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Interaction of retinal guanylate cyclase with the α subunit of transducin: potential role in transducin localization

Derek H. ROSENZWEIG^{*,1}, K. SAIDAS NAIR^{*,1}, Konstantin LEVAY^{*}, Igor V. PESHENKO[†], John W. CRABB^{‡,§}, Alexander M. DIZHOOR[†], and Vladlen Z. SLEPAK^{*,2}

^{*}Department of Molecular and Cellular Pharmacology and Neuroscience Program University of Miami Miller School of Medicine, Miami, FL 33136, U.S.A.

[†]Hafters Research Laboratories, Pennsylvania College of Optometry, Elkins Park, PA 19027, U.S.A.

[‡]Department of Ophthalmic Research, Cole Eye Institute Cleveland Clinic, Cleveland, OH 44195, U.S.A.

[§]Department of Cell Biology, Lerner Research Institute Cleveland Clinic, Cleveland, OH 44195, U.S.A.

Abstract

Vertebrate phototransduction is mediated by cGMP, which is generated by retGC (retinal guanylate cyclase) and degraded by cGMP phosphodiesterase. Light stimulates cGMP hydrolysis via the G-protein transducin, which directly binds to and activates phosphodiesterase. Bright light also causes relocalization of transducin from the OS (outer segments) of the rod cells to the inner compartments. In the present study, we show experimental evidence for a previously unknown interaction between $G_{\alpha t}$ (the transducin α subunit) and retGC. $G_{\alpha t}$ co-immunoprecipitates with retGC from the retina or from co-transfected COS-7 cells. The retGC– $G_{\alpha t}$ complex is also present in cones. The interaction also occurs in mice lacking RGS9 (regulator of G-protein signalling 9), a protein previously shown to associate with both $G_{\alpha t}$ and retGC. The $G_{\alpha t}$ –retGC interaction is mediated primarily by the kinase homology domain of retGC, which binds GDP-bound $G_{\alpha t}$ stronger than the GTP[S] (GTP γ S; guanosine 5'-[γ -thio]triphosphate) form. Neither $G_{\alpha t}$ nor $G_{\beta\gamma}$ affect retGC-mediated cGMP synthesis, regardless of the presence of GCAP (guanylate cyclase activating protein) and Ca^{2+} . The rate of light-dependent transducin redistribution from the OS to the inner segments is markedly accelerated in the retGC-1-knockout mice, while the migration of transducin to the OS after the onset of darkness is delayed. Supplementation of permeabilized photoreceptors with cGMP does not affect transducin translocation. Taken together, these results suggest that the protein–protein interaction between $G_{\alpha t}$ and retGC represents a novel mechanism regulating light-dependent translocation of transducin in rod photoreceptors.

Keywords

cGMP; G-protein; photoreceptor; subcellular localization

INTRODUCTION

In vertebrate photoreceptors, light-activated rhodopsin initiates a photoresponse by inducing the exchange of GDP for GTP on the α subunit of the heterotrimeric G-protein transducin ($G_{\alpha t}$). GTP-bound $G_{\alpha t}$ activates cGMP PDE6 (phosphodiesterase 6), which reduces the cytoplasmic concentration of cGMP, leading to closure of cGMP-gated Na^+ and Ca^{2+} channels [1,2]. The G-protein is inactivated upon hydrolysis of GTP, the molecular event accelerated by RGS9 (regulator of G-protein signalling 9), a protein complex that directly binds to $G_{\alpha t}$. In addition to rhodopsin, PDE6 and RGS9, transducin can interact with centrins [3,4], LGN [5,6], phosducin [7,8] cytoskeleton components [9] and other proteins [10].

Restoration of cGMP levels and reopening of the cGMP-gated ion channels are key events in photoreceptor recovery. Mammalian retinas contain two isoforms of guanylate cyclase, retGC-1 and retGC-2 (for simplicity we will use retGC when referring to retGC-1). These approx. 115 kDa transmembrane enzymes are products of two distinct genes [11] and are regulated by GCAP (guanylate cyclase activating protein)-1 and GCAP-2. GCAPs are Ca^{2+}/Mg^{2+} exchanging proteins in a light-dependent manner [12-14].

Photoreceptors have an OS (outer segment), where phototransduction takes place, an inner segment containing common cellular organelles, the nucleus and an axon ending with the synaptic terminal. Light induces redistribution of some signal-transduction proteins between the OS and the other compartments (for reviews see [15,16]). Up to 90% of transducin subunits can migrate from the OS to the inner compartments of rods in bright light [17]. In the present study, we present experimental evidence that $G_{\alpha t}$ can directly interact with retGC without altering cyclase activity, and that this interaction plays a role in subcellular localization of transducin in rods.

EXPERIMENTAL

Animal research was conducted in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee. RGS9-knockout mice [18] were provided by Dr Ching-Kang Chen (Virginia Commonwealth University). *Nrl*^{-/-} (where *Nrl* is neural retina leucine zipper) mice were provided by Dr Anand Swaroop (University of Michigan). A mouse line lacking retGC-1 (GCE null) were originally received from Dr David Garbers (UT Southwestern) [19] and were developed into an SPF colony with Charles River Laboratories maintained at Pennsylvania College of Optometry. Bovine and mouse OS were purified as previously described [20,21].

Antibodies specific for rod and cone $G_{\alpha t}$ subunits were from Santa Cruz Biotechnology (SC389 and SC390 respectively). Antibodies against RGS9 and $G_{\beta 5}$ have been described previously [22]. We used three antibodies raised against retGC-1. Two different rabbit polyclonal antibodies were specific for the CAT (catalytic) domain: a new antibody against the fragment Met⁷⁴⁷-Ser¹⁰⁵², and a previously described antibody (7957CAT) [23]. The third anti-retGC antibody used in the present study was specific for the KHD (kinase homology domain; 6344KHD) [23]. The 7957CAT and 6344KHD antibodies were a gift from Dr James B. Hurley (University of Washington). Antibodies against $G_{\beta 1}$ ($\beta N1$) were a gift from Dr Melvin I. Simon (California Institute of Technology).

