blotting—Protein samples were prepared in 4 × SDS sample buffer, and subjected to 4–12% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane using iBlot Western blot kit (Invitrogen) and analyzed using rabbit anti-Ga1t K-20 (SCBT)(1:2000 dilution), anti-Gβ1 C-16 (SCBT) (1:250 dilution), anti-recoverin P26 (1:3000), and anti-His probe H-15 (SCBT) (1:3000) antibodies. The antibody-antigen complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000 dilution) secondary antibody and enhanced chemiluminescence (ECL) reagents obtained from GE Healthcare.

GTPγS Binding Assay—Samples of hypotonic ROS extract containing 2 µM GTP alone or in mixture with 4 µM UNC119 were incubated for 5 min at 25 °C and reconstituted with bleached uROS membranes (100 nM rhodopsin). Binding reactions were started with the addition of 5 µM [35S]GTPγS. Aliquots were withdrawn at the indicated times, mixed with 1 ml of ice-cold 20 mM Tris-HCL (pH 8.0) buffer containing 130 mM NaCl, 2 mM MgSO4, and 1 mM GTP, passed through Whatman cellulose nitrate filters (0.45 µm), and washed three times with 3 ml of the same buffer without GTP. Bound [35S]GTPγS was measured by scintillation counting. The data were fit with Equation 1.

$$Y = Y_{max} \times (1 - \exp(-kt))$$  \hspace{1cm} (Eq 1)

Single Turnover GTPase Assays—GTPase assays were carried out in suspensions of uROS (10 µM R*) reconstituted with hypotonic ROS extract containing 1 µM G, in 20 mM HEPES buffer (pH 7.4), containing 100 mM NaCl and 8 mM MgSO4 similar as described (44). The GTPase reactions were initiated by addition of 100 nM [γ-32P]GTP. After a 5-s interval, the reaction was stopped or allowed to proceed with or without the addition of UNC119 (3 or 12 µM) for the indicated time intervals. The reactions were quenched by addition of 100 µl of 7% perchloric acid. Nucleotides were then precipitated using charcoal, and free [32P]P formed was measured by liquid scintillation counting. After subtraction of fraction of GTP hydrolyzed at 5 s, the data were fit with Equation 1, where k is the rate constant of GTP hydrolysis.

Fast Kinetic BRET Assay—Agonist-dependent cellular measurements of bioluminescence resonance energy transfer (BRET) between masGRKct-Rluc8 and GB1γ2-Venus were performed to visualize the action of G protein signaling in living cells as previously described with slight modification (45, 46). 293T/17 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), MEM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO2. For transfection, cells were seeded into 6-cm dishes at a density of 4 × 106 cells/dish. After 4 h, expression constructs (total 5 µg/dish) were transfected into the cells using Lipofectamine LTX (8 µl/dish) and PLUS (5 µl/dish) reagents. D2 receptors, Gαo, Venus155–239-Gβ6, Venus1–155-Gγ3, masGRKct-Rluc8, myc-tagged UNC119 constructs were transfected at a 1:2:1:1:1:1:6 ratio. BRET sensor constructs were gifts from Dr. Nevin A. Lambert (Department of Pharmacology and Toxicology, Medical College of Georgia). Empty vector was used to normalize the amount of transfected DNA. The cells were used for experiments at 16–24 h after transfection. BRET measurements were made using a microplate reader (POLARStar Omega; BMG Labtech) equipped with two emission photomultiplier tubes, allowing us to detect two emissions simultaneously with highest possible resolution of 50 ms for every data point. All measurements were performed at room temperature.

