Regulation of the catalytic and allosteric properties of photoreceptor phosphodiesterase (PDE6) by the glutamic acid-rich protein-2 (GARP2)

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REGULATION OF THE CATALYTIC AND ALLOSTERIC PROPERTIES OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BY THE GLUTAMIC ACID-RICH PROTEIN-2 (GARP2)

BY

WEI YAO

B.S., Jinan University, 2007

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Biochemistry

September, 2013
This dissertation has been examined and approved.

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DEDICATION

This dissertation is dedicated to my wonderful family, especially my loving parents, Yukuan Yao and Guirong Song, as well as my sister, Jing Yao. I will always appreciate their constant love, encouragement and support through every step of my life. I also dedicate this dissertation to all my friends for their support and help.
ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Dr. Rick Cote, who has not only taught me to strive for excellence as a researcher but who has also inspired me to be a decent person. It is my pleasure and good fortune to have such a wonderful advisor, whose generous support, great patience and sapient guidance led me to achieve the goal of publishing my dissertation.

I would like to thank my committee members, Dr. Thomas Laue, Dr. Kelley Thomas, Dr. Vaughn Cooper and Dr. Roy Planalp, for their time, encouragement, and expertise throughout my doctoral research.

I would like to acknowledge everyone in Cote lab for their help, support and advice to me whenever I encountered difficulties in my research and in my life: Xiujun Zhang, Sue Matte, Karyn Cahill, Xiongzhuo Gao, Hannah Gitschier, Kevin Schuster, Katie Kozacka and Mary Awad. I would also like to thank Kyle Burton and Rose Hadley from Dr. Planalp’s lab for their cooperation, as well as Melanie Shields for her proofreading of my dissertation.

This research was funded by a grant from the National Institutes of Health (EY05798), and University of New Hampshire Dissertation Year Fellowship (2012-2013).
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ABSTRACT

REGULATION OF THE CATALYTIC AND ALLOSTERIC PROPERTIES OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BY THE GLUTAMIC ACID-RICH PROTEIN-2 (GARP2)

by

Wei Yao

University of New Hampshire, September, 2013

The photoreceptor phosphodiesterase (PDE6) must be precisely regulated to control the sensitivity, amplitude, and kinetics of the photoresponse during excitation, termination and adaptation to light stimulation in rod and cone photoreceptors. The central hypothesis of this thesis is that one PDE6 binding partner, the glutamic acid-rich protein (GARP2), may regulate PDE6 to reduce its “dark noise”, enhance its sensitivity and conserve metabolic energy during rod photoresponse saturation.

The first aim of this research is to better understand the unique biochemical and biophysical characteristics of GARP2 in order to reveal its functional attributes for regulating PDE6 during phototransduction in rod photoreceptors. We first improved immunological methods to better characterize GARP2. We then developed approaches to purify PDE6 free of GARP2; we also generated recombinant GARP2 to permit study of
its biochemical and biophysical properties, and discovered that recombinant GARP2 suppresses the basal activity of PDE6, and behaves as a natively unfolded protein in solution.

The second aim is to identify the interacting sites between GARP2 and PDE6 and determine the regulatory mechanism of GARP2 on PDE6 in visual phototransduction. We demonstrated that the N-terminal half of the inhibitory γ subunit of PDE6 (Py), interacts with high affinity for GARP2. The C-terminal portion of GARP2 is most effective in suppressing the basal activity of PDE6, whereas the central region of GARP2 reduces cGMP binding to PDE6 GAF domains. This suggests that GARP2 alters both the allosteric and catalytic properties of PDE6 to regulate PDE6 activity and lifetime through multiple interacting sites with the PDE6 holoenzyme. GARP2 may play an important role in lowering the level of dark noise, and reducing cGMP metabolic flux during rod photoresponse saturation under bright light.

The final aim of this research is to investigate the effects of zinc on the catalytic mechanism and structural stability of PDE6. Several different zinc-chelator systems were used to make solutions with defined concentrations of free zinc. We demonstrate that low concentrations of zinc are able to activate PDE6 catalytic activity, while high concentrations cause the loss of PDE6 activity.
1. **Physiology of Rod and Cone Photoreceptor Cells**

The vision of vertebrates is initiated in the highly differentiated photoreceptor cells in the retina. Typically, there are two classes of photoreceptor cells in vertebrates, rods and cones, which exhibit different functional features of the light response and these functions are well suited to the ecological needs of vertebrates [Fig. 1-1. (Rodieck, 1998)].

1.1 Dark noise and single-photon detection in rod photoreceptors

In darkness, rods are depolarized by the influx of Na\(^+\) and Ca\(^{2+}\) through the cGMP-gated channels in the plasma membrane. Dark noise is the spontaneous
fluctuations in the electrical signal of the retina's photoreceptors in the dark that is generated in the outer segment (Baylor et al., 1980), a specialized organelle dedicated to phototransduction. Noise in the dark circulating current consists of three components: discrete photon-like events arising from thermal activation of the photopigment, continuous noise arising within the phototransduction enzymatic cascade, and fluctuations arising from gating and/or blocking transitions in the cGMP-activated channel. Of these, the first two components are considered the major sources of noise in the rod photoreceptor (Baylor et al., 1980; Rieke et al., 1996).

In rods, the amount of dark noise limits the behavioral sensitivity of rod vision (Baylor et al., 1984; Rieke et al., 1998): This is because large noise fluctuations may be mistaken for single photon responses and the noise may cause some single photon responses to be undetected. Rieke and Baylor (1996) showed that the smaller, continuous dark noise is generated by cGMP concentration fluctuations that result from spontaneous activation of the enzyme responsible for hydrolysis of cGMP, phosphodiesterase (PDE6). The spontaneous activation of PDE6 that results in this dark noise appears to be an intrinsic property of PDE6, and not due to activation by the phototransduction cascade (Rieke et al., 1996). Since the continuous component of dark noise is low in rods, a single photon photoresponse is large enough in amplitude to exceed the threshold for detection.

Cone photoreceptors have more dark noise than rods (Schneeweis et al., 1999) and the behavioral thresholds of cone vision are higher. Previous studies found that bleaching the photopigment reduced the dark noise in primate cones, suggesting that some noise originated from spontaneous pigment activation (Schnapf et al., 1990). In addition, the frequency composition of the dark noise was different from that of the dim
flash response (Schnapf et al., 1990; Schneeweis et al., 1999), suggesting an additional contribution to the noise downstream besides spontaneous photopigment activation. In salamander cones, the dominant noise in long wavelength-sensitive cones originated from the spontaneous activation of the photopigment, whereas the major noise of short wavelength-sensitive cones (in which the photopigment was relatively stable), arose in the transduction cascade (Rieke et al., 2000).

The difference in the level and the dominant sources of the dark noise in rod and cone photoreceptors suggests that different regulatory mechanisms must exist to regulate the absolute sensitivity in these two different classes of photoreceptor cells in the retina.

1.2 Light response in photoreceptor cells

Rod photoreceptors operate in dim illumination conditions by being able to reliably detect and absorb a single photon of light to generate an electrical response under fully dark-adapted conditions (Baylor et al., 1979). However, they undergo photoresponse saturation in moderately bright light conditions. Rods exhibit relatively slow photoresponses and their spatial distribution throughout the retina limits the acuity of vision. In contrast, cone photoreceptors are less sensitive to light than rods and operate under bright light conditions. A remarkable property of cones is that they can remain photosensitive without being saturated even under the condition of extremely bright light (Boynton et al., 1970). Cones are less responsive than rods to photons but their response is much faster. In addition, there are multiple classes of cone cells in the retina, each expressing a distinctive photopigment, which allows many animals to be able to discriminate colors.

1.3 Energy metabolism in rods and cones
As the most energetically demanding system in the brain, the retina uses ATP as energy to support multiple neuronal functions. In photoreceptor cells, the generation of energy relies more on oxidative than glycolytic metabolism and a greater consumption of energy is required in the dark than in the light (Ames, III et al., 1992).

In darkness, the ATP expenditure of a mouse rod is estimated to be about $9 \times 10^7$ ATP s$^{-1}$ which is used primarily by the Na$^+/K^+$ ATPase in the inner segment to pump out excess Na$^+$ entering via the cGMP-gated channels in the outer segments. This ongoing entry of cations in the outer segment and their efflux out of the inner segment constitutes the circulating dark current (Baylor, 1996). Because mammalian rods and cones exhibit similar dark currents (Nikonov et al., 2006), the ATP expenditure of a cone in the dark is assumed to be similar to that of a rod. In bright light, the saturation of the light response in rods causes the cGMP channels to close and decreases the total energy consumption to $2 \times 10^7$ ATP s$^{-1}$ (Okawa et al., 2008). In cones, the light response never allows the influx of Na$^+$ through the cGMP-gated channels to fall further than about half that in the dark. As a result, the consumption of ATP of a cone in light is much greater than that in a rod. The 75% light-induced reduction in energy consumption in rods during photoreceptor response saturation provides an energy-efficient mechanism for high-sensitivity vision in dim light and reduced energy consumption in bright light.

The subcellular components in rods and cones also provide some evidence for their different energy consumptions. As an important enzyme for energy generation in mitochondria, the cytochrome c oxidase expression levels reflect the oxidative capacity of cells in order to match local energy demand (Wong-Riley, 2010). In the ellipsoid portions of photoreceptor inner segments, the mitochondria with highly active
cytochrome c oxidase are much larger and more abundant in cones than those in rods, resulting in a three-fold greater total surface area of inner mitochondrial membrane in cones than that in rods (Wong-Riley, 2010). This observation also indicates that cone photoreceptors utilize more electron transport chain enzymes to generate more ATP (Perkins et al., 2003; Okawa et al., 2008) to meet its higher level of energy metabolism. The efficient manner of energy consumption in rods may help us understand the evolutionary meaning of the duplex retina in vertebrates and also explain why there are relatively few cones in some diurnal animals (Okawa et al., 2008).

2. **Cellular Biology of Rod and Cone Photoreceptor Cells**

2.1 Morphology of rods and cones (Fig. 1-2.)

The cellular structure of rods and cones is highly specialized for light capture and signal transmission (Rodieck, 1998). In structure, rods and cones are quite similar and both rods and cones consist of three major compartments: the outer segment, the inner segment and the synaptic terminal. The outer segment of photoreceptor cells consists of
membranous disks which are developed from the highly expanded and convoluted plasma membrane. The visual pigments which absorb photons and trigger the visual signaling pathway, as well as some other signaling components needed to generate the electrical response, are embedded on the tightly stacked disks. Rods and cones display different structures in their outer segments. In rods, the outer segment is a stack of thousands of completely internalized, physically distinct disk membranes, whereas the outer segment in cones consists of continuous infoldings of the plasma membrane (Collin et al., 2004). The inner segments of both rods and cones are similar and they contain nuclei and other organelles required for normal cellular metabolism. In the visual signaling pathway, the electrical signal generated in the outer segment is transferred to the synaptic terminal and results in neurotransmitter release. The specialized structure of photoreceptor cells allows the transmission of light energy into a biochemical response and the subsequent of the electrical communication between photoreceptor cells and neighboring retinal neurons.

3. **Visual Phototransduction Pathway in Photoreceptor Cells**

The absorption of a photon by a visual pigment molecule (opsin) initializes visual transduction in photoreceptor cells. Visual transduction occurs in the outer segments of rods and cones and efficiently converts the light signal to an electric response, transferring it to other neurons. The phototransduction cascade consists of several proteins residing in the outer segment, including opsin, transducin, photoreceptor
phosphodiesterase (PDE6), regulator of G-protein signaling 9 (RGS9-1), type 5 G-protein β-subunit (Gβ5-L), guanylate cyclase, opsin kinase and arrestin.

3.1 Visual excitation (Fig. 1-3.)

The excitation of visual transduction begins when a photon causes cis-trans-isomerization of the chromophore 11-cis-retinal on rhodopsin (opsin in cones), which induces a conformational transition into the active state of rhodopsin, R*. The active R* is able to bind to and activate a photoreceptor-specific, heterotrimeric G protein, transducin (Burns et al., 2001). The α subunit of transducin (Tα) binds guanine

Fig. 1-3. Visual excitation in photoreceptor cells. The components of phototransduction cascade are localized on the disk membranes. Visual excitation is initiated with the absorption of photons by rhodopsin (R). The photoactivated rhodopsin (R*) binds to and activates transducin (Tαβγ) by exchanging GDP for GTP. The activated α-subunit of transducin (Tα*) dissociates from the β- and γ-subunits and binds to the photoreceptor phosphodiesterase (PDE6). The inhibitory γ-subunit of PDE6 is displaced by Tα*, and PDE6 is activated to hydrolyze cGMP. The drop of the cGMP level causes the closure of cGMP-gated ion channels and the hyperpolarization of the plasma membrane.
nucleotides and attaches to the outer segment membrane by its acylated N-terminus; the isoprenyl moiety of the γ-subunit of transducin anchors both γ- and β- subunits to the membranes. The binding of R* to transducin allows bound GDP to exchange for GTP. Upon the binding of GTP, Ta becomes activated (Ta*), and then dissociates from the Tβγ dimer (Arshavsky et al., 2002). Active Ta* is able to bind to its effector, photoreceptor phosphodiesterase (PDE6) which causes displacement of the inhibitory γ-subunit of PDE6. The displacement of this inhibitory subunit (Py) from the catalytic dimer (Paβ) allows the exposure of the catalytic pocket on PDE6 and triggers the rapid hydrolysis of cGMP. The rapid drop in cGMP concentration in the cytoplasm during light activation results in the dissociation of cGMP from the CNG ion channel in the plasma membrane, followed by closure of the channel. After the CNG ion channel closes, the inward cation current is eliminated; this results in the hyperpolarization of the membrane and generates a transient photoresponse. The photoresponse persists until the phototransduction proteins become deactivated.