Plasmid vectors for mammalian cell expression of retGC1 and rod $G_{\alpha t}$ were from Dr J. Hurley (University of Washington) and the Guthrie Institute respectively. Other reagents were from Sigma.

MS analysis

A 115 kDa protein band was excised from the gel, and in-gel digestion with trypsin was performed as in the Crabb laboratory protocol (<http://www.lerner.ccf.org/eye/crabb/protocols/silverdigest.php>). Briefly, the gel slice was placed in a low-adhesion microcentrifuge tube and washed with 60% acetonitrile solution with TFA (trifluoroacetic acid). Washed and de-stained gel pieces were dried using a vacuum centrifuge, and then rehydrated and trypsinized overnight at 37°C. Proteins were extracted in 60% acetonitrile with TFA, freeze-dried and analysed as described previously [24]. LC-MS/MS (liquid chromatography–tandem MS) was performed with a QTOF (quadrupole time-of-flight) mass spectrometer (QTOF2; Waters). Proteins were identified from MS/MS data [using the ProteinLynx Global Server (Waters) and Mascot (Matrix Science) search engines, and the Swiss Prot (<http://www.expasy.org>; provided in the public domain by Swiss Institute of Bioinformatics, Geneva, Switzerland) and National Center for Biotechnology (National Institutes of Health, Bethesda, MD, U.S.A.) protein sequence databases].

Immunoprecipitation

Immunoprecipitation, SDS/PAGE and immunoblots were all carried out as previously described [5] with minor alterations. All steps were carried out on ice. A 200 μ l aliquot of frozen bovine OS (~500 μ M rhodopsin concentration), which was purified and stored using the standard procedure [20,21], was thawed on ice and centrifuged (10000 *g* for 30 min at 4 °C) to form a 25 μ l pellet. The pellet was resuspended in 175 μ l of lysis buffer [10 mM Tris/HCl (pH 7.6), 5 mM MgCl₂, 150 mM KCl, 1 mM DTT (dithiothreitol) and complete protease inhibitor cocktail (Roche)]. This OS suspension was divided into 50 μ l portions and incubated in light or dark in the presence of different nucleotides, as required by a particular experiment (see the Results section). After the treatment, Triton X-100 was added to a final concentration of 1% (v/v), incubated for 1 min on ice and centrifuged at 10 000 *g* in a table-top Beckman centrifuge at 4°C to remove the insoluble fraction (i.e. cytoskeleton). The obtained supernatants were then mixed with 25 μ l of Protein-A–Sephrose beads for 2 h. This step ('pre-clearing') reduces non-specific protein binding to the beads. The pre-cleared lysates were then incubated with the beads containing anti-rod G_{at} antibodies. To immobilize the antibody, 2 μ g of the rod G_{at}-specific IgG (SC389) was added to 25 μ l of Protein A beads in a volume of 100 μ l and was incubated for 2 h at 4°C with mixing. The normal rabbit IgG was used to prepare the control Protein A beads. For immunoprecipitation, the pre-cleared lysate was incubated with the beads overnight, continuously rotating at 4°C. The beads were then briefly centrifuged and the supernatant was collected as the unbound material (~200 μ l) for Western blot analysis. The resin was washed twice with 500 μ l of the lysis buffer containing 1% (v/v) Triton X-100. The collected beads were then mixed with 20 μ l of 2 × SDS/PAGE sample buffer, and was incubated at 65°C in a heat block for 5 min. The beads were centrifuged again and the supernatant was collected as the specifically eluted material (eluate). The unbound and eluted fractions were resolved by SDS/PAGE (10% gels) and analysed by Western blot.

To ensure that the ECL (enhanced chemiluminescence) signal obtained from the immunoprecipitated fractions fell within the linear range of detection, we typically loaded the gel with 10 μ l of unbound and 10 μ l of eluted material. As a result, the eluted fraction was 10-fold concentrated relative to the unbound fraction. To estimate the amount of co-precipitated proteins, we compared the signal intensity of scanned Western blot films to the signals of serial dilutions of the OS lysate (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/417/bj4170803add.htm>).

COS-7 cell culture and transfection

Transient transfection of COS-7 cells was performed using a common procedure with Lipofectamine™ as described previously [22]. The cells were harvested at 48 h after transfection.

Guanylate cyclase activity assay

RetGC activity was assayed as previously described in [12]. Briefly, the assay mixture (25 μ l) contained 30 mM Mops/KOH (pH 7.2), 60 mM KCl, 4 mM NaCl, 1 mM DTT, 5 mM free Mg^{2+} , 2 mM Ca/EGTA buffer, 0.3 mM ATP, 4 μ M cGMP, 1 mM GTP, 1 μ Ci of [α - 32 P]GTP, 0.1 μ Ci of [8- 3 H]cGMP, GCAP-1 and HEK (human embryonic kidney)-293 cell membranes. The reaction mixture was incubated for 40 min at 30°C, stopped by heating for 2.5 min at 95°C, and the aliquots were analysed by TLC using fluorescent PEI–cellulose (polyethyleneimine–cellulose) plates (Merck) as described previously [12]. The results shown are representative from three to seven independent experiments producing virtually identical results. Rod transducin subunits investigated for the interaction with guanylate cyclase were purified according to the method of Bigay and Chabre [25]. GCAP-1 was expressed in *Escherichia coli* and purified as described previously [26] with some modifications [27].

