2008-12-04

Light-Induced Relocalization of the Photoreceptor G Protein Transducin is Mediated by Binding Partner-Restricted Diffusion: New Insights into G Protein Subunit Dissociation

Derek H. Rosenzweig
University of Miami, drosenzweig@med.miami.edu

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
Rosenzweig, Derek H., "Light-Induced Relocalization of the Photoreceptor G Protein Transducin is Mediated by Binding Partner-Restricted Diffusion: New Insights into G Protein Subunit Dissociation" (2008). Open Access Dissertations. 172.
https://scholarlyrepository.miami.edu/oa_dissertations/172

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
UNIVERSITY OF MIAMI

LIGHT-INDUCED RELOCALIZATION OF THE PHOTORECEPTOR G PROTEIN TRANSDUCIN IS MEDIATED BY BINDING PARTNER RESTRICTED DIFFUSION: NEW INSIGHTS INTO G PROTEIN SUBUNIT DISSOCIATION

By
Derek H. Rosenzweig

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida
December 2008
LIGHT-INDUCED REDISTRIBUTION OF THE PHOTORECEPTOR G PROTEIN
TRANSDUCIN IS MEDIATED BY BINDING PARTNER-RESTRICTED
DIFFUSION: NEW INSIGHTS INTO G PROTEIN SUBUNIT DISSOCIATION

Derek H. Rosenzweig

Approved:

Vladlen Z. Slepak, Ph.D.
Professor of Molecular & Cellular
Pharmacology

Terri A. Scandura, Ph.D.
Dean of the Graduate School

Charles W. Luetje, Ph.D.
Professor of Molecular & Cellular
Pharmacology

Jeffrey L. Goldberg, M.D.,Ph.D.
Assistant Professor of
Ophthalmology and Cell Biology
and Anatomy

John L. Bixby, Ph.D.
Professor of Molecular & Cellular
Pharmacology and Neuroscience;
Associate Dean for Graduate Studies;
Director UM Neuroscience Center

Donald F. Ready, Ph.D.
Professor of Biological Sciences
Purdue University
Abstract of a doctoral dissertation at the University of Miami.

Dissertation supervised by Professor Vladlen Z. Slepak. No. of pages in text: (133).

Phototransduction is a well characterized system for study of G protein coupled receptor (GPCR) signaling. The GPCR rhodopsin couples to the heterotrimeric G protein transducin. Light-stimulated activation of transducin in turn activates phosphodiesterase (PDE), leading to closure to cGMP-gated channels and inhibition of glutamate release. Rod and cone photoreceptors are highly polarized neurons consisting of the outer segment (OS) where phototransduction biochemistry occurs, the inner segment containing mitochondria and other organelles, the nuclear layer, an axon, and a glutamatergic synapse. Upon illumination, activated G protein transducin redistributes from the rod OS (where it is localized in the dark) to the inner compartments of the cell. Interestingly, cone transducin does not translocate in light. Opposite to this, visual arrestin migrates from the inner compartments to the OS, where it binds to rhodopsin.

Previous reports from other groups and our lab argue for either an active or passive mechanism for transducin and arrestin redistribution. Our lab has shown that arrestin migration occurs by diffusion which is restricted by molecular
sinks (Nair et al, 2005b). The focus of my dissertation was to unravel the molecular mechanism of rod transducin translocation.

Specifically, I found energy (ATP) was not required for transducin movement within photoreceptors. Also, I found that the disc membranes of the rod outer segments as well as protein-protein interactions with retinal guanylate cyclase serve to restrict transducin diffusion through the cell. In addition, I used the insights gained from these studies of transducin to re-examine the relationship of other G proteins’ subcellular localization and signal transduction. Ultimately, I found that most G proteins do not undergo subunit dissociation under physiological activating conditions.
# TABLE OF CONTENTS

LIST OF FIGURES .......................................................................................................... vi
LIST OF ABBREVIATIONS ............................................................................................. ix

CHAPTER 1

INTRODUCTION

- G protein mediated signal transduction ............................................................... 1
- Photoreceptor Morphology .................................................................................... 3
- Phototransduction .................................................................................................. 5
- Photoreceptor physiology ..................................................................................... 7
- Light-induced redistribution of photoreceptor proteins ........................................ 8
- G protein redistribution in invertebrates ............................................................. 13
- Cone cell biology is different compared to rods ................................................. 14
- Protein-protein interactions of transducin ......................................................... 15
- Subunit dissociation and localization of non-photoreceptor G proteins .......... 16

CHAPTER 2

MATERIALS AND METHODS

- Animals and tissue preparation ......................................................................... 19
- Immunofluorescence ............................................................................................ 20
- Ex vivo experiments ............................................................................................... 22
- Biochemical assays ............................................................................................... 23
- Retinoid analyses ................................................................................................ 25
- Analysis of N-myristoylation ............................................................................... 26
- Mass spectrometry analysis of excised protein bands ........................................ 27
- Immunoprecipitation ............................................................................................ 28
- Cell culture, transfection and membrane fractionation ....................................... 29
- Guanylate cyclase activity assay .......................................................................... 30
- GST pull down assay ........................................................................................... 31
- Thin layer chromatography analysis of nucleotides .......................................... 32
- Preparation of lipid rafts from mouse ROS ....................................................... 32
- Assessment of calcium mobilization in CHO-K1 cells ...................................... 33
- Antibodies, peptides, and plasmids ..................................................................... 34
CHAPTER 3
RESULTS

I. Transducin Localization is Determined by Subunit Dissociation and Diffusion ............... 35
   GTP, but not ATP, is required for transducin redistribution .................. 35
   Cone transducin in wild type and manipulated photoreceptors .......... 40
   Are cone photoreceptors fully activated? .............................................. 42
   Membrane association of transducin .................................................. 49
   N-acylation and localization of transducin ............................................. 51
   Subunit dissociation facilitates transducin dispersion ........................ 53

II. Retinal guanylate cyclase interacts with the alpha subunit of transducin
   and facilitates its translocation in retinal rods ........................................ 60
   RetGC co-precipitates with bovine rod Gαt ........................................ 60
   RetGC co-precipitates with mouse rod and cone Gαt subunits .......... 65
   Reconstituted Gαt:retGC interaction in COS-7 cells ................................. 67
   Transducin has no affect on retGC activity ........................................ 69
   Gαt interacts with retGC at the kinase homology domain .................. 69
   Does retGC coordinate Gαt localization to lipid rafts? ................ 72
   Light induced transducin redistribution in mice lacking retGC-1 ........ 75
   The role of cGMP in transducin distribution .................................. 77

III. Persistent dissociation of non-photoreceptor G protein subunits
   alters subcellular localization and disrupts signal transduction .............. 82
   mSIGK causes membrane detachment of Gi, Go, and Gq .................... 82
   mSIGK induces subunit dissociation of non-photoreceptor G proteins . 84
   Redistribution of Gβ1 in CHO-K1 and COS-7 cells ............................. 86
   Subunit dissociation alters signal transduction in CHO-K1 cells ........ 89
CHAPTER 4
DISCUSSION

I. A model for light-induced transducin translocation ................................................................. 93

Transducin translocates to the inner compartments via subunit dissociation, membrane detachment and diffusion ......................................................... 94
Subunit association, membrane binding, and localization ........................................ 96
Transducin redistribution ................................................................................ 100
G protein subunit dissociation ........................................................................ 102

II. Binding partners of transducin restrict its dissociation and localization ...................... 102

Identification of $G_\alpha_t$:retGC complex ................................................................. 103
RetGC is binding partner that restricts diffusion of transducin ........................ 105
The $G_\alpha_t$:retGC complex and the diffusion model ...................................... 108

III. Subcellular localization of non-photoreceptor G proteins and regulation of signal transduction ............................................................. 111

Localization of endogenously expressed Gi, Go, and Gq ................................ 113
Subunit dissociation causes G protein redistribution in cell lines .......... 115
How does G protein subunit dissociation affect signaling? ...................... 116
Pharmacological implications ........................................................................ 118

REFERENCES ................................................................................................................. 122
### LIST OF FIGURES

#### CHAPTER 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Agonist-induced G protein signal transduction</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>The phototransduction cascade within a rod cell</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>Light-induced protein redistribution in rod cells</td>
<td>11</td>
</tr>
</tbody>
</table>

#### CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Preparation of mouse eyecups</td>
<td>20</td>
</tr>
<tr>
<td>2.2</td>
<td>Delivery of small molecules to mouse eyecups</td>
<td>23</td>
</tr>
</tbody>
</table>

#### CHAPTER 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Permeabilization of retinas allows small molecule delivery</td>
<td>36</td>
</tr>
<tr>
<td>3.2</td>
<td>Light-dependent movement of rod transducin does not require ATP</td>
<td>37</td>
</tr>
<tr>
<td>3.3</td>
<td>Relocalization of rod transducin is governed by GTP binding and hydrolysis</td>
<td>38</td>
</tr>
<tr>
<td>3.4</td>
<td>Relocalization of rod transducin is faster in RGS9(^{-}) mice, which lack GTPase activity on G(\alpha_t)</td>
<td>39</td>
</tr>
<tr>
<td>3.5</td>
<td>Behavior of cone Gt in cones and rods</td>
<td>41</td>
</tr>
<tr>
<td>3.6</td>
<td>UV light exposure of Nrl(^{-}) mouse eyecups</td>
<td>43</td>
</tr>
<tr>
<td>3.7</td>
<td>Retinoid analysis in Nrl(^{-}) retinas under UV or sunlight illumination</td>
<td>44</td>
</tr>
<tr>
<td>3.8</td>
<td>Cone transducin binds GTP(\gamma_S) and undergoes subunit dissociation in detergent solution</td>
<td>46</td>
</tr>
<tr>
<td>3.9</td>
<td>Cone transducin binds GTP(\gamma_S) and is protected from trypsinolysis</td>
<td>48</td>
</tr>
<tr>
<td>3.10</td>
<td>Cone transducin binds GTP(\gamma_S) but does not detach from OS membranes</td>
<td>50</td>
</tr>
<tr>
<td>3.11</td>
<td>Distinct N-acylation of rod and cone transducin subunits</td>
<td>52</td>
</tr>
</tbody>
</table>
Figure 3.12 Translocation of rod transducin is facilitated by the synthetic Gβ-binding peptide mSIRK ......................................................54

Figure 3.13 Translocation cone transducin is facilitated by the synthetic Gβ-binding peptide mSIRK ......................................................54

Figure 3.14 Quantification of the series of data from peptide induced translocation ..............................................................................55

Figure 3.15 Translocation of cone transducin is facilitated by the synthetic Gβ-binding peptide mSIRK in Nrl⁻/⁻ mice ..................................56

Figure 3.16 Subunit dissociation underlies the difference between rod and cone transducins in membrane detachment .......................57

Figure 3.17 mSIRK facilitates transducin subunit dissociation in detergent solutions..................................................................................58

Figure 3.18 A ~115 kDa band consistently co-precipitates with Gαt..........................60

Figure 3.19 Co-immunoprecipitation of Gαt with retGC in bovine ROS .....................62

Figure 3.20 retGC Co-immunoprecipitates with Gαt in mouse ROS ......................65

Figure 3.21 Co-immunoprecipitation of retGC with cone Gαt ..................................66

Figure 3.22 Reconstitution of ret-GC with rod Gαt in transfected COS-7 cells ..............................................................................................67

Figure 3.23 Transducin does not affect catalytic activity of retGC .........................68

Figure 3.24 Preferential binding of Gαt to the kinase homology domain of retGC .......................................................................................70

Figure 3.25 Addition of recombinant KHD to the Gαt IP .............................................71

Figure 3.26 Preparation of detergent resistant membranes (Lipid Rafts) from mouse outer segments .........................................................72

Figure 3.27 Transducin migrates to lipid rafts in retGC null mouse OS .....................73

Figure 3.28 Rate of light-induced transducin migration to the inner compartments of rods in retGC-1 knockout mice ...............................74

Figure 3.29 Time-course of transducin migration to the rod OS in retGC-1 null mice .....................................................................................75

Figure 3.30 Rate of light-induced Gβγ migration in retGC-1 knockout mice ......................76
Figure 3.31 Degradation of cGMP is greatly accelerated by GTPγS in bovine ROS ................................................................. 78
Figure 3.32 Cyclic nucleotides can be delivered to permeabilized eyecups ........................................................................... 79
Figure 3.33 Cyclic nucleotides remain stable throughout the duration of experimentation ...................................................... 80
Figure 3.34 cGMP has no effect on transducin translocation .................................................. 81
Figure 3.35 mSIGK induced membrane detachment of G proteins in native tissue ........................................................ 83
Figure 3.36 Quantification of mSIGK induced membrane detachment of G proteins ........................................................ 83
Figure 3.37 mSIGK, but not GTPγS, induces G protein membrane detachment in cell lines ...................................................... 84
Figure 3.38 Heterotrimeric G protein subunits dissociate in presence of mSIGK in a detergent solution ........................................... 85
Figure 3.39 mSIGK induced relocalization of Gβ1 CHO-k1 cells ........................................ 87
Figure 3.40 mSIGK induced relocalization of Gβ1 COS-7 cells ........................................ 88
Figure 3.41 Subunit dissociation induces calcium mobilization in CHO-K1 cells ........................................................ 89
Figure 3.42 Quantification of mSIGK-induced Ca^{2+} mobilization in CHO-K1 cells ......................... 90
Figure 3.43 mSIGK induces receptor independent ERK activation in CHO-K1 cells via subunit dissociation ................................. 91

CHAPTER 4
Figure 4.1 Model of the mechanism of light-induced transducin dispersion ........................................................................... 97
Figure 4.2 Hypothesis for retGC-facilitated targeting of transducin to OS membranes ............................................................ 110
ABBREVIATIONS

**G protein** – guanine nucleotide binding regulatory protein

**GPCR** - G protein coupled receptor

**Gα** – guanine nucleotide binding protein alpha subunit

**Gβγ** – guanine nucleotide binding protein beta/gamma subunits

**GTP** – guanosine 5’-triphosphate

**GDP** – guanosine 5’-diphosphate

**cGMP** – guanosine 3′,5′-cyclic monophosphate

**ATP** – adenosine 5’-triphosphate

**RGS** – regulator of G protein signaling

**retGC** – retinal guanylate cyclase

**PDE** – phosphodiesterase

**mSIGK** – myristoyl-SIGKALNILGYPDYD

**mSIRK** – myristoyl-SIRKALNILGYPDYD

**L9A** – myristoyl-SIRKALNIAGYPDYD (inactive, mutant of mSIRK)

**CAT** – catalytic domain of retGC

**KHD** – kinase homology domain of retGC

**8-Br** – 8-bromo-guanosine 3′,5′-monophosphate

**DMEM** – Dulbecco’s modified eagle media

**COS-7** – CV-1 Origin SV40 cells

**CHO-K1** – Chinese hamster ovary cells

**kDa** – kilo Daltons

**Da** – Daltons

**OS** – outer segments

**IS** – inner segments

**ONL** – outer nuclear layer

**ST** – synaptic terminal
OPL – outer plexiform layer (ST)
ROS – rod outer segments
GRK – G protein receptor kinase
GAP – GTPase activating protein
GCAP – guanylate cyclase activating protein
RPE – retina pigment epithelial
LGN - Leu-Gly-Asn repeat-enriched proteins
AMF – Al\(^{3+}\), Mg, F in solution
FRET – fluorescence resonance energy transfer
PBS – phosphate buffered saline
BSA – bovine serum albumin
DTT – dithiothreitol
HPLC – high pressure liquid chromatography
CID – collision induced dissociation
LC MS/MS – liquid chromatography followed by tandem mass spectrometry
GFP – green fluorescent protein
BODIPY – BOron-Difluoride-DIPYrrmethene (fluorescent dye)
GTP\(_{\gamma}S\) – non-hydrolyzable GTP
Nrl – neural retina leucine zipper
E – eluted fractions in immunoprecipitations
U – unbound fractions in immunoprecipitations
IP - immunoprecipitation
Chapter I
INTRODUCTION

G protein Mediated Signal Transduction

G protein coupled receptors (GPCRs) represent one of the largest families of cell surface receptors, responsible for relaying extracellular stimuli such as hormones, neurotransmitters, and odorants to stimulate a physiological cellular response. These seven-pass transmembrane proteins couple to the large family of heterotrimeric G proteins, which consist of an alpha, beta, and gamma subunit (Gαβγ). GPCRs act as guanine nucleotide exchange factors (GEFs) to heterotrimeric G proteins: upon agonist stimulation, conformational changes on the GPCR induce exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the alpha subunit of the coupled G protein (Limbird et al., 1980; Kwok-Keung Fung and Stryer, 1980; Northup et al., 1982; Smigel et al., 1982). The accepted model for G protein activation proposes that the GTP-bound alpha subunit of the G protein (Gα) dissociates from the beta/gamma subunit (Gβγ) (Northup et al., 1983). The dissociated subunits are free to interact with and stimulate target effector proteins. In turn, the activated effector (usually an enzyme or ion channel) regulates a second messenger, leading to a physiological response within the cell (Figure 1.1).

Within mammals, there are over 1000 GPCRs (the human genome encodes about 800 GPCR genes), each one evolving to effectively bind and respond to a particular agonist. Coupling to the thousands of receptors are the heterotrimeric G proteins. There are 15 known Gα subunits, 5 Gβ subtypes, and
11 Gγ subunits—which combine into the various heterotrimers. Their function is to amplify the signals from thousands of inputs (Downes and Gautam, 1999). The amino acid sequences for the α, β, and γ subunits are highly conserved when compared across mammalian species. Since G protein mediated signaling is involved in many physiological processes, the study of structure and function of GPCRs and their ligands and ligand-binding sites are of major pharmacological relevance (Milligan, 2006). Moreover, the GPCRs are the targets of more than 25% of the marketed drugs (Hopkins and Groom, 2002).

Figure 1.1 Agonist induced signal transduction by GPCRs and G proteins. Agonist (yellow ovals) binds to the receptor (pink) on the extracellular side of the plasma membrane. Agonist-bound receptor induces exchange of GTP for GDP on the alpha subunit (orange) of the trimeric G protein. GTP-bound alpha subunit dissociates from Gβγ (blue and purple), and it activates an effector (light blue). The effector modulates the concentration of second messengers (red triangles), which leads to a physiological response.

Signals transmitted by GPCRs can be terminated in two ways. The first of these mechanisms is that Gα subunits contain an intrinsic ability to hydrolyze
GTP to GDP (GTPase activity) (Mixon et al., 1995; Coleman et al., 1994). This inherent GTPase activity is greatly accelerated by members of the regulators of G protein signaling (RGS proteins), which act as GTPase activating proteins, or GAPs by directly interacting with GTP-bound Gα subunits (Berstein et al., 1992; Arshavsky and Bownds, 1992; Chen et al., 1996). Second, the receptors themselves can be shut down. Activated GPCRs are substrates for G protein receptor kinases (GRKs) which phosphorylate the receptor (Weller et al., 1975; Benovic et al., 1987), making the receptor a high affinity binding site for arrestins (Lohse et al., 1990; Wilden et al., 1986). Binding of arrestin blocks further stimulation of the G protein, and it can also induce receptor internalization, thus preventing further signaling (Goodman et al., 1996; Lin et al., 1997).

Photoreceptor Morphology

The vertebrate retina is made up of layers of specialized neurons. In the outer-most layer, the photoreceptors consist of rods and cones. Rods are highly sensitive, functioning under low illumination. Cones are much less sensitive, functioning under high levels of illumination and are responsible for color vision. Photoreceptors are polarized neurons consisting of the light sensitive dendrite-like structure called the outer segment (OS), an inner segment containing mainly mitochondria and other organelles, a nuclear layer, and an axon ending in a glutamate releasing synapse. The prototypical GPCR, rhodopsin, is localized exclusively in the OS and is activated by light. Rhodopsin was the first GPCR to
be cloned and sequenced (Hargrave and Fong, 1977; Ferretti et al., 1986), as well as the first to be crystallized (Palczewski et al., 2000).