3.2 Inactivation of visual excitation (Fig. 1.4)

The recovery to the dark-adapted state in photoreceptor cells following visual excitation is a precisely regulated procedure in which all the activated phototransduction proteins in the excitation pathway must be deactivated in order to restore the dark-adapted cGMP concentration and to reopen the CNG ion channels. The deactivation of each component in the excitation pathway in phototransduction is controlled by an individual mechanism.

Rhodopsin is deactivated through phosphorylation at several C-terminal sites by a specific G protein-coupled receptor kinase (Maeda et al., 2003). Following rhodopsin
phosphorylation, arrestin binds and deactivates rhodopsin (Fig. 1-4A). The high-affinity binding of arrestin to rhodopsin also blocks the interacting region between rhodopsin and transducin. The phosphorylation of rhodopsin is regulated by recoverin, a calcium-binding protein whose activity is also well controlled through the level of calcium in photoreceptor cells (Hurley, 1994).

The inactivation of Ta* requires hydrolysis of its bound GTP by accelerating its intrinsic GTPase activity and it is defined as the rate-limiting step in the recovery state of rod phototransduction. The GTPase rate of transducin is determined by a complex of proteins including Ta*-GTP, RGS9-1, Gβ5-L and the RGS9-1 anchor protein (R9AP) (Anderson et al., 2009). The inhibitory γ-subunit of PDE6 is also found to play an

![Image of Fig. 1-4. Inactivation of visual excitation.](image)

**Fig. 1-4. Inactivation of visual excitation.**

**A.** Activated rhodopsin (R*) is phosphorylated at several C-terminal sites by rhodopsin kinase (RK) and the phosphorylation allows the binding of arrestin (Arr), which deactivates rhodopsin and blocks the interacting sites between rhodopsin and transducin. **B.** Activated transducin (Ta*) is deactivated by the hydrolysis of bound GTP through its intrinsic GTPase activity. The RGS9-1 complex (including RGS9-1, Gβ5L, and R9AP) accelerates the GTPase rate of Ta*. Deactivated Ta dissociates from PDE6 and rebinds to Tβγ.
important role in this deactivation process by binding itself to RGS9-1 and enhancing the affinity of the RGS9-1 protein complex (Guo et al., 2011). After Ta hydrolyzes the bound GTP, it reforms the heterotrimer with Tβγ and returns to its inactive state. The inhibitory γ-subunit then binds back to the catalytic dimer of PDE6 and deactivates it.

As the central effector enzyme in phototransduction, PDE6 has to be precisely deactivated after light illumination to tightly control the excitation and recovery states in photoreceptors. The deactivation of PDE6 is processed through the releasing of the inhibitory γ-subunit (Py) from the inactive α-subunit of transducin. Py rebinds to the catalytic dimer of PDE6 and re-inhibits the enzyme activity of PDE6. There exists a feedback regulation mechanism in which the strength of the interaction of Py for transducin versus the catalytic dimer of PDE6 is modulated by the binding state of cGMP to the non-catalytic binding sites on the PDE6 GAF domain (Norton et al., 2000). Another deactivation mechanism is found in transgenic mice overexpressing Py, which suggests that free Py is able to bind and inhibit Ta* activated PDE6 (Tsang et al., 2006). Other regulatory mechanisms are required to completely explain the deactivation mechanism of PDE6 and studies on PDE6 binding partners may provide insight into the mechanism of PDE6 inactivation.

3.3 Light adaptation in photoreceptor cells

In addition to excitation and inactivation in phototransduction, photoreceptor cells employ another process termed light adaptation to avoid photoresponse saturation under bright light and to control the amplitude and kinetics of the photoresponse in presence of constant illumination. In light adaptation, the reactions in excitation and inactivation are desensitized so that the photoreceptor cells can detect and respond to the light intensity
over a wider range of illumination. Previous studies have found that several desensitization reactions are mediated by calcium in light adaptation. Guanylate cyclase (GC) can be regulated by calcium level through Ca\(^{2+}\) binding proteins, GC-activating proteins (GCAPs). When the intracellular Ca\(^{2+}\) level is reduced upon light activation, GCAPs stimulate GC to synthesize cGMP. This Ca\(^{2+}\)/GCAP-dependent regulation of GC activity is a powerful feedback mechanism in which the rate of cGMP synthesis increases as Ca\(^{2+}\) levels fall during the response to light, helping to restore cGMP levels and allowing the cGMP channels to reopen (Stephen et al., 2008). The drop of Ca\(^{2+}\) concentration also affects the function of another Ca\(^{2+}\) binding protein, recoverin. The Ca\(^{2+}\) bound recoverin inhibits the phosphorylation of rhodopsin by GRK1 (Klenchin et al., 1995). Interestingly, this inhibition by recoverin of rhodopsin phosphorylation requires greater and lengthier changes in intracellular Ca\(^{2+}\) concentration, which suggests that the regulation of calcium by recoverin only occurs in light adaptation but not in excitation of visual transduction. Another calcium-dependent mechanism in light adaptation involves calmodulin or calmodulin-like proteins to regulate the sensitivity of CNG channels. In the light, the fall of Ca\(^{2+}\) causes the dissociation of calmodulin from the channel and increases the sensitivity of the channel to cGMP, so the channel is able to operate at a lower cGMP level during light adaptation (Hsu et al., 1993).

However, a study on the current of light-adapted transgenic mouse rods in which GCAPs have been ablated suggests that a novel Ca\(^{2+}\)-dependent modulation of PDE6 may be occurring (Chen et al., 2010). Furthermore, the observation that acceleration of the falling phase of the light response in Py-overexpressed transgenic rods indicates that the decline of light-activated PDE6 activity is responsible for the decline of the light
response (Tsang et al., 2006). Both studies suggest that currently accepted Ca$^{2+}$-dependent mechanisms for light adaptation cannot explain the physiological data and that and the modulation of PDE6 may also play a critical role in light adaptation. However, the mechanism of PDE6 regulation during light adaptation is unknown and further work is needed to provide biochemical evidence for Ca$^{2+}$-dependent or Ca$^{2+}$-independent regulatory mechanism (Fain, 2011).

### 3.4 Dark-adaptation

Dark adaptation is defined as the slow recovery of visual sensitivity after exposure to a strong light. Although the detailed cellular and molecular mechanism of dark adaptation is unclear, some predictions based on physiological and biochemical experiments have been made to partially explain it. Lamb and Pugh reported that the dark recovery time in photoreceptors is dependent on the level of the bleaching of visual pigments (dependent on the light intensity and exposure time) and the rate of visual pigment regeneration (Lamb et al., 2004).

Rhodopsin is a member of the superfamily of seven-helix, G-protein-coupled receptor proteins (GPCRs) and in its inactive state, there is a light-absorbing chromophore (11-cis retinal form) bound to it, while the absorption of a photon can isomerize the chromophore to the all-trans configuration and activates rhodopsin to interact with transducin, a G protein (Palczewski, 2012). After the photoresponse, the all-trans retinal dissociates from the opsin to regenerate rhodopsin. The regeneration of rhodopsin consists of the synthesis of 11-cis retinoid in the retinal pigment epithelium, the delivery of it to the outer segment in photoreceptor cells and the removal and storage...
of all-trans retinal. It is believed that the regeneration of visual pigments is the rate-limiting steps in dark adaptation (Lamb et al., 2004).

In the dark-adapted state, the circulating "dark current" in photoreceptor cells is maintained by the influx of Na\(^+\) and Ca\(^{2+}\) through the partially opened CNG ion channels in the outer segment plasma membrane. The extrusion of Na\(^+\) is accomplished by a Na\(^+\)/K\(^+\)-ATPase on the inner segment membrane and the efflux of K\(^+\) by the K\(^+\) channels on the inner segment membrane. The Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchanger localized on the outer segment membrane and some other ion channels in the inner segment are also involved in regulating ion conduction and transport in photoreceptors (Cote, 2008; Matulef et al., 2003; Molday et al., 2000).

In summary, the biochemical and physiological features in excitation, recovery and adaptation in phototransduction have been studied for decades, and multiple collaborative or independent regulatory mechanisms involved in this sophisticated signaling pathway have been discovered. However, we still lack sufficient knowledge to completely explain some physiological phenomena. How is PDE6 modulated during light adaptation? What regulatory mechanisms reduce energy consumption in rod photoreceptors exposed to bright light and how do cones differ from rods? How is the spontaneous activation of PDE6 in rods suppressed to reduce dark noise and enhance the sensitivity of rods to single photons? As the central effector of visual transduction, PDE6 plays a critical role. The central hypothesis of this thesis is that novel PDE6 interacting proteins may contribute to regulation of the enzymatic activity of the rod enzyme in its dark-adapted and light-activated states.
4. Structure and Functions of PDE6

4.1 The PDE superfamily

In mammals, the cyclic nucleotide phosphodiesterase (PDE) superfamily consists of 11 family members derived from more than 20 genes, which are distributed in various types of tissues. Functionally, PDEs catalyze the hydrolysis of cAMP and cGMP to produce 5'-AMP and 5'-GMP. In coordination with adenylate cyclase (AC) and guanylate cyclase (GC), PDEs regulate the cellular levels of these second messengers in multiple signaling pathways. In the PDE superfamily, some members are highly specific for hydrolysis of cAMP (PDE4, 7 and 8) or cGMP (PDE5, 6 and 9), while the others hydrolyze both cAMP and cGMP (PDE1, 2, 3, 10 and 11) with certain preferences (Bender et al., 2006). The activity of PDEs is precisely modulated in cells to be responsible for various stimulations such as cytokines, oxidative influences, and light. Dysfunctions of PDEs have been reported to be closely related with a large number of diseases such as erectile dysfunction (ED; PDE5) and retinitis pigmentosa (RP) (PDE6). Several PDE inhibitors have been used clinically, for example, the inhibitors of PDE5 are used to for the treatment of ED (Barnett et al., 2006) and more PDE inhibitors are under development to provide treatments for many diseases.

Among the PDE superfamily members, PDE6 and PDE5 are most closely related. PDE5 is abundant in vascular and airway smooth muscle, platelets, cerebellar Purkinje cells, gastrointestinal epithelial cells and endothelial cells (Francis et al., 2006; Francis et al., 2011). PDE6 is expressed primarily in retinal photoreceptors in great abundance and is also found in the pineal gland and certain melanoma cells (Cote, 2006). Both PDE5 and PDE6 contain GAF domains (named for their occurrence in cGMP binding PDEs,
certain adenylate cyclases and the *Escherichia coli* FhlA protein) in their regulatory regions, share similar amino acid sequence, strongly prefer cGMP over cAMP as a substrate and present overlapping pharmacological profiles for inhibitors (Zhang et al., 2005b). However, PDE6 is unique in its inhibitory subunit, catalytic efficiency and regulatory mechanisms as presented below.

4.2 The structure and function of PDE6

Physiologically, the PDE6 family is important for visual transduction in the retina. Mutations in each of the genes for the catalytic subunits (PDE6A, PDE6B and PDE6C) or the inhibitory γ subunits (PDE6G and PDE6H) have all been found to associate with retinal degenerative diseases (Ferrari et al., 2011). For example, 10% of retinitis pigmentosa (RP) cases involve defects of cyclic nucleotide metabolism due to a gene defect in PDE6. In order to provide a fundamental study for treatment of retinal diseases, the structure, function and regulatory mechanisms of PDE6 have to be better understood.

The primary sequences of PDE6 expressed in rods and cones are highly conserved while they present distinct structural features. Rod PDE6 is a heterotetramer composed of two catalytic subunits, α (gene name, PDE6A) and β (PDE6B), forming a catalytic dimer, as well as two high-affinity associated inhibitory γ-subunits (PDE6G). Cone PDE6 is also a heterotetramer but it has two identical catalytic α' subunits (gene name, PDE6C) and two inhibitory γ'-subunits (PDE6H). The different structures of PDE6 in rods and cones may reflect their distinct cellular functions in visual transduction.

4.2.1 The catalytic subunits of rod PDE6

The atomic-level structure of rod PDE6 is not resolved since its catalytic subunits cannot be heterologously expressed in a soluble and active state in biochemical quantities.
and it is difficult to purify large quantities of native PDE6 from photoreceptor cells (Zhang et al., 2004b; Muradov et al., 2006). So far, two negative-staining electron microscopy (EM) density maps of PDE6 at low resolution (18Å and 28Å) have been reported (Goc et al., 2010; Kameni Tcheudji et al., 2001). The crystal structures of a nearly full-length PDE2 (another GAF domain containing PDE), a chimeric PDE5/PDE6 catalytic domain and a cone PDE6 GAFa domain have been resolved (Pandit et al., 2009; Barren et al., 2009; Martinez et al., 2008). Each PDE6 catalytic subunit consists of N-terminal tandem GAF domains (GAFa and GAFb), a catalytic domain and a C-terminal motif that is post-translationally modified by isoprenylation.

The GAF domains (GAFa and GAFb) on the N-terminus of PDE6 are capable of inducing allosteric changes that can regulate enzymatic activity (Cote et al., 2010). This allosteric change is found to be an indirect regulation (Arshavsky et al., 1992b; Mou et al., 2001) in which the inhibitory γ subunit mediates the feedback and cross talk between the GAF domain and catalytic domain. Direct interactions of the N-terminal half of the γ-subunit with GAFa and GAFb have been identified by crosslinking experiments (Muradov et al., 2002; Guo et al., 2008). In addition, the GAFa domain contains a high-affinity cGMP binding site (Yamazaki et al., 1980; Gillespie et al., 1988; Gillespie et al., 1989a; Cote et al., 1993) and the high-affinity binding of cGMP to the GAFa nucleotide binding pocket is stabilized by the central, polycationic region of the γ subunit (Mou et
The function of GAFb is unclear while some observations suggest an important role for GAFb in linking allosteric changes in GAFa to the regulation of the catalytic domain. In addition to allosteric regulation and ligand binding, the GAF domains are also involved in the dimerization of the catalytic dimer (Muradov et al., 2003).