Preparation of the Fluorescence Probes—Purified [MYR]-GGCGASAEEK was dissolved in 100 mM HEPES (pH 7.3) buffer containing 50% acetonitrile (final concentration 0.4 mM), mixed with 5-fold excess of BC and incubated for 1 h at 25 °C. The reaction was quenched with 5 mM DTT. The product, [MYR]-GC[BC]GASAEEK, was purified by reverse phase HPLC and confirmed by MALDI mass spectrometry. Conjugation of AMCA-X to cysteine farnesyl methyl ester was performed using respectively 6 mM and 2 mM solutions of the reagents in 25% acetonitrile, 25% DMF, and 100 mM HEPES buffer (pH7.3). The product, farnesyl-Cys-AMCA, was purified by reverse phase HPLC.
Fluorescence Assays—Assays were performed on a F-2000 or F-2500 Fluorescence Spectrophotometer (Hitachi) in 1 ml of 100 mM HEPES buffer (pH 7.3). Direct changes in fluorescence of [MYR]-GC[BC]GASAEK (25 nm) on addition of various concentrations of UNC119, UNC11955–240, and PrBP/δ were monitored with excitation at 445 nm and emission at 490 nm. The concentration of labeled peptide was determined using ε445 = 53,000. The Kd values were calculated by fitting the data to Equation 2,

\[
\frac{F}{F_0} = 1 + \frac{F_{\text{max}} - F}{1 + 10^{\log K_d - X}}
\]

where \(F_0\) is a basal fluorescence of [MYR]-GC[BC]GASAEK, \(F\) is the fluorescence after addition of binding protein, \(F/F_{\text{max}}\) is the maximal relative increase of fluorescence, and \(X\) is the logarithm of concentration of added protein.

Changes in fluorescence of farnesyl-Cys-AMCA (500 nM) on addition of various concentrations of UNC119, UNC11955–240, and PrBP/δ were monitored with excitation at 350 nm and emission at 435 nm. When [farnesyl-Cys-AMCA] is comparable to \(K_d\), Equation 3 for binding with ligand depletion was used to fit the data,

\[
F/F_{\text{max}} = 1 + \left(\frac{F_{\text{max}} - F}{F_{\text{max}} - F_0}\right) \times 0.5 \times (B_m + K_d + X) - \sqrt{(B_m - K_d + X)^2 - 4 \times B_m \times X}
\]

where \(F_0\) is a basal fluorescence of [farnesyl-Cys-AMCA], \(F\) is the fluorescence after addition of binding protein, \(F/F_{\text{max}}\) is the maximal relative increase of fluorescence, \(B_m\) is a concentration of farnesyl-Cys-AMCA, and \(X\) is a concentration of added protein. Fluorescence Resonance Energy Transfer assay of [farnesyl-Cys-AMCA] binding to PrBP/δ was performed with excitation at 280 nm and emission at 435 nm. The data were fit with Equation 4 for binding with ligand depletion.

\[
F = ((F_{\text{max}} - F_0)/B_m) \times 0.5 \times (B_m + K_d + X) - \sqrt{(B_m - K_d + X)^2 - 4 \times B_m \times X}
\]

Fitting of the experimental data was performed with nonlinear least squares criteria using GraphPad Prizm Software. The Kd values are expressed as mean ± S.E. for three independent measurements.

RESULTS

Interaction of UNC119 (HRG4) with Transducin Is Dependent on the N-terminal Lipid Modification and Independent of the Activation State of Gt1—For the initial characterization of UNC119 interactions with transducin we utilized the N-terminally truncated Strep-tagged UNC119, UNC11955–240 which includes the sequence homologous to PrBP/δ. Pull-down experiments using UNC11955–240 demonstrate that it binds to Gαt1GDP purified from bovine ROS (Fig. 1). To determine if myristoylation of Gt1 is required for the interaction with UNC11955–240, we constructed chimeric Gt1 (Gt1*) corresponding to the N-terminally His-tagged Chi8 (41), but with the His8-tag placed within the helical domain of Gt1 between Met115 and Pro116. Myristylated Gt1* (myrGt1*) was obtained using co-expression with N-myristoyl transferase (NMT) (42). In the pull-down assay, myrGt1* bound UNC11955–240 similar to bovine Gt1GDP. Only trace amounts of nonmyristoylated Gt1* precipitated with the UNC11955–240 resin (Fig. 1). Thus, the interaction of UNC11955–240 with Gt1 is largely dependent on the N-acylation of the protein.