Photoreceptors are highly metabolic, requiring maintenance of cholesterol levels in OS, rhodopsin levels and OS length, phospholipid oxidation in the OS, pigment turnover, as well as many other processes (Fliesler and Schroepfer, 1986; Schremser and Williams, 1995; Sun et al., 2006; Flannery et al., 1990). These terminally differentiated neurons must remain stable and viable throughout the lifetime of an organism. Rod cells typically contain around one hundred disc membranes within the OS, whereas cones contain many invaginations or extended folds of the plasma membrane. Disc membranes of the rod OS are constantly being formed at the base of the OS, and they migrate towards the distal end of the cell over the course of several days. When they reach the distal tip of the OS, they are absorbed by the retinal pigment epithelial cells (RPE). This process is regulated by circadian rhythms, illumination and temperature (Nguyen-Legros and Hicks, 2000). If any part of this process is compromised, the result is ultimately photoreceptor degeneration. Light converts large amounts of 11-cis-retinal pigment into all-trans-retinal in the OS, of which the rods and cones cannot regenerate. To constantly maintain the pigments within the photoreceptors, the all-trans-retinal bi-product is taken up into the retinal pigment epithelium at the distal ends of the photoreceptors where the disc membrane shedding and phagocytosis occurs. In the RPE, the all-trans-retinal undergoes further metabolic breakdown, and finally is converted back to 11-cis-retinal to be delivered back to the photoreceptors (McBee et al., 2001).
Phototransduction

The details of the phototransduction cascade are highlighted in Figure 1.2. Photo-active rhodopsin initiates the photo-response by inducing the exchange of GDP for GTP on the $\alpha$ subunit of the heterotrimeric G protein transducin (G$\alpha$). The nucleotide exchange is achieved when the C-terminus of the activated receptor induces a conformational change in regions near the nucleotide binding site on the G$\alpha$ subunit. Evidence for this has been shown in studies of the effects of mutations in those particular regions on GDP release (Thomas et al., 1993; Marin et al., 2001; Oldham et al., 2006). GTP-bound G$\alpha$ dissociates from G$\beta\gamma$ and activates cyclic GMP phosphodiesterase (PDE6), which reduces the cytosolic concentration of cyclic GMP. The reduction of cGMP causes closure of the cGMP-gated Na$^+$ and Ca$^{2+}$ ion channels, hyperpolarization of the cell and suppression of glutamate release at the synapse (Baylor, 1996; Arshavsky et al., 2002).
Figure 1.2 The phototransduction cascade within a rod outer segment. Photoreceptor outer segments are packed with disc membranes containing the biochemical machinery for signaling. Light acts as the agonist for the GPCR rhodopsin. Activated rhodopsin stimulates GTP exchange on the G protein transducin. GTP-bound α transducin activates cGMP phosphodiesterase PDE6, which hydrolyzes cellular cGMP. The reduction of cGMP causes closure of the cGMP-gated ion channels in the plasma membrane blocking entry of cat-ions, thus hyperpolarizing the cell. As a result of the reduction in Ca^{2+} concentration, retinal guanylate cyclase (GC) becomes active and restores cGMP levels. Also, the reduced Ca^{2+} concentration leads to activation of rhodopsin kinase (Rh Kinase), which phosphorylates rhodopsin, and recruits arrestin binding to block further activation of transducin.

Transducin is inactivated upon hydrolysis of GTP, the molecular event accelerated by a regulator of G protein signaling (Gβ5L-RGS9), a protein complex that directly binds to Gαt (He et al., 1998; Skiba et al., 2001). The phototransduction cascade is also regulated by cellular calcium. Light-induced
closure of cGMP ion channels causes the reduction of cellular calcium levels. The suppression of rhodopsin kinase (GRK1) by the calcium binding protein, recoverin, is then relieved by the lack of Ca\textsuperscript{2+}. Rhodopsin kinase is then able to phosphorylate activated rhodopsin (Binder et al., 1996; Chen et al., 1995). Phosphorylated rhodopsin becomes a high-affinity target for the binding of visual arrestin. Arrestin binding prevents further stimulation of transducin and allows for recovery of cGMP levels (Gurevich and Benovic, 1995).

Restoration of cGMP levels and reopening of the cGMP-gated ion channels are key events in photoreceptor recovery. Mammalian rod photoreceptors contain two isoforms of guanylate cyclase, retGC-1 and retGC-2 (for simplicity I will use retGC in the text when referring to retGC-1 cyclases in general). Cones express only retGC-1. These approximately 115 kDa transmembrane enzymes are products of two distinct genes (Yang and Garbers, 1997) and are regulated by guanylate cyclase activating proteins, GCAP-1 and GCAP-2. GCAPs are Ca\textsuperscript{2+}/Mg\textsuperscript{2+} exchanging proteins which become active in a light- and calcium dependent manner (Peshenko and Dizhoor, 2007; Dizhoor and Hurley, 1999; Palczewski et al., 2004).

*Photoreceptor Physiology*

Much information that is known on photoreceptor physiology comes from studies on rod cells because until very recently there was a lack of ability to generate large amounts of purified cone cells or cone cell proteins. For example, mouse cone mouse photoreceptors represent approximately 3% of the total
photoreceptor population in the retina (Carter-Dawson et al., 1978). Early studies of photoreceptor physiology showed a relationship between intensity of light and changes in rod cell membrane currents. Suction electrodes were used to measure the change in membrane current in single rod cells exposed to increasing intensities of flashes of light (Baylor et al., 1978). Saturating flashes of light was found to cause suppression of the steady inward current normally observed in darkness. The kinetics of each step in the activation of the photoresponse has been quantified (Lamb and Pugh, 1992). As mentioned earlier, the response can be measured electrically upon closure of cGMP-gated ion channels and hyperpolarization of the cell. Interestingly, it has been suggested since over a half-century ago that a single photon can be registered by the retina (Hecht et al., 1942). Rod cells achieve this high-sensitivity by a high gain amplification mechanism. One active rhodopsin can activate 50-100 molecules of the G protein transducin, which in turn, can activate around 1000 PDE molecules leading to a great reduction in cellular cGMP and rapid closure of the cGMP channels (Leskov et al., 2000; Yee and Liebman, 1978). The physiological responses of cone photoreceptors are not as well understood and are only recently becoming clear (Dunn et al., 2007; Kefalov et al., 2005; Nikonov et al., 2006; Daniele et al., 2005).

Light-induced redistribution of photoreceptor proteins

The physiological state of a neuron is influenced by the distribution of signaling proteins within it. Some processes, such as axonal transport and
mitochondrial import, use energy from ATP hydrolysis to distribute proteins. However, simple diffusion and protein-protein or protein-lipid interactions also can determine distribution. For instance, scaffolding proteins influence the distributions of cAMP signaling enzymes (McConnachie et al., 2006), and protein-membrane interactions determine the sub-cellular distribution of the small G protein Ras (Feig, 2006).

Two decades ago, it was noted that light stimulates redistribution of two proteins (transducin and arrestin) in opposite directions within photoreceptors (Whelan and McGinnis, 1988). These immunohistochemical observations were criticized as being a result of epitope masking. Arshavsky and colleagues used two independent methods to demonstrate that up to 90% of transducin migrates from the rod OS to the inner compartments in bright illumination (Sokolov et al., 2002). The mechanism of this “massive” redistribution of transducin, however, was still not determined.

Several studies were able to lend valuable insights and speculations on the mechanism of transducin and arrestin redistribution. For example, it was shown that light mediated arrestin movement occurs in absence of transducin signaling and even in absence of rhodopsin phosphorylation (Mendez et al., 2003). Within 30 minutes of the onset of bright light, transducin and arrestin redistribute maximally to their respective compartments. Movement back to their respective compartments after the onset of dark is slower with transducin requiring up to 200 minutes and arrestin requiring 30 minutes (Sokolov et al., 2002; Elias et al., 2004; Nair et al., 2005b). The ideas of phospho-rhodopsin-
independent and transducin-independent movement of arrestin led investigators to hypothesize that transport mechanisms for both arrestin and transducin redistribution play a role in light adaptation.

What is the significance of light-induced protein redistribution in photoreceptors? Movement of arrestin seemingly regulates binding to rhodopsin and termination of signaling. Interestingly, transducin redistribution has been shown to correlate with a ten-fold decrease in the gain of phototransduction in rats and mice (Sokolov et al., 2002; Kassai et al., 2005), while visual arrestin translocation has been proven to modulate rhodopsin activity and play a role in light adaptation (reviewed in Arshavsky, 2002). It is clear, however, that redistribution of both arrestin and Gq in the drosophila eye (discussed later) plays a strong role in light adaptation (Frechter et al., 2007; Lee and Montell, 2004). Translocation of these signaling molecules may also serve as a cell survival mechanism to prevent over-signaling under extreme illuminating conditions (Calvert et al., 2006).

More recently, Arshavsky and colleagues reported that the calcium binding protein, recoverin can also undergo light mediated redistribution between subcellular compartments of rods (Strissel et al., 2005). Other calcium binding proteins (GCAP-1 and GCAP-2) were not shown to undergo redistribution. The authors suggest that recoverin translocation affects light adaptation because its departure from the OS may allow higher activity of rhodopsin kinase. However, the significance and mechanism of light induced recoverin redistribution remain to be understood. Understandably, a fundamental question arises: how do such
large amounts of protein move from one compartment of the rod to another (transducin is expressed at ~100-300 μM in rods as calculated from [Harosi, 1975; Lamb and Pugh, 2006])? By what means does light induced translocation of these signaling proteins occur? How does this “massive” redistribution of proteins occur so rapidly? Figure 1.3 illustrates light-induced protein redistribution in retinal rods.

![Diagram of protein redistribution](image)

**Figure 1.3 Light-induced protein redistribution in rod cells.** In dark, visual arrestin is localized to the inner compartments. Upon illumination, arrestin translocates to the outer segments. Transducin displays opposite behavior. In dark, transducin localizes to the outer segments, and light causes translocation of up to 90% to the inner compartments. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; ST, synaptic terminal.

While much data exists demonstrating the phenomenon of light-induced protein redistribution in rods, little conclusive data has been shown for the actual mechanisms of the protein redistribution. Arguments favoring either active transport or diffusion as the mechanism for protein migration in photoreceptors have been discussed (Marszalek et al., 2000; Lee et al., 2003; Lee and Montell,
What drives arrestin redistribution in light and dark conditions? Our lab has previously reported that movement of arrestin does not require molecular motors; it occurs even without ATP (Nair et al., 2005b). If arrestin movement occurs by the random process of diffusion, then how is it directed to the OS in light, and to the inner compartments in dark? Our lab hypothesized that arrestin migration depends on the availability of molecular “sinks” to direct or restrict arrestin to the different compartments. In dark, non-phosphorylated and inactive rhodopsin has a low affinity for arrestin (Vishnivetskiy et al., 2007), and microtubules within the inner compartments bind all of arrestin (Nair et al., 2004). Light-active, phosphorylated rhodopsin has a high affinity for visual arrestin (Vishnivetskiy et al., 2007), and on the course of tens of minutes it binds all of the arrestin – localizing to the ROS. On the other hand, knowledge of the means by which transducin migrated between the subcellular compartments of the rod is limited. This dissertation aims to specifically address the mechanism of light stimulated transducin redistribution in retinal rods.

There are two possible mechanisms to reasonably explain the mechanism of transducin redistribution: 1) transducin movement could require energy dependent molecular motors; 2) movement may occur by diffusion of the molecules. If transducin movement occurs by an active mechanism, then an ATP-binding protein such as a molecular motor should be identifiable as a direct binding partner of transducin. At the moment, no such interaction has been described. If transducin movement occurs by diffusion, then what could regulate
this process? Logically, a simple mechanism could involve interacting binding
partners which change affinities in a light-dependent manner based on the
activation status of transducin.

Indeed, Calvert et al. (2006) hypothesized a link between transducin
subunit dissociation, diffusion, and redistribution. The widely held view that
heterotrimeric G-protein complexes dissociate during activation (Gilman, 1987)
primarily derives from early studies of rod transducin. Transducin subunits
dissociate and detach from membranes during activation (Kuhn, 1980; Fung et
al., 1981). I hypothesize that protein-protein interactions and diffusion are the key
to transducin redistribution, and in the following chapters I will provide direct
experimental evidence that supports this hypothesis and elucidates the
mechanism of transducin redistribution.

G protein redistribution in invertebrates

Light-induced redistribution of signaling proteins has also been observed
in invertebrates. For example, much like in mammalian photoreceptors, arrestin
translocates from inner compartments of Drosophila photoreceptors to the
rhabdomere (equivalent to the OS in vertebrates). A genetic study in Drosophila
has suggested that arrestin movement in the fly photoreceptors requires active
transport. When the researchers tested arrestin translocation in a mutant fly
which lacked the motor protein myosin III (NINAC), they found a lack of light
induced arrestin translocation (Lee and Montell, 2004). However, another group
did not confirm this observation. When the gene for the active transporter NINAC
was disrupted here, arrestin redistribution was still able to occur (Satoh and Ready, 2005). While the reason for the differing results is unclear (possibly due to different microscopy methods), the latter data suggest diffusion as the mechanism for arrestin movement in Drosophila photoreceptors.

Unlike vertebrates, flies do not express transducin in their photoreceptors. Instead, they express Gq which, similarly to mammalian rods, undergoes light stimulated subcellular redistribution from the rhabdomeres (Kosloff et al., 2003). The movement of Gq in these cells has also been shown to correlate with light adaptation. In flies, localization of G proteins is highly important for physiological function, and much like in mammalian cells, expression of Gβγ in invertebrates is required for proper localization in Drosophila photoreceptors. In other words, formation of the G protein heterotrimer is required for proper localization and function within the cells. Another study showed that light-dependent translocation of Gq was diminished in a mutant NINAC (myosin III transporter), suggesting active transport as the mechanism of Gq movement (Cronin et al., 2004). Taken together, the data suggest that the mechanism of light-driven relocalization of proteins in drosophila photoreceptors remains to be elucidated.

*Cone cell biology is different compared to rods*

One important issue in my studies comes from the comparison of mammalian rods and cones. In cones, transducin does not undergo light mediated redistribution and remains in the OS even under intense illumination (Elias et al., 2004; Kennedy et al., 2004; Coleman and Semple-Rowland, 2005).
Rods and cones express different visual pigments. Moreover, rods and cones express different genes for transducin (rods express rod G\(\alpha\)t and G\(\beta\)1\(\gamma\)1; cones express cone G\(\alpha\)t and G\(\beta\)3\(\gamma\)8). Other proteins such as retGC-1 and RGS9 are the same in rods and cones. It has been suggested that a shorter lifetime of light-activated cone visual pigment or activated cone transducin may explain the different distributions of transducin in cones versus rods (Lobanova et al., 2007). In this study, I propose that factors influencing the rate of transducin activation and inactivation are not crucial. Instead, I propose that transducin in cones does not redistribute in light simply because its subunits do not effectively dissociate even during complete activation.

**Protein-protein interactions of transducin**

The model for light-induced transducin redistribution presented in my dissertation posits that activated transducin detaches from the disc membranes of the rod OS and diffuses throughout the photoreceptor. What is the means by which transducin localizes to the OS in dark? I hypothesize that the hundreds of disc membranes (which almost completely fill the OS) are the target of rod transducin. Also, I hypothesize that localization of transducin is also influenced by protein-protein interactions in both the OS and the inner compartments. Indeed, transducin is known to interact with several proteins. In addition to rhodopsin, PDE6 and RGS9, transducin can interact with centrins (Wolfrum et al., 2002; Giessel et al., 2006), LGN (Nair et al., 2005a; Kerov et al., 2005a), phosducin (Yoshida et al., 2004; Sokolov et al., 2004) cytoskeleton components
(Peterson et al., 2005) and even the “house-keeping” protein GAPDH (Chen et al., 2008). Of all the known binding partners of Gαt, only rhodopsin and the transducin βγ subunits are as abundant as Gαt. Based on the evidence available, it is easy and fitting to speculate that these interacting partners of transducin could influence its ability to redistribute. To further develop this hypothesis, I have searched for novel binding partners of transducin and tested for their ability to regulate translocation of transducin in the photoreceptors.

**Subunit dissociation and localization of non-photoreceptor G proteins**

During the 1980s, Gilman and coworkers found that heterotrimeric G protein subunits could dissociate upon activation in a detergent solution. Their studies focused mainly on the stimulatory G protein, Gs (Gilman, 1987). However, the model of G protein subunit dissociation arose primarily from studies on transducin (a unique G protein which can be purified from native tissues upon addition of GTP nucleotides) (Fung et al., 1981; Abood et al., 1982; Hurley 1980). As will be discussed in this dissertation, cone transducin does not dissociate upon activation (see results, Figures 3.7-3.9). If cone transducin does not dissociate, then it raises the following question: do G protein subunits in general truly undergo subunit dissociation upon activation? Remarkably, this fundamental question remains a topic of debate (Lambert, 2008).

The main evidence supporting G protein subunit dissociation came from early studies of the apparent molecular weight of the G proteins, which showed
that activated G proteins were "lighter" than the inactivated proteins (Sternweis et al., 1981). However, these seminal biochemical experiments were performed using detergent-solubilized membrane extracts or purified native G proteins (Northup et al., 1983), which were permanently activated by GTPγS or AMF (Al3+, Mg2+, F−). Other types of experiments indicated that in their native environment in the presence of GTP the subunits do not physically separate. This caveat was appreciated by many scientists and was discussed, for example, by Levitzki (2002). Biophysical studies utilizing G proteins fused to fluorescent molecules to study fluorescence resonance energy transfer (FRET) in intact cells did not resolve the question as some experiments supported the dissociation model (Janetopoulos et al., 2001) while others pointed to a rearrangement of subunits rather than dissociation (Bunemann et al., 2003; Frank et al., 2005). Regardless of the conclusion, experimentation with G protein subunits fused to fluorescent proteins has inherent problems with tagging and expression levels of the ectopically expressed molecules, and does not necessarily describe how G protein activation is occurring in vivo.

A strong line of evidence for subunit dissociation in native cells came from the studies of the rod photoreceptor G protein, transducin (Hurley and Stryer, 1982; Wensel and Stryer, 1986). Rod transducin is the unique heterotrimeric G protein, in that it can detach from the membranes upon GTP binding, without detergent. In this study I show, however, that cone transducin does not effectively dissociate even if it is fully activated and binds GTPγS. As mentioned
earlier, cone transducin consists of different $\alpha$, $\beta$ and $\gamma$ subunits compared to its rod counterpart. Subunit dissociation of cone transducin and other natively expressed G proteins therefore remains to be understood completely.

While the main focus of this dissertation is on the mechanism of transducin redistribution, I have also applied my hypotheses and ideas generated in the study of photoreceptors to other G proteins expressed in other cell types. I will investigate the behavior and subcellular localization of endogenously expressed heterotrimeric G proteins under various activating conditions. Here, I propose a link between G protein subunit dissociation, membrane detachment, subcellular redistribution, and the effects on signal transduction. I hypothesize that most G proteins do not undergo subunit dissociation upon activation. Further, I will test how forcibly displacing $G\alpha$ from $G\beta\gamma$ influences subcellular localization and signaling capacity of cells.
Chapter II

MATERIALS AND METHODS

Animals and tissue preparation

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. After the required period of dark or light adaptation, mice were anesthetized with isofluorane and killed by cervical dislocation, and the eyes were enucleated. The eyecups or retinas were prepared by removing the cornea and lens in a dark room under a dissection microscope as shown in Figure 2.1 (Nair et al., 2005b) and maintained in culture in DMEM supplemented with or depleted of certain ingredients as required by a particular experiment. Explicit details for my eyecup preparations are given in Figure 2.1.

RGS9 knockout mice (Chen et al., 2000), were provided by Ching-Kang Chen (University of Utah, Salt Lake City, UT). The mouse line lacking retGC-1 (GCE null) were originally received from David Garbers (Yang et al., 1999) and developed into an SPF colony with Charles River Laboratories maintained at Pennsylvania College of Optometry. Nrl<sup>-/-</sup> mice were provided by Anand Swaroop (Yoshida et al., 2004). Gnat2<sup>+/Gnat1<sup>-/-</sup></sup> eyecups and retinas, which express cone G<sub>αt</sub> within rod cells, were a gift from Janis Lem at Tufts University School of Medicine, Boston, MA.
Handling, breeding, weaning, and other care of all mouse lines used in our lab was performed by Kelly Wang, a senior Research Associate in our lab.

**Figure 2.1 Preparation of mouse eyecups.** All steps are done in warmed DMEM media under a stereo dissecting scope. A, Freshly enucleated mouse eye is handled with fine tip forceps carefully poking or holding the cornea and optic nerve for manipulations. B, After poking and holding the cornea with the forceps, a dissecting micro-scissors are used to cut around the cornea. C, After cutting the cornea, the cornea, iris, lens and vitreous are gently pulled out using a second forceps while holding the optic nerve with the first forceps. Typically, eyecups can remain viable for experimentation for up to 3.5 hours in warmed DMEM. However, rough handling of the eyecups result in damaged and dead photoreceptors.