The structure of the catalytic domain of PDE6 has been analyzed based on the PDE5 catalytic domain as a structurally homologous modeling template. It is likely to consist of 16 α-helices (Conti, 2004) and two metal ions (zinc and magnesium) (He et al., 2000a) that are coordinated by a group of six invariant residues present in all 11 PDE families to form an integral part of the active site of PDE6. PDE6 presents a 500-fold greater catalytic efficiency for cGMP as compared to PDE5. The light-activated PDE6 hydrolyzes cGMP with a catalytic constant \( k_{\text{cat}} \) of 5500 s\(^{-1}\) in bovine rod photoreceptors. The catalytic efficiency \( k_{\text{cat}}/K_m = 4 \times 10^8 \text{M}^{-1}\text{s}^{-1} \) for cGMP approaches the diffusion-controlled limit (Cote, 2006), which is essential for light-induced changes in cGMP levels to occur on the millisecond timescale needed for the physiological response of rods and cones to illumination (D'Amours et al., 1999; Arshavsky et al., 2002). The regulation of the catalytic activity by the inhibitory γ-subunit is a unique feature of the PDE6 catalytic domain as compared with PDE5. The removal of both γ subunits in PDE6 activates the enzyme 300-fold (Mou et al., 1999).
Rod PDE6 is a heterotetramer composed of two catalytic subunits (α and β) forming a catalytic dimer, and two inhibitory γ-subunits. Each of the catalytic subunits contains a catalytic domain and a noncatalytic cGMP-binding site (occupied by circles) in the regulatory GAF domain. The inhibitory γ-subunit binds to PDE6 with high affinity and blocks the active site to prevent cGMP hydrolysis in its nonactivated state.

Divalent cations such as Mg$^{2+}$ and Zn$^{2+}$ are important for the catalytic activity of PDE6. Previous studies found that the concentration of Mg$^{2+}$ changes the apparent $K_m$ of PDE6 for cGMP (Srivastava et al., 1995) and rod PDE6 requires tightly-bound zinc for its catalytic activity (He et al., 2000a). The stoichiometry between PDE6 and endogenous zinc has been determined to be 3-4 g atoms of zinc per mole PDE6, which suggests that there are two tightly bound zincs per catalytic subunit (He et al., 2000a). The loss of PDE6 activity caused by the removal of the high-affinity bound zinc was able to be fully recovered by magnesium, manganese or cobalt. Dipicolinic acid (DPA) and EDTA can remove the majority of the tightly-bound zinc, which causes the complete loss of activity of PDE6 even in the presence of magnesium, but the lost activity could be restored by the addition of zinc (He et al., 2000a).

The C-terminus of PDE6 catalytic subunits presents one CAAX motif (A, aliphatic; X, any amino acid) which is subjected to post-translational modification. The α-subunit of rod PDE6 is farnesylated at the first cysteine residue of the terminal sequence CC(I/V)Q, whereas the β-subunit has a geranylgeranyl group bound to the cysteine of the terminal sequence C(C/R)IL (Qin et al., 1992; Anant et al., 1992).
prenylated, carboxymethylated C-termini are hydrophobic and are responsible for anchoring PDE6 to the outer segment disk membrane (Catty et al., 1991).

4.2.2 The inhibitory γ subunit of PDE6

The PDE6 family is unique in having a high-affinity inhibitory γ subunit (Pγ) associated to the catalytic dimer. The 87-amino acid rod inhibitory subunit is a natively unfolded protein with little secondary structure (Berger et al., 1997; Uversky et al., 2002), while it is remarkable for the variety of regulatory functions it performs as well as the multitude of proteins with which it interacts in addition to the catalytic subunits of PDE6 (Guo et al., 2008). Primarily, Pγ regulates cGMP hydrolytic rates by controlling access of cGMP to the catalytic pocket of PDE6 through the interaction between the last few C-terminal residues of Pγ and the catalytic domain (Granovsky et al., 1997; Zhang et al., 2010b). There is also an allosterically mediated inhibition of catalysis that occurs in the absence of the C-terminal residues of Pγ (Zhang et al., 2010a). The central region of Pγ, with high affinity for the catalytic dimer (Mou et al., 2001), is found to enhance the binding affinity of cGMP to the noncatalytic binding sites within the regulatory domain of PDE6 (Cote et al., 1994).

In addition to the PDE6 catalytic dimer, Pγ also interacts with other phototransduction proteins and mediates important regulatory mechanisms of PDE6. The N-terminal region of Pγ (a.a. 24-45) has been reported to interact with transducin α-subunit. This interaction increases cGMP dissociation from noncatalytic cGMP binding sites on PDE6 (Morrison et al., 1989; Muradov et al., 2010; Zhang et al., 2012). During the activation of PDE6 by transducin, the glycine-rich region of Pγ (a.a. 45-62) is required as a "docking site" to stabilize the binding of Tα* and thereby develop additional
interactions with the C-terminal Py (a.a. 71-86) that lead to de-inhibition of PDE6 catalysis (Zhang et al., 2012; Artemyev et al., 1992). In the recovery state of phototransduction, Py serves to facilitate the formation of a tighter RGS9-1 complex to potentiate the GTPase accelerating function of RGS9-1 (He et al., 1998; Hu et al., 2002). Previous work suggests that C-terminal region of Py can bind to RGS9-1 (Slep et al., 2001) and recent work in our lab reveals that the region of a.a. 55-62 is also important for GTPase acceleration of the Ta*/RGS9-1 complex (Zhang et al., 2012).

5. PDE6 Interacting Proteins

As referred to in previous sections, transducin is a PDE6 interacting protein in photoreceptor cells. In visual excitation, the rhodopsin-activated transducin (Ta*-GTP) binds to PDE6 holoenzyme and displaces the inhibitory γ-subunit from the catalytic pocket, which leads to the activation of PDE6. Multiple interacting sites have been identified between transducin and Py (Granovsky et al., 2001; Slep et al., 2001; Zhang et al., 2012). It has been assumed that transducin can activate PDE6 in a 1:1 molar ratio (Wensel et al., 1990; Leskov et al., 2000), but the model of transducin activation of PDE6 in which transducin is hypothesized to relieve γ-subunit inhibition at either one or both catalytic sites of PDE6 is unclear.

In addition to transducin, the prenyl binding protein (PrBP/δ) is considered a PDE6 interacting protein. PrBP/δ was originally found to be co-purified with soluble bovine rod and cone PDE6 and referred to as the δ-subunit of PDE6 (Gillespie et al., 1989b). This 17-kDa protein is ubiquitously expressed and interacts with numerous proteins, most of which are post-translationally modified with farnesyl or geranylgeranyl.
groups (Zhang et al., 2004a). In photoreceptor cells, PrBP/δ transports prenylated proteins including PDE6, opsin and the retinitis pigmentosa G protein regulator (Florio et al., 1996;Gillespie et al., 1989b;Zhang et al., 2005a) from the inner segment to the outer segment where phototransduction occurs (Karan et al., 2008). In vitro, it has been shown that PrBP/δ binds PDE6 with high affinity through the specific interaction with the farnesylated and geranylgeranylated C-termini of rod PDE6 catalytic subunits resulting in the release of PDE6 from its disk membrane attachment site (Goc et al., 2010;Norton et al., 2005). Although the binding of PrBP/δ to PDE6 has no effect on the catalytic activity of PDE6, it has been found to reduce the ability of activated transducin to activate PDE6 (Norton et al., 2005) and enhances the nucleotide exchange at the non-catalytic cGMP binding sites of PDE6 (Mou et al., 1999;Gillespie et al., 1989b). PrBP/δ may play a role in negative feedback regulation of PDE6 activation, perhaps during prolonged light adaptation (Cote, 2008).

Another PDE6 interacting protein is aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1), a photoreceptor-specific chaperone of PDE6. AIPL1 is expressed in the retina and the pineal gland (Van der Spuy et al., 2002) and mutations on the Aipl1 gene result in one of the most clinically severe forms of Leber congenital amaurosis [LCA type 4; (den Hollander et al., 2008)]. In structure, AIPL1 shares similar sequence information and domain organization with the ubiquitously expressed aryl hydrocarbon receptor-interacting protein (AIP) (Sohocki et al., 2000). AIPL1 consists of an N-terminal FK506-binding protein (FKBP)-like domain and a C-terminal tetratricopeptide repeat (TPR)-domain (Sohocki et al., 2000;Das et al., 1998) and these domains are believed to be related with its chaperone activity (Van der Spuy, 2006). AIPL1 is considered as an
essential component of a retina-specific chaperone complex for the reason that it was found to interact with Hsp90 and Hsp70 (Hidalgo-de-Quintana et al., 2008; Schwartz et al., 2006; Kosmaoglou et al., 2008). Transgenic research found that the expression level and activity of PDE6 were largely reduced in the AIPL1 knockout mice, which suggests that AIPL1 is critical for the expression and function of PDE6 (Ramamurthy et al., 2004). Previous studies suggest that AIPL1 functions on the farnesylated modification of the rod PDE6 α subunit and stabilizes the PDE6 holoenzyme structure by properly assembling it to avoid rapid protein degradation (Kolandaivelu et al., 2009). Recently, it has been reported that AIPL1 is able to directly bind the farnesyl lipid moiety and the potential farnesyl-binding sites have been identified on AIPL1, which suggests that the function of AIPL1 on PDE6 may require the binding of AIPL1 to the farnesylated PDE6 α subunit (Majumder et al., 2013). However, further research is needed to fully understand the mechanism of AIPL1 action on PDE6 in photoreceptor cells.

In addition, the Regulator of G-protein Signaling 9-1 (RGS9-1) is considered as a PDE6 interacting protein. The molecular organization of RGS9-1 consists of a catalytic RGS domain that functions to stimulate GTP hydrolysis on the G-protein α-subunit (He et al., 2000b; Skiba et al., 2001), a G protein gamma-like (GGL) domain which specifically interacts with Gβ5L, and a DEP (Disheveled, Egl-10, Pleckstrin)/DHEX (DEP helical extension) domain that mediates its interactions with RGS9 anchor protein (R9AP) on the membrane (Anderson et al., 2009). In photoreceptor cells, the RGS9-1 complex, including RGS9-1, Ta*-GTP, Gβ5L and R9AP (Anderson et al., 2009), plays an important role in recovery after light activation ceases. The process requires the inactivation of Ta*-GTP through the intrinsic GTPase activity of Ta; the GTPase rate is
considered the rate-limiting step in the recovery state of photoresponse (Krispel et al., 2006). It is believed that RGS9-1 modulates the rapid deactivation process of Ta*-GTP by accelerating its GTPase rate, in which the association of RGS9-1 with Gβ5L and R9AP is required (Keresztes et al., 2004; Krispel et al., 2003). Previous studies have reported that the C-terminal region of Py can bind to RGS9-1 (Slepak et al., 1995; Slep et al., 2001; Arshavsky et al., 1994) and the N-terminal half of Py can associate with the transducin/RGS9-1 complex (Guo et al., 2011). Recent work in our lab confirmed that the maximal acceleration of the GTPase rate of the Ta*/RGS9-1 complex requires the C-terminal region (Ile-86, Thr-65 and Val-66) of Py and the amino acids 55-62 of Py facilitate the anchoring of Py to the Ta*/RGS9-1 complex and stabilize the interaction (Zhang et al., 2012). All these observations suggest that the interaction between Py and RGS9 serves to form a tighter RGS9-1 complex to potentiate the GTPase accelerating function of RGS9-1 (Hu et al., 2002).

6. Characteristics of the Glutamic Acid-Rich Protein2 (GARP2)

6.1 The cGMP-gated cation (CNG) channel in rod photoreceptor cells

As an essential component of the visual signaling pathway, the photoreceptor cGMP-gated cation (CNG) channel plays a major role in light-dependent regulation of the ion flow into rod or cone outer segments (Sarfare et al., 2007; Kaupp et al., 2002; Matulef et al., 2003), as well as in maintenance of the structural integrity of rod outer segments. Photoreceptor CNG channels come from the superfamily of voltage-gated channels and are non-selective cation channels (Kaupp et al., 2002); in rods, 90% of the current is carried by Na^+ and 10% by Ca^{2+}. The rod CNG channel is a
heterotetramer composed of three \( \alpha \)-subunits (gene name, \textit{CNGA1}) and one \( \beta \)-subunit (gene name, \textit{CNGB1}), while the cone CNG channel consists of two cone \( \alpha \)-subunits (\textit{CNGA3}) and two cone \( \beta \)-subunits (\textit{CNGB3}) subunits (Weitz et al., 2002; Zhong et al., 2002; Shuart et al., 2011; Weitz et al., 2002). The structure of CNGA1 and CNGB1 is composed of six transmembrane domains, a pore loop region, a cGMP binding domain and a C-terminal region (Shuart et al., 2011). CNGB1 has a unique glutamic acid and proline-rich region at the N-terminus (GARP region), and as well as two Ca\(^{2+}/\text{CaM} \) (calmodulin) binding domains (CaM-1 and CaM-2) (Sarfare et al., 2007). In addition to the GARP region on CNGB1, there are two other forms of GARP—GARP1 and GARP2—in rod photoreceptors that represent alternative splicing products of the CNGB1 gene. In structure, CNGB1, GARP1 and GARP2 share the conserved N-terminus.

![Diagram of Cyclic nucleotide-gated channel \( \beta \) subunit (CNGB1)](attachment)

1394 a.a.

**Cyclic nucleotide-gated channel \( \beta \) subunit (CNGB1)**

1

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<th>CaM</th>
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590 a.a.

GARP1

590

GARP2

299 a.a.

RVVAAGSL

Fig. 1-7. The GARP (glutamic acid-rich protein) family.

The \( \beta \)-subunit of cyclic nucleotide-gated channel (CNGB1), GARP1 and GARP2 have the same N-terminal GARP' region (GARP'). Both CNGB1 and GARP1 have the glutamate-rich domain (Glu-rich). CNGB1 also has a channel region including a calmodulin binding domain (CaM), transmembrane domain (\( \beta' \) domain) and a cGMP binding domain (cGMP). GARP1 and GARP2 express different C-terminal ends, which are distinct from the sequence of CNGB1.
while the translation of the CNGB1 gene was terminated at different exons—human CNGB1: exon1-33; human GARP2: exon1-12 with exon 12a; human GARP1: exon 1-16 and exon β4b (Ardell et al., 2000).