GST (glutathione transferase) pulldown assay

cDNAs corresponding to the KHD (nucleotides 1462–2391) and CAT (nucleotides 2686–3183) of retGC-1 (GenBank® accession number AJ222657) were PCR-amplified and cloned into the GST-tag expression vector pGEX-6P1 at BamHI and NotI sites. GST-fusion proteins were expressed in *E. coli* origami cells (Calbiochem/Novagen) and purified on glutathione–Sepharose 4B (Invitrogen). Bacteria were induced with 0.5 mM IPTG (isopropyl β -D-thiogalactoside) for 4 h at 30°C, and lysed in the BugBuster buffer (Novagen) supplemented with protease inhibitor cocktail (Roche) and 1% *N*-laurylsarcosine. After complete solubilization of the bacterial pellet, Triton X-100 was added at a final concentration of 2.5% (v/v). Lysates were diluted 5-fold with ice-cold PBS and centrifuged for 30 min at 20000 *g* at 4°C. Supernatants were incubated with glutathione–Sepharose 4B overnight at 4°C and washed extensively with ice-cold wash buffer [PBS (pH 7.6), 0.5% Triton X-100 and protease inhibitors]. Beads with 20 μ g of GST, GST–KHD, and GST–CAT proteins were incubated for 4–6 h in a binding buffer [PBS (pH 7.6), 1% (w/v) BSA, 0.5% Triton X-100 and protease inhibitors) with $G_{\alpha t}$. After incubation, beads were washed and the bound proteins were eluted with SDS/PAGE sample buffer and analysed by Western blot.

Subcellular localization of transducin

Analysis of transducin localization in rods was performed using immunofluorescence microscopy as described previously [21,28]. Briefly, enucleated mouse eyes were fixed with paraformaldehyde, dissected and the retinas were embedded in agar. The retinas were then sectioned on a vibratome to obtain 100 μ m sections, which then were probed with antibodies against rod transducin (SC389 at 1:500 dilution). The stained sections were imaged on a laser confocal microscope, and the images were analysed using Metamorph software version 6.1. The integrated immunofluorescence intensity was measured to calculate the percentage of total transducin present in the inner compartments, as described in detail previously [21].

Ex vivo experimentation

Mouse eyecups or explanted retinas were prepared and subjected to acute culture *ex vivo* as described in detail previously [21,28]. Prior to dissection, mice were dark-adapted for at least 3 h, and the dissections were performed under dim red light. For permeabilization, the

retinas were treated with 100 ng/ml α -toxin (α -haemolysin, Sigma) in DMEM (Dulbecco's modified Eagle's medium). To study the effects of cGMP and other nucleotides on transducin localization, permeabilized retinas or eyecups were washed with DMEM and transferred to the medium containing various nucleotides at a 100 μ M concentration. The total time of the explanted retinas *ex vivo* did not exceed 2 h. Following the incubations, the tissue was fixed, embedded in agar, sectioned and analysed by immunofluorescence microscopy.

TLC analysis of nucleotides

Eyecups were permeabilized and incubated in 50 μ l of DMEM containing cGMP, GTP, GTP[S] (GTP γ S; guanosine 5'-[γ -thio]-triphosphate) or GDP, depending on the purpose of the assay [see the Results section, Figure 8 and Supplementary Figure S1 (at <http://www.BiochemJ.org/bj/417/bj4170803add.htm>)], in the wells of a microtitre plate, with one whole eyecup per well. Aliquots (5 μ l) of the medium were collected at the specified time points and spotted on to PEI-cellulose. The spots were dried at room temperature (20 $^{\circ}$ C) and the nucleotides were resolved with 0.5 M LiCl solution, which acted as the mobile phase. The nucleotides were visualized using a 265 nm UV hand-held illuminator. The location of the nucleotides, which appeared as dark spots on the fluorescent background, was registered by a hand-held digital camera (Canon) and then marked with a pencil. The spots were cut out, and the nucleotides were extracted in 0.5 M LiCl, and measured by absorbance at 260 nm in a spectrophotometer. The concentration of the eluted nucleotide was calculated using a calibration curve obtained with the known nucleotide amounts.

RESULTS

RetGC co-immunoprecipitates with bovine rod $G_{\alpha t}$

We immunoprecipitated detergent-solubilized bovine OS fractions using antibodies against $G_{\alpha t}$. As a control for the non-specific binding to Protein A–Sepharose beads, we blocked $G_{\alpha t}$ antibodies with the synthetic peptide used as the antigen in the production of the antibody, or used normal rabbit IgG bound to the similarly treated Protein A beads. The eluates from these beads were resolved by SDS/PAGE, and the gels were stained with silver nitrate (Figure 1A). The pattern of detected proteins slightly varied, but in all experiments, we consistently observed a protein band of approx. 115 kDa, which specifically co-precipitated with $G_{\alpha t}$. This band was excised from the gel and subjected to MS analysis. Seven distinct tryptic peptides generated from the 115 kDa protein identified it as bovine retGC-1. As a control, the band corresponding to $G_{\alpha t}$ (39 kDa) was analysed and unambiguously identified as the α subunit of bovine rod transducin; no presence of other polypeptides was detected above the background level. We confirmed the identity of the 115 kDa protein by immunoblot using anti-retGC antibodies [23] (Figures 1B and 1C). The specificity of the $G_{\alpha t}$ -mediated retGC interaction was verified using Protein A beads coupled with normal IgG. To further confirm the $G_{\alpha t}$ –retGC interaction, we co-immunoprecipitated the $G_{\alpha t}$ –retGC complex using antibodies against retGC-1 (Figures 1B and 1C). We found that in this reciprocal immunoprecipitation, a small fraction of $G_{\alpha t}$ was specifically absorbed from the lysates (Figure 1C). We next compared the efficiency of retGC co-immunoprecipitation with anti- $G_{\alpha t}$ antibodies to that of the $G_{\beta 5 L}$ –RGS9 complex, a known binding partner of $G_{\alpha t}$. We found that $G_{\beta 5}$ –RGS9 co-immunoprecipitated with $G_{\alpha t}$ under our conditions, but did not co-precipitate with retGC. In most experiments, RGS9 (55 kDa) was completely obscured by the IgG bands present in the eluted fraction. Therefore we used antibodies specific to $G_{\beta 5}$ to reveal the complex. Interestingly, we did not detect $G_{\beta 1}$ in our $G_{\alpha t}$ immunoprecipitates, possibly because the anti- $G_{\alpha t}$ antibodies preferentially bind to $G_{\beta \gamma}$ -free $G_{\alpha t}$ or because binding of the antibody causes transducin to dissociate.