*Immunofluorescence.*

The tissues were fixed with 4% paraformaldehyde, embedded in agar (3.5% agar in PBS), sectioned with a vibratome, and immune-stained with antibodies against the various proteins described in each experiment. For
example, antibodies against rod Gαt were and diluted 1:500 in buffer. The primary antibody incubation was 1.5 hours in buffer (PBS, 0.1% TritonX-100, and 1% BSA) Antibodies against rod and cone Gα subunits were from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was cyanine 3-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). The secondary antibody incubation time was 1.5 hours in the same buffer described. The preparations were washed extensively, and then mounted on a cover slip using Antifade reagent (Invitrogen, Carlsbad, CA) and visualized first in the open field with a Nikon (Tokyo, Japan) TE3000 fluorescence microscope. The images were taken on a Zeiss (Oberkochen, Germany) LSM 510 laser scanning confocal microscope.

Quantification of the relative amount of transducin in the OS and the inner compartments was determined using MetaMorph software (Universal Imaging, Downingtown, PA). Briefly, an area of the visual field was selected (see Figure 3.4) to include the entire photoreceptors length, from the OS to synaptic termini. The total fluorescence, $F_t$, was measured. Within the same selected area, we recorded the fluorescent signal from the regions corresponding only to the OS or the inner compartments [inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL)], which we designated $F_o$ and $F_i$, respectively. To normalize to data between independent experiments, we determined the $F_i/F_t$ or $F_o/F_t$ ratio.

For every image, three separate areas were selected, and the mean and SD were determined. The exposure time and other parameters were set so that
none of the areas within the field was saturated. For rod photoreceptors, analysis of a single confocal plane or the collapsed series of confocal images produced essentially the same results. For image presentation purposes only, so that the relatively dim fluorescence in the inner compartments could be clearly visible in the print, the OS areas had to be saturated. It should be noted here also that, although it may appear that the immunofluorescence intensity in the inner compartments is low compared with the OS, the combined area of IS, ONL, and OPL is approximately 5 times larger than that of OS, and so the total amount of the antigen in the inner compartments is higher than it may appear from the visual assessment of image intensity.

For all relocalization experiments using time points, fixed eyecups were coded (and a “key” was made for the code) in order to remove bias from analysis of retinal sections for the various time points and various mouse models. For example, in Figure 3.28, fixed eyecups were coded and labeled by Dr. Dizhoor in Elkins Park, PA. The coded eyecups were sent to Miami, and I sectioned, stained and analyzed transducin localization on the coded eyecups. After the data was obtained, Dr. Dizhoor sent the “key” to the code so that I could generate a graph of the data. In this way, the experiments were done blindly, removing bias as much as possible.

Ex vivo experiments

Eyecups or retinas were dissected under the dim red light and incubated in DMEM as described above and previously (Nair et al., 2005b). ATP depletion
was performed by incubating the specimen for 15 min in DMEM free of glucose and sodium pyruvate and supplemented with 2 mM deoxyglucose and 5 mM KCN, pH 7.5. For permeabilization, retinas were incubated with 50 μg/ml α-toxin (Hemolysin; Sigma, St. Louis, MO) for 45 minutes at room temperature. The nucleotides were added at the concentration of 0.1 mM. GDP, GTP, GDPβS, GTPγS, cGMP, and 8-bromo-cGMP were from Sigma, and BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene fluorophore)–GTPγS was from Invitrogen. The sample incubated with BODIPY–GTPγS was washed for at least 1 minute in the medium or isotonic buffer to remove excess fluorescence before fixing.

![Diagram of α-toxin binding to plasma membranes](image)

**Figure 2.2 Delivery of small molecules to permeabilized photoreceptors.** α-toxin binds to plasma membranes and creates small pores, thus permeabilizing the cells. The pores which are formed allow small molecules (depicted in pink) and peptides less than 2,000 Daltons to enter the cytosol of the cells. Using this method, it is possible to manipulate the nucleotide content of the photoreceptors as well as perform other pharmacological manipulations.

**Biochemical Assays**

SDS-PAGE, Western blot, and preparation of bovine and mouse OS were performed as described previously in our lab (Nair et al., 2004). Bovine OS
membranes were prepared to the standard protocol of (Kuhn 1977). To prepare mouse OS, dark adapted retinas (typically 10 retinas) were dissected and placed in 0.2ml of 8% Optiprep (Sigma) in isotonic buffer with protease inhibitors. The suspension was vortexed on medium speed for 1 minute and then centrifuged at 4,000 rpm for 1 minute. The supernatant was collected as the OS fraction, and the retinas were resuspended in 0.2ml of Optiprep buffer and vortexed and centrifuged two more times. The collected OS fractions (~600μl) was diluted 2-fold with ice-cold buffer and centrifuged at 14,000rpm for 5 minutes to pellet the OS membranes. The supernatant was discarded and the pellet was resuspended in 0.2 ml of buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM DTT, and protease inhibitors) and used as required for particular experiments.

For transducin membrane association assays, the OS (~5-10μl pellet) were resuspended in 100 μl of buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM DTT and protease inhibitors) with or without the nucleotide as required by a specific experiment, incubated in light or dark for 15 minutes, and centrifuged at 14,000rpm for 10 minutes at 4 degrees. The supernatant was collected as the soluble fraction and the pellet was resuspended to the same volume as the supernatant. Both samples were analyzed by Western blot using specific antibodies as indicated in Results. For the trypsin protection assay, the OS membranes were incubated with GTPγS, and then trypsin was added at varying concentrations ranging from 100–500 ng, as required by the experiment (see Figure 3.9). After 15 minutes incubation at 20°C, the reactions were stopped by
addition of SDS-containing sample buffer for SDS-PAGE and Western blot analysis.

To study subunit dissociation by size-exclusion chromatography, bovine OS membranes were incubated in light in the presence or absence of GTP\(_{\gamma}\)S, and then sodium cholate was added to the final concentration of 1%. The slurry was centrifuged at 40,000 rpm for 30 minutes, and the extract was resolved on a 1 X 25 cm Superdex 75 column. The column was equilibrated with the buffer containing 1% cholate, and the collected fractions were analyzed by Western blot to detect rod or cone G\(\alpha\) subunits, or other G protein subunits. In the experiments with mSIRK/mSIGK (myristoylated G\(\beta\)-binding peptide -- see Results sections I and III; Goubaeva et al., 2003) and to fractionate the extracts from dissected mouse retinas and mouse brain membranes (see Figures 3.17 and 3.38), 4-mm-diameter columns were used to reduce the volume. The volume of collected fractions was 0.3 ml for the 1 cm column and 60 \(\mu\)l for the 4 mm columns. Blue dextran, hemoglobin, and cytochrome c (Sigma) were used as internal molecular weight standards.

**Retinoid Analyses**

Eyecups prepared in culture medium in darkness were exposed to 5 minutes of additional darkness, 5 minutes of direct sunlight, or 5 minutes of illumination from a hand-held UV illuminator placed 0.5 cm directly above the eyecup in a culture dish. The power output of the UV illuminator is described in the text. After 5 minutes of dark or illumination, eyecups were immediately frozen
on dry ice. All subsequent steps were performed in dim red light. The frozen eyecups (four per sample) were thawed, homogenized, extracted, and analyzed by normal-phase HPLC by Greg Garwin at the University of Washington, Seattle, WA as described previously (Garwin and Saari, 2000). An internal standard, Etretinate (all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7,-dimethyl-2,4,6,8-nonatetraen-1-ethyl ester), was used as described previously (Saari et al., 2002).

**Analysis of N-myristoylation**

Samples for mass spectrometry analysis were prepared by the method of the Crabb laboratory (http://www.lerner.ccf.org/eye/crabb/protocols/). The membrane and soluble fractions obtained from bovine or mouse OS were resolved by SDS-PAGE and stained by Coomassie blue. The bands corresponding to rod or cone Gα subunits were excised, washed with ethanol/water solutions, and dried using a Speedvac. The dried gel slices were rehydrated overnight in a Tris-HCl, pH 7.4, buffer containing 0.01 mg/ml trypsin. The hydrolizate was extracted in a 60:40 acetonitrile solution, separated from the residual gel, lyophilized, and stored at -20°C. They were then dissolved in water (HPLC purity) with 5–10% of acetonitrile (HPLC purity) and subjected to reverse-phase liquid chromatography followed by mass spectrometry performed in the lab of James Hurley by Junhua Wei at the University of Washington, Seattle, WA as described previously (Kennedy et al., 2001; Lee et al., 2002).

The synthetic N-myristoylated (C14:0) peptides and N-terminal tryptic peptides (GAGASAEK and GSGISAEDK for rod and cone Gαt, respectively)
were purchased from Alpha Diagnostic (Austin, TX) and were used as standards to optimize the HPLC gradient, mass spectrometer settings, and collisionally induced dissociation (CID) tandem mass spectrometry conditions. Selected ion monitoring strongly improves the detection sensitivity of small amount of peptides in a digestion mixture. Data from the mass spectrometer were filtered through narrow mass windows (1 Da) centered on the predicted masses of the various fatty acylated N-terminal peptides of the rod and cone transducin being analyzed. To obtain relative amounts of the various fatty acyl species in a given sample, we integrated the area under peaks confirmed by CID to be transducin N termini. The relative amount of each species was calculated as a fraction of the total amount of rod or cone transducin peptide in membrane-bound or GTPγS solubilized fraction.

Mass Spectrometry Analysis of Excised Protein Bands

A 115 kDa protein band was excised from the Coomassie-stained gel, and digestion with trypsin was performed using the Crabb laboratory protocol (http://www.lerner.ccf.org/eye/crabb/protocols/silverdigest.php). Briefly, the gel slice was placed in a low-adhesion micro centrifuge tube and washed with 60% acetonitrile solution with TFA. 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, lyophilized and analyzed as described previously (Crabb et al., 2002) by John Crabb in at the
Cleveland Clinic, Cleveland, OH. LC MS/MS was performed with a quadrupole time-of-flight mass spectrometer (QTOF2; Waters Corp., Milford, MA). Proteins were identified from MS/MS data (ProteinLynx Global Server; Waters Corp., 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] protein sequence databases).

**Immunoprecipitation**

Immunoprecipitation (IP) was carried as previously described in our lab (Nair et al., 2005a) with minor alterations. All steps were carried out on ice. A 200 μl aliquot of frozen bovine OS (~500 μM rhodopsin concentration), which was purified and stored using the standard procedure (Nair et al., 2004; Rosenzweig et al., 2007), was thawed on ice and centrifuged to form a 25 μl pellet. The pellet was resuspended in 175 μl of the lysis buffer (10mM Tris, pH 7.6, 5mM MgCl₂, 150mM KCl, 1 mM DTT, and Complete Protease Inhibitor Cocktail, Rosche). This OS suspension was divided into 50μl portions and incubated in light or dark in the presence of different nucleotides, as required by a particular experiment (see Results section II). After the treatment, Triton X-100 was added to a final concentration of 1%, incubated for 1 minute on ice and centrifuged at 14,000 rpm in a table-top Beckman centrifuge at 4°C to remove the insoluble fraction (i.e., cytoskeleton). The obtained supernatants were then mixed with 25μl of Protein-A Sepharose beads for 2 hours. 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 $G_{\alpha t}$ antibodies.

To immobilize the antibody, 2 μg of the rod $G_{\alpha t}$-specific IgG (SC389) were added to 25μl of protein-A beads in the volume of 100 μl and incubated for 2 hours 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 collected as the unbound material (~200 μl) for western blot analysis. The resin was washed twice with 500 μl of the lysis buffer containing 1% Triton X-100. The collected beads were then mixed with 20 μl of 2x SDS-PAGE sample buffer, and incubated at 65°C in a heat block for 5 minutes. The beads were centrifuged again and the supernatant was collected as the specifically eluted material (eluate). The unbound and eluted fractions were resolved by 10% SDS-PAGE and analyzed by western blot.

To ensure that the ECL signal obtained from the IP fractions fell within the linear range of detection, I typically loaded the gel with 10 μl of unbound and 10 μl 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, I compared the signal intensity of scanned western blot films to the signals of serial dilutions of the OS lysate (Figure 3.19 B).
Cell culture, transfection, and Membrane Fractionation

Transient transfection of COS-7 cells was performed using a common procedure with Lipofectamine as described in our lab earlier (Witherow et al., 2000). The cells were harvested at 48 hours after transfection. Membrane fractions from COS-7 and CHO-K1 cells were obtained by harvesting and homogenizing cells in hypotonic buffer (5mM Tris-HCl, pH 7.6, 0.1mM MgCl$_2$, 1mM DTT, and Protease Inhibitors Cocktail, ROCHE) and centrifuging at 100,000Xg for 30 minutes at 4°C. The supernatant was discarded as the cytosolic fractions, and the membrane pellet was resuspended in buffer (10mM Tris, pH 7.6, 5mM MgCl$_2$, 150mM KCl, 1 mM DTT, and Complete Protease Inhibitor Cocktail, Rosche) and treated as required by a particular experiment.

Guanylate Cyclase Activity Assay

RetGC activity was assayed as previously described (Peshenko and Dizhoo, 2007) in the lab of Alexander Dizhoo by Igor Peshenko at the Pennsylvania College of Optometry in Elkins Park, PA. Briefly, the assay mixture (25 μl) 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 μM cGMP, 1 mM GTP, 1 μCi of [$\alpha$-$^{32}$P] GTP, 0.1 μCi of [8-3H] cGMP, GCAP-1 and HEK 293 cell membranes. The reaction mixture was incubated for 40 min at 30°C, stopped by heating for 2.5 minutes at 95°C, and the aliquots were analyzed by thin layer chromatography using fluorescent polyethylenimine cellulose plates (Merck) as described previously (Peterson et al., 2005; Lobanova et al., 2007). The data
shown are representative from 3-7 independent experiments producing virtually identical results. Rod transducin subunits used to investigate for their interaction with guanylate cyclase were purified according to the method of Bigay and Chabre (1994).

**GST Pull-Down Assay**

This assay was performed with the help of Konstantin Levay, a Research Professor in our lab. cDNAs corresponding to Kinase Homology Domain (nucleotides 1462-2391) and Catalytic Domain (nucleotides 2686-3183) of retGC1 (GenBank Accession number AJ222657) were PCR amplified and cloned into the GST-tag expression vector pGEX-6P1 at BamH1 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 for 4 hours 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 to the final concentration 2.5%. Lysates were diluted 5-fold with ice-cold PBS and centrifuged for 30 min at 20,000g 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; protease inhibitors). Beads with 20 μg of GST, GST-KHD, and GST-CAT proteins were incubated for 4-6 hours in a binding buffer (PBS pH 7.6; 1% BSA; 0.5% Triton X-100; protease inhibitors) with Gαt. After incubation, beads were washed and the
bound proteins eluted with SDS-PAGE sample buffer and analyzed by western blot.

*Thin Layer Chromatography (TLC) analysis of nucleotides*

Explanted retinas were acutely cultured and permeabilized as described above. Fifty μl of DMEM media containing cGMP, 8-bromo-cGMP, GTP, GTPγS or GDP, depending on the purpose of the assay, (see Results section II) was added to the retinas. Five μl aliquots of the media were collected at the specified time points and spotted onto PEI cellulose (Merck). The spots were dried at room temperature and the nucleotides were resolved with 0.5M 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 by pencil. The spots were cut out, and the nucleotides were extracted in 0.5M 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.

*Preparation of Lipid Rafts from Mouse ROS*

Mouse OS were prepared from dark adapted wild type or retGC null mouse retinas, as described above. The OS were washed with buffer (10mM Tris, pH 7.6, 5mM MgCl₂, 150mM KCl, 1 mM DTT, and Complete Protease Inhibitor Cocktail, Rosche) containing 0.5% triton and immediately mixed with
equal volume of 80% sucrose. The 40% sucrose/ROS slurry was laid on a sucrose step gradient (5%, 30%, and 40%) and centrifuged at 100,000Xg overnight. Twelve 50μl fractions were collected and analyzed by western blot for transducin co-migration with the specific raft marker caveolin-1, which typically is found in fractions 3-5 at the 5%/30% gradient interface.

Assessment of Calcium Mobilization in CHO-K1 Cells

Calcium mobilization assays were conducted as described previously in our lab (Narayanan et al., 2007), and performance of this assay was greatly assisted by Simone L. Sandiford, a graduate student in our lab. Briefly, CHO-K1 cells were grown on cover slips. After 24 hours, the cover slides were washed with 2% FBS in HBSS. Cells were then incubated in 2% FBS in HBSS containing 1 μM fura-2AM for 45 minutes at room temperature in the dark. To permit the de-esterification of fura-2AM, the cells were incubated in filtered Locke's buffer for 30 minutes. The cover slips were placed in a flow chamber and were stage-mounted on a Nikon TE2000 inverted fluorescence microscope. The cells were continuously perfused with Locke's buffer and stimulated with 100 μM ATP to activate Gq-coupled adenosine receptors. Images were collected in real time every 2 seconds using a 20X UV objective lens and recorded using Metafluor software. The excitation wavelengths were 340 and 380 nm. The emission was set at 510 nm. The cytosolic free Ca^{2+} concentration was calculated from the fluorescence measurements using the fura-2 Ca^{2+} imaging calibration kit (Molecular Probes).
Antibodies, Peptides and Plasmids

Antibodies specific for rod and cone Gαt subunits were from Santa Cruz Biotech (SC389 and SC390, respectively). Antibodies against RGS9 and Gβ5 were described previously in our lab (Witherow et al., 2000). I used three antibodies raised against retGC1. Two different rabbit polyclonal antibodies were specific for the catalytic domain: a new antibody against the fragment Met_{747}-Ser_{1052}, and a previously described antibody (7957CAT) (Laura and Hurley, 1998). The third anti-retGC antibody used in this study was specific for the kinase homology domain (6344KHD) (Laura and Hurley, 1998). The 7957CAT and 6344KHD antibodies were a gift from James B. Hurley. Antibodies against Gβ1 (βN1) were a gift from Melvin I. Simon. Antibodies specific for Gαq, Gαi-1, Gαo, and Gβ3 were from Santa Cruz Biotech.

The myristoylated synthetic peptides, mSIRK, mSIGK, and L9A (control) were gifts from Alan Smrcka (Goubaeva et al., 2003).

Plasmid vectors for mammalian cell expression of retGC1 and rod Gαt were from J. Hurley (gift) and Guthrie Institute, respectively. Other reagents were from Sigma.
Chapter 3

Results

I. Transducin Localization is Determined by Subunit Dissociation and Diffusion

GTP, but not ATP, is required for transducin redistribution

As described in the introduction of this dissertation, the debate on whether light induced translocation of signaling proteins in photoreceptors is governed by an active or passive mechanism has not been resolved (for review, see Calvert et al., 2006; Artemyev, 2008; Strissel and Arshavsky, 2004; Slepak and Hurley, 2008). In our laboratory, this question was addressed by Saidas Nair, and he deprived mouse retinas of energy to show that ATP is not needed for redistribution of visual arrestin (Nair et al., 2005b). ATP was depleted from freshly dissected mouse retinas by incubating them in glucose-free medium in the presence of deoxyglucose and KCN. ATP depletion did not interfere with redistribution of arrestin, but it blocked light-induced redistribution of rod transducin (Nair et al., 2005b). Initially, the significance of that finding was unclear. ATP could be needed to either support active transport or make GTP to activate transducin.

To distinguish these roles of ATP, I supplied GTP analogs to the cytoplasm of ATP-depleted photoreceptors. How could nucleotides be introduced into the photoreceptors? Since the nucleotides cannot cross the plasma membranes, our lab developed the idea to deliver the analogs into photoreceptors using staphylococcal α-toxin (Figure 2.2). α-toxin is a polypeptide
that generates pores in the membrane that allow molecules less than 2000 Da to enter cells (Bhakdi et al., 1996; Otto-Bruc et al., 1998).