In rods, the CNG channel is activated and opened by binding of cGMP to the cyclic nucleotide binding domain. During light activation, the drop in cGMP levels caused by hydrolysis of PDE6 allows the release of cGMP from the channel and results in the closure of the channel, which leads to hyperpolarization of the membrane potential of the rod photoreceptor. In addition to cGMP, the rod CNG channel is modulated by Ca^{2+} concentration through Ca^{2+}/calmodulin binding to CNGB1. Under dark conditions, the intracellular Ca^{2+} and cGMP concentrations are high and Ca^{2+}/calmodulin binds to the channel and the open channel presents a low affinity for cGMP binding. When light activation causes the closure of the channel and the drop of intracellular Ca^{2+} concentration, Ca^{2+} dissociates from the CaM and the affinity of cGMP to the channel increases. The Ca^{2+}/calmodulin modulation plays important role in the regulation of cGMP affinity which may be a feedback mechanism in light adaptation (Kaupp et al., 2002).

5.2 The biochemical properties and functions of GARPs

CNGB1, GARP1 and GARP2 are only found in the outer segments of rod photoreceptor cells but not in cones. GARP2 is more abundant than CNGB1 in rods, whereas GARP1 is present in low abundance based on immunoblots. In structure, GARP1 is 65 kDa and its amino acid sequence is almost identical to the GARP region of rod CNGB1; GARP2 is 32 kDa and its amino acid sequence corresponds to the first 291 amino acids of GARP1 but it presents a unique C-terminus of eight amino acids.
Although GARP2 lacks the so-called GARP region (a.a. 355-465 on CNGB1), it still exhibits an abnormally high content of glutamate residues (Batra-Safferling et al., 2006). The large percentage of negatively charged residues on GARPs causes the higher apparent molecular weight on SDS-PAGE. Both GARP1 and GARP2 are considered as "natively unfolded" proteins with less secondary and tertiary structure as detected by biochemical and biophysical analysis (Batra-Safferling et al., 2006). In rods, biochemical assays suggest that CNGB1 and GARP2 interact with peripherin-2 in the rim region of the outer segment disk membrane (Poetsch et al., 2001). There also exists an interaction between GARPs and guanylate cyclase (GC), as well as the retina-specific ATP-binding cassette transporter (ABCR) (Korschen et al., 1999).

Several transgenic studies have demonstrated the critical roles for GARPs in maintenance of ROS structural integrity as well as normal retinal function. In CNGB1-deleted transgenic mice (in which the exon 26 of CNGB1 gene was deleted to disrupt CNGB1 expression but normal GARP1 and GARP2 were expressed), the CNGA1 subunit was rapidly degraded, the visual responses of rod photoreceptors were completely abolished, the length of the rod outer segment was shorter (ROS), the number of ROS was less, and the degeneration of the retina was faster (Huttl et al., 2005). In another transgenic mouse system in which all GARPs (CNGB1, GARP1 and GARP2) were ablated, the expression of CNGA1 subunit, GC1 and ABCA4 were greatly reduced, ROS was shorter and misaligned with abnormal elongated disks, and the sensitivity of rod photoreceptor cells to light was reduced (Zhang et al., 2009). Both of these transgenic studies suggested that the interaction between GARP2 and peripherin-2 is critical for normal disk morphogenesis and integrity of the rod outer segment structure.
The roles of GARP2 and peripherin-2 in the structure of ROS have been further evaluated through in situ bimolecular fluorescence complementation (BiFC) in transgenic *Xenopus laevis* rods. In this study, the interaction between CNGB1 and peripherin-2 was observed in rod inner segments initially before trafficking to outer segments, while GARP2-peripherin-2 interaction was only observed at sites of disk morphogenesis. This suggests that GARP2-peripherin-2 interaction directly participates in structuring disks and that the CNGB1-peripherin-2 complex functions to localize plasma membrane ion channels (Ritter et al., 2011).

As a rod-specific protein, GARP2 has been identified as a high-affinity PDE6 binding protein that inhibits the basal activity of the PDE6 holoenzyme by up to 80 percent (Korschen et al., 1999; Pentia et al., 2006). However, the precise regulatory mechanisms of GARP2 on the activity and life time of PDE6 in phototransduction are unclear. Recently, transgenic mice with overexpressed GARP2 were found to exhibit increased phototransduction gain, which suggests a potential role of GARP2 for modulating transducin activation (McKeon et al., 2012). The overall goal of this thesis is to evaluate the function of GARP2 in rod photoreceptors. To achieve this, I have biochemically characterized GARP2 and evaluated its ability to modulate the activity and regulation of PDE6.
CHAPTER 2

CHARACTERIZATION OF THE BIOCHEMICAL AND

BIOPHYSICAL PROPERTIES OF GARP2

Abstract

Glutamic Acid-Rich Protein-2 (GARP2) is a rod photoreceptor-specific protein of 299 amino acids with an unusually high content of glutamate residues. GARP2 has been reported to interact with several membrane-associated phototransduction proteins including phosphodiesterase (PDE6) in rod photoreceptors. As a high-affinity PDE6 binding partner, GARP2 has been demonstrated to suppress the basal activity of nonactivated PDE6 but has no effect on activated PDE6. Considering the functional differences between rod and cone photoreceptors, GARP2 may play an important role in rod phototransduction, such as detecting single photons in the dark-adapted state and/or regulating visual signaling pathways during rod photoresponse saturation. The goal of this research is to characterize the properties of GARP2 and discover the functional relevance of GARP2 for regulating PDE6 during visual transduction. Here we show that an improved immunological method can be utilized to detect GARP2. We developed methods in which native GARP2 can be selectively separated from PDE6 on rod outer segment (ROS) membranes. Finally, we prepared purified, recombinant GARP2 and show that it has similar biophysical properties as native GARP2. We propose that these
biochemical and biophysical characteristics of GARP2 may be responsible for the regulation of PDE6 in rod photoreceptors.

**Introduction**

PDE6 is the central effector enzyme of visual transduction, and cGMP hydrolysis by PDE6 must be precisely regulated to control the sensitivity, amplitude, and kinetics of the photoresponse during excitation, termination and adaptation to light stimulation of photoreceptor cells. Rod PDE6 is a heterotetramer composed of two catalytic subunits (α and β) as well as two high-affinity associated inhibitory γ subunits. Cone PDE6 is also a heterotetramer but it has two identical catalytic α' subunits with two inhibitory γ' subunits. The different structures of PDE6 in rods and cones may reflect their distinctive cellular functions in visual transduction. Each PDE6 catalytic subunit consists of N-terminal tandem GAF domains (GAFa and GAFb), a catalytic domain and a C-terminal membrane-associated motif. The GAF domains (GAFa and GAFb) of the catalytic dimer containing two non-catalytic cGMP binding sites (Yamazaki et al., 1980; Gillespie et al., 1988; Gillespie et al., 1989a; Cote et al., 1993) are able to induce allosteric changes upon ligand binding to regulate the catalytic activity of the catalytic domain (Arshavsky et al., 1992b; Mou et al., 2001). The regulation of the catalytic activity by the inhibitory γ subunit, Pγ, is a unique feature of PDE6, in which both γ subunits can be displaced by transducin to activate PDE6 upon light stimulation. The inhibitory γ subunit is a natively unfolded protein with 87 amino acids (Berger et al., 1997; Uversky et al., 2002), and it is remarkable for its variety of regulatory functions (Guo et al., 2008). First, Pγ regulates cGMP hydrolytic rates by controlling access of cGMP to the catalytic pocket of PDE6.
(Granovsky et al., 1997; Zhang et al., 2010b). Second, Py enhances the binding affinity of cGMP to the noncatalytic binding sites within the regulatory domain of PDE6 (Cote et al., 1994). Third, Py also interacts with other phototransduction proteins including transducin and RGS9-1 to mediate important regulatory mechanisms of PDE6 during excitation and recovery of the photoresponse (Zhang et al., 2012; He et al., 1998; Slep et al., 2001).

The activation mechanism of PDE6 by transducin is well studied. Following photoactivation of rhodopsin, activated transducin (Tα*-GTP) binds to the PDE6 holoenzyme and displaces Py from the catalytic pocket, which leads to the activation of PDE6. Multiple interacting sites have been identified between transducin and Py (Granovsky et al., 2001; Slep et al., 2001; Zhang et al., 2012). In contrast, much less is known about other mechanisms that are thought to regulate PDE6 during recovery from light stimulation and during prolonged light adaptation.

Glutamic Acid-Rich Protein-2 (GARP2) is considered to be a potential regulatory protein of PDE6. GARP2 is a splice variant of the rod cGMP-gated channel β-subunit [CNGB1; (Sugimoto et al., 1991)], which is found to be restricted to the outer segment of rod photoreceptors, but not in cones. GARP2 is a 299-amino acid protein, of which the first 291 amino acids correspond to those of CNGB1, along with a unique eight-amino acid C-terminus. Although GARP2 lacks the "GARP region" (a.a. 355-465 of CNGB1), it still exhibits abnormally high content of glutamate residues (Batra-Safferling et al., 2006). As a result of the high number of negatively charged residues, a greater apparent molecular weight (~60 kDa) on SDS-PAGE is observed than the calculated molecular weight of GARP2 (32 kDa). The high content of proline and glutamate residue also contributes to the low hydrophobicity of GARP2 as an acidic protein (Batra-Safferling et
al., 2006). GARP2 is considered to be a “natively unfolded” protein with low amount of secondary and tertiary structure. Some biochemical assays suggest that GARP2 interacts with peripherin-2 in the rim region of the outer segment disk membrane in the rods (Poetsch et al., 2001). GARP2 also interacts with other phototransduction proteins including PDE6, guanylate cyclase (GC), and retina-specific ATP-binding cassette transporter (ABCR) (Korschen et al., 1999).

Although GARP2 is abundant in the outer segments of rods, its functions in rod photoreceptor cells are not clear. Using transgenic mice, several studies have demonstrated that GARPs are critical for maintaining the structural integrity of ROS and the normal physiological functions of the retina. In CNGB1-deleted transgenic mice, in which CNGB1 expression was disrupted but normal GARP1 and GARP2 were expressed, it was observed that the CNGA1 subunit was rapidly degraded, the retina degenerated faster, and the visual response of rod photoreceptor cells was completely abolished. In addition, the length of ROS was shorter and the number of ROS was less, while the morphological organization of discs and the rim regions of ROS were found to be normal (Huttl et al., 2005). With another transgenic mouse system in which the expression of all GARPs (CNGB1, GARP1 and GARP2) was abolished, the sensitivity of rod photoreceptor cells to light was greatly reduced, the expression of CNGA1 subunit, GC1 and ABCA4 were greatly reduced, and ROS were shorter and maligned, with abnormal elongated disks (Zhang et al., 2009). Recently, transgenic mice with overexpressed GARP2 were found to exhibit an increased phototransduction gain, which suggests a potential role of GARP2 in transducin activation (McKeon et al., 2012). In situ bimolecular fluorescence complementation (BiFC) in transgenic *Xenopus laevis* rods
suggested that GARP2-peripherin-2 interactions may directly participate in structuring disks of the rod outer segment, and that the CNGB1-peripherin-2 complex may function to localize plasma membrane ion channels (Ritter et al., 2011).

As a rod-specific protein, GARP2 has been identified as a high-affinity PDE6 binding protein that inhibits the basal activity of PDE6 holoenzyme by up to 80 percent (Pentia et al., 2006). However, the precise regulatory mechanisms of GARP2 on the activity and lifetime of PDE6 in phototransduction are still unclear. We propose that GARP2 is involved in the unique phototransduction function of rods, in which GARP2 may regulate PDE6 to reduce its “dark noise”, enhance its sensitivity and reduce cGMP metabolic flux to conserve metabolic energy during rod photoresponse saturation. The overall goal of this chapter is to better understand the biochemical and biophysical characteristics of GARP2 in order to discover the functional relevance of GARP2 for regulating PDE6 during visual transduction.

**Materials and Methods**

*Materials*—Bovine retinas were purchased from W.L. Lawson, Inc. The Superdex 200 and Mono-Q columns and the Butyl-Sepharose chromatography media were from GE Healthcare. The Ni-nitriloacetic acid (NTA) His-Bind resin was obtained from Novagen and glutathione-agarose was from Thermo-Fisher/Pierce. Filtration and ultrafiltration products were from Millipore. Mouse monoclonal anti-GARP2 antibody (8G8) to the first 15 amino acid of GARP2 was a generous gift from Dr. Robert Molday (University of British Columbia). Rabbit polyclonal anti-GARP antibody to bovine
sequences common to GARP1, GARP2 and the rod β-subunit CNGB1 (FPc) was a kind gift of Dr. Benjamin Kaupp (Institut für Biologische Informationsverarbeitung, Jülich, Germany). Chicken anti-GARP2 antibody (SP) to the first 15 amino acid of GARP2 was a generous gift from Dr. Steven Pittler (University of Alabama at Birmingham). Rabbit anti-GARP2 antibody against the last 8 amino acid of GARP2 (ABR) is purchased from Pierce. Affinity-purified anti-peptide rabbit polyclonal antibodies directed to the PDE6 GAFb domain (NC397-64), the C-terminus of the Py-subunit of PDE6 (CT-9710) and to full-length bovine PrBP/δ (FL-R98) were prepared in our laboratory. Monoclonal mouse ROS antibody against PDE6 holoenzyme was affinity purified in our lab. Full-length recombinant bovine PrBP/δ was kindly provided by Dr. Karyn Cahill and Hannah Gitschier. Mouse Anti-His tag antibody was obtained from Cell Signaling. All other chemicals were obtained from Sigma Chemical Co. or from Thermo-Fisher.