Interactions of UNC119 with holotransducin and activated Gαt1GTPγS were investigated using extracts of ROS membranes with GTP and GTPγS. Following extraction with GTP, Gαt1 hydrolyzes the nucleotide leading to the formation of heterotrimeric Gαt1GDP, Gβ1γ1, or Gt. The pull-down experiments showed that UNC11955–240 interacted with Gαt1GDP from heterotrimeric Gt and Gαt1GTPγS (Fig. 2A). The ratio of Gβ1γ1 to Gt1 in the pull-down complex was markedly reduced compared with that in the ROS extracts (Fig. 2A). The trace amounts of Gβ1γ1 were precipitated not as a complex with Gt1, but rather due to nonspecific interaction with the StrepTactin agarose resin (Fig. 2B). Therefore, interaction with UNC119 with Gt1 appears to be dissociation of Gαt1 and Gβ1γ1. Recoverin, a prominent myristoylated protein in photoreceptor cells, was present in the ROS extracts, but negligible in the pull-down preparations (Fig. 2C).

Interaction of UNC119 with Native Transducin—Extraction of ROS with GTP yields “reconstituted” transducin whose properties differ from properties of “native” transducin (43). “Native” transducin is obtained without exposure to photobleached R* and dissociation-reassociation of Gt1 and Gβ1γ1 (43). To probe the interaction of UNC119 with “native” Gt1, the UNC11955–240 pull-downs were carried out using the hypotonic extract of “dark” ROS membranes. Similarly to the pull-downs from the GTP and GTPγS ROS extracts, Gαt1 and minor quantities of Gβ1γ1 were pelleted from the hypotonic ROS extract with the StrepII-UNC11955–240 resin (Fig. 3A). Thus, UNC11955–240 was able to compete with Gβ1γ1 for the interaction with Gt1 in “native” transducin as well. Next, we examined the effect of UNC119 on “native” Gt1 on “dark” ROS membranes. Addition of UNC119 or UNC11955–240 to ROS membranes in isotonic buffer caused significant release of Gt1 and Gβ1γ1 into soluble fraction (Fig. 3B). The levels of recovery in the soluble fraction remained unchanged in the presence of UNC119 (Fig. 3B). These data indicate that UNC119 interacts with membrane-bound Gt1 and detaches Gt1 and Gβ1γ1 from the membrane.

Further analysis of UNC119 interactions with “reconstituted” Gt1, Gαt1GTPγS, and “native” transducin was performed.
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by native gel electrophoresis. Gt1 from the GTP- and hypotonic ROS extracts (Fig. 4, A and C) migrated similarly and slightly faster than Gt1 in the GTPγS extract (Fig. 4B). In the all three extract preparations, Gβγ did not co-migrate with Gt1, as it was previously shown for the “reconstituted” Gt (43). Addition of UNC119 to each of the ROS extracts strikingly shifted positions of Gt1 on the gel toward the position of UNC119. The Gt1 band shifted to similar slow migrating bands in the GTP- or hypotonic ROS extract and isotonic UNC119 extract of dark ROS membranes (Fig. 4, A, C, and D). A somewhat different Gt1 pattern was seen in the GTPγS extract with addition of UNC119 (Fig. 4B). Migration of Gβγ on native gel was not significantly altered in the presence of UNC119 (Fig. 4).

Effects of UNC119 on G-protein Activation and Inactivation—The apparent ability of UNC119 to interact with heterotrimeric Gi and dissociate Gt1 and Gβγ suggests that it might interfere with transducin activation by R*. Indeed, the GTPγS-binding assays indicated that UNC119 inhibits transducin activation by R*-containing uROS (Fig. 5A). UNC119 is also capable of interaction with the active GTPγS-bound conformation of Gt1 and may affect the GTP hydrolysis by Gt1. Single-turnover GTPase assay revealed no effect of UNC119 on the Gt1 GTPase catalytic rate (Fig. 5B).

To investigate the function of UNC119 in living cells, we employed a fast kinetic BRET assay of heterotrimeric Go activation/inactivation by the D2 dopamine receptor D2R in transfected HEK293 cells (45, 46). In this study we tested the effect of UNC119 proteins on D2R-Gt- mediated signaling by measuring amplitude as well as kinetics of agonist-induced interaction between Gβγ-Venus and its effector construct, masGRKct-RLuc8, as a readout of G protein activation and deactivation state. We first observed that the dopamine-induced maximum amplitude was decreased in UNC119-expressing cells (Fig. 6B). Also, UNC119 significantly reduced the rate of Gi activation, but had very little effect on the G-protein inactivation (Fig. 6, A and C). In our time-resolved measurements, UNC119 exerts an effect on the D2-Gi signaling that is supporting the observations of negative regulation of GEF activity by UNC119 described in biochemical experiments (Fig. 5A).