Figure 3.1 Permeabilization of retinas allows small molecule delivery. A, Mouse retinas were incubated in DMEM in ambient light in the presence of 100 µM BODIPY-GTPγS with or without α-toxin, then fixed, sectioned, and visualized by fluorescence microscopy. A parallel set of retinas was tested for the potential loss of protein from the permeabilized cells. After the incubation with α-toxin, the retinas were separated from the medium by centrifugation at 500 x g for 30 s. The supernatant (S) containing the medium and the pellet (P) were analyzed by Western blot using antibodies to Gt (39 kDa) and recoverin (24 kDa). To test the distribution of a marker cytosolic protein, GFP (27 kDa), retinas from GFP-expressing mice were also subjected to this assay. B, Retinas were permeabilized with α-toxin in the presence of BODIPY-GTPγS in the dark and then either illuminated or left in the dark for additional 30 min. Rod Gt immunofluorescence is shown in red, and BODIPY fluorescence is green.

Figure 3.1A shows that α-toxin allows entry of a fluorescently tagged nonhydrolyzable analog of GTP, BODIPY-GTPγS, but proteins such as rod
transducin and recoverin do not escape via the α-toxin pores. Green fluorescent protein (GFP) expressed in rods of a transgenic mouse also is retained in the

Figure 3.2 Light-dependent movement of rod transducin does not require ATP. Mouse retinas were permeabilized with α-toxin in glucose-free DMEM supplemented with 2 mM deoxyglucose and 5 mM KCN to deplete ATP. They were then incubated in the presence or absence of BODIPY-GTPγS and subsequently illuminated for 30 minutes at 500 lux before fixing. Localization of rod transducin subunits and BODIPY-GTPγS was determined by fluorescence microscopy. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer.

cells treated with α-toxin. BODIPY-GTPγS accumulates in photoreceptors when they are illuminated (Figure 3.1B) evidently because of the rhodopsin-catalyzed exchange of endogenous GDP for the fluorescent nucleotide. Most importantly, BODIPY-GTPγS treatment in light induces dispersion of rod transducin α and βγ subunits to all compartments of the photoreceptor cell even in ATP-depleted rods
(Figure 3.2). This shows that transducin must be activated for redistribution to occur but that ATP and active transport are not required. In other words, no molecular motors are necessary for transducin movement.

![Figure 3.3 Relocalization of rod transducin is governed by GTP binding and hydrolysis. A, Retinas were permeabilized in dark and incubated with 100 μM GTP in glucose-free DMEM or 100 μM GDPβS in DMEM supplemented with 1 mM ATP. Retinas were then illuminated, fixed, and probed for rod Gt localization. B, Two retinas were permeabilized in ambient light in the presence of GTPγS and another pair in the presence of GTP. One retina from each pair was subsequently transferred to the dark for an additional 2 h, whereas the control retina remained in light, after which they were fixed and analyzed for rod Ga localization. These are representative of three independent experiments.](image)
Figure 3.4 Relocalization of rod transducin is faster in RGS9<sup>−/−</sup> mice, which lack GTPase activity on G<sub>αt</sub>. Eyecups from dark-adapted wild-type and RGS9 knock-out (RGS9<sup>−/−</sup>) mice were prepared under dim red light and transferred to 500 lux illumination. At the indicated times, the tissue was fixed and subsequently sliced and stained with anti-G<sub>αt</sub> antibody and analyzed by immunofluorescence microscopy. The two side-by-side images exemplify the difference between wild type (WT) and RGS9<sup>−/−</sup> mice at a single time point (5 min) after the onset of light. The grayscale panel illustrates how the fluorescence in the inner compartments (F<sub>i</sub>, red box) and total fluorescence (F<sub>t</sub>, green box) are selected to determine the relative distribution of G<sub>t</sub> (F<sub>i</sub>/F<sub>t</sub>) using LSM and Metamorph software. The graph below shows the mean ± SD F<sub>i</sub>/F<sub>t</sub> ratio for wild-type (white squares) and RGS9<sup>−/−</sup> (black triangles) mice from three independent experiments.

Nonfluorescent GTP or GTPγS delivered to permeabilized retinas exerted the same effect on rod transducin localization (Figure 3.3 A, B), and GDPβS blocked light-induced redistribution (Figure 3.3 A). Both GTP and GTPγS caused dispersion transducin from the OS in light. However, return to the OS after the subsequent incubation in darkness only occurred when GTP was used. GTPγS prevented re-sequestration of transducin in the OS in darkness (Figure 3.3 B). These results show that transducin must return to its GDP-bound state to become once again sequestered within the OS. To confirm this conclusion in live
animals, I tested the kinetics of light-induced transducin redistribution in mice lacking RGS9, a member of regulator of G protein signaling (RGS) family that acts as the GTPase-activating protein for transducin (He et al., 1998; Chen et al., 2000). I expected that activated transducin would accumulate faster than normal because the rate of its inactivation in $RGS9^{-/-}$ rods is slowed. Consistent with this, redistribution of rod transducin occurs faster in $RGS9^{-/-}$ rods than in wild-type retinas (Figure 3.4).

**Cone transducin in wild-type and genetically manipulated photoreceptors**

In agreement with previous observations (Elias et al., 2004; Kennedy et al., 2004; Coleman and Semple-Rowland, 2005), I found that light does not stimulate redistribution of cone transducin in cones (Figure 3.5 A). In retinas lacking the Nrl (neural retina leucine zipper) transcription factor ($Nrl^{-/-}$), all photoreceptors have morphological, biochemical, and physiological characteristics of cones (Mears et al., 2001; Daniele et al., 2005). I examined cone transducin in $Nrl^{-/-}$ cones and found that it does not redistribute to inner compartments in light, similar to its behavior in wild-type cones (Figure 3.5 B). Cones are known to express higher levels of RGS9 than rods (Cowan et al., 1998). Because RGS9 attenuates rod transducin relocalization (Figure 3.4), I hypothesized that the high level of RGS9 activity in cones could be responsible for the permanent sequestration of cone transducin in the OS of cones. However, I found that the absence of RGS9 does not alter the localization of cone transducin in cones (Figure 3.5 C).
Figure 3.5 Behavior of cone Gt in cones and rods. A, Wild-type mice were dark adapted for 12 hours and sacrificed, either immediately (Dark) or after an exposure to light (Light). Retinal sections were stained with the antibody against the α subunit of cone Gt. The right shows the merged image with the phase contrast of the same (light-adapted) sample. Shown are the results obtained during 3 hours illumination at ambient light (500 lux). An increase in light intensity up to 100,000 lux (direct sunlight, dilated eyes) did not affect the distribution of cone transducin. B, Localization of cone Gt in the dark- and light-adapted Nrl–/– mice, which contain only cone-like cells. C, Localization of cone Gt in the RGS9–/– mice. D, Retinas of wild-type mice were permeabilized with α-toxin in the dark in the presence of GDP or GTPγS, illuminated for 10 minutes at 5000 lux and then for 30 min at 500 lux, and subsequently fixed and stained for cone Gt. Exposure of retinas to direct sunlight for up to 30 min or irradiation with 385 nm light source from a distance of 0.5 cm resulted in the similar distribution of cone Gt. E, Localization of cone Gαt and rod Gβ subunit (Gβ1) in the dark- and light-adapted (500 lux, 30 min) Gnat2+, Gnat1–/– mice, which express cone Gαt in rod cells that lack rod Gαt.
Next, I tested whether the irreversible binding of GTPγS can cause redistribution of cone transducin in cones. I consistently observed that GTPγS introduced into illuminated cones using α-toxin does induce approximately 10% of cone Gαt to move to the cone cell inner compartments (Figure 3.5 D). The dramatic difference in spatial redistribution of cone and rod transducins could be determined by either the type of Gαt protein (since rod and cone transducins are products of different genes) or the context of the cell in which it is expressed. To resolve this, I used Gnat2⁺,Gnat1⁻/⁻ (for guanine nucleotide binding protein, α transducin) mice in which cone Gαt is expressed in rods in a rod transducin-deficient background (Calvert et al., 2000). In striking contrast to its behavior in cones, cone Gαt undergoes light-dependent redistribution in Gnat2⁺,Gnat1⁻/⁻ rods (Figure 3.5 E). Furthermore, the presence of cone Gαt in rods supports the light-dependent translocation of endogenous rod Gβγ. I conclude that the ability of transducin to change its distribution is determined by the cellular environment in the rod rather than by intrinsic properties of the α subunit.

*Are cone photoreceptors fully activated?*

Cones are less sensitive to light than rods, so the apparent inability of cone transducin to effectively redistribute in light could be explained by either inefficient activation of cone opsin or inefficient receptor–G-protein coupling. Nrl⁻/⁻ retinas are dominated by UV opsin, so I employed two types of light sources that
Figure 3.6 UV light exposure of Nrl\(^{-/-}\) mouse eyecups. Eyecups from Nrl\(^{-/-}\) mice were exposed to UV light (365 nM) from a hand-held light source for the indicated times. Under these conditions cone transducin does not redistribute to the inner compartments more than ~10%.

have a strong UV output to ensure that cone transducin was fully activated in these experiments. One source was a 360 nm UV illuminator, and the other was direct sunlight. The UV illuminator was placed within 0.5 cm of the dissected eyecups. Its output peaked at 350 ± 20 nm, and its power was 1.5 mW/cm\(^2\). This corresponds to about 3 x 10\(^7\) 350 nm photons per seconds per square micrometer. The extent of bleaching of cone opsin was evaluated using retinas from Nrl\(^{-/-}\) mice exposed to the UV illuminator, and corresponding movement of cone G\(\alpha\)t was also determined (figure 3.6). Because the spectral characteristics of UV opsin and all-\textit{trans} retinal are very similar, it was necessary to evaluate the extent of UV pigment bleaching by analyzing retinoids with HPLC (Garwin and Saari, 2000). Approximately 75% of the 11-\textit{cis}-retinal chromophore in Nrl\(^{-/-}\) retinas is converted into all-\textit{trans} retinal, retinol, or retinyl esters after 5 min of exposure to either the UV illuminator or direct sunlight (Figure 3.7).
Figure 3.7 Retinoid analysis in Nrl<sup>−/−</sup> retinas under UV or sunlight illumination. Nearly complete bleaching of cone visual pigment in Nrl<sup>−/−</sup> retinas. A, HPLC traces showing retinoid analyses from three conditions. Eyecups were prepared from dark-adapted mice and exposed to darkness, 5 minutes direct sunlight, or 5 minutes of illumination from a 350 nm UV lamp. Retinoids were extracted and analyzed by HPLC by Greg Garwin and John Saari at University of Washington. Note the significant reduction of peaks 3 and 5 (syn and anti-11-cis-retinal oxime) and the corresponding increases in all-trans retinoids (peaks 4, 6, 7). Component 1, retinyl esters; 2, internal standard; 3, syn 11-cis-retinal oxime; 4, syn all-trans-retinal oxime; 5, anti-11-cis-retinal oxime; 6, all-trans-retinol; 7, anti-all-trans-retinal oxime. B, Quantification of the results shown in A. "11-cis-" refers to peaks 3 and 5, and "all-trans" refers to peaks 4, 6, and 7. The black bars represent dark-adapted eyecups (n = 2 samples, four eyes each); the white bars represent eyecups illuminated with sunlight (n = 2 samples, 4 eyes each), and the gray bars represent eyecups illuminated with UV light (n = 1 sample, 4 eyes).
I also evaluated the extent of cone pigment activation by measuring the extent to which the UV opsin C terminus is phosphorylated during its activation. My collaborator, Junhua Wei (in the lab of Jim Hurley, University of Washington, Seattle, WA) measured the extent of phosphorylation of the UV opsin C terminus (Lee et al., 2002) after exposure to 1, 5, and 10 min of UV illumination or sunlight. With both sources of illumination, 30–35% of UV opsin was phosphorylated with one, two, or three phosphates in a steady state reached within 1 min exposure to the illumination (data not shown). These results show that, in the steady-state cycle of activation and inactivation in Nrl−/− cones, 75% of cone pigment is bleached and at least 30% have undergone phosphorylation during its active lifetime. Cone transducin did not relocalize under any of the tested illumination conditions.

To establish whether the high level of cone opsin stimulation under my experimental illumination conditions translates into efficient activation of cone transducin, I also directly evaluated cone transducin activation. I used GTPγS binding as an integrator of activation events to evaluate the extent of cone transducin activation under the same experimental conditions which were used to determine cone transducin localization (Figure 3.5). Size-exclusion chromatography can be used to detect a reduction in the apparent molecular weight of a G-protein that indicates dissociation of activated Gα and Gβγ subunits.
Figure 3.8 Cone transducin binds GTPγS and undergoes subunit dissociation in detergent solution. A, Bovine OS membranes were incubated in light with GDP or GTPγS and solubilized with 1% sodium cholate. Blue dextran (BD) and hemoglobin (Hb; 60 kDa as the tetramer) were added to the extracts before fractionation on a 1 x 25 cm Superdex 75 size exclusion column. The fractions were probed for rod and cone Gαt subunits. B, Freshly dissected retinas from Nrl<sup>−/−</sup> mice were permeabilized with α-toxin (α-Tx) in the dark in the presence of GTPγS or GDPβS, then illuminated for 5 minutes at 1000 lux, and rinsed in 5 ml of medium to wash out free nucleotide. In a control testing the integrity of nonpermeabilized cell membranes, α-toxin was omitted from the media before the subsequent incubation with GTPγS. The retinas were then homogenized, solubilized with 1% cholate, and fractionated on a 0.4 x 24 cm Superdex 75 column.
Activated cone transducin binds tightly to membranes (Figure 3.10). Therefore, as with all other heterotrimeric G-proteins, except rod transducin, analysis of cone transducin by chromatography requires membrane solubilization with mild detergent, typically 1% sodium cholate (Northup et al., 1983). Dissected retinas from Nrl−/− mice were treated with α-toxin in the presence of either GDP or GTPγS, exposed to direct sunlight, rinsed to remove unbound nucleotide, and then solubilized with cholate. The extracts were then analyzed by gel filtration.

My results showed a dramatic reduction in the apparent molecular weight of GTPγS-treated cone transducin, characteristic of fully activated G-proteins (Figure 3.8 B). When the retinas were incubated with GTPγS in the absence of α-toxin, when they were incubated with GDP instead of GTPγS, or when they were incubated in darkness (data not shown), cone transducin remained in the heterotrimeric form. In another set of experiments, I compared the behavior of rod and cone transducin in isolated bovine OS membranes. The membranes were illuminated in the presence of either GDP or GTPγS, extracted with 1% cholate, and fractionated by gel filtration (Figure 3.8 A). Treatment with GTPγS caused a complete shift of the elution volume of both rod and cone transducin, demonstrating that both of the G-proteins bound GTPγS. The similarity of the results with permeabilized retinas and with isolated membranes indicates that, in both preparations, the bulk of cone transducin was stimulated to bind GTPγS.
Figure 3.9 Cone transducin binds GTPγS and is protected from trypsinolysis. Limited trypsinolysis assay. OS membranes from wild-type (WT), Nrl^−/−, and Gnat2^+, Gnat1^−/− mice were incubated with increasing amounts of trypsin, in the presence of GDP or GTPγS. The reaction was stopped by addition of SDS sample buffer, and the samples were analyzed by Western blot.

Using another independent method, a detergent-free protease protection assay, I was able to confirm that GTPγS binds to cone transducin. Membranes from wild-type, Nrl^−/−, or Gnat2^+, Gnat1^−/− mice were incubated with GDP or GTPγS in light and subsequently treated with increasing concentrations of trypsin (Figure 3.9). Cone Gαt undergoes proteolytic fragmentation, consistent with it being bound to GTPγS. Altogether, the analyses of cone opsin activation (Figure 3.7) and the nucleotide-binding state of the G-protein (Figure 3.9) show that cone
transducin is fully activated in my experiments, yet does not undergo light mediated redistribution.

**Membrane association of rod and cone transducins**

Reasonably, if rod transducin moves between the cellular compartments by diffusion, then the prerequisite for its exit from the OS would be release from disc membranes to the cytosol. To account for the different behaviors of cone and rod transducins, rod transducin would dissociate from membranes more effectively than cone transducin. To test this idea, I compared the distributions of rod and cone transducins between soluble and particulate fractions from homogenates of either acutely cultured live mouse retinas (Figure 3.10 A) or purified OS membranes (Figure 3.10 B). As expected, rod transducin was solubilized readily from either whole retina or OS membranes by GTPγS and light. In contrast, the bulk of cone transducin remained in the particulate fractions in wild-type, *Nrl*-/-, and *RGS9*-/- photoreceptors (Figure 3.10). Importantly, I found that cone Gαt can be eluted readily by GTPγS from membranes prepared from *Gnat2*,*Gnat1*-/- mice. This is consistent with the ability of cone transducin to redistribute when expressed in rods (Figure 3.5 E). Thus, the ability of cone transducin to leave the OS correlates with its ability to detach from membranes.
Figure 3.10 Cone transducin binds GTPγS but does not detach from OS membranes. A, Whole retinas were dissected from dark-adapted wild-type mice and either kept in the dark or illuminated. The retinas were homogenized and centrifuged, and the pellet (P) and supernatant (S) were probed with antibodies for rod and cone Gα subunits. Shown is the result of a 15 minutes illumination at 1000 lux; the exposure of the retinas to direct sunlight resulted in the same distribution of cone and rod transducins. B, OS membranes were isolated in the dark from wild-type (WT), Nrl−/−, Gnat2+/−, Gnat1−/−, and RGS9−/− mice. The membrane suspension was illuminated at 1000 lux by a fluorescent lamp or by direct sunlight in the presence of GDP or GTPγS and then centrifuged to obtain membrane (M) and soluble (S) fractions. The pellet (P) and supernatant (S) were probed with antibodies for rod and cone Gα subunits.
N-acylation and localization of rod and cone transducin

Unlike other heterotrimeric G-proteins, which are acylated at their N termini with a myristoyl (C14:0) residue, rod transducin is acylated heterogeneously with C14:0, C14:1, C14:2, and C12:0 fatty acyl residues (Kokame et al., 1992; Neubert et al., 1992). I hypothesized that the difference between membrane association of cone and rod transducins could be caused by differences in their lipid modifications. To examine N-acylation of rod and cone Gα subunits, we isolated them using SDS-PAGE, digested the excised protein bands with trypsin, and subjected the N-terminal peptides to reverse-phase chromatography and tandem mass spectrometry (Figure 3.11). Rod transducin was eluted from OS membranes with light and GTPγS, and I compared the fatty acid compositions of the soluble and membrane-bound forms of Gαt. Both the soluble and membrane fractions contained all four species of rod Gαt: C14:0, C14:1, C14:2, and C12:0. There was only a slight enrichment of the membrane-bound Gαt with more hydrophobic acyl groups and only a slight enrichment of the soluble Gαt with the less hydrophobic species. To obtain sufficient amounts of cone Gαt, I took advantage of its high expression in the Nrl−/− and Gnat2+, Gnat1−/− animals. I found that cone Gαt is modified exclusively with C14:0 in Nrl−/− mice. Surprisingly, cone Gαt in Gnat2+, Gnat1−/− retinas, in which it can be solubilized, is also modified only by C14:0.
Figure 3.11 Distinct N-acylation of rod and cone transducin subunits. OS membranes were isolated from dark-adapted wild-type (WT), Nrl–/–, and Gnat2+, Gnat1–/– mice, treated with 100 µM GTPγS, and centrifuged to obtain the soluble and membrane fractions. The fractions were resolved on SDS-PAGE and stained with Coomassie blue. The bands corresponding to rod and cone Gαt were excised, and the slices were digested with trypsin and extracted. The resulting mixture of peptides was analyzed by Junhua Wei at University of Washington by HPLC and mass spectrometry to determine the amount of the N-acylated N-terminal peptides. A, Ion chromatograms monitoring narrow mass windows (1 Da) corresponding to rod and/or cone transducin N-terminal peptides with fatty-acyl residues from the soluble fractions obtained from wild-type, Nrl–/–, and Gnat2+, Gnat1–/– retinas, respectively. The y-axis for each chromatogram represents the relative abundance of each peak and is normalized to the amplitude of the maximum signal in the whole ion chromatogram (molecular and fragment ion chromatograms have individual maxima). The x-axis represents the elution time from the C18 reversed-phase column. The numbers to the right of each peak represent the relative fraction of each species calculated by integrating the area under each peak. B, The four fatty acyl N-terminal transducin peptides in membrane and soluble fractions from wild-type, Nrl–/–, and Gnat2+, Gnat1–/– retina were quantified by integrating the areas under peaks corresponding to the peptides. The level of each species is expressed as the fraction of the rod or cone Gα peptide detected in the membrane (M) or soluble (S) fractions, respectively. Each data point is the average of at least four separate determinations. The error bars represent SD.
These findings show that (1) the distribution of fatty acids is likely determined by the amino acid sequence of $\alpha$ and not by its environment and (2) the membrane affinity of $\alpha$ in cones versus rods is determined by a factor other than differential N-myristoylation.