To estimate the amounts of the proteins in the immunoprecipitate fractions, we compared the intensity of the Western blot signals with serial dilutions of the retinal lysate (Figure 1D and Supplementary Figure S1). These analyses showed that anti- $G_{\alpha t}$ antibodies precipitated approx. 10% of rod $G_{\alpha t}$ from bovine OS under the conditions used in the present study. Surprisingly, almost an equal proportion of retGC (~8.0%) co-immunoprecipitated with $G_{\alpha t}$. Assuming a 1:1 stoichiometry in the $G_{\alpha t}$ –retGC complex, this result indicated that approx. 80% of retGC present in the OS lysate was bound to $G_{\alpha t}$.

Immunoprecipitation of retGC was more efficient than that of $G_{\alpha t}$, and we could immunoprecipitate approx. 50% of retGC (Figures 1B and 1C). Approx. 1.5% of total transducin co-precipitated with anti-retGC antibodies. Because the ratio of expression levels between retGC and transducin was previously calculated to be approx. 1:15 [29], most of the transducin must be present in a retGC-free form. Therefore one should expect the fraction of $G_{\alpha t}$ co-immunoprecipitated with retGC to be very small. If 100% of retGC was bound to $G_{\alpha t}$, the maximal possible fraction of co-immunoprecipitated $G_{\alpha t}$ should be only one-fifteenth (6.6%) of total $G_{\alpha t}$. Considering the 50% efficiency of retGC immunoprecipitation with the anti-retGC antibodies (Figure 1B), the maximal amount of $G_{\alpha t}$ in co-immunoprecipitates should be 3.3%, which is comparable with our result of ~1.5% (Figure 1C). Thus, based on the estimate from the co-immunoprecipitation using anti-retGC antibodies, approx. 45% of retGC is complexed with $G_{\alpha t}$. With the understanding of the limitations of quantitative Western blot methods, our estimates from reciprocal co-immunoprecipitation suggest that a remarkably large fraction of retGC (45–80%) is bound to $G_{\alpha t}$. These estimates are consistent with the staining pattern of the 115 kDa band relative to $G_{\alpha t}$ in the silver-stained gels (Figure 1A).

A parallel analysis of co-immunoprecipitation of $G_{\alpha t}$ with the $G_{\beta 5L}$ –RGS9 complex showed that approx. 1% of $G_{\beta 5L}$ –RGS9 present in the OS lysate bound to the beads with anti- $G_{\alpha t}$ antibodies. Since the efficiency of $G_{\alpha t}$ immunoprecipitation is approx. 10%, this indicated that 10% of total $G_{\beta 5L}$ –RGS9 was associated with $G_{\alpha t}$. Considering the RGS9/transducin ratio to be 1:85 [30], this suggested that approx. 0.1% of $G_{\alpha t}$ absorbed on to the anti- $G_{\alpha t}$ beads was represented by its complex with $G_{\beta 5L}$ –RGS9.

Thus our results indicate that a large (45–85%) pool of retGC is associated with $G_{\alpha t}$. Like RGS9– $G_{\alpha t}$, the retGC– $G_{\alpha t}$ complex represents only a small fraction of transducin, which is much more abundant than either retGC or $G_{\beta 5L}$ –RGS9.

RetGC co-immunoprecipitates with mouse rod and cone $G_{\alpha t}$ subunits

We investigated the $G_{\alpha t}$ –retGC interaction using available mouse models. First, we established that retGC co-immunoprecipitated with rod $G_{\alpha t}$ from wild-type mouse OS (Figure 2A). As expected, RGS9 and $G_{\beta 5L}$ were also present in these $G_{\alpha t}$ immunoprecipitates. We then tested whether retGC co-precipitated with rod $G_{\alpha t}$ from retinas of mice lacking RGS9. Our results showed that co-immunoprecipitation of retGC and $G_{\alpha t}$ still occurred (Figure 2A), indicating that the RGS9– $G_{\beta 5L}$ complex is not required for the $G_{\alpha t}$ –retGC interaction. To determine whether the $G_{\alpha t}$ –retGC interaction occurs in cones, we used mice lacking the transcription factor Nrl, in which retinas contain only cone-like photoreceptors [31,32]. Figure 2(B) demonstrates that retGC co-precipitates with the cone $G_{\alpha t}$ and the RGS9– $G_{\beta 5L}$ complex. As in the experiments with bovine OS, in experiments on mouse OS, $G_{\alpha t}$ and retGC co-precipitated equally well from light- or dark-adapted retinas, and $G_{\beta 1}$ did co-precipitate with the mouse $G_{\alpha t}$ –retGC complex.

Reconstitution of the $G_{\alpha t}$ -retGC interaction in COS-7 cells

We transiently transfected rod $G_{\alpha t}$ and retGC cDNAs into COS-7 cells and performed immunoprecipitation under two different conditions. First, $G_{\alpha t}$ and retGC cDNAs were co-transfected into the same pool of cells, and then the membrane fractions were solubilized and subjected to immunoprecipitation. Secondly, retGC and $G_{\alpha t}$ were transfected separately, and the cell lysates obtained were mixed just prior to the immunoprecipitation. We found that $G_{\alpha t}$ and retGC co-immunoprecipitated only if the two proteins were co-expressed within the same cells. The $G_{\alpha t}$ -retGC complex could be reciprocally precipitated with either anti- $G_{\alpha t}$ or anti-retGC antibodies (Figure 3). The efficiency of immunoprecipitation (~50%) from co-transfected cells was similar for retGC and $G_{\alpha t}$. This is consistent with the idea that the expression ratio of these two proteins in COS-7 cells was nearly stoichiometrical in contrast with photoreceptors, where transducin is expressed in large excess.