Comparison of the Lipid Binding Properties of UNC119 and PrBP/δ—To quantitatively assess the interactions of UNC119 with Goi, myristoylated N-terminal Goi peptide, [MYR]-GCGASAEEK with Ala3 substituted for Cys was synthesized to allow the labeling with a fluorescence probe, BC. Addition of both UNC119 and UNC11955–240 to [MYR]-GC-GASAEEK led to large, dose-dependent increases of the probe fluorescence (Fig. 7). From the fluorescence binding curves, the Kd values for UNC119 and UNC11955–240 were 185 ± 15 nM and 270 ± 10 nM, respectively (Fig. 7B). The binding of BC-labeled peptide to UNC119 was specific since no fluorescence change occurred on addition of UNC119 preincubated with excess unlabeled [MYR]-GCGASAEEK (not shown). Comparable affinities of UNC119 and UNC11955–240 for the Goi N terminus suggest that it binds primarily to the UNC119 region homologous to PrBP/δ. Next, we tested if PrBP/δ interacts with [MYR]-GC-[BC]GASAEEK. Three lines of evidence indicated that PrBP/δ does not appreciably bind the N terminus of Goi; 1) addition of PrBP/δ did not cause change in fluorescence of [MYR]-GC-[BC]GASAEEK; 2) titration of the BC-labeled peptide with UNC11955–240 in the presence of excess PrBP/δ did not significantly alter the affinity of UNC11955–240 for the probe; and 3) preincubation of PrBP/δ with the unlabeled peptide [MYR]-GCGASAEEK did not affect PrBP/δ binding with farnesyl-Cys-AMCA (not shown).
Homology between UNC119 and PrBP/δ suggests that UNC119 may potentially bind prenylated proteins. This possibility was examined with the binding assay employing fluorescently labeled farnesylated Cys probe, farnesyl-Cys-AMCA. Binding of UNC119 and UNC11955–240 to farnesyl-Cys-AMCA increased the probe fluorescence in a dose dependent manner (Fig. 8). No fluorescence increase was observed when UNC119 was preincubated with unlabeled farnesylated probe (not shown). The $K_d$ of UNC119 binding to farnesyl-Cys-AMCA was estimated at 3.8 μM, which is an order of magnitude higher than the $K_d$ for the UNC119 interaction with the myristoylated N terminus of Goαt1. The same assay yielded a $K_d$ of 0.55 μM for farnesyl-Cys-AMCA binding to PrBP/δ (Fig. 8). A comparable binding affinity ($K_d$ 0.36 μM) was obtained for farnesyl-Cys-AMCA binding to PrBP/δ using the Trp-fluorescence energy transfer assay (supplemental Fig. S1). This affinity is similar to the affinity of dansyl farnesyl binding to PrBP/δ reported previously (47). Thus, farnesyl-Cys-AMCA is an appropriate reporter of the interactions of UNC119 and PrBP/δ with farnesylated probes.

DISCUSSION

The energy independence and the massive amounts of transducin returning to the rod outer segment during dark adaptation following exposure to bright light favor some sort of a diffusion mechanism as a transducin transport mode (4, 12). Appreciable inter-disc diffusion of heterotrimeric Goαt modified with two lipid anchors might be possible when one or both of the lipids are sequestered by lipid-binding proteins (21). Indeed, Zhang et al. have recently demonstrated that UNC119 interacts with the N-acylated Goαt1GTP, and that the Goαt trans-

FIGURE 4. UNC119 alters mobility of Goαt1 upon native gel electrophoresis. Samples of ROS extracts with GTP (A), GTP•S (B), hypotonic buffer (C), and isotonic buffer containing UNC119 (D) alone or preincubated for 30 min with 5 μM His6-UNC119 (A–C), were subjected to native PAGE and analyzed by immunoblotting with anti-Goαt1. The blots were then stripped and re-probed with anti-Goβ, and anti-His6 antibodies.