*Subunit dissociation facilitates dispersion of transducin*

The tight association of cone $\alpha$ with membranes could be attributable to association with a cone-specific membrane protein. An obvious candidate for this protein would be RGS9, which is more abundant in cones than in rods (Cowan et al., 1998), but my finding that cone $\alpha$ still associates with membranes in $RGS9^{-/-}$ retinas (Figure 3.10 B) rules out this possibility. Another binding partner that plays a major role in membrane association of $\alpha$ is the $\beta\gamma$ complex. To dissect the role of $\beta\gamma$ in membrane binding and redistribution of transducin, I performed a set of experiments in which dissociation of $\alpha$ and $\beta\gamma$ subunits was facilitated by synthetic myristoylated peptides, mSIRK and mSIGK. These novel reagents interfere with G-protein subunit association by binding directly to $\beta$ at the same site in which $\alpha$ binds to $\beta$ (Davis et al., 2005; Bonacci et al., 2006; Goubaeva et al., 2003). The effects of these two peptides in this study were indistinguishable. Therefore, I will refer to either peptide as “mSIRK” in the following discussion.
Figure 3.12 Translocation of rod transducin is facilitated by the synthetic Gβ-binding peptide mSIRK. Dark-adapted retinas were permeabilized with α-toxin and incubated in the dark in the presence or absence of 50 µM mSIRK. After 1 hour, the retinas were fixed, and the localization of rod Gαt and Gβ1 was determined. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer (synaptic terminal).

Figure 3.13 Translocation cone transducin is facilitated by the synthetic Gβ-binding peptide mSIRK. A, Dark-adapted retinas were permeabilized with α-toxin and treated with 50 µM mSIRK or its inactive mutant (L9A) peptide in the presence or absence of 100 µM GTPγS. Retinas were then exposed to sunlight for 15 min, fixed, sectioned, and stained with antibodies against cone Gαt. B, Localization of cone Gβ (Gβ3 immunoreactivity) in permeabilized wild-type mouse retinas treated with mSIRK, L9 with GTPγS, and mSIRK with GTPγS, in light. This is representative of three independent experiments. OS, outer segment; IS, inner segment; ONL, outer nuclear layer.
As a negative control in my studies, I used a mutant form of mSIRK, L9A, which has a single amino acid substitution and is ineffective at binding to Gβ. Addition of mSIRK alone to permeabilized retinas induced redistribution of rod Gαt and Gβγ even in the absence of nucleotides and in darkness (Figure 3.12). In contrast, mSIRK alone did not affect localization of cone Gαt in wild-type or Nrl−/− mice in light or darkness.

Figure 3.14 Quantification of the series of data from peptide induced translocation. Rod or cone Gαt immunofluorescence in the entire photoreceptor (Ft) and immunofluorescence from the inner compartments (Fi) were determined using Metamorph software. The average F/Ft ratio (mean ± SD) in each experiment was determined within three areas of the retina. The data were collected in at least six independent groups of mice. Retinas were dissected under dim red light and permeabilized in the presence of GTPγS, mSIRK, L9A mutant, or mSIRK with GTPγS as indicated. For L9A plus GTPγS, n = 3. The concentration of mSIRK or L9A was 50 µM. Light intensities varied from 500 lux to direct sunlight, providing identical results. Control permeabilized retinas were kept in the dark.
Remarkably, the combination of mSIRK and GTPγS causes cone Gαt to diffuse and equilibrate throughout the cytoplasm of wild-type (Figure 3.13 A) and Nrt+/− (Figure 3.15) permeabilized cones. Up to 60% of total cone Gαt immunofluorescence was detected in the inner compartments in the presence of GTPγS and mSIRK, whereas GTPγS alone or in combination with L9A resulted in translocation of no more than 15% (Figure 3.13 A). I also monitored the localization of the cone Gβγ complex Gβ3γ8 and found that Gβ3 remained in cone OS under all tested conditions, in both wild-type and Nrt+/− retinas (Figures 3.13 B, 3.15).

Figure 3.15 Translocation of cone transducin is facilitated by the synthetic Gβ-binding peptide mSIRK in Nrt−/− mice. Retinas of Nrt−/− mice, which only contain cone-like photoreceptors were permeabilized and treated with mSIRK or mSIRK with GTPγS, in light (1000 lux or direct sunlight for 5 or 30 min produced identical results). Localization of cone Gαt and Gβ3 was determined by immunofluorescence microscopy. OS, outer segment; IS, inner segment; ONL, outer nuclear layer.
Figure 3.16 Subunit dissociation underlies the difference between rod and cone transducins in membrane detachment. A, OS membranes isolated from dark-adapted retinas were resuspended in a buffer containing 100 µM GDP, GTPγS, 50 µM mSIRK, or the mixture of mSIRK and GTPγS. After a 10 minute incubation at room temperature in light (1000 lux or sunlight), the suspension was centrifuged to obtain the supernatant (S) and membrane (M) fractions. These samples were analyzed by Western blot with antibodies against rod and cone Gαt, Gβ1, and Gβ3 subunits. B, The bar graph shows the amount of solubilized Gαt, as percentage of total, as mean ± SD from at least three independent experiments, in which the Western blots were scanned and analyzed by Scion densitometry (Frederick, MD) software.

Consistent with its effects on subcellular localization of rod and cone subunits, mSIRK promoted elution of rod Gα and Gβγ subunits from the membrane in the absence of GTPγS and facilitated solubilization of cone Gαt but not cone Gβ3 (Figure 3.16). The effects of mSIRK on subunit dissociation of rod and cone G-proteins were consistent with the effects of this peptide on transducin localization in cells and membrane association. In the size exclusion gel filtration assay, mSIRK caused the shift of at least 50% of the GDP-bound rod
Figure 3.17 mSIRK facilitates transducin subunit dissociation in detergent solutions. Bovine OS membranes were treated with 50 µM mSIRK and solubilized with 1% cholate. Fifty microliters of the extract were resolved by gel filtration on a 0.4 x 25 cm Superdex 75 column, which was pre-equilibrated with the buffer containing 25 µM mSIRK. The distribution of rod and cone Gαt in the collected fractions was determined by Western blot. The extracts from GDP- and GTPγS-treated membranes were resolved on the same column to determine the location of the heterotrimers and dissociated Gα subunits, respectively. BD, Blue dextran; Hb, hemoglobin.

transducin toward the size corresponding to the dissociated subunits (Figure 3.17). In contrast, mSIRK caused only some broadening of the cone transducin peak, so that the bulk of cone transducin remained in its heterotrimeric form.

In summary, I found that (1) subunit dissociation, not GTP binding per se, is the event that determines localization of transducin and (2) the α and βγ subunits of cone transducin associate with each other more tightly than the subunits of rod transducin. While GTP binding indeed causes subunit dissociation of rod transducin, only the addition of mSIRK plus GTPγS was able to cause cone transducin to dissociate and redistribute through the cell. Another
key result was that mSIRK alone, in dark, was able to cause rod transducin to dissociate and disperse (Figure 3.12). In Chapter 4 of this dissertation, I will present a more detailed model for the mechanism of transducin redistribution in rods and cones. Based on my findings, my conclusion is that rod transducin migrates through the cells because its subunits dissociate, detach from the membranes, and diffuse throughout the cell. Cone transducin does not redistribute because even when bound to GTPγS, its subunits do not effectively dissociate or detach from the membranes. Only addition of the Gβ-binding peptide causes dissociation and dispersion of cone transducin. As will be discussed, my model is similar the “sink” model of arrestin diffusion and translocation in photoreceptors (Nair et al., 2005b).
II. Retinal guanylate cyclase interacts with the alpha subunit of transducin and facilitates its translocation in retinal rods

Figure 3.18 A ~115 kDa band consistently co-precipitates with Gαt. Bovine OS were solubilized with 1% Triton X-100 and subjected to immunoprecipitation as described in Materials and Methods, using anti-Gαt IgG in the presence or absence of the blocking peptide (Santa Cruz SC389 P). The eluates from the beads were resolved on an 8% SDS PAGE gel and stained by silver. The indicated 115kD band was analyzed by mass spectrometry. The Gαt band was excised and analyzed as a control.

RetGC Co-Immunoprecipitates with bovine rod Gαt

To identify proteins that might facilitate transducin localization within rod photoreceptors, I immunoprecipitated Gαt from detergent-solubilized bovine OS fractions and analyzed the specifically-bound proteins. As a control for the non-
specific binding to Protein A Sepharose beads, I blocked the Gαt 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 by silver nitrate (Figure 3.18). The pattern of detected proteins slightly varied, but in all experiments, I consistently observed a protein band of approximately 115 kDa, which specifically co-precipitated with Gαt. This band was excised from the gel and subjected to mass spectrometry analysis. Seven distinct tryptic peptides generated from the 115 kDa protein reliably identified it as bovine retinal guanylate cyclase type 1 (RetGC-1). As a control, the band corresponding to Gαt (39 kDa) was analyzed and unambiguously identified as the alpha subunit of bovine rod transducin, and no presence of other polypeptides was detected above the background level.

I confirmed the identity of the 115 kDa protein by immune-blot using anti-retGC antibodies (Laura and Hurley, 1998) (Figure 3.19). The specificity of the Gαt-mediated retGC interaction was verified using Protein A beads coupled with normal IgG. To further confirm the Gαt:retGC interaction, I co-immunoprecipitated the Gαt:retGC complex using antibodies against retGC1 and probed for Gαt (Figure 3.19 A). In this reciprocal orientation, a small fraction of Gαt was specifically absorbed from the lysates. I next compared the efficiency of retGC co-IP with anti-Gαt antibodies to that of the Gβ5L-RGS9 complex, a known binding partner of Gαt. Gβ5L-RGS9 co-immunoprecipitated with Gαt under my
conditions, but not with retGC (Figure 3.19 A). In most experiments, resolution of RGS9 (55 kDa) was completely obscured by the IgG bands present in the eluted fraction. Therefore, I used antibodies specific to Gβ5 to reveal the complex, since Gβ5 and RGS9 are perpetually in complex. In contrast, Gβ1 was not detected in my immunoprecipitates, possibly because binding of the antibody to the Gα subunit causes dissociation of transducin under these conditions.

Figure 3.19 Co-immunoprecipitation of Gαt with retGC in bovine ROS. A, Bovine OS were solubilized with 1% Triton X-100 in a modified RIPA buffer and subjected to immunoprecipitation using anti-Gαt, anti-retGC, or control IgG antibodies. Eluates (E) from the beads and the unbound fraction (U) were subjected SDS-PAGE. Western blot analysis of IP fractions probed with anti-retGC-1, anti-Gαt, anti-Gβ1 and anti-Gβ5L antibodies. B, Serial dilutions of bovine OS were analyzed by western blot with anti-Gαt and anti-retGC antibodies. Developed films were scanned and analyzed by densitometry (Scion software) to be sure that western blot signals were within the linear range of detection.
To estimate the amounts of the proteins in the IP fractions, I compared the intensity of the western blot signals to serial dilutions of the retinal lysate (Figure 3.19 B). It is important to note that to detect these interactions the loading of the eluate (E) was ~10-fold over the unbound (U) fractions. These analyses showed that anti-G\(\alpha\)t antibodies precipitated approximately 10% of rod G\(\alpha\)t from bovine OS under my experimental conditions. Surprisingly, almost an equal proportion of retGC (about 8.0%) co-immunoprecipitated with G\(\alpha\)t. Assuming a 1:1 stoichiometry in G\(\alpha\)t:RetGC complex, this result indicated that about 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 I could pull down about 50% of retGC (Figure 3.19 A). About 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 approximately 1:15 (Hwang et al., 2003), most of 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 about 6.6% of total G\(\alpha\)t.

Considering the 50% efficiency of retGC IP with the anti-retGC antibodies (Figure 3.19 A), the maximal amount of G\(\alpha\)t in my co-IP should be about 3.3%, which is comparable to my result of about 1.5% (based on Figure 3.19 B). Thus, based on the estimate from the co-IP using anti-retGC antibodies, about 45% of retGC is complexed with G\(\alpha\)t. With the understanding of the limitations of
quantitative western blot methods, my estimates from reciprocal co-IP suggest that a remarkably large fraction of retGC (45-80%) is bound to Gαt. Moreover, these estimates are consistent with the distinctive detection of the 115 kDa band in the silver-stained gels (Figure 3.18).

A parallel analysis of co-immunoprecipitation of Gαt with the Gβ5L-RGS9 complex showed that about 1% of Gβ5L-RGS9 present in the OS lysate bound to the beads with anti-Gαt antibodies. Since my experimental efficiency of Gαt IP is about 10%, this indicated that 10% of total Gβ5L-RGS9 was associated with Gαt. Considering the RGS9:transducin ratio to be 1:85 (He et al., 1998), this suggested that approximately 0.1% of Gαt absorbed on the anti-Gαt beads was represented by its complex with Gβ5L-RGS9 (Figure 3.19 A).

Thus, my results indicate that a large (45-85%) pool of retGC is associated with Gαt. Like Gβ5L-RGS9-Gαt, the retGC:Gαt complex represents only a small fraction of transducin, which is much more abundant than either retGC or Gβ5-L-RGS9. In the discussion section of this dissertation I will propose a mechanistic link between transducin, the disc membranes in the rod OS, and retGC. I will discuss how they are possibly related to the “sink” model of light-induced redistribution of signaling proteins in rod photoreceptors. Other groups have demonstrated the importance of retGC in protein trafficking in the rod OS, as well as the relation of retGC to cone transducin localization in cone photoreceptors (Coleman and Semple-Rowland, 2005; Baehr et al., 2007).
Figure 3.20 retGC Co-immunoprecipitates with Gαt in mouse ROS; RGS9 is not required for the interaction. Mouse OS lysates from wild type and RGS9−/− mouse OS were subjected to immunoprecipitation and western blots were probed with anti-retGC-1, Gαt, RGS9, and Gβ5 antibodies.

RetGC Co-Immunoprecipitates with mouse rod and cone Gαt subunits

To test if this interaction can occur in mouse rods and cones, I investigated the Gαt:retGC interaction using available genetic mouse models. First, I established that retGC co-immunoprecipitated with rod Gαt from wild type mouse OS (Figure 3.20). RGS9 and Gβ5L, which can form a stable complex with Gαt (Hu and Wensel, 2002), were also found to be present in these immunoprecipitated fractions. I then tested if retGC co-precipitated with rod Gαt from retinas of mice lacking RGS9. My result showed that co-immunoprecipitation of retGC and Gαt still occurred in the RGS9−/− mouse OS (Figure 3.20). This result indicates that while the Gβ5L-RGS9 complex co-
precipitates with Gαt and retGC, it is not required for the Gαt:retGC interaction.

To determine if the Gαt:retGC interaction occurs in cones, I used mice lacking
the transcription factor Nrl, in which retinas contain only cone-like photoreceptors
(Daniele et al., 2002; Mears et al., 2001). Figure 3.21 demonstrates that retGC
co-precipitates with the cone Gαt and the Gβ5L-RGS9 complex. As in the
experiments with bovine OS (Figure 3.19), Gβ1 did not co-precipitate with the
mouse Gαt:retGC complex (not shown), and in all experiments on mouse OS,
Gαt and retGC co-precipitated equally well from light- or dark-adapted retinas.

![Nrl -/-](image)

**Figure 3.21 Co-immunoprecipitation of retGC with cone Gαt.** Cone Gαt was
immunoprecipitated from the OS prepared from the Nrl knockout mice, which contain only cone-
like photoreceptors. The fractions were probed with anti-cone Gαt, retGC, RGS9, and Gβ5
antibodies.
Reconstitution of Gαt:retGC Interaction in COS-7 cells

Rod Gαt and retGC cDNAs were transiently transfected into COS-7 cells, and I performed immunoprecipitation under two different conditions. First, Gαt and retGC cDNAs were co-transfected into the same pool of cells, and then the membrane fractions were solubilized and subjected to immunoprecipitation. Second, retGC and Gαt were transfected separately, and the obtained cell lysates were mixed just prior to the immunoprecipitation. I found that Gαt and retGC co-immunoprecipitated only if the two proteins were co-expressed within the same cells. The Gαt:retGC complex could be reciprocally precipitated with either anti-Gαt or anti-retGC antibodies (Figure 3.22).

Figure 3.22 Reconstitution of ret-GC with rod Gat in transfected COS-7 cells. Human rod Gat and Human retGC-1 cDNAs were transiently expressed in COS-7 cells. Cell lysates were immunoprecipitated with anti Gat (A), anti retGC (B) or control IgG.
The efficiency of immunoprecipitation (~50%) from co-transfected cells was similar for retGC and G\(\alpha\). This is consistent with the idea that the expression ratio of these two proteins in COS-7 cells was nearly stoichiometrical in contrast to photoreceptors, where transducin is expressed in large excess.

**Figure 3.23 Transducin does not affect catalytic activity of retGC.** Purified rod G\(\alpha\), G\(\beta\)\(\gamma\) of transducin or BSA was incubated with HEK293 membranes expressing retGC-1, in presence or absence of the recombinant activator of retGC, GCAP-1. Cyclic GMP production was measured using radiolabeled GTP as the substrate. This assay was performed by Igor Peshenko and Alexander Dizhoor at the Pennsylvania School of Optometry, Elkin's Park, PA.
Transducin has no effect on retGC activity

A previous report from another group suggested that transducin Gβγ complex could inhibit the activity of retGC (Wolbring et al., 1999). To investigate if either heterotrimeric transducin or its individual subunits could modulate retGC activity I purified rod Gαt, either GTPγS or GDP-bound, and Gβγ from bovine OS and reconstituted them with retGC-containing membranes. The enzymatic activity of retGC was measured in presence or absence of a sub-saturating concentration of recombinant GCAP-1, as described in Materials and Methods (also, see Peshenko and Dizhoor, 2007). I found that neither Gαt nor the Gβγ complex had any detectable effect on retGC activity under these conditions (Figure 3.23). The reason for this contradiction is unclear at this time.

Gαt Interacts with retGC at its Kinase Homology Domain

RetGC is usually regarded as having five domains defined in its structure: the extracellular (ECD) domain, which is exposed either to the lumen of OS discs or outside the OS plasma membrane; a short transmembrane (TM) region; a kinase homology domain (KHD); dimerization domain (DD) and a catalytic (CAT) domain (Figure 3.24 A) (Dizhoor and Hurley, 1999; Laura et al., 1996). In order to determine the site on retGC to which Gαt binds, GST-fusion proteins of the catalytic domain (GST-CAT) and kinase homology domain (GST-KHD) of retGC1 were generated. The GST-fusion proteins were immobilized on glutathione-agarose beads and tested for their ability to pull down purified transducin or
transducin from crude OS lysates from wild type and Nrl<sup>−/−</sup> mice. My results showed that GDP-G<sub>α</sub> bound much stronger to the KHD than to the CAT domain.

**Figure 3.24. Preferential binding of G<sub>α</sub> to the kinase homology domain of retGC.**

A, Schematic representation of the retGC domains: ECD-extracellular domain; TM-transmembrane domain; KHD-kinase homology domain; DD-dimerization domain; CAT-catalytic domain. B, Top: Coomassie-stained gel showing the purity of GST-KHD and GST-CAT products. Bottom: 20 μg GST-KHD, GST-CAT or GST were immobilized on glutathione beads, which were added to mouse retinal lysates containing approximately ~1 μg of G<sub>α</sub>. Extracts from Nrl<sup>−/−</sup> retinas were used for analysis of cone G<sub>α</sub> binding. The GST pull-down assays were performed as described in Materials and Methods, and the specifically bound rod and cone G<sub>α</sub> were detected by western blot. Representative of three independent experiments.
of retGC. I also found that binding of GTPγS to Gαt suppressed this interaction (Figure 3.24 B). I then tested if the purified GST-KHD fusion protein could block the interaction of Gαt with endogenous retGC. The Gαt IP was performed from bovine OS after adding an excess (0.1 mg/ml) of purified KHD to the lysate (Figure 3.25). Recombinant KHD bound to the immobilized Gαt under these conditions, however it did not reduce binding of endogenous retGC to Gαt. This suggested that GST-KHD bound primarily to the “free” Gαt, and that the full-length cyclase has a higher affinity to Gαt than the recombinant KHD domain.