Transducin has no effect on retGC activity *in vitro*

A previous report from another group suggested that the transducin $G_{\beta\gamma}$ complex could inhibit the activity of retGC [33]. To investigate whether either heterotrimeric transducin or its individual subunits could modulate retGC activity we purified $G_{\alpha t}$, either GTP[S] or GDP-bound, and $G_{\beta\gamma}$ from bovine OS and reconstituted them with retGC-containing membranes. We measured the enzymatic activity of retGC in the presence or absence of a sub-saturating concentration of recombinant GCAP-1, as described previously [12,34], and found that neither $G_{\alpha t}$ nor the $G_{\beta\gamma}$ complex had any detectable effect on retGC activity under these conditions (Figure 4).

$G_{\alpha t}$ interacts with retGC at its KHD

RetGC is usually regarded as having five domains defined in its structure: the ECD (extracellular domain), which is exposed either to the lumen of OS discs or outside the OS plasma membrane; a short TM (transmembrane) region; a KHD; a DD (dimerization domain) and a CAT domain (Figure 5A) [13,35]. In order to determine the site on retGC to which $G_{\alpha t}$ binds, we generated GST-fusion proteins of the CAT domain (GST-CAT) and KHD (GST-KHD) of retGC-1. The GST-fusion proteins were immobilized on glutathione-agarose beads and tested for their ability to pulldown purified transducin or transducin from crude OS lysates. The results of the present study showed that GDP- $G_{\alpha t}$ bound much stronger to the KHD than to the CAT domain of retGC. We also found that binding of GTP[S] to $G_{\alpha t}$ suppressed this interaction (Figure 5B). We then tested whether the purified GST-KHD or GST-CAT fusion proteins could block the interaction of $G_{\alpha t}$ with endogenous retGC. We found that neither GST-KHD (Figure 5C) nor GST-CAT (results not shown) reduced binding of endogenous retGC to $G_{\alpha t}$ in the co-immunoprecipitation assay. This suggested that GST-KHD bound primarily to the 'retGC-free' pool of $G_{\alpha t}$, and that full-length cyclase, which is a dimer, has a higher affinity to $G_{\alpha t}$.

Light-induced transducin redistribution occurs faster in mice lacking ret-GC1

Subcellular localization of transducin is determined by its interaction with the OS membranes [21]. Therefore we reasoned that the localization of transducin might be affected by retGC, which is a transmembrane protein localized in the OS. To test this idea, we examined light-dependent movement of transducin subunits in the rods of retGC-1-knockout mice [19]. We found that light-induced transducin redistribution from OS occurred substantially faster than in the wild-type animals (Figure 6). As a control in the present study we used mice lacking RGS9, a model where transducin movement is accelerated due to the increased lifetime of its GTP-bound state [21,36,37]. To our surprise, light-induced transducin relocation in the retGC-1 knockouts occurred even faster than in the RGS9 knockouts. Accordingly, the return of transducin from the inner compartments to the OS

after the onset of darkness was delayed compared with that in the wild-type mice (Figure 7). The results obtained on live mice sacrificed at different time points of light or dark adaptation were very similar to the results obtained on the eyecup preparations. The distribution of the rod transducin $G_{\beta\gamma}$ complex, which was monitored using anti- $G_{\beta1}$ antibodies, corresponded with that of $G_{\alpha t}$ under all of the conditions tested (results not shown).

Does cGMP play a role in transducin distribution?

The effect of retGC-1 knockout on transducin movement could theoretically result from the reduced guanylate cyclase activity (concentration of cGMP) in rods or from the absence of the protein–protein interaction between $G_{\alpha t}$ and retGC. To distinguish between these two possibilities, we studied transducin relocation in explanted permeabilized retinas in the presence or the absence of cGMP. We have previously shown that treatment of photoreceptors with *Staphylococcus aureus* α -toxin, a pore-generating polypeptide, allows the entry of nucleotides into the cells. Most proteins are too large to exit via the pores and remain inside. In these preparations transducin retains the ability to relocate in the light- and nucleotide-dependent manner, to the extent and with the kinetics similar to its redistribution in live animals [21]. In the present study, we used the same experimental paradigm to test the effect of cGMP and its poorly hydrolysed analogue 8-bromo-cGMP (Figure 8 and Supplementary Figure S2 at <http://www.BiochemJ.org/bj/417/bj4170803add.htm>).

To rule out the effects of PDE6-mediated cGMP degradation, we limited the study to the analysis of transducin migration to the OS, which occurs after the onset of darkness. We reasoned that it would be difficult to interpret the results of such an experiment if PDE6 is activated to its full extent. We expected that, if cGMP were involved in $G_{\alpha t}$ redistribution back to the OS, addition of a high concentration of cGMP could accelerate the migration. To monitor the hydrolysis of cGMP, aliquots of bathing media were analysed by thin-layer anion-exchange chromatography (Figure 8A and Supplementary Figure S2). We found that a fraction of cGMP was converted into GMP in a light- and GTP[S]-dependent manner (Supplementary Figure S2). Under the conditions used in the transducin relocation experiment (45 min in the dark) this fraction did not exceed 10%, indicating that there was an ample supply of cGMP available throughout the duration of the relocation experiment; degradation of 8-bromo-cGMP was negligible (Figure 8A).

The analysis of transducin translocation in the cGMP-treated eyecups by immunofluorescence microscopy showed that neither cGMP nor 8-bromo-cGMP affected the movement of $G_{\alpha t}$ to OS (Figure 8B). Treatment of the cells with GTP[S] used as a control for nucleotide entry into the cells resulted, as expected, in irreversible partitioning of transducin to the rod inner compartments. Thus our results strongly indicate that transducin relocation to the OS was not affected by cGMP, which also suggests that retGC-1 influences transducin movement, not through its cyclase activity, but through direct interaction with $G_{\alpha t}$.