FIGURE 5. UNC119 blocks R*-dependent transducin activation. A, rhodopsin-catalyzed GTP•S binding to Goα. Samples of hypotonic ROS extract containing 2 μM Goz, alone or in mixture with 4 μM UNC119 were incubated for 5 min at 25 °C and reconstituted with bleached uROS membranes (100 nm rhodopsin). Binding reactions were started with the addition of 5 μM [35S]GTP•S. Bound [35S]GTP•S was measured by the filter binding assay. B, single turnover GTPase assay. GTPase assays were carried out using 100 nm (γ-32P)GTP in suspensions of uROS (10 μM R*) reconstituted with hypotonic ROS extract containing 1 μM Goz, in the presence or absence of 12 μM UNC119 as described under “Experimental Procedures.” Results from one of three similar experiments are shown.

FIGURE 6. UNC119 reduces the amplitude and slows the activation kinetics of dopamine D2-Gαi signaling. A, normalized traces of dopamine evoked D2-Gαi signaling in the presence and the absence of UNC119. As shown under “Experimental Procedures,” dopamine D2 receptor, Goαi, and BRET sensors were co-expressed with or without Myc-tagged UNC119 in HEK293 cells. BRET ratios are plotted against time during sequential applications of dopamine and haloperidol. Traces are normalized to the response immediately before addition of haloperidol. B, maximal amplitudes elicited by 1 μM dopamine in the presence or absence of UNC119. C, effects of UNC119 on the activation and deactivation time constants of the D2-Gαi signaling. The activation and deactivation phases were fitted by a single exponential function and the time constants were obtained (mean ± S.E.). D, whole protein extracts were prepared from cells and expression of UNC119 was analyzed by Western blotting (WB) with anti-Myc antibody. WB with anti-β-actin antibody served as an internal control for the amount of loaded samples. Data in A–C represent quadruplicate measurements.
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A

B

FIGURE 7. Binding of UNC119 to myristoylated N-terminal peptide of G\(_{\alpha}\)

A, emission spectrum of [MYR]-GC[BC]GASAEEK alone (50 nm) and in the
presence of 500 nm UNC119. B, changes in fluorescence of [MYR]-GC[BC]GASAEEK (25 nm) on addition of various concentrations of UNC119 and UNC11955–240
were monitored with excitation at 445 nm and emission at 490 nm. The relative changes F/Fo were fit to sigmoidal dose-response.

FIGURE 8. Binding of UNC119 to farnesyl-Cys-AMCA probe. A, emission spectrum of farnesyl-Cys-AMCA alone (500 nm) and in the presence of 1 \(\mu\)M UNC11955–240. B, changes in fluorescence of farnesyl-Cys-AMCA (500 nm) on addition of various concentrations of UNC11955–240 and PrBP/δ were monitored with excitation at 350 nm and emission at 435 nm. The relative changes F/Fo were fit to equation for binding with ligand depletion.