Figure 3.25 Addition of recombinant KHD to Gαt IP. Immunoprecipitation of rod Gαt was performed as described in Figure 3.19, except that here, 20 μg of GST-KHD was added to the lysate during the pre-clearing stage. An equivalent amount of GST was added to the control. Following the IP, the fractions were analyzed with anti-Gαt and anti-retGC1 antibodies. Specific anti-KHD antibodies (6344KHD) (Laura and Hurley, 1998) were used to detect GST-KHD in the fractions. Representative of two independent experiments.
Detergent resistant membranes (Lipid Rafts) can be prepared from mouse outer segments using a standard protocol for bovine ROS. Bovine and mouse rod outer segments were washed with buffer containing 0.5% triton and immediately mixed with equal volume of 80% sucrose. The 40% sucrose/ROS slurry was laid on a sucrose step gradient and centrifuged at 100,000Xg overnight. Twelve 50μl fractions were collected top-down, and analyzed by western blot for transducin and the raft marker caveolin-1. Transducin co-migrates in the raft fractions 3-5, which contain Cav-1.

Does retGC coordinate transducin localization to lipid rafts?

Our lab has previously demonstrated that illumination of photoreceptors triggers translocation of transducin and RGS9 to detergent resistant membranes (DRM) or lipid rafts (Nair et al., 2002). It was shown that transducin and RGS9 co-localize with other makers for lipid rafts, such as caveolin-1, rhodopsin, and retinal guanylate cyclase. Since I have found that transducin and retGC can interact, I hypothesized that perhaps retGC is coordinating transducin localization to the lipid rafts. To test this hypothesis, I prepared raft fractions from isolated wild-type and retGC−/− OS and probed for Gαt distribution. Figure 3.26 shows the
preparation of rafts in light from mouse OS compared to those from bovine OS, where in both preparations, Gαt co-migrates similarly to the raft fraction. The raft fractions from mouse OS preparations were typically found to be in fractions 3-5 on the sucrose gradient as evident by the raft marker caveolin-1. In mice lacking retGC, Gαt was still able to localize to lipid rafts suggesting that Gαt localizes to the DRM by a different mechanism other than direct binding to retGC (Figure 3.27).

![Figure 3.27](image)

**Figure 3.27 Transducin migrates to lipid rafts in retGC null mouse OS.** Mouse rod outer segments isolated from wild type (WT) and retGC null (GC⁻/⁻) were washed with buffer containing 0.5% Triton X-100 and immediately mixed with equal volume of 80% sucrose. The 40% sucrose/ROS slurry was laid on a sucrose step gradient and centrifuged at 100,000Xg overnight. Twelve 50μl fractions were collected and analyzed by western blot for transducin and the raft marker caveolin-1.
Figure 3.28 Rate of light-induced transducin migration in retGC-1 knockout mice. Eyecups were prepared from wild-type, retGC-1 knockout and RGS9 knockout mice under infrared light. The eyecups were then illuminated at 500 lux for the indicated times prior to fixation. The retinas were sectioned, stained with the antibodies against Gat and analyzed by confocal microscopy. A. Representative sequence of sections from wild-type (WT) and retGC-1-null (GC⁻/⁻) mice. OS, outer segments, IS, inner segments, ONL, outer nuclear layer, ST, synaptic termini. B. Quantitative analysis of relative fluorescence intensity in the inner compartments. Representative of four independent experiments for retGC-1-null and WT, and three experiments for RGS9-null. In each experiment, fluorescence was analyzed from three randomly selected areas per retinal section using Metamorph software. Error bars represent mean ± standard deviation.
Light-induced transducin redistribution in mice lacking ret-GC1

Since I found that subcellular localization of transducin is determined by its interaction with the OS membranes, as described above (Rosenzweig et al., 2007), I therefore reasoned that the localization of transducin might be affected by retGC, which is a transmembrane protein localized in the OS. To test this idea, I examined light-dependent movement of transducin subunits in the rods of retGC-1 knockout mice (Yang et al., 1999). I found that light-induced transducin re-distribution from OS occurred substantially faster than in the wild-type animals (Figure 3.28).

Figure 3.29 Time-course of transducin migration to the OS in retGC-1 mice. Eyecups from wild-type and retGC+/− were prepared and illuminated for 30 minutes at 500 lux to induce transducin translocation. The retinas were then placed in dark for the indicated times, fixed, sectioned and analyzed for localization of Gαt. A, Representative sequence of sections. B, Quantitative analysis of relative fluorescence intensity in the inner compartments for the series of time points of dark adaptation. Error bars represent mean ± standard deviation; n=4 for retGC-1-null and WT mice and n=3 for the control RGS9 null mice.
As a control in this study mice lacking RGS9 were used, a model where transducin movement is accelerated due to the increased lifetime of its GTP-bound state (Figure 3.4) (Rosenzweig et al., 2007; Lobanova et al., 2007; Kerov et al., 2005b). To my surprise, light-induced transducin relocalization in the retGC-1 knockouts occurred even faster than in the RGS9⁻/⁻ mice. Accordingly, the return of transducin from the inner compartments to the OS after the onset of darkness was delayed compared to that in the wild-type mice (Figure 3.29). The data 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.

Figure 3.30 Rate of light-induced Gβγ migration in retGC-1 knockout mice. Eyecups were prepared from wild-type and retGC-1 knockout mice under infrared light. The eyecups were then illuminated at 500 lux for the indicated times prior to fixation. The retinas were sectioned, stained with the antibodies against Gβ1 and analyzed by immunofluorescence microscopy. A, Representative 5 minute time point of sections from wild-type (WT) and retGC-1-null (GC⁻/-) mice. OS, outer segments, IS, inner segments, ONL, outer nuclear layer. B, Quantitative analysis of relative fluorescence intensity in the inner compartments for a series of time points of illumination using Metamorph software. Error bars represent mean ± standard deviation.
The distribution of the rod transducin Gβγ complex, which was monitored using anti-Gβ1 antibodies, corresponded to that of Gαt under all the tested conditions. Indeed, Figure 3.30 shows the similarity in migration patterns of Gβγ when comparing wild-type and retGC knockout mice. The most striking difference in the amount of Gβ1 redistributing to the inner compartments is found after just 5 minutes exposure to ambient light. Transducin migrates to the inner compartments in retGC null mice at least twice as fast as in the wild-type mice.

*The role of cGMP in transducin distribution*

The effect of retGC-1 knockout on transducin movement could theoretically result from (1) the reduced guanylate cyclase activity (concentration of cGMP) in rods or from (2) the absence of the protein-protein interactions between Gαt and retGC. To distinguish between these two possibilities, I examined transducin relocalization in explanted permeabilized retinas in the presence or the absence of cGMP. I have previously shown that treatment of photoreceptors with *S. aureus* α-toxin, a pore-generating polypeptide, allows the entry of nucleotides into the photoreceptor cells (Figure 3.1). Most endogenous proteins are too large to exit via the pores and remain inside. In these preparations transducin retains the ability to re-localize in the light- and nucleotide-dependent manner, to the extent and with the kinetics similar to its redistribution in live animals (Rosenzweig et al., 2007). Here, I used the same experimental paradigm to test the effect of cGMP and its poorly hydrolyzed analog 8-bromo-cGMP on transducin relocalization. I 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 analyzed by thin layer anion-exchange chromatography (Figures 3.31-3.33).

![Degradation of cGMP](image)

**Figure 3.31** Degradation of cGMP is greatly accelerated by GTP\(\gamma\)S in bovine ROS. *Left*, Bovine OS (20\(\mu\)g of total protein) were incubated with 100\(\mu\)M cGMP or 8-bromo-cGMP for 5 min, in room light, in the presence or absence of 100\(\mu\)M GTP\(\gamma\)S. Treated ROS was immediately pellet by centrifugation, and 5ml aliquots were taken and loaded to TLC nitrocellulose membrane and visualized with a handheld UV lamp (~254 nM). *Right*, Quantitation of nucleotide degradation was determined by spectrophotometric absorbance (~260 nM) of nucleotides cut and eluted from the nitrocellulose membrane. Degradation was calculated by dividing the GMP breakdown product into the estimated total cyclic nucleotide added to the samples. Error bars represent mean ± standard deviation from 3 independent experiments.

The stock nucleotides were diluted to the experimental concentration of 100\(\mu\)M. As a control, the nucleotides were then delivered to inactive (GDP) and activated (GTP\(\gamma\)S) bovine ROS to test their ability to be hydrolyzed by PDE6.
(Figure 3.31). As expected, activated bovine ROS is able to completely hydrolyze cGMP into GMP, yet only a fraction of 8-bromo cGMP is hydrolyzed. To ensure delivery of cyclic nucleotides to permeabilized eyecups, cGMP and 8-bromo cGMP degradation was analyzed after delivery to light adapted eyecups treated with or without α-toxin and GTPγS. As expected, both nucleotides did not degrade when α-toxin was not present (Figure 3.32). However, in presence of α-toxin and GTPγS, some degradation of cGMP was detected, suggesting that cyclic nucleotides enter permeabilized cells and are subject to PDE6 activity.

![Eyecup Controls](image.png)

**Figure 3.32 Cyclic nucleotides can be delivered to permeabilized eyecups.** Cyclic nucleotides (100μM) were delivered to eyecups and incubated for 30 minutes in light in the presence or absence of α-toxin. 5μl aliquots of the bathing media were taken and the nucleotides were analyzed by TLC and spectrophotometry. cGMP undergoes partial degradation in the permeabilized eyecups. Error bars represent mean ± standard deviation from 4 independent experiments.

To rule out the effects of PDE6-mediated cGMP degradation, I limited my study to the analysis of transducin migration to the OS, which occurs after the
onset of darkness. Reasonably, it would be difficult to interpret the results of such an experiment if PDE6 is activated to its full extent in light. In these experiments, only a fraction of cGMP was converted to GMP in light and GTP-dependent manner (Figure 3.33). Under the same conditions used in the transducin relocalization experiment (45 minutes time point; Figure 3.29) this hydrolyzed fraction did not exceed 10%, indicating that there was an ample supply of cGMP available throughout the duration of the relocalization experiment. Degradation of 8-Br-cGMP was found to be negligible.

Figure 3.33 Cyclic nucleotides remain stable throughout the duration of experimentation. Eyecups were prepared from dark-adapted wild type mice under dim red light and incubated for 30 min with α-toxin. They were then illuminated for 30 minutes at 500 lux in the presence of indicated nucleotides, including GDP, GTP, cGMP and 8-bromo-cGMP (8-Br). They were then transferred into a fresh medium supplemented with the nucleotides and finally placed in dark 45 minutes, which caused approximately 75% of transducin to return to OS (see Figure 3.29). After 45 minutes in darkness, 5 μl of media was collected and analyzed for the presence of the nucleotides using TLC. The positions of cGMP, 8-bromo-cGMP (8-Br), GMP, GDP or GTP are indicated with arrows. The bar graph shows the results of spectrophotometric analysis of the nucleotides eluted from the spots. The values represent the absorption at 260 nm of remaining cGMP and 8-Br compared to sum of the residual cGMP with GMP product, which was set as 100%. Data (mean ± standard deviation) from 4 independent experiments.
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\(\alpha\) to OS (Figure 3.34). Treatment of the cells with GTP\(\gamma\)S was used as a control for nucleotide entry into the cells, and irreversible redistribution of transducin to the rod inner compartments was observed. Thus, my results strongly indicate that transducin relocalization to the OS was not affected by cGMP, which also suggests that retGC-1 influences transducin movement not through its enzymatic activity, but through the direct interaction with G\(\alpha\).

**Figure 3.34 cGMP has no effect on transducin translocation.** Eyecups were prepared from dark-adapted wild type mice under dim red light and incubated for 30 minutes with \(\alpha\)-toxin as described in the previous figure. They were then illuminated for 30 minutes at 500 lux in the presence of indicated nucleotides, including GDP, GTP\(\gamma\)S, cGMP and 8-bromo-cGMP (8-Br). They were then transferred into a fresh medium supplemented with the nucleotides and finally placed in dark 45 minutes, which caused approximately 75% of transducin to return to OS. Localization of rod G\(\alpha\)t in the treated eyecups was analyzed after 45 minutes of onset of dark using immunofluorescence microscopy of the retinal sections as described in Figure 3.4. The data (mean \(\pm\) standard deviation, n=5) show the amount of G\(\alpha\)t immunofluorescence in inner compartments as the percentage of the total immunofluorescence signal.
III. Persistent dissociation of non-photoreceptor G protein Subunits alters subcellular localization and disrupts signal transduction

*mSIGK causes membrane detachment of Gi, Go, and Gq*

As described in Chapter 3, part I, the synthetic peptide mSIGK caused rod transducin subunit dissociation and detachment from outer segment (OS) membranes, even in the absence of GTP binding and activation (Figures 3.12-3.16) (Rosenzweig et al., 2007). Detachment of cone transducin from cone OS membranes was only achieved in presence of mSIGK and GTPγS.

I next investigated if other endogenously expressed G proteins in other cell types behave similarly to rod or cone transducin. I treated mouse brain membrane fractions as well as COS-7 and CHO-K1 membranes with mSIGK in the presence of various guanine nucleotides. In the brain membrane fractions, treatment with mSIGK and GTPγS caused up to ~25% membrane detachment of Gαi, Gαo, Gαq, and Gβ1 (Figures 3.35, 3.36). Treatment with the nucleotides alone or peptide alone did not induce membrane detachment. Treatment of COS-7 and CHO-K1 membrane fractions with the nucleotides in conjunction with the peptide yielded similar results for endogenously expressed Gαq and Gβ1 (Figure 3.37). Treatment with GTPγS alone, which presumably causes complete G protein activation, did not induce more than ~5% membrane detachment of any of the G protein subunits tested. These results correlate with my observations of the effects of GTPγS and the peptide on cone transducin (Figure 3.16).
Figure 3.35 mSIGK induced membrane detachment of G proteins in native tissue. Mouse brain membrane fractions were treated with GDP, GTPγS, L9A, mSIGK, or mSIGK + GTPγS in buffer suspension for 30 minutes. Samples were centrifuged at maximum speed for 30 minutes at 4°C, and membrane pellets (P) and soluble fractions (S) were analyzed by SDS-PAGE and western blot for protein distribution using specific antibodies to the indicated G protein subunits.

Figure 3.36 Quantitation of mSIGK induced membrane detachment of G proteins. Mouse brain membrane fractions were treated with GDP, GTPγS, L9A, mSIGK, or mSIGK + GTPγS in buffer suspension for 30 minutes. The bar graph shows the amount of solubilized G proteins, as percentage of total. Data is mean ± SD from 6 independent experiments, in which the Western blots were scanned and analyzed by Scion (Frederick, MD) software.
**Figure 3.37** mSIGK, but not GTPγS, induces G protein membrane detachment in cell lines. Membrane fractions prepared from COS-7 and CHO-K1 cells were treated with GDP, GTPγS, L9A, mSIGK, or mSIGK + GTPγS in isotonic buffer suspension for 30 minutes. Samples were then fractionated to membrane pellet (P) and soluble fractions (S) by centrifugation at maximum speed for 30 minutes at 4°C. Endogenous G protein subunit distribution was analyzed by SDS-PAGE and western blot using specific antibodies for the Gαq and Gβ1 subunits.

*mSIGK induces subunit dissociation of non photoreceptor G proteins*

Since mSIGK was able to induce subunit dissociation of rod and cone transducin (Figure 3.17), I tested if mSIGK could cause subunit dissociation of other typical G proteins, as determined by size-exclusion chromatography. I investigated the effect of the mSIGK peptide on the apparent molecular weights of Gi, Go and Gq in detergent extracts of mouse brain membranes fractions. Figure 3.38 shows that treatment with mSIGK alone resulted in a molecular weight shift in elution patterns for Gαi, Gαo, Gαq, and Gβ1, similar to the pattern observed with GTPγS treatment. The observed shift to a smaller molecular weight indicated that the peptide can induce subunit dissociation in a detergent
solution for all of the G proteins tested. For a control, treatment of the membrane fractions with GDP revealed an elution pattern of higher molecular weight, indicating intact heterotrimers of the G proteins tested.

Figure 3.38 Heterotrimeric G protein subunits dissociate in presence of mSIGK in a detergent solution. Mouse brain membranes were incubated with GDP, GTPγS or mSIGK and solubilized with 1% sodium cholate. Blue dextran (BD) and hemoglobin (Hb; 68 kDa as the tetramer) were added to the extracts as a marker before fractionation on a 0.4 x 25 cm Superdex 75 column, which was pre-equilibrated with the buffer containing 25 µM mSIGK. The distribution of the G protein subunits in the collected fractions was determined by Western blot using specific antibodies to the indicated G protein subunits. After washing with 4 volumes of buffer, the extracts from GDP- and GTPγS-treated membranes were resolved on the same column to determine the location of the heterotrimers and dissociated Gα and Gβγ subunits, respectively.
Redistribution of Gβ1 in CHO-K1 and COS-7 cells

My previous results demonstrated that when transducin G protein subunits physically dissociate, they detach from the membranes and are free to diffuse and redistribute through the photoreceptor cell (Chapter 3, part I) (Rosenzweig et al., 2007). To test if mSIGK causes G protein redistribution within cells, COS-7 and CHO-K1 cells were treated with DMSO (control), L9A control peptide, or mSIGK for 20 minutes in DMEM medium. Fixed and permeabilized cells were probed for localization of the endogenous Gβ1. The treated cells were also stained with Phalloidin, which binds to filamentous actin, allowing for visualization of the actin filaments within the cells. I found that when treated with controls, both cell lines displayed Gβ1 localization on the plasma membrane rim and at focal points at the ends of filamentous actin, with little cytosolic signal. This pattern was found in ~80% of the cells randomly selected for visualization. In cells treated with mSIGK, I observed minor redistribution of Gβ1, where there was less fluorescent signal on the membranes, less localization to focal points, and slightly more diffuse cytoplasmic signal (Figures 3.39, 3.40). This pattern was consistently observed in more than 50% of the mSIGK treated cells; however, the extent of redistribution was never seen to be as great that of transducin in treated rod or cone photoreceptors. Unfortunately, I was unable to consistently detect quantifiable amounts of Gβ1 redistribution under these experimental conditions, perhaps owing to the low expression levels of endogenous G proteins in the COS and CHO cell lines. Further experiments using overexpressed tagged subunits may yield quantifiable results.
Figure 3.39 mSIGK induced relocalization of Gβ1 CHO-k1 cells. CHO-K1 cells grown on cover slides were treated with DMSO (cntrl), mSIGK, or L9A. Cells were fixed and permeabilized and stained with phalloidin (red panels, for filamentous actin) and specific antibodies against Gβ1 (green panels). mSIGK treatment causes the cytosolic signal of Gβ1 to increase, which correlates with peptide induced membrane detachment of Gβ1 in Figure 3.37. Images were taken using a laser scanning confocal microscope, LSM software. Red arrows point to punctate staining and yellow arrows point to diffuse staining.
Figure 3.40 mSIGK induced relocalization of Gβ1 COS-7 cells. COS-7 cells grown on cover slides were treated with DMSO (cntrl), mSIGK, or L9A. Cells were fixed and permeabilized and stained with phalloidin (red panels, for filamentous actin) and specific antibodies against Gβ1 (green panels). The result here is similar to that in CHO-K1 cells, and seemingly correlates with mSIGK induced ~25% membrane detachment of Gβ1. Red arrows point to punctate staining and yellow arrows point to diffuse staining.
**Subunit dissociation alters signal transduction in CHO-K1 cells**

It was previously shown that mSIGK could mobilize calcium signals in various cell lines, presumably by facilitating or attenuating Gβγ signaling (Goubaeva et al., 2003). I tested if the mSIGK induced subunit dissociation and redistribution of Gβ1 correlated with receptor independent Gq mediated calcium signals in CHO-K1 cells. CHO-K1 cells were incubated with the calcium binding dye, Fura-2AM, and then treated with DMSO, L9A, or mSIGK for 15 minutes. The cover slips were transferred to a perfusion chamber which is equipped for fluorescence microscopy. Cells treated with DMSO or L9A displayed similar Gq mediated calcium signals when perfused with ATP.