DISCUSSION

Recent years have been marked by the discovery of novel functions and novel binding partners of heterotrimeric G-proteins. In addition to their receptors, effector enzymes, and RGS proteins, G-proteins were shown to associate with AGS (activator of G-protein signalling), Ric-8, centrin and other proteins [4,38–40]. The functional significance of these novel interactions is under investigation in a number of laboratories. One of the original goals of the present study was to search for new binding partners that could potentially play a role in the subcellular redistribution of transducin between different compartments of the

rod. In the course of the study we unexpectedly discovered that a direct protein–protein interaction occurs between $G_{\alpha t}$ and retGC.

We first detected the rod $G_{\alpha t}$ –retGC interaction through co-immunoprecipitation of this complex from native retinal tissue, and the following evidence argues that this interaction is specific: (i) retGC does not bind to Protein A beads coupled with control IgG, and binding of retGC to beads coupled with the anti-transducin antibody was blocked by the $G_{\alpha t}$ -specific antigenic peptide; (ii) the $G_{\alpha t}$ –retGC complex can be reciprocally immunoprecipitated with both anti- $G_{\alpha t}$ and anti-retGC antibodies; (iii) co-immunoprecipitation was detected using different preparations (bovine and mouse rod OS, $Nrl^{-/-}$ cone OS and COS-7 cells expressing recombinant retGC and $G_{\alpha t}$); (iv) the results of co-immunoprecipitation are supported by the GST-fusion pulldown using specific domains of retGC; (v) the $G_{\alpha t}$ –retGC complex formation depended on the nucleotide-bound state of $G_{\alpha t}$, with its GDP-bound form being the preferred conformation; and (vi) the efficiency of $G_{\alpha t}$ co-immunoprecipitation with retGC was better than that of its known binding partner, the $G_{\beta 5L}$ –RGS9 complex.

The results of the present study indicate that $G_{\alpha t}$ and retGC can interact with each other directly. Previous work by Yamazaki and co-workers [41–43] implied that RGS9 can bind to retGC and regulate its activity. We reasoned that the $G_{\alpha t}$ –retGC interaction could be mediated by RGS9/ $G_{\beta 5L}$. Indeed, RGS9 and $G_{\beta 5L}$ were present in our $G_{\alpha t}$ co-immunoprecipitation fractions along with retGC. However, we did not detect $G_{\beta 5L}$ in the immunoprecipitates with anti-retGC antibodies (Figure 1C), and found no major difference in the efficiency of the co-immunoprecipitation of the $G_{\alpha t}$ –retGC complex obtained from wild-type and RGS9-knockout mice (Figure 2). We conclude that RGS9 is not required for the binding of transducin to retGC, and it apparently co-precipitates with $G_{\alpha t}$ independently. Another observation that supports direct $G_{\alpha t}$ –retGC interaction is that purified GST-fused individual retGC domains also bound transducin from retinal lysates or purified transducin (Figure 5). On the other hand, we found that if $G_{\alpha t}$ and the full-length retGC were separately expressed in COS-7 cells, the interaction did not occur when the two cell lysates were simply mixed together. This indicates that, in order to form a stable complex in COS-7 cells, $G_{\alpha t}$ and the full-length retGC must associate before their solubilization in detergent. For example, the two native proteins must be correctly oriented within a cell, perhaps with participation of other membrane and/or cytoskeleton components. It is possible that once $G_{\alpha t}$ and full-length retGC form the complex, other proteins and membranes are no longer required to maintain the apparent high affinity of the interaction in detergent solution. This notion is supported by our observation that recombinant KHD domain cannot displace retGC from its pre-existing native complex with $G_{\alpha t}$ (Figure 5C).

What is the potential significance of the $G_{\alpha t}$ –retGC interaction? First, we tested whether transducin subunits could modulate the catalytic activity of retGC (Figure 4). We found that, regardless of $G_{\alpha t}$ activation status (GDP compared with GTP[S]-bound) or the presence of the retGC activating protein GCAP-1, transducin did not modulate retGC activity. These results are in contrast with an earlier report on modulation of retGC activity by the $G_{\beta \gamma}$ complex of transducin [33]. The reason for this discrepancy is not immediately apparent, but we concluded the interaction with $G_{\alpha t}$ is unlikely to be a part of the cyclase regulation itself and instead may rather contribute to a function different from phototransduction. We therefore tested its potential involvement in a control of transducin distribution between the OS and inner compartments.

The knockout of the retGC-1 gene is known to result in mislocalization of transducin in cones, which primarily express retGC-1 [44]. In a retGC-1/retGC-2 double knockout, rod $G_{\alpha t}$ localization is ‘patchy’, and transducin is poorly expressed or undergoes faster

degradation [45]. There could be many potential explanations for these effects, including gross changes associated with cell death resulting from cGMP deficiency. In those studies the researchers did not observe a major effect of retGC-1 knockout on rod transducin localization apparently because they focused on localization of transducin in completely light- or dark-adapted states [45]. However, analysis of transducin migration kinetics presented in the present study revealed a robust effect. Light-induced movement from the OS was markedly faster than in both wild-type and RGS9-null mice, while the return of transducin to the OS in the dark was delayed (Figures 6 and 7). Supplementation of permeabilized cells with cGMP or its slowly hydrolysing analogue had no effect on transducin movement back to the OS (Figure 8), indicating that the observed effect of retGC-1 knockout can be explained by the protein–protein interaction between transducin and retGC-1, rather than a reduction in cyclase activity.