**Rod Outer Segment (ROS) Membrane Isolation and Purification**—ROS membranes from bovine retina were prepared as described previously (Pentia et al., 2005). Briefly, ROS was isolated from frozen bovine retinas on a discontinuous sucrose gradient under dark conditions and stored at -80 °C. ROS membranes were homogenized in an isotonic buffer (10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride) using a glass, handheld homogenizer. The soluble proteins and ROS membrane were separated by centrifugation.

**Purification of PDE6 Holoenzyme (Pαβγγ) and PDE6 Catalytic dimer (Pαβ)—** Rod PDE6 holoenzyme was extracted with a hypotonic buffer (5 mM Tris, pH 7.5, 1 mM EDTA-2Na, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride) from ROS homogenates and purified by Mono-Q anion exchange chromatography in a linear salt
gradient followed by Superdex 200 gel filtration chromatography. The purified PDE6 was concentrated by ultrafiltration, mixed with 50% glycerol and stored at -20 °C (Pentia et al., 2005). The catalytic activity of PDE6 was assayed with a colorimetric method (Cote, 2000). The PDE6 catalytic dimer (Paβ) was prepared from the PDE6 holoenzyme by digesting the inhibitory Py subunits by a limited trypsin proteolysis as described previously (Hurley et al., 1982; Pentia et al., 2005). Briefly, a time course of trypsinization on PDE6 was performed to digest 90% of the Py subunit without altering the apparent molecular weight of the catalytic subunits. The Paβ was purified with a Mono Q ion-exchange column under a linear gradient from 100 mM NaCl to 1 M NaCl in 10 mM Tris, pH 7.5. The PDE6 concentration was determined as described previously (Cote, 2000).

Isolate GARP2 from PDE6 with Hydrophobic Interaction Chromatography (HIC)—Purified ROS membranes were homogenized in the hypotonic buffer and the soluble fractions were separated from membranes by centrifugation. The hypotonic extraction was repeated three times. The pooled hypotonic extract was then adjusted to 500 mM ammonium sulfate and loaded to a butyl-Sepharose column (HIC). The unbound proteins were washed through the column using 500 mM ammonium sulfate in 5 mM Tris, pH 7.5, and bound proteins were eluted by a step gradient with 400 mM, 150 mM and no ammonium sulfate in 5 mM Tris, pH 7.5, and 1 mM dithiothreitol. Slot blot analysis was used to identify GARP2-containing fractions with a GARP2 antibody as well as PDE6-containing fractions with PDE6 antibody. If necessary, the pooled GARP2-containing fractions can be loaded to the butyl-Sepharose column again to improve the
performance. Pooled GARP2 was further purified with Mono Q ion-exchange chromatography.

*Preparation of GARP2-free ROS Membranes and GARP2-free PDE6 Using Triton X-100—* Under dark conditions, sucrose purified ROS membranes were suspended and homogenized with an insulin syringe in isotonic buffer. The soluble proteins were removed by the centrifugation at 130,000 x g for 5 min. The ROS membranes were exposed to light and washed with the hypotonic buffer containing 10 mM Mg^{2+} (HM) to tightly bind PDE6 on ROS membrane. The washed ROS membranes were extracted with HM buffer supplied with 0.1% Triton X-100 (v/v) and spun down by centrifugation at 130,000 x g for 5 min. The supernatant contains most of the GARP2 and the pellet is GARP2-free ROS membrane with PDE6 bound to it. The ROS membrane was extracted with Triton X-100 three times. In order to obtain GARP2-free PDE6, the GARP2-free ROS membranes were washed with HM buffer with no detergent three times to remove the excess Triton X-100, followed by standard hypotonic extraction as described previously (Pentia, 2005). The hypotonic extract was further purified with Mono Q and gel filtration to achieve GARP2-free PDE6.

*Purification of Recombinant PrBP/δ—* The coding sequence for bovine PrBP/δ was cloned into pET-47b (+) vector to generate (His)$_6$-PrBP/δ. Recombinant (His)$_6$-PrBP/δ was expressed in *E. coli* BL21(DE3) cells following induction with 0.5 mM isopropylthiogalactoside at 37 °C for 3 hours. (His)$_6$-PrBP/δ was purified using Ni-NTA affinity purification and the recombinant tag was removed by incubating with HRV3C protease for 16 hours at 4 °C. The bovine PrBP/δ coding sequence was also inserted to pGEX6p vector to generate PrBP/δ with a glutathione S-transferase (GST) fusion tag.
GST-PrBP/δ was expressed under the same conditions as the (His)₆-PrBP/δ and purified by affinity chromatography on a glutathione-agarose column, and the GST tag was removed by incubation with HRV3C protease followed by repurification of the PrBP/δ and storage at 4 °C until use. The protein concentration of recombinant PrBP/δ was determined with Bradford protein assay as previously described (Bradford, 1976).

*Isolation of PDE6 from GAR2-associated ROS Membranes with PrBP/δ*—Under dark conditions, the ROS pellet was homogenized in isotonic buffer and spun down by centrifugation at 16,000 x g for 30 min to remove the soluble proteins. Meanwhile, rhodopsin spectra were examined to determine the rhodopsin concentration on the ROS homogenate and thereby estimate the concentration of PDE6 on ROS membrane. The washed ROS membranes were exposed to light and resuspended in isotonic buffer supplemented with 20-fold excess of recombinant PrBP/δ [(His)₆-PrBP/δ, GST-PrBP/δ or PrBP/δ cleaved affinity tag] relative to PDE6. The ROS homogenate was incubated with PrBP/δ for 40 min at 4 °C with resuspension every 10 min using a 10 ml syringe to maximally increase the contact between PDE6 and PrBP/δ. The homogenate was spun down at 16,000 x g for 30 min to collect the soluble PDE6 from ROS membrane. The extraction with PrBP/δ was repeated to increase the yield of released PDE6. The PrBP/δ released PDE6 was further purified by Mono Q and gel filtration chromatography.

*Separation of PDE6 from GAR2 with Proteolysis Method*—The purified ROS membranes were homogenized in isotonic buffer in the dark followed by rhodopsin spectrum scan to estimate PDE6 concentration. The ROS membrane was exposed to light and resuspended with the isotonic buffer with 1 µg/ml trypsin (250 µl ROS homogenate
with PDE6 concentration at 250 nM). The time course proteolysis was processed on ice to reduce the significant damage to the structure of the PDE6 catalytic dimer as well as the inhibitory subunits. After 30 min, 60 min and 90 min incubation, the trypsin proteolysis was quenched by adding 10 µg/ml soybean trypsin inhibitor (STI). The ROS homogenate after trypsin treatment was centrifuged at 16,000 x g for 30 min to isolate the soluble fractions from ROS membrane.

*Expression and Purification of (His)_6-GARP2s and GST-GARP2*—Full-length GARP2 and three fragments of GARP2 (amino acids 1-150, 75-225 and 151-299) were generated by inserting the corresponding bovine GARP2 coding sequences into the pET47b vector which provides an N-terminal 6xHis affinity tag. Recombinant protein was expressed in *E. coli* (DE3) Rosetta cells (EMD Lifesciences for full-length, 1-150 a.a. and 75-225 a.a. truncation mutats) or Arctic Express (Agilent Technology for 151-299 a.a. mutation) by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20 °C for 3 hours (Rosetta cells) or at 10 °C for 18 h (Arctic Express cells). After harvesting two liters of cell culture by centrifugation at 8000 x g for 5 min (approximately 5 g pellet), cells were resuspended in 25 ml Ni-NTA binding buffer (20 mM Tris, pH 8.0, 100 mM NaCl) with EDTA-free protease inhibitor cocktail (300 µl per 5 g cell pellet). Cells were then lysed by 30 sonication bursts (10 sec each with 20 sec rest) on ice. After centrifuging the cell lysate at 16,000 x g for 50 min, the supernatant containing recombinant GARP2 was purified using His-Bind affinity resin. The column was washed with 60 mM imidazole prior to elution of His-tagged GARP2 with 250 mM imidazole.

Full-length GARP2 was also subcloned into the pGEX6P1 expression vector (containing a GST fusion partner) and expressed in Rosetta cells after IPTG induction for
3 hours at 20 °C. GST-GARP2 was affinity purified using immobilized glutathione beads, and subsequently treated with HRV3C protease to remove the fusion tag. The concentrations of all recombinant proteins were determined with a colorimetric protein assay with BSA as the standard protein (Bradford, 1976).

**Immunoprecipitation Assay with ROS1 Antibody**—Co-immunoprecipitation of GARP2 with PDE6 holoenzyme was carried out using Pierce Direct IP Kit (Thermo) and ROS1 antibody against bovine rod PDE6 holoenzyme (Hurwitz et al., 1984b). First, ROS1 coupled AminoLink Plus beads were prepared per the kit instructions. Briefly, 200 µl of the bead slurry was incubated with 200 µg of ROS1 antibody in a modified coupling buffer (0.5 mM sodium phosphate, 7.5 mM sodium chloride; pH 7.2) with sodium cyanoborohydride supplement (3 µl sodium cyanoborohydride stock solution in each 200 µl reaction system) at room temperature for 2 hours. The coupling reaction was quenched by adding 1 M Tris-HCl and the beads were washed with 1 M NaCl to remove the excess sodium cyanoborohydride and equilibrated with the coupling buffer before use. In the immunoprecipitation, 20 µl ROS1-coupled beads were incubated with the recombinant GARP2 and GARP2-free PDE6 (500-fold molar excess GARP2 over 5 nmol PDE6 holoenzyme) in binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl₂) for 2 hours at 4 °C. After washing the beads three times with binding buffer, the immunoprecipitated product was eluted with 2x gel loading buffer and subjected to SDS-PAGE followed by Western blot to detect the specific interaction.

**Pull Down Assay**—His-tagged or GST-tagged GARP2 were pre-incubated overnight at 4 °C with Ni-NTA beads or immobilized glutathione beads, respectively. Excess recombinant GARP2 was removed by washing the beads with binding buffer for
three times and the washed beads were incubated with potential binding partners for 2 hours at 4 °C by rotating the mixture. The unbound fraction and the beads were gently separated by centrifugation at 1000 x g for 1 min. After removing the supernatant, the beads were washed three times with washing buffer and proteins bound to the beads were eluted in 2x gel loading buffer. To control for nonspecific binding, identical samples were incubated with empty Ni-NTA beads or immobilized glutathione beads without recombinant GARP2 bound. The total loading material, bound proteins, and unbound proteins were subjected to SDS-PAGE followed by Western blotting to determine specific protein-protein interactions. In the pull-down assays using Ni-NTA beads, the binding buffer is 25 mM imidazole, 20 mM Tris and 100 mM NaCl, pH 8.0; the washing buffer is 60 mM imidazole, 20 mM Tris, 300 mM NaCl with 0.015% Triton X-100, pH 8.0. In the pull-down assay using immobilized glutathione beads, the binding buffer contains 50 mM Tris and 150 mM NaCl, pH 7.5; the washing buffer contains 50 mM Tris, 300 mM NaCl and 0.015% Triton X-100, pH 7.5.

Labeling the Recombinant GARP2 with IAF—Purified (His)$_6$-GARP2 or GARP2 (cleaved of the GST moiety) were concentrated, and the buffer was exchanged using Centricon ultrafiltration devices (Millipore # UC903024). The solution used for the labeling reaction was as follows: 10 mM Tris, pH 7.5, 100 mM NaCl, and 2 mM MgCl$_2$. Protein disulfide bonds were reduced with a 10-fold molar excess of Tris (2-carboxyethyl) phosphine-HCl. After the pH was adjusted to 7.5–8.0 with 100 mM Tris base, reduced proteins were incubated overnight at 4 °C in darkness with a 20-fold excess of 5-iodoacetamidofluorescein (5-IAF) suspended in DMSO. Excess label was removed by
ultrafiltration and gel filtration chromatography as previously described. The degree of
label incorporation was determined spectrophotometrically (Kingsbury et al., 2011).

Analytical Ultracentrifugation and Data Analysis—Experiments were performed
in an Optima XLI analytical ultracentrifuge (Beckman-Coulter) equipped with a
fluorescence detection system (AVIV Biomedical) (MacGregor et al., 2004). Sedimentation velocity studies were typically run at 50,000 rpm at 20 °C using a Beckman An-50 Ti rotor with double-sector cells with sapphire windows (Spin Analytical). Fluorescence scans (reported in arbitrary units) were acquired at 2-min intervals for all samples simultaneously. Analysis of 250–500 scans of each sample was performed according to a c(s) distribution model using the program Sedfit (Schuck et al., 2002) to determine sedimentation coefficients of each protein or complex. Green fluorescent protein (GFP) (2.6 S) was used as an internal standard. The Stokes radius (RS) and prolate axial ratio (a/b) were calculated with the program SEDNTERP (Laue et al., 1992) using the observed sedimentation coefficient.

SDS-PAGE and Western Blotting—SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) in 4-12% Bis-Tris gel. The immunoblotting procedure followed the protocols in Gallagher (Gallagher, 1998).

Collection of CNGB1 Protein Sequences and Multiple Sequence Alignment—Full-
length CNGB1 vertebrate sequences (Appendix I) including the predicted protein sequences from genomic DNA sequencing were obtained from NCBI and Ensembl Genome Browser. Sequences were initially aligned using ClustalW and manually adjusted as needed. A phylogenetic tree was constructed from the alignment.
Results and Discussion

Development of the immunological approaches to characterize GARP2. GARP2 has no enzyme activity with which to track its presence and it also shows anomalous electrophoretic mobility in which the apparent MW in SDS-PAGE is 50-60 kDa instead of the calculated 32 kDa. The abnormal apparent MW may be caused by its high content of negatively-charged glutamate residues which affect the distribution of SDS on GARP2 protein during SDS-PAGE. For these reasons, immunological approaches are needed to identify and quantify GARP2. In this section, analysis was carried out with the Odyssey Li-Cor Infrared Imaging System (see Methods). Purified rod outer segment (ROS) membranes or purified recombinant GARP2 were subjected to SDS-PAGE and then protein transferred to nitrocellulose for immunological detection. The major goal of this section is to optimize the conditions for immunological detection of GARP2 using four primary antibodies to GARP2.