**Figure 3.41 Subunit dissociation induces calcium mobilization in CHO-K1 cells.** Fura-2AM loaded CHO-K1 cells were treated with DMSO (cntrl), L9A, or mSIGK for 15 minutes, and placed in a perfusion chamber for fluorescence microscopy. Plotted data indicating the change in cytosolic calcium concentration from DMSO (gray squares), L9A (blue triangles), and mSIGK (red circles) treated cells over time. The green bar represents time of perfusion with adenosine receptor agonist, ATP.
(agonist for endogenous adenosine receptors) consistent with previous observations (Figure 3.41) (Narayanan et al., 2007). However, cells treated with mSIGK showed a large increase in basal calcium release, with only a small response to agonist stimulation. This suggests that subunit dissociation and redistribution of Gαq and Gβγ can activate signaling cascades independent of receptor activation or guanine nucleotide exchange, thus disrupting the physiological homeostasis of the cell.

![Figure 3.42 Quantitation of mSIGK-induced Ca²⁺ mobilization in CHO-K1 cells. Data represented as a percent of receptor response. DMSO-cntrl was set as 100% response, and data of L9A and mSIGK treated cells are presented as % response of control. Operation of the flow rate and image capture was performed in our lab by Simone L. Sandiford.](image)

In the same study by Smrcka and colleagues (2003), it was shown that the mSIGK peptide could also stimulate the MAP kinase pathway independently of receptor activation in various cell lines. Increased levels of phospho-ERK were
found within minutes of peptide application, and they concluded that this was caused by the “liberation” of the Gβγ subunits from Gαq (Goubaeva et al., 2003). I reasoned that this described “liberation” of subunits was indeed subunit dissociation, and I tested if my observations of mSIGK-induced membrane detachment, subunit dissociation and redistribution of G proteins correlated with ERK activation in CHO-K1 cells. Consistent with the findings of Smrcka’s group, I found the peak phospho-ERK to be detected within 5 minutes of mSIGK application. Detection of phospho-ERK was diminished greatly after 15 minutes (Figure 3.43). Treatment of the cells with DMSO or the L9A control peptide did not stimulate ERK activation above basal levels.

Figure 3.43 mSIGK induces receptor independent ERK activation in CHO-K1 cells via subunit dissociation. CHO-K1 cells were treated with DMSO (cntrl), L9A, or mSIGK for the indicated times. Total ERK (bottom panel) and phosphor-ERK (top panel) was detected by Western blot. Peak ERK activation by mSIGK is observed at 5 minutes of incubation. Lysis and analysis of treated cells was performed in our lab in collaboration with Konstantin Levay.
In summary, I found that (1) most G protein subunits remain tightly sequestered to the cellular membranes, even when bound to GTP\(\gamma\)S. This correlates very strongly with my observations of cone transducin (figure 3.10) (Rosenzweig et al., 2007); (2) use of the synthetic peptide mSIGK and GTP\(\gamma\)S causes membrane detachment of many G proteins via subunit dissociation consistent with my results with transducin as well as other reports (Figures 3.35-3.38) (Goubaeva et al., 2003); (3) treatment of cells with mSIGK induces partial redistribution of endogenous G\(\beta1\) in COS-7 and CHO-K1 cells, although the effectiveness of microscopic detection was insufficient to conclusively measure the extent of redistribution; (4) mSIGK-induced subunit dissociation and redistribution of G protein subunits correlates with receptor-independent activation of signaling cascades such as Gq mediated calcium mobilization and G\(\beta\gamma\) mediated ERK activation. In other words, GDP-bound “free” G\(\alpha\) and “free” G\(\beta\gamma\) can have functional effects.

These results confirm the results of Alan Smrcka and colleges (Goubaeva et al., 2003; Bonacci et al., 2006), and they add to them by showing that subunit dissociation, membrane detachment, and subcellular redistribution of G proteins correlates with receptor independent activation of signaling cascades. Based on my observations with cone transducin and other G proteins’ distribution patterns in the activated state (GTP\(\gamma\)S-bound) I conclude that under normal physiological conditions, most G proteins do not dissociate upon GTP binding and remain tightly bound to the membranes in the trimeric form.
Chapter 4

Discussion

I. A model for light-induced transducin translocation

Rod and cone photoreceptors are highly polarized neurons consisting of the outer segment (OS) where phototransduction biochemistry occurs, the inner segment containing mitochondria and other organelles, the nuclear layer, and an axon ending with a glutamatergic synapse.

In darkness, rod transducin is localized exclusively to the OS. Upon illumination, activated transducin migrates from the rod OS to the inner compartments of the cell. Interestingly, cone transducin does not redistribute, even under intense illumination. My focus was to unravel the molecular mechanism of rod transducin translocation. I found that ATP is not required for transducin movement, and therefore molecular motor-driven transport does not play a major role in this process. Instead, according to my findings, transducin subunit dissociation is the only molecular event necessary and sufficient to drive transducin redistribution, regardless of illumination. I have also confirmed that protein-protein and protein-lipid interactions restrict transducin localization in different conditions of illumination. Finally, I used the insights gained from my studies of transducin to further examine the relationship of other G proteins' (such as Gq and Gi) subcellular localization and signal transduction in non-photoreceptor cells. The results of my studies provide experimental evidence
which supports the notion that most G proteins, with the exception of rod transducin, do not undergo subunit dissociation upon activation.

Transducin translocates to the inner compartments via subunit dissociation, membrane detachment, and diffusion

My dissertation research answered two fundamental questions about transducin localization: (1) why does the distribution of transducin in rods change in light versus darkness? and (2) why does the distribution of transducin in cones not depend on light? Three experimental approaches were a key to this study. First, to deplete photoreceptors of energy, I incubated retinas without glucose and with KCN. This treatment causes loss of essentially all ATP in the retina, and it blocks rhodopsin phosphorylation (Nair et al., 2005b), a biochemical reaction with a low $K_m$ (~2 μM) for ATP (Palczewski et al., 1988). Second, I permeabilized photoreceptors with $\alpha$-toxin. The $\alpha$-toxin-treated cells take up nucleotides (BODIPY-GTP$\gamma$S) and retain proteins (transducin and recoverin) (Figure 3.1). Third, to promote dissociation of transducin subunits in photoreceptors, I used a novel reagent, a small peptide that competes with $G\alpha$ for a binding site on $G\beta$ called mSIRK (Figures 3.12-3.17).

Classic in vitro studies of phototransduction suggest a simple explanation for the light-dependent localization of transducin in rods. Activation (binding of GTP to $G\alpha_t$) lowers the affinity of transducin $\alpha$ for $\beta\gamma$ (Fung et al., 1981), and it weakens the affinity of rod transducin for membranes (Kuhn, 1980). Recent evidence indicates transducin subunits also dissociate under physiological conditions (Sokolov et al., 2002). Although they did not provide experimental
evidence, Calvert et al. (2006) theorized a link between the dissociation state of transducin and its distribution. Immediately after activation, the subunits dissociate, leave OS membranes, and enter the cytoplasm. Cytosolic proteins equilibrate throughout all compartments of a photoreceptor within ~ 5 minutes (Peet et al., 2004; Nair et al., 2005b). Here I provide direct experimental evidence showing that if all of the transducin in a rod is activated, its subunits will dissociate and diffuse so that cytoplasmic concentrations of transducin in all compartments of the cell become equal. We called this model the “dissociate/disperse” hypothesis (Rosenzweig et al., 2007; Slepak and Hurley, 2008).

To test this hypothesis, I simplified the system by stabilizing either the active or inactive forms of transducin using specific guanine nucleotides introduced into photoreceptors permeabilized by α-toxin. I found that GTPγS stabilizes the activated state, and GDPβS stabilizes the inactive state. GTPγS causes transducin to disperse from the OS (Figures 3.1–3.3), and GDPβS keeps it sequestered in the OS (Figure 3.3 A). These findings provide simple and direct experimental support for the dissociate/disperse hypothesis. They also provided an experimental framework to investigate the reason for the different localization of activated transducin in rods and cones, as well as similar analyses of non-photoreceptor G proteins (Figures 3.37, 3.39. 3.40).
Subunit association, membrane binding, and localization

Transducin localization in photoreceptors correlates with its membrane affinity. In dark, it binds to the stacks of disc membranes which dominate the OS. Rod transducin readily detaches from membranes (Figures 3.10, 3.16) and diffuses throughout the cell. Cone transducin in wild type and Nrl⁻/⁻ cones remains bound to membranes (Figures 3.10, 3.16), and it stays mostly in the OS (Figures 3.5, 3.15), even when fully activated (Figures 3.5 D, 3.6). The different membrane affinities and distributions of transducins in rods versus cones could reflect differences inherent in either the type of G-protein or the type of cell in which it is expressed. To resolve this, I examined membrane binding and localization of cone Gαt expressed in rods in place of rod Gαt. I found that activated cone Gαt in rods has a weak affinity for membranes, and it disperses throughout the rod cell when activated (Figures 3.5 E, 3.10 B). Therefore, the ability to disperse transducin is a property of the cellular environment, not the type of Gαt. What could be the factors responsible for the strikingly different behavior of Gαt in rods versus in cones?

One reason for the rod versus cone difference may be related to lipid modification of Gαt. The affinity of a G-protein for membranes is influenced by N-acylation of its α subunit (Bigay et al., 1994; Wang et al., 1999; Chen and Manning., 2001). Rod transducin is modified by a mixture of fatty acyl residues, including C14:2, C14:1, and C12:0. I hypothesized that Gαt in cones is modified only with the more hydrophobic C14:0, but, when expressed in rods, it is acylated
with the less hydrophobic residues. Indeed, cone G\(\alpha t\) in Nrl\(^{-}\) cones is modified exclusively by C14:0 (Figure3.11). This appeared to be a reasonable explanation for the enhanced membrane affinity of activated cone G\(\alpha t\). However, two findings argue against that explanation.

Figure 4.1 Model of the mechanism of light-induced transducin dispersion. In the dark, the G\(\alpha\) subunit of rod transducin (blue) is associated with G\(\beta 1\gamma 1\) (light green and brown), and the heterotrimer is associated with the OS disc membranes. Similarly, cone G\(\alpha t\) (green) is associated with G\(\beta 3\gamma 8\), (red and violet) and bound to cone OS membranes. Upon stimulation by light, both cone and rod G\(\alpha\) bind GTP and undergo a conformational change to the active state (symbolized by a lighter color). For rod transducin this causes the subunits to dissociate, release from the membrane and disperse throughout the cytosol of the entire rod cell. Cone transducin G\(\alpha t\)-GTP does not physically dissociate from G\(\beta\gamma\), so the heterotrimer stays attached to the membrane sequestered in the OS.
First, C14:0 rod transducin also partitions to the cytosol and is only slightly enriched in membranes (Figure 3.11). Second, cone Gαt is still modified exclusively by C14:0 when expressed in rods (Figure 3.11). These findings show that the type of N-terminal acylation on Gαt is a characteristic of the type of transducin, and it does not determine either the affinity of Gαt for membranes or the localization of Gαt.

Another reason for the difference between rod and cone transducin may be due to the type of prenylation of Gγ subunits. Prenylation of Gγ influences the localization of rod Gβγ. Replacing the farnesyl (C15) modification normally present at the C terminus of the rod Gγ1 with a geranylgeranyl (C20) residue prevents light-induced dispersal of Gβ1γ1 from the OS (Kassai et al., 2005). However, the type of modification on Gγ1 does not affect the distribution of rod Gαt in rods (Kassai et al., 2005). It is, therefore, unlikely that differences in prenylation of rod Gγ1 and cone Gγ8 are solely responsible for the different distributions of cone Gαt when expressed in rods versus when it is expressed in cones.

Cone Gβγ, however, may be modified with geranylgeranyl which could be the reason for the observed OS membrane retention (Figures 3.13 – 3.16). It was shown earlier though that bovine cone Gγ is modified with a farnesyl residue (Chen et al, 2003). Surprisingly though, only a small fraction of membrane soluble cone Gβγ was analyzed (extracted by the Gβγ binding protein phosducin). Since I have shown that even under the most rigorous dissociating conditions,
more than 90% of cone Gβγ remains within the OS membranes (Figure 3.16), it remains unknown which lipid modifies the large membrane-bound fraction of cone Gβγ. It is quite possible that it is indeed modified by geranylgeranyl, and further investigation is required to resolve this issue. Regardless, prenylation of Gγ and myristoylation of Gα is essential for heterotrimer formation and membrane association (Wedegaertner et al., 1995; Casey, 1995). Based on my results, both rod and cone Gα seemingly have a low affinity to the rod Gβ1γ1 (Figures 3.4, 3.5).

I also found that the type of Gβγ complex may help explain the difference in behaviors of rod and cone transducin. The Gβγ complex in rods is assembled from Gβ1 and Gγ1. In cones, it is Gβ3 and Gγ8 (Fung et al., 1992; Ong et al., 1995). Figures 3.12 – 3.16 reveal a relationship between localization of activated Gα and its affinity for Gβγ where rod Gα has a lower affinity for the rod Gβγ (particularly, notice how when cone Gα is “forced” to redistribute or detach from membranes, cone Gβγ remains in the OS and in membrane fractions). When either rod or cone Gα is fully and stably activated by GTPγS, an equilibrium must be reached in which the cytosolic concentration of soluble Gα is equal in all compartments of the photoreceptor cell (Figures 3.1-3.3). This equilibrium is obvious in rods (Figure 3.14), but, in cones under these conditions, there is an excess of cone Gα in the outer segment (Figure 3.14). I hypothesized that this excess must represent a trimeric complex of GTPγS-activated cone Gα with Gβ3γ8 bound to membranes (Figure 3.13 B). In order to test my hypothesis, I
needed a novel approach to facilitate the subunit dissociation of cone transducin which could then address if Gα dissociation from Gβγ causes transducin redistribution.

Our lab developed the idea to use mSIRK, a peptide that promotes G-protein dissociation (Smrcka and Scott, 2002; Davis et al., 2005). If the excess cone Gαt in the OS is associated with Gβγ, mSIRK, a peptide that competes with Gα for binding to Gβγ, would disrupt the complex and Gαt would diffuse through the cell. If excess cone Gαt were in the OS for a different reason, it would not be affected by mSIRK. mSIRK does cause redistribution of excess cone Gαt from the OS (Figures 3.13 B, 3.14, 3.15). Therefore, the excess cone Gαt in the OS does represent the fraction of GTPγS-bound cone Gαt associated with Gβ3γ8 (Figures 3.13 A, 3.16). In rods, the equilibrium state is different: nearly all Gαt–GTPγS is dissociated from Gβ1γ1 so the subunits are dispersed throughout the rod. Consistent with this, mSIRK does not affect the distribution of activated rod Gαt (Figure 3.14).

**Transducin redistribution**

In darkness, the membrane-rich OS of rods and cones retain nearly all of the inactive heterotrimeric transducin. Light stimulates transducin to bind GTP and assume its active conformation. The affinity of activated rod Gαt for its partner, Gβ1γ1, is weak. Dissociated rod Gαt and Gβγ subunits have a low affinity for membranes so they disperse throughout the cytoplasm of all
compartments of the rod. Activated cone transducin essentially remains heterotrimeric. Through the use of mSIRK, it was revealed that cone transducin is retained in the OS because the heterotrimer has a high affinity for membranes and because Gβ3γ8 itself has a high membrane affinity (Figures 3.13 B, 3.14, 3.15).

In a photoreceptor operating in constant light under physiological conditions, activation of transducin is neither complete nor stable. Instead of equilibrium, a steady state is established throughout the photoreceptor. The subcellular distribution of rod G-protein in this steady state and the rate at which this state is reached depend on relative rates of activation, deactivation, and diffusion. Impairment of GTP hydrolysis by GTPγS binding, by RGS9 gene inactivation, or by the Q200L mutation in Gαt (Kerov et al., 2005b) favors the GTP-bound dissociated and dispersed state. Accordingly, GDPβS (Figure 3.3 A) or overexpression of RGS9 (Lobanova et al., 2007) shifts transducin toward the associated state, which concentrates in the OS. Transducin migration throughout the rod cell requires tens of minutes (Sokolov et al., 2002; Elias et al., 2004), which is much slower than unimpeded diffusion of a soluble protein in rods (Nair et al., 2005b) and cones (Rosenzweig et al., 2007 supplemental data). Transducin binding partners, such as phosducin (Sokolov et al., 2004), centrin (Wolfrum et al., 2002), Leu-Gly-Asn repeat (LGN) protein (Kerov et al., 2005a; Nair et al., 2005a), and cytoskeletal elements (Peterson et al., 2005; Reidel et al., 2008), may act as diffusion barriers that impede relocalization. This will be further discussed in detail in section II of this Discussion.
G-protein subunit dissociation

Whether or not G protein subunits dissociate is a fundamental question that has been discussed for many years (Lambert, 2008). Dissociation of rod transducin has served as a general model for G-protein activation (Gilman, 1987; Levitzki and Klein, 2002). I have shown that the propensity of activated cone transducin to dissociate is so low that a significant proportion of it remains heterotrimeric and bound to membranes even when it is fully activated in cone cells. From these results I infer that many G-proteins, like cone transducin, remain associated during activation in their cellular environment. Section III of the Discussion will deal primarily with the question: do G proteins physically dissociate upon activation?

II. Binding partners of transducin restrict its dissociation and localization

Recent years were 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, Ric-8, centrin and other proteins (Giessl et al., 2006; Blumer et al., 2007; Wilkie et al., 2005; McCudden et al., 2005). The functional significance of these novel interactions is under investigation in a number of laboratories. For novel binding partners of transducin, many of the interactions have been proposed to be involved in light-induced transducin redistribution between the photoreceptor
compartments. One of the initial goals of this study was to search for new binding partners that could potentially influence the subcellular redistribution of transducin within the rod. In the course of this study I unexpectedly discovered the direct protein-protein interaction between Gαt and retinal guanylate cyclase (retGC).

Identification of the Gαt:retGC complex

I first detected the rod Gαt:retGC interaction through co-immunoprecipitation of this complex from native retinal tissue, and the following evidence argues that this interaction is specific: (1) 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 Gαt-specific antigenic peptide; (2) the Gαt:retGC complex can be reciprocally immunoprecipitated with both anti-Gαt and anti-retGC antibodies; (3) co-immunoprecipitation was detected using different preparations: bovine and mouse rod OS, Nrl−/− cone OS, and COS-7 cells expressing recombinant retGC and Gαt; (4) the results of co-immunoprecipitation are supported by the GST fusion pull down using specific domains of retGC; (5) the efficacy of Gαt:retGC complex formation depended on the nucleotide-bound state of Gαt, with its GDP-bound form being the preferred conformation; (6) the efficiency of Gαt co-immunoprecipitation with retGC was better than that of its known binding partner, Gβ5L-RGS9 complex.
My data indicate that Gαt and retGC can interact with each other directly. Previous work by Yamazaki and colleagues implied that RGS9 can bind to retGC and regulate its activity (Seno et al., 1998; Yu et al., 2001; Bondarenko et al., 2002). I reasoned that the Gαt:retGC interaction could be mediated by RGS9/Gβ5L. Indeed, RGS9 and Gβ5L were present in the Gαt co-immunoprecipitation fractions along with retGC (Figures 3.19 A, 3.20). However, I did not detect Gβ5L in the immunoprecipitates with anti-retGC antibodies (Figure 3.19 C), and found no major difference in the efficiency of the co-immunoprecipitation of the Gαt:retGC complex obtained from wild type and RGS9 knockout mice (Figure 3.20). I conclude that RGS9 is not required for the binding of transducin to retGC, and it apparently co-precipitates with Gαt independently.

Another observation that supports direct Gαt:retGC interaction is that purified GST-fused individual retGC domains also bound purified transducin (Figure 3.24). At the same time, I found that if Gαt 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, Gαt and the full-length retGC must associate at the membrane 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 Gαt and full-length retGC form the complex, other proteins and membranes are no
longer required to maintain the interaction and such complex remains sufficiently detergent-resistant. This notion is supported by my observation that recombinant KHD domain cannot displace retGC from its pre-existing native complex with Gαt (Figure 3.25). In addition to these observations, I used quantitative western blot to determine that a majority (approximately 40-80%) of retGC is bound to transducin in rod photoreceptors. With such a large fraction of retGC interacting with transducin, it is reasonable to propose that it can influence each others' activity.