The present study showed (i) the effect of retGC-1-knockout on transducin localization, and (ii) the direct retGC– $G_{\alpha t}$ interaction; however, the causal relationship between the two phenomena remains a hypothesis. The idea that a binding partner localized in the OS should facilitate localization of a diffusing protein within the OS is consistent with our model of transducin and arrestin relocation in rods [16]. According to this model, diffusion of signal transduction proteins is restricted in cell compartments by their interacting partners ('sinks') such as rhodopsin for arrestin [28] and disc membranes for transducin [21]. OS membrane attachment, which requires lipidation of both $G_{\alpha t}$ and $G_{\beta\gamma}$ subunits and is regulated by the subunit dissociation, is the crucial factor in transducin localization. If lipidation or subunit association is disrupted, transducin loses its ability to concentrate in the OS [21,46,47]. Binding of $G_{\alpha t}$ to retGC identified in the present study is certainly not as crucial as membrane association. It should be viewed as one of the mechanisms that contribute to transducin translocation at the quantitative level, as do GTP hydrolysis [21,36,37], interaction with phosducin [8], the recently reported interaction with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [10] and cytoskeletal elements [9,48]. Interestingly, among these mechanisms, the interaction with retGC appears to have the most profound effect on transducin migration (Figures 6 and 7). At the moment, we cannot provide a complete mechanistic explanation to this finding. RetGC cannot act as a 'sink' for transducin because it is expressed in rods at a much lower level than transducin, whereas the sink must be as abundant as the migrating protein. We can speculate that the initial docking with retGC facilitates a conformation of transducin that favours its anchoring into the membrane bilayer, for example by exposing the N-myristoyl group of $G_{\alpha t}$. Such an action could explain how retGC can influence localization of the much more plentiful transducin. Along with the sheer abundance of disc membranes in the rod, this hypothetical mechanism could also explain why, after the onset of darkness, diffusing transducin is targeted to the OS rather than other membranes in the cell. For this mechanism to be feasible, binding of transducin with retGC should be reversible, however our co-immunoprecipitation results indicate very strong binding. It is possible that the interaction is more dynamic *in situ*, where transducin can interact with lipids instead of retGC. When the membrane is solubilized for co-immunoprecipitation, $G_{\alpha t}$ becomes trapped in a high-affinity complex with retGC. One can also speculate that a dynamic interaction between transducin and cyclase might be influenced by the formation of larger protein complexes involving retGC [4,41,49–51]. Regardless of the underlying mechanism, the correlation between the $G_{\alpha t}$ –retGC interaction and localization of transducin is very intriguing and deserves further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CAT domain	catalytic domain
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
GCAP	guanylate cyclase activating protein
GST	glutathione transferase
GTP[S]	GTP γ S, guanosine 5'-[γ -thio]triphosphate
HEK	human embryonic kidney
KHD	kinase homology domain
MS/MS	tandem MS
OS	outer segment
PDE6	phosphodiesterase 6
PEI-cellulose	polyethyleneimine-cellulose
retGC	retinal guanylate cyclase
RGS9	regulator of G-protein signalling 9
TFA	trifluoroacetic acid

Figure 1. Co-immunoprecipitation of retGC with $G_{\alpha t}$

(A) Bovine OS were solubilized with 1% Triton X-100 and subjected to immunoprecipitation as described in the Experimental section, using an anti- $G_{\alpha t}$ IgG in the presence or absence of the blocking peptide (Santa Cruz Biotechnology; SC389 P). The eluates from the beads were resolved on a gel and silver-stained. The indicated 115 kDa band was analysed by MS. The $G_{\alpha t}$ band was excised and analysed as a control. The molecular mass in kDa is indicated on the left-hand side of the gel. (B) Immunoprecipitation of $G_{\alpha t}$ and retGC from bovine OS using anti- $G_{\alpha t}$ and anti-retGC antibodies. Shown are representative Western blots analysing the eluted (E), unbound (U) fractions and the input (I) OS lysate. The eluate is 10-fold more concentrated compared with the input and the unbound fractions (see the Experimental section for more details). ctrl, control. (C) Co-immunoprecipitation of photoreceptor proteins with rod $G_{\alpha t}$ and retGC from bovine OS. The unbound (U) and eluted (E) fractions were probed with the specific antibodies recognizing retGC-1, $G_{\alpha t}$, $G_{\beta 1}$ and $G_{\beta 5}$. Protein A beads with control IgG were used as a negative control. (D) The relative amount (percentage of total) of rod $G_{\alpha t}$, $G_{\beta 1}$, $G_{\beta 5}$ and retGC immunoprecipitated with an anti- $G_{\alpha t}$ antibody was determined by densitometry of the scanned Western blots. Quantification of protein amounts in co-immunoprecipitation fractions was performed by comparison with serial dilutions of OS lysate as described in Supplementary Figure S1 (at <http://www.BiochemJ.org/bj/417/bj4170803add.htm>). The values shown are the scanned arbitrary units in the eluate fraction divided by the sum of eluate and unbound fractions and multiplied by 100% (means $\pm$ S.D.), determined in independent experiments with $n = 10$ for $G_{\alpha t}$, $n = 8$ for retGC and $n = 3$ for $G_{\beta 5}$ or $G_{\beta 1}$. IP, immunoprecipitation.

Figure 2. Co-immunoprecipitation of retGC with $G_{\alpha t}$ from mouse photoreceptors
(A) Rod $G_{\alpha t}$ was immunoprecipitated from mouse OS lysates prepared from wild-type or RGS9-knockout mice. The immunoprecipitation fractions were probed with anti- $G_{\alpha t}$, anti-retGC, anti-RGS9 and anti- $G_{\beta 5}$ antibodies. Results shown are representative of at least three experiments. **(B)** The OS lysates were prepared from the photoreceptors of *Nrl*-knockout mice, and immunoprecipitated using antibodies specific to cone $G_{\alpha t}$. The immunoprecipitation fractions were probed with anti-cone $G_{\alpha t}$, anti-retGC, anti-RGS9 and anti- $G_{\beta 5}$ antibodies. In both **(A)** and **(B)**, normal IgG was used as the negative control for immunoprecipitation. Results shown are representative of three independent experiments. E, eluate fraction; IP, immunoprecipitation; U, unbound fraction.