Optimize the conditions for immunologic assay to detect GARP2. Most of the conditions in each step of the immunological assay (i.e., SDS-PAGE, the transfer procedure and antibody probing) were tested and optimized here. Considering the abnormal migration of GARP2 in SDS-PAGE, we first tested three different SDS-PAGE systems (4-12% Bis-Tris gel in MES or MOPS buffer; or 10-20% Tris-HCl gel in a Tris/glycine/SDS buffer), respectively. Following SDS-PAGE and transfer proteins to the membrane, immunoblots were processed with PDE6 and GARP2 antibodies to compare the performance of the SDS-PAGE conditions. As shown in Fig. 2-1, PDE6 NC antibody recognized PDE6 at ~100 kDa and two forms of GARP2 (32 kDa and 45 kDa) were detected by GARP2 FPc antibody. GARP2 was detected at 32 kDa in all three SDS-
PAGE systems, but the signal was strongest in the MOPS buffer system. The Bis-Tris gel system was found to be more sensitive to GARP2 and PDE6 detection compared with the Tris-HCl gel system. With respect to the two different running buffers (MES and MOPS), which are designed for resolving different sizes of proteins in the Bis-Tris SDS-PAGE system, we found that the GARP2 signal at 32 kDa was comparable to the 50 kDa in the MOPS buffer, while GARP2 signal at 32 kDa was weaker than at 45 kDa in the MES buffer. Overall, the MES running buffer was found to be more sensitive for detecting lower levels of GARP2 and PDE6. In view of these results, we considered the 4-12% Bis-Tris SDS-PAGE with MES running buffer to be most suitable for GARP2 detection.

We next compared nitrocellulose and polyvinylidene difluoride (PVDF) membranes for immunoblot detection of GARP2 (Fig. 2-2). When equal amounts of GARP2 were loaded, PVDF displayed a stronger signal against GARP2 but it also presented much higher background than nitrocellulose did under the same conditions. We concluded than nitrocellulose was preferred for subsequent experiments.

We also tested 5% and 20% methanol in the transfer buffer on both kinds of membranes. We found that the 20% methanol transfer buffer was more effective for transferring GARP2 onto the nitrocellulose membrane; in contrast, no significant difference was detected when PVDF membranes were tested with different concentrations of methanol (data not shown).

After examining the conditions for transfer, the components in the blocking buffer were also studied. Commercial Li-Cor blocking buffer, 2% BSA in Tris-buffered saline buffer (TBS), and 5% non-fat dry milk in TBS were each incubated with nitrocellulose or PVDF membranes. In terms of reducing the background and in the intensity of the
**Fig. 2-1. Comparison of SDS-PAGE running systems.**

Three SDS-PAGE running systems (see methods) were tested with ROS homogenates to detect native GARP2 with a Western Blot assay. The ROS pellet was homogenized and washed with isotonic buffer in the dark and the concentration of rhodopsin in ROS was determined with a spectroscopic bleaching assay (Bownds et al., 1971). Homogenized ROS containing decreasing amounts of rhodopsin were separately loaded on each SDS-PAGE gel. After transfer of the proteins to nitrocellulose membrane, GARP2 FPc Ab, and PDE6 NC Ab were used to probe GARP2 and PDE6 in ROS. The amount of rhodopsin used was: 1: 0.75; 2: 0.3; 3: 0.1; and 4: 0.05 nmol.

**Fig. 2-2. Evaluation of nitrocellulose and PVDF membranes with 5% non-fat dry milk and 2% BSA as blocking buffer.**

ROS homogenates containing 0.1 nmol rhodopsin was loaded onto each lane of SDS-PAGE. The proteins from SDS-PAGE gel were transferred to nitrocellulose (N) or PVDF (P) membranes under the same transferring conditions. Each kind of membrane was cut into small pieces and blocked with 5% non-fat dry milk (M) or 2% BSA (B) in TBS buffer, respectively. For each membrane blocked with each blocking buffer, three GARP2 antibodies (ABR, FPc and SP) were used to detect GARP2. γ CT64 antibody was also applied to each membrane as the control. PM: PVDF with milk; PB: PVDF with BSA. NM: Nitrocellulose with milk; NB: Nitrocellulose with BSA.

GARP2 signal, all three different kinds of blocking buffer performed similarly (data not...
In summary, the optimal transfer conditions were determined to utilize nitrocellulose membranes, with 20% methanol in the transfer buffer. All of the tested blocking buffers did not differ in the ability to detect GARP2 on immunoblots.

**Characterization of the different antibodies against GARP2.** Four kinds of GARP2 primary antibodies (ABR, FPc, SP and 8G8) were used against different regions of GARP2 in this study. As shown in Fig. 2-3A, FPc antibody recognizes the entire GARP2 amino acid sequence. The ABR antibody (Thermo) is designed to recognize the unique C-terminus of GARP2 (CDVQTRVVAAGSL). The SP antibody and 8G8 antibody are designed to detect the N-terminal sequence (GWVQRVLPQPPGTPC) common to all three GARP-containing proteins.

As shown in Fig. 2-3B, all four antibodies detected several molecular forms of GARP proteins present in ROS under the optimized immunoblot conditions. However, each individual antibody showed different ability to recognize the different forms of GARP-containing proteins as well as different levels of background staining. Since GARP2 shares the same N-terminal region with GARP1 and CNGB1, antibodies directed
to this conserved region were less specific. The performance of these four primary antibodies is presented and summarized on Fig. 2-3B and Table 2-1.

In summary, four different anti-GARP2 antibodies that recognize different amino acid sequences on GARP2 have been characterized. By using immunological analysis combined with the Odyssey Li-Cor Infrared Imaging System under the various optimized conditions, nanogram quantities of GARP2 can be detected using recombinant GARP2 as the standard. It suggests that this improved immunological method can provide the best approach to assay the presence and the concentration of GARP2. Among these GARP2 primary antibodies, FPc antibody is ideal to use to detect low protein level of GARP2 because of its high sensitivity; 8G8 antibody generated from mouse is preferred to be used in double color detection of Li-Cor infrared imaging system when it is needed to be combined with rabbit antibodies directed to other proteins.

Fig. 2-3B. Characterization of four primary antibodies direct to GARP2.
ROS homogenates containing 0.1 nmol (1) or 0.75 nmol (2) rhodopsin were loaded onto an SDS-PAGE gel. After transferring and blocking the nitrocellulose membranes under the same conditions, each GARP2 primary antibody (ABR, SP and 8G8 at 1000-fold dilution; FPc at 3000-fold dilution) was incubated with the membrane. The Western blot signal was obtained by Li-Cor scanner. Py CT64 antibody was used as control for first three antibodies. The circles on the figure indicate the presumed GARP2 signals. The performance of these primary antibodies is described in Table 2-1.
Table 2-1 Summary of four GARP2 primary antibodies on immunoblot

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<th>SP</th>
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<tr>
<td></td>
<td>Rabbit</td>
<td>Rabbit</td>
<td>Chicken</td>
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<tr>
<td><strong>Epitope on GARP2</strong></td>
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**Background**

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**Specificity for GARP2**

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<th>GARP2</th>
<th>GARP1, GARP2 and CNGB1</th>
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 development of methods to separate PDE6 from GARP2. As a membrane-associated protein, PDE6 attaches to the ROS membrane through its hydrophobic, prenylated and carboxymethylated C-terminus (Catty et al., 1991). The standard procedures for PDE6 purification start with hypotonic extraction of PDE6 from ROS membranes followed by protein purification using ion exchange chromatography and gel filtration chromatography (see Methods). Previous work in our lab demonstrated that GARP2 co-purifies with PDE6 during these standard purification protocols (Pentia et al., 2006), suggesting that GARP2 is a high-affinity PDE6 binding protein. Thus, additional treatment is needed to disrupt PDE6-GARP2 interactions. In this section, several methods have been developed to selectively separate these two proteins for further study.

High salt disrupts GARP2-PDE6 interactions and allows separation by hydrophobic interaction chromatography (HIC). In order to obtain purified native
GARP2, a high concentration of ammonium sulfate is applied to disrupt the high affinity interaction between GARP2 and PDE6. Following the high salt treatment, the mixture of GARP2 and PDE6 is subjected to hydrophobic interaction chromatography (HIC). HIC utilizes noncovalent interactions between proteins and the hydrophobic surface of the HIC matrix to separate the proteins. This reversible interaction is significantly changed by the presence of specific concentrations of salts in the running buffer. Usually, a high concentration of salt enhances the interaction between proteins and HIC medium while a low concentration of salt causes the interaction to weaken. In HIC, the proteins with the lowest degree of hydrophobicity are eluted first and the most hydrophobic proteins, which need a greater reduction in salt concentration to reverse the interaction, elute last.

In this study, purified ROS membranes were homogenized and washed with isotonic buffer to remove the soluble protein followed by washing the ROS membranes in a hypotonic buffer with a high concentration of Mg^{2+} to bind PDE6 tightly to ROS membrane. The complex of PDE6 and GARP2 was extracted with hypotonic buffer and supplied with a high concentration (500 mM) of ammonium sulfate to disrupt PDE6-GARP2 interactions. The mixture of GARP2 and PDE6 in the high salt solution was loaded onto the hydrophobic interaction chromatography column (butyl-Sepharose). The column was washed with a step-gradient of ammonium sulfate (500 mM, 400 mM, 150 mM and 0 mM). As shown on Fig. 2-4, it was found that 500 mM ammonium sulfate (AS) was able to dissociate GARP2 from its binding sites on PDE6 and PDE6 was detected at 150 mM AS based on its catalytic activity, while GARP2-containing fractions were identified in fractions lacking AS, based on Slot Blot analysis. Further purification of PDE6 or GARP2 was performed on an anion exchange column (Mono Q). The harsh AS
treatment may shorten the active lifetime of these proteins, although we can isolate native GARP2 from PDE6.

Low concentration of detergent can solubilize GARP2 from ROS membrane without releasing PDE6. We also examined whether detergent could disrupt the interactions between GARP2 and PDE6. During the standard PDE6 purification procedure, purified, disrupted ROS were first washed in isotonic buffer (IS) to remove the soluble proteins. The ROS membranes were then washed in 10 mM Mg\(^{2+}\) hypotonic buffer (HM) to make the rod PDE6 tightly associate with ROS membrane while releasing other membrane associated proteins. Finally, PDE6 was eluted from the membranes with
hypotonic buffer lacking Mg$^{2+}$. Usually, GARP2 is co-extracted with PDE6 under this hypotonic condition (Pentia et al., 2005).

In this section, we tested two detergents [CHAPS (a zwitterionic detergent) and Triton X-100 (a non-ionic detergent)] with an isotonic buffer. ROS homogenates were incubated in a series of CHAPS concentrations (3 mM, 5 mM, 10 mM, 30 mM and 50 mM) as well as a series of Triton X-100 concentrations (0.2%, 0.3% and 1%). Following centrifugation, the amount of released PDE6 in each soluble fraction was determined in a PDE6 activity assay and the amount of GARP2 was tracked using an immunoblot assay. We observed that 1% Triton X-100 extracted most of GARP2 from ROS membranes, while ~70% of the PDE6 remained membrane-bound; In contrast, CHAPS released more than 30% of PDE6 into the supernatant under conditions that solubilized most of GARP2 *(data not shown).* For this reason, we focused subsequent efforts on Triton X-100.

In order to reduce the amount of PDE6 released with GARP2 by Triton X-100, we replaced the isotonic buffer with high Mg$^{2+}$ buffer. As shown in Fig. 2-5, low concentrations of Triton X-100 (e.g., 0.1%) can solubilize all of the GARP2 from ROS membranes without affecting membrane attachment of PDE6 (< 10% released). This method confirms that the high-affinity interaction between GARP2 and PDE6 can be disrupted by detergent to permit isolation of GARP2-free PDE6 without affecting PDE6 catalytic activity.
Fig. 2-5. Low concentration of detergent selectively solubilizes GARP2 from ROS membranes.

Aliquots of ROS homogenate were extracted twice with the indicated concentration of Triton X-100 in high Mg2+ hypotonic buffer. After centrifugation soluble and pellet fractions were analyzed by immunoblot assay. **A.** GARP2 localization upon Triton X-100 addition. On the immunoblot, 1st extracted supernatant (1S), 2nd extracted supernatant (2S) and the extracted ROS membrane (M) were probed with GARP2 FPC and SP primary antibodies to track its location in the presence of Triton X-100; the circles show the GARP2 signal remaining on ROS membrane. **B.** Solubilization of PDE6 by Triton X-100. The percent of released PDE6 at each Triton concentration was determined with a PDE6 activity assay; the arrow indicates the optimized concentration of Triton.

PrBP/δ binds to and solubilizes PDE6 from ROS membranes without solubilizing GARP2. The second approach we developed utilized the PDE6 binding protein, prenyl binding protein (PrBP/δ) to specifically release PDE6 from the rod outer segment membrane. PrBP/δ comprises a single domain that serves to covalently bind prenyl groups attached to proteins, specifically farnesyl- and geranylgeranyl-containing proteins (Hanzal-Bayer et al., 2002). The binding of PrBP/δ to a prenylated protein allows the protein to be solubilized from the membrane. *In vitro*, PrBP/δ has been shown to interact with the α and β subunits of PDE6 as well as some other phototransduction proteins.
Previous work in our lab has shown that recombinant (His)$_6$-PrBP/δ interacts with PDE6 in solution and that recombinant frog PrBP/δ can solubilize PDE6 from frog ROS membranes (Norton et al., 2005).