port in the dark is impaired in the UNC119 (MRG4) knock-out mice (28). A “restricted” diffusion model has been proposed in which the rate-limiting step is defined by very slow spontaneous GTP/GDP exchange on Gt in the IS (28). UNC119 then interacts with and stabilizes Gt by inhibiting intrinsic GTPase activity and allowing the UNC119-Gt-GTP complex to diffuse to the OS. Diffusion of Gt released by spontaneous activation is facilitated by PrBP/δ (28). Although a slow spontaneous nucleotide exchange in the absence of R* (49) was observed in isolated preparations of Gt (48), it is unclear that it occurs in Gt docked to IS membranes under in vivo like conditions. Our results suggest an alternative model for transducin transport to the OS in the dark (supplemental Fig. S2). UNC119 is capable of interacting with heterotrimeric Gt (Figs. 2–4). It facilitates transducin release from the membrane accompanied by dissociation of UNC119-Gt and G\(_{\beta}\)\(_{\gamma}\). The co-crystal structure of UNC119 with the lauroylated N-terminal Gt peptide shows UNC119 interactions with the lipid as well as the N-terminal 6–10 residues of Gt (28). Thus, UNC119 apparently disrupts the interaction of transducin subunits by sterically occluding the G\(_{\beta}\)\(_{\gamma}\)-binding site within the N-terminal \(\alpha\)-N-helix of Gt (49). We found that UNC119 interacts weakly with farnesylated probe, farnesyl-Cys-AMCA (Fig. 8). Still, in agreement with the previous study, UNC119 did not appreciably bind farnesylated Gt (28). In contrast, PrBP/δ bound farnesyl-Cys-AMCA much more potently then UNC119 (Fig. 8). G\(_{\beta}\)\(_{\gamma}\) is partially mislocalized in PrBP/δ knock-out mice (26). Therefore, PrBP/δ is a probable facilitator of diffusion of G\(_{\beta}\)\(_{\gamma}\) released from Gt by UNC119. Phosducin, another photoreceptor G\(_{\beta}\)\(_{\gamma}\)-binding protein, seems to play a different role. Phosducin assists light-dependent translocation of transducin from the OS to the IS by sequestering Gβ\(_{\gamma}\) (22). However, in the IS, phosducin is phosphorylated in the dark and releases G\(_{\beta}\)\(_{\gamma}\) (23, 50). Thus, phosphorylation of phosducin serves as a trigger for the formation of heterotrimeric Gt in the IS (2, 23). Formation and membrane association of Gt act as a transducin IS “sink” and as a starting point for the Gt to the OS return (2, 28). Supporting this hypothesis, transducin return to the OS in the dark is delayed in mice expressing phosphorylation-deficient phosducin (51).

Our model of transducin return to the OS in the dark does not assume the requirement for Gt spontaneous activation in the IS. The kinetics of transducin IS→OS translocation are much slower then the light-induced OS→IS translocation (4). What is the rate-limiting step in this process? One possibility is that the molar ratio of UNC119 to light-translocated Gt in the IS is low. As a result, only a small fraction of Gt is solubilized by UNC119 from IS membranes and is diffusing at any given moment during dark adaptation. The levels of UNC119 in the OS are clearly much lower than they are in the IS (35). Accordingly, in the OS the equilibrium between UNC119-Gt and G\(_{\alpha}\)\(_{\beta}\)\(_{\gamma}\) shifts toward the heterotrimer which associates with disc membranes. It remains to be investigated whether UNC119 assists the axial diffusion of Gt in the OS. In addition to transducin trafficking, UNC119 may play important roles in regulation of vesicle and ciliary trafficking processes in photo-
receptor cells. Protein myristoylation may mediate UNC119 binding to known partners such as Arf-like proteins 2 and 3 (Arl2/3) (52, 53). Arl2/3 are present in the IS and the connecting cilium, respectively, and are shown to regulate microtubule dependent processes (52, 54, 55).

Besides transducin, other G proteins from the G1 family (Gαo, Gαf, Goγm) are myristoylated (16, 17). UNC119 inhibited Rα-dependent activation of Gi and D2R-dependent activation of Gαo, indicating that UNC119 may inhibit GPCR-dependent activation of the Gi family G proteins (Figs. 5 and 6). The mechanism presumably involves G-protein membrane extraction and subunit dissociation. We found no effect of UNC119 on the Gαi1 GTase activity under single turnover conditions that allow measurements of GTP hydrolysis independent of the GTP binding reaction. Furthermore, UNC119 did not affect inactivation of Goi in cultured cells (Fig. 6). These results contrast the reported inhibitory effect of UNC119 on transducin GTase activity observed by multi-turnover GTase assay (28).

The ability of UNC119 to inhibit Rα-dependent GTPγS binding to Gi, possibly explains this discrepancy (Fig. 5). Low-level expression of UNC119 was reported in adrenal gland, cerebellum, cultured fibroblasts, T-cells, lung, and kidney (32, 33). Our findings suggest that apart from its role as a cofactor of G-protein trafficking in ciliary sensory cells (28), UNC119 may modulate signal transduction from GPCRs to G proteins in different cell types and tissues.

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