Figure 3. Reconstitution of ret-GC with rod $G_{\alpha t}$ in transfected COS-7 cells

Human rod $G_{\alpha t}$ and retGC-1 cDNAs were transiently expressed in COS-7 cells. Cell lysates were immunoprecipitated with anti- $G_{\alpha t}$ (A), anti-retGC (B) or control IgG. The immunoprecipitation (IP) fractions were analysed with anti- $G_{\alpha t}$ and anti-retGC antibodies. Results are representative of three independent experiments. E, eluate fraction; U, unbound fraction.

Figure 4. Transducin does not affect the catalytic activity of retGC

Purified rod $G_{\alpha t}$, $G_{\beta\gamma}$ subunit complex of transducin or BSA was incubated with HEK-293 membranes expressing retGC-1, in the presence or absence of recombinant GCAP-1. cGMP production was measured using radiolabelled GTP as the substrate, as described in the Experimental section. Values (means $\pm$ S.D.) from three independent experiments using two different preparations of transducin subunits.

Figure 5. Preferential binding of $G_{\alpha t}$ to the KHD of retGC

(A) Schematic representation of the retGC domains. DD, dimerization domain; ECD, extracellular domain; TM domain, transmembrane domain. (B) Top panel: Coomassie-Blue-stained gel showing the purity of GST–KHD and GST–CAT products. Bottom panel: 20 μ g of GST–KHD, GST–CAT or GST were immobilized on glutathione beads, which were added to mouse retinal lysates containing approx. 1 μ g of $G_{\alpha t}$. Identical results were obtained with purified transducin subunits (results not shown). Extracts from *Nrl*^{−/−} retinas were used for analysis of cone $G_{\alpha t}$ binding. The GST-pulldown assays were performed as described in the Experimental section, and the specifically bound rod and cone $G_{\alpha t}$ were detected by Western blot analysis. Results are representative of three independent experiments. The molecular mass in kDa is indicated on the left-hand side of the gel. (C) Immunoprecipitation of rod $G_{\alpha t}$ was performed as described in Figure 1, except that here, 20 μ g of GST–KHD (final concentration 0.1 mg/ml) was added to the lysate during the pre-clearing stage. An equivalent amount of GST was added to the control. Following the immunoprecipitation, the fractions were analysed with anti- $G_{\alpha t}$ and anti-retGC1 antibodies. Specific anti-KHD antibodies (6344KHD) [23] were used to detect GST–KHD in the fractions. Results are representative of two independent experiments. IP, immunoprecipitation.

Figure 6. Rate of light-induced transducin migration in retGC-1-knockout mice

Eye cups were prepared from wild-type, retGC-1-knockout and RGS9-knockout mice under IR light. The eye cups were then illuminated at 500 lux for the indicated times prior to fixation. The retinas were sectioned, stained with the antibodies against $G_{\alpha t}$ and analysed by laser confocal microscopy as described in the Experimental section. **(A)** Representative sequence of sections from wild-type and retGC-1-null mice. IS, inner segments, ONL, outer nuclear layer, ST, synaptic termini. **(B)** Quantitative analysis of relative fluorescence intensity in the inner compartments. The ratio of combined fluorescence signal from the inner compartments (IS + ONL + ST) to the total fluorescence from the end of OS to the ST was determined as described previously [21]. Results are representative of four independent experiments for retGC-1-null and wild-type mice, and three experiments for RGS9-null mice. In each experiment, fluorescence was analysed from three randomly selected areas per retinal section. Error bars represent means $\pm$ S.D. GC $^{-/-}$, retGC-1-null mice; RGS $^{-/-}$, RGS9-null mice; WT, wild-type.

Figure 7. Time course of transducin migration to the OS in retGC-1 mice

Eyecups were prepared as in Figure 6 and illuminated for 30 min at 500 lux to induce transducin translocation. The retinas were then placed in the dark for the indicated times, fixed, sectioned and analysed for localization of $G_{\alpha t}$. **(A)** Representative sequence of sections. IS, inner segments, ONL, outer nuclear layer, ST, synaptic termini. **(B)** Quantitative analysis of relative fluorescence intensity in the inner compartments was performed as in Figure 6(B). Error bars represent the mean $\pm$ S.D.; $n = 4$ for retGC-1-null and wild-type mice and $n = 3$ for RGS9-null mice. GC^{-/-}, retGC-1-null mice; RGS^{-/-}, RGS9-null mice; WT, wild-type.

Figure 8. cGMP has no effect on transducin translocation

Eyecups were prepared from dark-adapted wild-type mice under dim red light and incubated for 30 min with α -toxin as described in the Experimental section. They were then illuminated for 30 min at 500 lux in the presence of indicated nucleotides, including GDP, GTP[S], cGMP and 8-bromo-cGMP. They were then transferred into a fresh medium supplemented with the nucleotides and finally placed in the dark for 45 min, which caused approx. 75% of transducin to return to the OS (see Figure 7). (A) After 45 min in darkness, 5 μ l of medium was collected and analysed for the presence of the nucleotides using TLC. The positions of cGMP, 8-bromo-cGMP, GMP, GDP or GTP are indicated with arrows. The histogram shows the results of spectrophotometric analysis of the nucleotides eluted from the spots. The values represent the absorption at 260 nm of the remaining cGMP and 8-bromo-cGMP compared with the sum of the residual cGMP with GMP product, which was set as 100%. Values are means $\pm$ S.D. from four independent experiments. See the text and Supplementary Figure S2 (at <http://www.BiochemJ.org/bj/417/bj4170803add.htm>) for details. (B) Localization of rod G_{at} in the treated eyecups was analysed after 45 min incubation in darkness using immunofluorescence microscopy of the retinal sections as described in Figures 6 and 7. The results (means $\pm$ S.D., $n = 5$) show the amount of G_{at} immunofluorescence in inner compartments as the percentage of the total immunofluorescence signal. 8-Br, 8-bromo-cGMP.