In order to isolate PDE6 and GARP2, bovine PrBP/δ with a 6xHis fusion tag was incubated with bovine ROS homogenates at the increasing ratio of (His)$_6$-PrBP/δ per PDE6 (Fig. 2-6). A PDE6 activity assay was utilized to evaluate the amount of PDE6 released from ROS membranes and immunoblot analysis was used to track PDE6 and GARP2 co-localization. As shown on Fig. 2-6B, the PDE6 activity assay shows that greater than 90% of PDE6 was solubilized with a 20-fold molar excess of (His)$_6$-PrBP/δ over PDE6. This result agrees with the detected PDE6 immunological assay of the supernatant fraction (Fig. 2-6A). As for GARP2, no significant amount of GARP2 was detected by immunoblot in the soluble fraction even with a 50-fold molar excess of (His)$_6$-PrBP/δ (Fig. 2-6A).

To further elucidate the specific binding of PrBP/δ to PDE6, GST-PrBP/δ and PrBP/δ (cleaved product of GST-PrBP/δ) were also compared and no significant difference was observed under the same experimental conditions (data not shown). For the (His)$_6$-PrBP/δ, shorter incubation times with ROS homogenates were also tested and we found that 30 min incubation is sufficient to release more than 95% of the PDE6 from ROS membranes at the ratio of 20 (His)$_6$-PrBP/δ per PDE6 (data not shown). To determine how much of the (His)$_6$-PrBP/δ remained bound to PDE6 after solubilization, the solubilized fraction was loaded onto Ni-NTA beads. The SDS-PAGE analysis on the elution fraction from Ni-NTA beads suggested that (His)$_6$-PrBP remains bound to PDE6 (data not shown). This method has been optimized to purify GARP2-free PDE6 in large
scale (see Methods) and the yield of PDE6 is about two-fold greater that PDE6 purified under the standard hypotonic extraction method (Pentia et al., 2005). The purity of PDE6 is also improved, but the high-affinity bound PrBP/δ failed to be removed from PDE6 in further purification including ion exchange and gel filtration. These results suggest that PrBP/δ can be used as a helpful tool to release PDE6 from the rod outer segment membrane without solubilizing GARP2 from its membrane-associated state.

*Mild proteolysis treatment is used to dissociate PDE6 from ROS membrane without effects on GARP2.* In order to avoid the potential effects of PrBP/δ binding to PDE6, another approach to separate PDE6 and GARP2 was evaluated that used gentle proteolysis. The catalytic subunits of PDE6 anchor the holoenzyme to ROS membranes through their prenylated, carboxymethylated C-termini (Catty et al., 1991). The hydrophobic C-terminal regions of α and β subunits present several trypsin cleavage sites, which allows trypsin digestion under mild conditions without further disrupting the structure of PDE6 holoenzyme. In this study, 1 μg/ml trypsin in isotonic buffer was incubated with ROS homogenate at 4 °C followed by quenching the protease reaction at each time point (30 min, 60 min and 90 min) by adding 10 μg/ml soybean trypsin inhibitor (STI). The ROS homogenates were spun down to separate the soluble and membrane fractions. An immunoblot assay with both GARP2 and PDE6 antibodies was performed to track the location of PDE6 and GARP2 after this treatment. As shown in Fig. 2-7A, increasing amounts of PDE6 were detected in the supernatant fractions upon longer incubation time with trypsin, but no significant amount of GARP2 was released to the supernatant fractions even after 90 min proteolysis treatment. The efficiency of trypsin to release PDE6 was also determined by PDE6 activity assay. As shown on Fig.
2-7B, the percent of total released PDE6 increased as the proteolysis incubation time increased, which agrees with the results of the immunoblot. In addition, there was no shift on the band representing the PDE6 catalytic subunit observed on the immunoblot before and after trypsin digestion, which indicates that this proteolytic process did not cause damage to catalytic subunits of PDE6. These observations suggest that a low concentration of trypsin for digestion is able to release PDE6 from ROS membrane without disrupting the membrane association of GARP2.

Because the inhibitory γ subunit of PDE6 is also sensitive to trypsin and the trypsin digestion of Py allows PDE6 to be activated, further evaluation was required to test the state of PDE6 after trypsin treatment. A PDE6 activity assay for measuring non-activated PDE6 was performed to determine the percent of non-activated PDE6 in the released PDE6 fractions. As presented in Fig. 2-7B, only 15% of released PDE6 was activated after a 90 min proteolysis (represented as triangles), which suggested that using this mild proteolysis condition, most of the PDE6 can be isolated from ROS membranes without disrupting its structure or activating it. In summary, we conclude that after incubation for 60 min under 4 °C at a low concentration of trypsin (1 μg/ml), ROS membranes are able to release 70% of PDE6 without activating it, whereas no significant amount of GARP2 was detected in the extracted fraction.

To summarize, several methods have been successfully developed to separate GARP2 and PDE6, including HIC (yielding GARP2-free PDE6 and PDE6-free GARP2), selective detergent extraction (yielding PDE6-free GARP2 and GARP2-free ROS membranes), mild treatment with proteases (resulting in GARP2-free PDE6 and PDE6-free ROS membranes), and use of the PDE6 binding protein, PrBP/δ (providing GARP2-
**Fig. 2-6. Prenyl binding protein (PrBP/δ) selectively solubilizes PDE6 but not GARP2.**

Purified ROS membranes were homogenized and washed with isotonic buffer in the dark. A rhodopsin spectrum was used to estimate the PDE6 concentration. ROS membranes were incubated with isotonic buffer supplemented with 10, 20 or 50 PrBP/δ per PDE6 (mole/mole) and the mixture separated by centrifugation. **A.** Protein localization upon PrBP/δ addition. Western blot with PDE6 NC64; GARP2 FPc and PrBP/δ FL antibodies were used to track the proteins (T: total ROS homogenate; S: solubilized fraction; M: extracted ROS membrane). **B.** Solubilized PDE6 as a function of PrBP/δ concentration. PDE6 solubilization was quantified by an activity assay, normalized to the total PDE6 activity present in the sample.

**Fig. 2-7. Mild protease treatment selectively releases PDE6 from ROS membranes.** ROS homogenates (containing 300 nM PDE6) were incubated in an isotonic buffer containing trypsin for 30, 60 or 90 min, and the proteolysis reaction was quenched by soybean trypsin inhibitor. **A.** PDE6 and GARP2 localization during proteolysis. Western blot with PDE6 NC64 antibody was utilized to examining the integrity of PDE6, and GARP2 FPc was used to track the location of GARP2 after protease treatment (T: total ROS homogenate; S: solubilized fraction and M: ROS membrane). The circles indicate the released PDE6 and membrane-associated GARP2. **B.** Time course of released PDE6 from membranes during proteolysis. The percent of PDE6 at each time point was determined by PDE6 activity assay. The percent of released PDE6 in the total (circles) and the percent of activated PDE6 in released PDE6 (triangles) indicates that this proteolysis condition can digest the isoprenylated C-termini anchor of PDE6 to disk membranes without activating it and release >70% PDE6 from membrane attachment while leaving GARP2 bound to ROS membranes.
free PDE6 and PDE6-free ROS membranes). These approaches provide useful tools to prepare PDE6-free GARP2 and PDE6 lacking of GARP2 for work described elsewhere in this thesis.

**Preparation and purification of recombinant GARP2 and GARP2 fragments.** Although several separation methods were optimized in last section to isolate GARP2 from PDE6, purification of biochemical quantities of native GARP2 from photoreceptor cell extracts has proven very challenging. As an alternative, expressing recombinant proteins generated by inserting bovine GARP2 gene into an *E. coli* expression vector has been optimized to achieve greater quantities of GARP2. In addition, inclusion of affinity tags on recombinant GARP2 (rGARP2) permits pull-down assays in which the protein with the affinity tag can be coupled to a specific resin as a bait to evaluate its interaction with other proteins. In this section, two affinity tags [i.e., 6xHis and glutathione S-transferase (GST)] were each subcloned with the bovine GARP2 gene, and the conditions for the expression and purification of these rGARP2 proteins were optimized.

*Expression and purification of 6xHis tagged GARP2 proteins in E. coli.* A 6xHis tagged GARP2 (bovine GARP2 gene) was previously generated in our lab, but errors in the GARP2 DNA sequence were discovered during plasmid sequencing, and the protein product of this plasmid has not been corrected. Thus, PCR primers were redesigned and the bovine GARP2 gene was cloned into a pET-47b vector. The pET-47b vector was for rGARP2 expression because it permits affinity purification with the histidine-tagged N-terminus and subsequent cleavage with HRV3C protease when needed. Other proteases were also evaluated but we experimentally determined that all but HRV3C can degrade GARP2.
The pET47b-GARP2 clone was first expressed in BL21(DE3) cells, but very little expressed protein was detected with assay with GARP2 antibodies, even after various expression conditions were tested. Further study found that the predominant codons (CCA and CCC) for the amino acid proline in mammalian GARP2 sequence are very low frequency codons in the E. coli expression system. However, these 2 codons are used for 34 of the 51 proline residues in GARP2. Additionally, another low frequency codon was used for 7 of the 11 arginine residues. In order to increase the expression efficiency, E. coli Rosetta (DE3) cells were used, because the Rosetta strain contains a plasmid that expresses the tRNAs for these low frequency codons, thereby enhancing the expression of eukaryotic proteins.

The induction conditions for 6xHis tag GARP2 expression were optimized by varying the concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG), the temperature and the incubation time. IPTG at 0.2 mM, 0.5 mM and 1 mM were tested to induce GARP2 expression and it was found that 1 mM resulted in the highest expression level of (His)₆-GARP2. It was also found that part of the expressed (His)₆-GARP2 was degraded in the intact cells induced for 3 hours at 30 °C. It is possible that the natively unfolded GARP2 is very sensitive to proteases present in the E. coli host cell or some special characteristics of GARP2 itself cause it to be targeted for degradation as a foreign protein. In order to reduce the degradation of (His)₆-GARP2, induction conditions for (His)₆-GARP2 expression were tested with a time course (2 hours to 18 hours) at several temperatures (15 °C, 20 °C, 30 °C and 37 °C). Intact cells harvested for each condition were tested for the amount of GARP2 using immunoblot assays with both GARP2 antibodies and His tag specific antibody. The results indicated that low temperatures
(15°C or 20 °C) and shorter times of incubation (2~3 hours) were helpful in reducing the degradation of the protein. In the end, the optimized expression conditions used for (His)₆-GARP2 were 1 mM IPTG at 20°C for 2 hrs.

To disrupt the bacterial cells, several conditions such as the addition of detergent (B-PER) in the lysate buffer, freeze/thaw cycles or sonication were evaluated before large scale purification. Comparison of the lysing efficiency as well as the extent of GARP2 let us to adopt the following cell lysis conditions: ~3 mg cell pellet, 15 cycles (10 sec sonication and 30 sec rest on ice) of sonication for a total of 4 min of sonication.

We optimized conditions for the Ni-NTA affinity purification method as well: 10 mM imidazole in the binding buffer, washing the nonspecifically bound proteins with 60 mM imidazole, and eluting (His)₆-GARP2 with 250 mM imidazole. As shown in Fig. 2-8, Ni-NTA affinity purification results in (His)₆-GARP2 (apparent MW at 50 kDa) with 90% purity. The yield (~0.7 mg per liter bacterial culture) was modest compared to other recombinant proteins used in the lab. The purity was evaluated by SDS-PAGE and immunoblots with both anti-His and anti-GARP2 antibodies. We were unable to remove 6xHis tag from the purified recombinant protein for reasons that remain unclear.

In addition to generating full-length GARP2 (299 a.a.), we created constructs of various regions of bovine GARP2 (also inserted into the pET47b vector). The truncated forms of GARP2 we expressed were: N-terminal region (1-150 a.a.), the central region (75-225 a.a.) and the C-terminal region (151-299 a.a.). These (His)₆-GARP2 fragments were expressed and purified using similar conditions to those for full-length GARP2. Similar to full-length GARP2, the apparent MW of each fragment on SDS-PAGE did not agreed with the calculated MW (~17 kDa), which may be the result of the high content of
Fig. 2-8. Affinity purification on (His)$_6$-GARP2.
A. Coomassie-stained SDS-PAGE on Ni-NTA affinity purification. The total cell lysate after sonication and the eluted, purified (His)$_6$-GARP2 fractions were loaded onto SDS-PAGE followed by staining with Coomassie blue. B. Western blot to test purified 6xHis-GARP2. Three GARP2 primary antibodies (ABR, FPc and SP) and one primary antibody to the 6xHis tag were used to visualize rGARP2 on the immunoblots.

Fig. 2-9. Affinity purification on (His)$_6$-GARP2 fragments.
A. Coomassie-stained SDS-PAGE of affinity purified (His)$_6$-GARP2 fragments. For each (His)$_6$-GARP2 fragment, 2 µg and 5 µg purified protein were loaded on SDS-PAGE to evaluate their purity. NT: N-terminal (His)$_6$-GARP2 (1-150 a.a.); CT: C-terminal (His)$_6$-GARP2 (151-299 a.a.); and CENT: central (His)$_6$-GARP2 (75-225 a.a.). B. Western blot to evaluate purified His-GARP2 fragments. Same amount of different His-GARP2 proteins (1: NT (His)$_6$-GARP2; 2: CT (His)$_6$-GARP2; 3: CENT (His)$_6$-GARP2; 4: Full-length (His)$_6$-GARP2) were tested on immunoblots with GARP2 ABR, FPc, and 8G8 primary antibodies.

negatively charged amino acids found in GARP2. As shown in Fig. 2-9, N-terminal
GARP2 was found at ~27 kDa on SDS-PAGE and is recognized with GARP2 FPc and 8G8 antibodies. The C-terminal GARP2 fragment migrates at 25 kDa and is recognized with GARP2 FPc and ABR antibody. The central region of GARP2 appears at ~22 kDa on SDS-PAGE and is recognized with GARP2 FPc antibody. These three recombinant GARP2 fragments were expressed and purified with a yield of ~0.5 mg per liter cell culture.