RetGC is a binding partner that restricts diffusion of transducin

What is the potential significance of the Gαt:retGC interaction? First, I tested if transducin subunits could modulate the catalytic activity of retGC (Figure 3.23). In collaboration with Igor Peshenko and Alexander Dizhoor at the Pennsylvania College of Optometry, we found that regardless of the Gαt activation status (GDP vs. GTPγS-bound) or the presence of retGC activating protein GCAP-1, transducin did not modulate retGC activity. These results are at variance with an earlier report on modulation of retGC activity by Gβγ complex of transducin (Wolbring et al., 1999). The reason for this discrepancy is not immediately apparent, but I have concluded the interaction with Gαt is unlikely a part of the cyclase regulation per se, and instead may rather contribute to a function different from phototransduction. Second, I therefore tested the potential
involvement of this interaction in the modulation of transducin redistribution between the OS and inner compartments.

Retinal rods contain two isoforms of retGC (retGC-1 and retGC-2), whereas cones express only retGC-1. The knockout of retGC-1 gene is known to result in mislocalization of transducin in cones (Yu et al., 2001). In a retGC-1/retGC-2 double knock out, rod Gα localization is "patchy", and transducin is poorly expressed or undergoes faster degradation (Baehr et al., 2007). 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 (Baehr et al., 2007). In my study, I investigated not only the dark or light endpoints, but also the kinetics of translocation. My study revealed a robust effect of retGC knockout on the rate of transducin migration out of or into the OS.

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 3.28, 3.29). Supplementation of permeabilized photoreceptor cells with cGMP or its slowly hydrolyzing analog had no effect on transducin movement back to the outer segment (Figures 3.32 - 3.34), 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
cyclosome activity. Additionally, I have proven that under these experimental conditions, the delivered nucleotides are not degraded, and therefore they remain present in the cells yet do not influence redistribution of transducin (Figures 3.32 – 3.34).

How does the direct interaction of retGC with transducin have such a remarkable effect on transducin redistribution? Our lab previously reported that transducin and RGS9 translocate to the detergent resistant membranes (DRM) or lipid rafts in a light dependent marker (Nair et al., 2002). It was also shown that transducin localization to lipid rafts correlated with localization of other lipid raft markers such as caveolin-1 and retGC. I tested the hypothesis that retGC within lipid rafts directs transducin localization to the lipid rafts, thus restricting transducin diffusion to the inner compartments. I used the available mouse model of retGC knockout and standardized the procedure of lipid raft isolation for mouse rod OS membranes (Figure 3.26). A comparison of transducin localization in lipid raft preparations from wild type and retGC−/− OS revealed that retGC is not required for transducin localization to the lipid rafts (Figure 3.27). Therefore transducin localizes to the DRM by some other mechanism. I have also concluded that lipid rafts do not play a role in the ability of retGC to restrict transducin diffusion to the inner compartments.
**Gαt:retGC complex and the diffusion model**

This section of my study shows (1) the effect of retGC-1 knockout on transducin localization, and (2) the direct Gαt:retGC interaction. However, I did not establish the causal relationship between the two phenomena during my dissertation research, and therefore it remains a hypothesis. At the same time, the idea that a binding partner localized in the OS should facilitate localization of a diffusing protein within the OS is consistent with the model of transducin and arrestin relocalization in rods (Slepak and Hurley, 2008) as well as models presented by others (Reidel et al., 2008; Karan et al., 2008). According to my model, diffusion of signal transduction proteins is restricted in cell compartments by their interacting partners (“sinks”) such as rhodopsin and tubulin for arrestin (Nair et al., 2005b) and disc membranes for transducin (Figure 4.1) (Slepak and Hurley, 2008; Rosenzweig et al., 2007).

However, it is important to note that in order for a molecule to be a “sink” for another, both molecules must be expressed in similar concentrations. OS membrane attachment of transducin, which requires lipidation of both Gαt and Gβγ subunits and is regulated by 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 (Figures 3.12-3.16) (Rosenzweig et al., 2007; Kassai et al., 2005; Kerov et al., 2007). The binding of Gαt to retGC identified in this study is certainly not as crucial as membrane association and therefore cannot be viewed as a “sink” for transducin. It should be viewed,
however, as one of the mechanisms that contribute to transducin translocation at the quantitative level, as do GTP hydrolysis (Figure 3.4) (Rosenzweig et al., 2007; Lobanova et al., 2007; Kerov et al., 2005b), interaction with phosducin (Sokolov et al., 2004) and other factors (Peterson et al., 2005; Chen et al., 2007).

Interestingly, among these mechanisms, the interaction with retGC appears to have the most profound effect on transducin migration (Figures 3.28, 3.29). At the moment I have not provided 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. It is more likely that retGC facilitates the attachment of transducin to the disc membranes. I speculate that the initial docking with retGC facilitates a conformational change of transducin that favors its anchoring into the membrane bilayer, possibly by exposing the N-myristoyl group of Gαt. 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. One can also speculate that a dynamic interaction between transducin and the cyclase might be related to formation of larger protein complexes involving retGC (Giessl et al., 2006; Seno et al., 1998; Hallet et al., 1996; Korschen et al., 1999; Fleischman et al., 1980). Regardless of the
underlying mechanism, the correlation between \( G_{\alpha t}:\text{retGC} \) interaction and localization of transducin is very intriguing and deserves further investigation.

Figure 4.2 Hypothesis for retGC-facilitated targeting of transducin to OS membranes. In light, up to 90% of transducin translocates to the inner compartments of rod photoreceptors. After onset of darkness, the equilibrium of activated \( G_{\alpha t} + G_{\beta\gamma} \) shifts towards the inactive \( G_{\alpha\beta\gamma} \) trimer as GTP hydrolyzes to GDP. The diffusing trimeric form of transducin which is now in the inner compartments has a high affinity for the disc membranes of the OS and begins to bind and accumulate there. In this model, retGC acts as a facilitator to transducin membrane binding, possibly by binding directly to the soluble trimeric transducin, and then by presenting it to the disc membranes. Perhaps upon the binding of retGC, transducin assumes a conformation more suitable for the fatty acids on it’s \( \alpha \) and \( \gamma \) subunits to reach the hydrophobic membranes. This model is a hypothesis that seeks to explain the currently available data, and it remains to be tested experimentally.
III. Subcellular localization of non-photoreceptor G proteins and regulation of signal transduction

As mentioned earlier, a strong line of evidence for subunit dissociation in native cells came from early studies of the Stryer lab on the rod photoreceptor G protein, transducin (Hurley and Stryer, 1982; Wensel and Stryer, 1986). Moreover, the work of the Gilman lab in the early 1980s showed that isolated Gα subunits could stimulate adenylate cyclase enzymes, while isolated Gβγ subunits could inhibit the stimulation by Gα (Northup et al., 1983). Along with that data, they observed changes in the resolving patterns of the G proteins in a gel matrix and changes in apparent molecular weight of the G proteins dependent on guanine nucleotides. This indicated the ability for the subunits to dissociate (Sternweis et al., 1981; Northup et al., 1983) and led to the current model of G protein subunit dissociation (Gilman, 1987).

The notion that G protein subunits undergo dissociation upon activation has been questioned in recent years (Levitzki and Klein, 2002; Lambert, 2008), as no direct evidence of G protein subunit dissociation at the plasma membranes in vivo has ever been shown. The use of fluorescently tagged G protein subunits has allowed for optical visualization of real-time subunit dissociation events via techniques such as FRET and BRET (Bunemann et al., 2003; Frank et al., 2005). These reports however, have suggested that G protein do not necessarily dissociate upon activation, and the question remains open if G proteins actually dissociate in cells.
In this dissertation, I investigated the mechanism of light-induced transducin redistribution. By comparing the behavior of rod and cone transducins, I found that the dissociation state of transducin governs its localization within the photoreceptor. I have obtained *in vitro* and *in situ* data demonstrating that activation causes rod transducin subunits to dissociate, detach from the membranes, and diffuse throughout the cell. Conversely, activated cone transducin remains in the trimeric state, bound to the membranes of the outer segments. In the last part of my dissertation work, I applied the same experimental approach as that of the study of transducin to study other G proteins. I investigated subunit dissociation of the non-photoreceptor G proteins Gi, Go, Gq, and Gβ1 using isolated membranes fractions from mouse brain and cultured CHO-K1 and COS-7 cells.

G proteins and their receptors have been studied extensively. However, much of that research has been conducted using *in vitro* and recombinant techniques and little is known about G protein function in their natural environment. Therefore, in my experiments I purposely avoided expressing G protein subunits ectopically and instead focused on the analysis of the behavior of endogenous G proteins. The crucial molecular tool used in my study was again the synthetic peptide, mSIGK, which is known to facilitate G protein dissociation by virtue of binding to Gβ subunits and competing with Gα (Goubaeva et al., 2003). This peptide (and its analog, mSIRK) was
discovered in a phage display screen by Alan Smrcka and colleagues (Scott et al., 2001).

**Localization of endogenously expressed Gi, Go, and Gq**

I assessed subcellular localization of G proteins under different activating conditions by two approaches; (1) cell fractionation into crude membrane and cytosolic fractions (Figures 3.35 – 3.38) and (2) by immunofluorescence confocal microscopy (Figures 3.39, 3.40). The immunofluorescence assays relied on the ability to distinguish differences in membrane and cytosolic signal detection, and therefore the use of neurons which contain relatively little cytosolic space, was not desirable. I took advantage of COS-7 and CHO-K1 cell lines, which have larger cytosolic space, express several endogenous G proteins, and are morphologically more suitable to examine G protein subunit redistribution using immunofluorescence microscopy.

I found that similar to transducin in cones, G proteins expressed in brain can detach from the membrane fractions only in the presence of mSIGK and GTPγS (Figure 3.35). Also consistent with my observations for cone transducin, binding of GTPγS alone had a very limited effect, and less than 10% of Gα or Gβ was detected to detach from brain membranes (Figure 3.36). The efficiency of GTPγS binding was assessed by size exclusion chromatography. The gel filtration assay showed that indeed all G proteins were fully bound to GTPγS and capable of subunit dissociation under the membrane-soluble conditions (Figure 3.38). In agreement with my observation of rod and cone transducin, subunit
dissociation of Gi, Go, and Gq was also observed upon treatment of mouse brain membrane lysates with mSIGK (Figure 3.38).

There could be several explanations to the observed behavior of the G proteins tested. One possible explanation is that Gα-GTPγS still binds to membranes while it is dissociated from Gβγ, either directly via lipid residues or by virtue of interaction with membrane anchor proteins. This would be consistent with the “collision coupling and lateral diffusion” model of heterotrimeric G protein activation, which suggests that active dissociated G protein subunits laterally diffuse along membranes via their lipid residue anchors until interacting with their target (reviewed in detail in Hein et al., 2005). However, the fact that mSIGK binding causes membrane detachment of both Gα and Gβγ and causes subunit dissociation in a detergent solution, argues that when the subunits dissociate they detach from the membranes and redistribute within the cell (Figures 3.12, 3.13, 3.15, 3.39, 3.40 and 4.1). Since mSIGK binds to Gβ (Davis et al., 2005), its effect on Gα can only be rationalized by a physical separation of Gα from Gβγ. It is unlikely that Gα-GTPγS binds to the membranes via a lipid modification or some unknown binding partner, and they remain bound to Gβγ. I therefore interpret these results as the lack of effective subunit dissociation of all of the G proteins tested.

It should be noted that only a fraction (about 20% - 25% estimated by density of western blot signals) of either Gα or Gβ1 could be eluted by mSIGK and GTPγS. The question remains, why do activated G proteins become “stuck”
in the membrane? One explanation is that there is in fact one fraction that is constantly membrane-bound and another fraction that dissociates, as another report has suggested (Digby et al., 2008). In the brain membranes the dissociable fraction is seemingly less than in COS-7 and CHO-K1 cell lines. Alternatively, the lack of membrane detachment can be explained perhaps by trapping of G protein subunits in membrane vesicles, incomplete membrane lysis, or other technical reasons.

*Subunit dissociation causes G protein redistribution in cell lines*

My results from mSIGK treatment of membrane fractionations were consistent with my observations of subcellular localization of the G protein subunits using immunofluorescence microscopy (Figures 3.37, 3.39, 3.40). Incubation of intact COS-7 and CHO-K1 cells with mSIGK (which is myristoylated, and can cross the plasma membrane) resulted in re-distribution of endogenous Gβ1 subunits from plasma membrane and focal points to the cytosol (Figures 3.39, 3.40). Importantly, mSIGK-induced Gβ1 redistribution occurred in the absence of receptor agonist or GTPγS binding – similar to my results with rod transducin (Figure 3.12). I attempted to visualize the localization of Gαq and Gαi (both endogenously expressed in COS and CHO cells) under these conditions, but was unable to consistently obtain conclusive data because of insufficient quality of the antibodies for immunohistochemistry. Therefore, only the results for endogenous Gβ1 localization are presented here.
My interpretation of the results are that a majority of mSIGK treated cells in a randomly selected field of view displayed increased Gβ1 signals within the cytoplasmic space of the cells, indicating redistribution. However, redistribution is difficult to quantify because the expression of Gβ1 is low, and detection of fluorescent signals by immuno-staining is close to the background fluorescence of the cells. These results though, correlate with my observation of peptide-induced membrane detachment of Gβ1 (Figure 3.37). Also, mSIGK treatment appeared to cause redistribution of Gβ1 to internal membrane or vesicular structures. This is consistent with other studies showing that activation of certain G proteins leads to relocalization of Gβγ to internal organelles (Slessareva et al., 2006; Akgoz et al., 2006; Saini et al., 2007). To discern if mSIGK causes Gβγ to relocalize to organelles such as the golgi complex, co-localization experiments with known markers of organelles must be performed. Moreover, the significance and function of the observed Gβγ relocalization remains to be addressed and deserves further attention.

How does G protein subunit dissociation affect signaling?

Previously, Alan Smrcka and colleges treated rat arterial smooth muscle cells with mSIRK and showed rapid activation of ERK, Jun N-terminal kinase, p38 MAP kinase, phospholipase C, and release of calcium from internal stores all in the absence of GPCR and G protein activation. Additionally, they showed that the peptide stimulated G protein subunit dissociation in absence of GTP exchange (Goubaeva et al., 2003). Here, I tested if mSIGK (functionally identical
to mSIRK) could produce these same results in COS-7 and CHO-K1 cell lines. I have conclusively confirmed those results of Goubaeva et al. (2003) and added the novel hypothesis that subunit dissociation and subcellular redistribution of Gα and Gβγ can influence signaling in a receptor independent manner.

Correct localization of G proteins is crucial to proper signal transduction in cells. For example, when G protein subunits are mutated so that they no longer are post-translationally modified by lipids Gα loses affinity of Gβγ (Linder et al., 1991), and they do not localize to the plasma membrane readily (Evanko et al., 2000). Also, if individual tagged Gα or Gβγ subunits are overexpressed in transfected cells or if mutant G protein subunits that cannot associate are co-expressed, they do not localize properly to the plasma membrane. The G proteins only display correct localization and proper cellular signaling when the wild type subunits are co-transfected together (Takida and Wedegaertner, 2003; Evanko et al., 2005).

If G proteins do not undergo subunit dissociation under physiological conditions, then how does “forced” subunit dissociation affect signal transduction at the physiological level? In their study, Smrcka and colleagues (Goubaeva et al., 2003) “liberated” Gβγ from Gα using the synthetic Gβ-binding peptide mSIRK which activated MAP kinases as well as calcium signals independent of GPCRs. This suggested that dissociation of the Gq heterotrimer by mSIRK induced Gα and Gβγ mediated signals independent of the receptor.
I was also able to generate both calcium mobilization and MAP kinase activation when I treated CHO-K1 cells with mSIGK (Figures 3.41 - 3.43). While my results mainly confirmed those of the Smrcka lab, I was able to correlate the subunit dissociation, membrane detachment, and subcellular redistribution of \( G_\alpha q \) and \( G_\beta 1 \) with the physiological effects of mSIGK (Figures 3.37 – 3.40). It is becoming apparent that redistribution of G protein subunits may have a role in signal transduction. A recent study published in *Cell* show a novel signaling role of G protein subunits at the yeast endosome (Slessareva et al., 2006). Also, G protein subunits have been shown to translocate to the Golgi where they may have some signaling function (Saini et al., 2007). Taken together, my results further strengthen this novel paradigm shift in G protein signal transduction.

**Pharmacological implications**

What is the significance of my findings on G protein localization, subunit dissociation and the relationship with signal transduction? I found that (1) most G proteins remain tightly associated and bound the membrane fractions, even in the presence of GTP\(_\gamma\)S; (2) mSIGK delivered with GTP\(_\gamma\)S causes partial membrane detachment of endogenous \( G_\alpha i \), \( G_\alpha o \), \( G_\alpha q \), and \( G_\beta 1 \); (3) G proteins can undergo subunit dissociation in detergent solution in presence of GTP\(_\gamma\)S or the peptide mSIGK; (4) treatment of cells with mSIGK causes subcellular redistribution of \( G_\beta 1 \), which correlates with membrane detachment; (5) treatment of CHO-K1 cells with mSIGK induced calcium mobilization and ERK activation in absence of receptor activation. The signal activation correlated with my results...
of mSIGK induced Gαq and Gβ1 membrane detachment as well as Gβ1 redistribution in CHO-K1 cells. These data suggest that under normal physiological activating conditions, most G proteins remain as a trimer, bound tightly to the membrane where they function normally. While my data goes against the current paradigm of G protein subunit dissociation, it is however in agreement with several studies which report that G protein subunits do not dissociate (Frank et al., 2005; Bunemann et al., 2003; Klein et al., 2000; Yuan et al., 2007; Adjobo-Hermans et al., 2006).

My dissertation focused on the general concept of subcellular localization of signaling proteins. Taken together, the significance of my findings can be described as follows: 1) light-dependent relocalization of rod transducin is an energy-independent (no ATP) process controlled by the oligomeric (Gαβγ) status of transducin 2) diffusion of transducin is fast (based on Nair et al., 2005b) and random; therefore it is restricted and guided by the abundant disc membranes and binding partners such as retGC 3) it is a simple mechanism, and the only amount of protein needed to move is what actually moves 4) binding of GTPγS to cone Gαt is not sufficient to induce (effective) subunit dissociation in situ 5) such a mechanism may be applicable to other signaling proteins which redistribute in a signal-dependent manner. Unfortunately, I was unable to experimentally address the physiological consequences of transducin redistribution. I can however, offer two speculations: One speculation is that light-induced transducin translocation directly plays a role in adaptation of retinal rods (Sokolov et al., 2002). While
movement of transducin to the inner compartments seemingly correlates with adaptation, it is important to note that under those illuminating conditions rod cells are saturated and no longer function effectively. Another speculation is that movement of rod transducin to the inner compartments serves a cytoprotective role. Since rods are extremely sensitive, under bright illumination transducin might migrate away from the biochemical machinery to avoid cellular stress due to “over-signaling” which may lead to cellular degeneration. To address this hypothesis, a mutant form of transducin that remains in the outer segments must be expressed in rods. Only then can we address both the adaptation and cellular viability of the rods under intense illumination over the lifetime of the animal in which it is expressed. For example, Fukada and co-workers have already described that when rod Gβγ is mutated to remain “stuck” in the OS, rod Gαt can still redistribute, yet the physiological responses to flashes of light are similar to wild type mice (Kassai et al., 2005). This suggests that movement of transducin may not play a role in adaptation.

The results of my dissertation work also support the notion that G protein subunits must associate in order to localize to the plasma membrane. Additionally, my data suggest G proteins remain associated during physiological activation conditions, and hence, subunit dissociation is not required for signal transduction. These findings also indicate that subunit dissociation of G proteins leads to the deregulation of GPCR signaling. The notion that G proteins do not necessarily dissociate upon activation is of great relevance to researchers developing small molecules and drugs that target the G protein system.
Generating novel drugs to either induce or inhibit G protein signaling without interfering with receptor function would be highly useful in advancing the selectivity of modulating downstream G protein targets while avoiding the issue of single GPCRs affecting multiple trimeric G proteins.
REFERENCES


Fleischman D, Denisevich M, Raveed D, and Pannbacker RG (1980) Association of guanylate cyclase with the axoneme of retinal rods. Biochim Biophys Acta 630, 176-186


Peshenko IV, Moiseyev GP, Olshevskaya EV, and Dizhoor AM (2004) Factors that determine Ca2+ sensitivity of photoreceptor guanylyl cyclase. Kinetic analysis of the interaction between the Ca2+-bound and the Ca2+-free guanylyl cyclase activating proteins (GCAPs) and recombinant photoreceptor guanylyl cyclase 1 (RetGC-1). Biochemistry 43, 13796-13804