Expression and purification of glutathione S-transferase (GST)-tagged GARP2 proteins in E. coli. In order to improve the yield of recombinant GARP2 and eliminate the effect of the affinity tag on the biochemical functions of recombinant GARP2, another construct was generated by inserting bovine GARP2 into pGEX6p vector to obtain GST-tagged GARP2. The pGEX6p vector provides both the GST tag for affinity purification and an HRV3C cleavage site for removing the tag at the N-terminal end.

The expression level of bovine GARP2 in pGEX6p was compared between two host cell lines, BL21 and Rosetta, and at various OD600 levels at the time of induction. As shown in Fig. 2-10, the expressed GST-GARP2 migrates at ~70 kDa although its calculated MW is about 58 kDa. For both cell lines, the cells induced at a higher OD600 expressed higher levels of GST-GARP2, but they also resulted in more degraded products. The Rosetta cell line provided more expressed protein than the standard BL21 strain. The optimal expression conditions used Rosetta host cells and induced the cells when the culture reached an OD600 of 0.6. The optimized induction conditions were similar to (His)6-GARP2, using 1 mM IPTG for 2 hours at 20 °C.

Glutathione affinity purification (see Methods) was performed to purify GST-GARP2, but the purity of the protein was low. As shown on the first lane on the
Coomassie-stained SDS-PAGE in Fig. 2-11, multiple bands under 70 kDa appeared in the affinity-purified GST-GARP2 sample, which indicated that the greatest percent of contaminating proteins should be truncated GST-GARP2 products generated during the expression and purification. In order to reduce the degradation level, the concentration of bacterial protease inhibitor cocktail was increased and some mammalian protease inhibitors were also added to the cell lysate buffer. However, no significant improvement was observed in the purified GST-GARP2 sample, so gel filtration was used to remove the truncated proteins. The affinity-purified GST-GARP2 was applied to a gel filtration column and the fractions (B5-B14) from the gel filtration purification were subjected to SDS-PAGE. As shown in Fig. 2-11, the fractions from B5 to B9 contained the most GST-GARP2 at 70 kDa, while the dominant proteins found in fractions after B9 were truncated products. These results suggest that gel filtration effectively purified GST-GARP2 (~70 kDa) from other fragments. The same fractions were also tested on immunoblot with both GARP2 antibody and GST tag antibody with results suggesting that GST-GARP2 was successfully purified by these methods and that the contamination from affinity purification was indeed truncated products with the GST tag, and that they can be successfully removed by gel filtration. The yield of purified GST-GARP2 is 1.5 mg per liter bacterial culture, which is two-fold higher than of 6xHis tagged GARP2.

In order to cleave the GST moiety, HRV3C digestion conditions were optimized to be 1 unit HRV3C enzyme per 25 µg GST-GARP2 at 4 °C for 6 hours. The digested mixture was loaded onto glutathione beads to separate cleaved GARP2 from GST. The MW of purified GARP2 on SDS-PAGE is ~50 kDa, similar to native GARP2 (Fig. 2-12).
**Fig. 2-10. Optimize the expression conditions for GST-GARP2.**

Rosetta and BL21 stains of *E. coli* were induced at 0.2, 0.4, 0.6 and 1.0 OD600 for 2 hours at 20 °C. Under each condition, the same volume of bacterial cells was lysed and both the intact whole cell (W) and the lysate (L) were loaded on SDS-PAGE followed by Western blot. GARP2 FPc antibody (red) and GST tag primary antibody (green) were utilized to indicate the expression level of GST-GARP2.

**Fig. 2-11. GST-GARP2 purification using immobilized glutathione and gel filtration.**

GST-GARP2 (~ 70 kDa) was expressed in Rosetta cells. Affinity purification on immobilized glutathione beads resulted in poor purity of the protein. The full-length GST-GARP2 was detected by both anti N- and C-terminal of GST-GARP2 [anti-GST tag antibody (green color) and GARP2 ABR antibody (red color)].

**Fig. 2-12. GST moiety cleaved from GARP2 by HRV3C protease.**

Cleaved GARP2 was separated from GST with immobilized glutathione beads. 1: GST-GARP2 before cleavage (~70kDa); 2: HRV3C-cleaved mixture; 3: Supernatant (GARP2 portion); 4: GST tag bound to beads.
In summary, we successfully purified milligram quantities of GARP2. Furthermore, recombinant GARP2s with two different "tags" allow them to be used in pull-down assays to study binding interactions with PDE6.

**Characterize the biochemical and biophysical properties of GARP2.** The ability to generate and purify greater quantities of recombinant GARP2 allows us to characterize the biochemical and biophysical properties of rGARP2 to reveal its regulatory function on the activity and lifetime of PDE6 in rods.

In order to study the biochemical functions of GARP2, purified (His)_6-GARP2 at specific concentrations were incubated with PDE6 holoenzyme, fully-activated PDE6 (Pαβ) and transducin-activated PDE6 (Ta*-GTPγS). The concentration of (His)_6-GARP2 was determined with a Bradford protein assay with BSA as the standard protein, and the activity of these three kinds of PDE6 were measured in a PDE6 activity assay. As shown in Fig. 2-13, the presence of 100-fold molar excess (His)_6-GARP2 per PDE6 reduced PDE6 basal activity by 40%, while the large excess of recombinant GARP2 showed no effect on the activity of Pαβ and Ta*-GTPγS activated PDE6. The same experiment has been repeated with purified GST-GARP2 and GARP2 after cleaving the GST tag, and similar results were obtained. This result agrees with previous work in our lab in which the addition of exogenous native GARP2 on ROS
membrane only suppressed the basal activity of PDE6 holoenzyme, not the activated forms of PDE6 (Pentia et al., 2006). The similar suppressing effect of native and recombinant GARP2 on PDE6 basal activity indicates that purified recombinant GARP2 presents similar biochemical properties to those of native GARP2.

In order to evaluate the interaction between GARP2 and PDE6, immunoprecipitation and pull-down assays were performed on PDE6 and recombinant GARP2. In the immunoprecipitation assay, ROS1, a specific antibody against PDE6 holoenzyme, was coupled to AminoLink beads and incubated with the hypotonic extract from ROS membranes (positive control) or the mixture of (His)_6-GARP2 and GARP2-free PDE6. The bound and unbound fractions were separated by centrifugation and loaded onto SDS-PAGE followed by an immunoblot with both GARP2 and PDE6 antibodies. As shown on Fig. 2-14, ROS1-coupled beads successfully immunoprecipitated PDE6 with its high-affinity bound native GARP2 in the ROS hypotonic extract sample, which agrees with a previous study (Pentia et al., 2006). Similarly, (His)_6-GARP2 was co-immunoprecipitated by ROS1-coupled beads with PDE6 lacking native GARP2, which demonstrates that the recombinant GARP2 can bind PDE6 with high affinity.

To further test the interaction between GARP2 and PDE6, (His)_6-GARP2 was coupled to Ni-NTA beads ("bait") followed by incubation with hypotonically purified PDE6 holoenzyme (containing endogenous GARP2). The nonspecifically bound proteins were washed out and the bound fractions were examined by immunoblot assay. Fig. 2-15 shows that purified PDE6 holoenzyme was detected in the (His)_6-GARP2 pulled down fraction and nonspecific binding of PDE6 was minimal on the empty control beads. A
similar pull down assay was repeated by coupling GST-GARP2 to glutathione beads to pull down GARP2-free PDE6 (see Methods). As shown in Fig. 2-15, GST-GARP2 can also pull down PDE6 lacking native endogenous GARP2. These results from the pull down assays indicate that the high-affinity interaction between GARP2 and rod PDE6 can be reconstituted with purified PDE6 and recombinant GARP2 (6xHis tag or GST tag). This pull down assay with affinity-tagged GARP2 can be utilized as a tool to study the interacting sites between GARP2 and PDE6, and also to identify other GARP2 interacting proteins in rod photoreceptors.

In addition to the biochemical characteristics of GARP2, the biophysical properties of GARP2 were determined in order to better understand the structure and function of GARP2. The hydrodynamic properties of GARP2 were studied with size-exclusion chromatography and analytical ultracentrifugation. In the core of gel filtration, the hydrodynamic features such as mass and shape are reflected by the elution volume of a protein from a size-exclusion column. We used purified GARP2 (GST-GARP2 lacking GST tag) and loaded it onto a size-exclusion column. GARP2 eluted at volumes suggesting an apparent MW of 200~250 kDa (data not shown), much larger than predicted by its amino acid sequence (32 kDa). This observation indicates that GARP2 may be present in an oligomeric state under those conditions or that GARP2 has a hydrodynamic radius much larger than that of a typical globular protein of this MW.

In order to examine whether GARP2 exists in an oligomeric state, sedimentation velocity analysis of GARP2 was performed. Sedimentation velocity measures the rate at which molecules move in response to centrifugal force to provide hydrodynamic information (size, shape and interactions of macromolecules) in solution. Purified (His)$_6$-
GARP2 or GARP2 moiety from GST-GARP2 was first labeled with 5-iodoacetimidofluorescein (5-IAF) and free 5-IAF dye was removed by gel filtration. As shown on Fig. 2-16A, (His)$_6$-GARP2 has a peak at ~1.3 S and multiple peaks at higher S values (3.5 S~6.5 S), suggesting that (His)$_6$-GARP2 exists in a monomer-multimer equilibrium state. In contrast, only one peak at 1.6 S was detected for GARP2 in which the GST tag has been removed (Fig. 2-16B). The different sedimentation velocity profiles between (His)$_6$-GARP2 and GARP2 indicated that the oligomeric state of (His)$_6$-GARP2 may be caused by the affinity tag instead of the GARP2 protein itself. The S value of GARP2 protein at 1.6 S corresponds to Stokes radius of 46 Å, which means that GARP2 is a natively unfolded protein, unlikely to have significant secondary or tertiary structure.

To summarize, the biochemical properties of recombinant GARP2s were evaluated by a PDE6 activity assay and a pull-down assay. The results suggest that they have similar properties to native GARP2 including suppressing the basal activity of PDE6 and binding to PDE6 with high affinity. Analytical ultracentrifugation also revealed that the sedimentation coefficient of GARP2 is consistent with GARP2 being a natively unfolded protein.
Fig. 2-14. Using PDE6 ROS1 antibody to immuneprecipitate GARP2.
The ROS1 antibody was incubated with a mixture of His-GARP2 and a PDE6 holoenzyme lacking endogenous GARP2 (GF-PDE6). ROS1 beads were also similarly incubated with the hypotonic extract from ROS membrane (ROS Hypo). The total mixture (L), unbound fraction (U) and immunoprecipitated fraction (B) were tested in a Western blot assay using PDE6 NC64 antibody and GARP2 FPc antibody.

Fig. 2-15. Using recombinant GARP2 to pull down PDE6. A. 1 nM PDE6 was mixed with 20 μM (His)₆-GARP2 immobilized on Ni-NTA beads. PDE6 NC64 antibody and γ CT97 antibody were used to detect PDE6 holoenzyme in Western blot assay. B. PDE6 NC64 antibody and GARP2 8G8 antibody were used in a Western blot assay in order to detect binding of PDE6 to immobilized GST-GARP2. Empty beads lacking recombinant GARP2 were used to evaluate the nonspecific binding to the beads. L: total loading sample; U: unbound fraction; B: beads pulled-down fractions.

Fig. 2-16. Sedimentation velocity of GARP2/5-IAF.
A. 10, 30 and 100 nM (His)₆-GARP2 labeled with 5-IAF dye were tested in AU-FDS at 60,000 RPM with 500 scan (1 min/scan). It has its first peak at ~1.3 S and multiple peaks at higher S values. B. 30 nM and 100 nM GARP2 was labeled with 5-IAF and applied to AU-FDS. Only one single peak presents as GARP2 at 1.6 S, which corresponds to Stokes radius of 46 Å. AU-FDS performed and analyzed by Sue Matte.
Phylogenetic analysis of CNGB1 gene and alternative splice products. The β subunit of the cGMP-gated channel gene in rod photoreceptors (CNGB1) is a member of the cyclic nucleotide-gated channel (CNG) gene family. In humans, there are six CNG gene members including four α subunits and two β subunits (Kaupp et al., 2002). CNGA1 and CNGB1 are expressed in rod photoreceptors, while CNGA3 and CNGB3 are expressed in cone photoreceptors. In addition to photoreceptors, other cell types also express CNG subunits, for example, CNGA2 is functional in olfactory sensory neurons and CNGA4 in taste receptor cells (Nordstrom et al., 2004).

In mammalian rod photoreceptors, the cyclic nucleotide gated channel consists of three α subunits (CNGA1) and one β (CNGB1) subunit (Shuart et al., 2011). The molecular characterization of CNGB1 in bovine, human and mouse indicated that CNGB1 presents an unusual bipartite structure, in which the N-terminal portion of CNGB1 is a glutamic acid-rich (GARP) region and the C-terminal region is the channel-like domain responsible for the cGMP regulated ion gating function (Korschen et al., 1995; Ardell et al., 1995; Colville et al., 1996; Colville et al., 1996). In addition to the GARP region on the N-terminal portion of CNGB1, there are also two soluble forms of glutamic acid-rich protein, GARP1 and GARP2, found in rods and they are determined to be the alternative splice products of the CNGB1 gene (Sugimoto et al., 1991). A genomic study on CNGB1 gene in Dr. Pittler's lab has mapped the CNGB1 locus encoding the GARP and CNGB1 transcripts to human chromosome 16q13 region and along with determining the gene structure of the human CNGB1 (Ardell et al., 2000). The human CNGB1 gene (~100 kb) consists of 33 exons including the GARP exons (1-16 exons) and the channel-like exons (17-33 exons). The transcript of the β subunit of the channel is
encoded by 1-33 exons; the GARP2 transcript is encoded by exon 1-12 with an additional 12a exon. While the GARP1 transcript is encoded by exon 1-16 and one alternative splicing exon β4b excluding exon 12a (Ardell et al., 2000). The presence of multiple alternative splicing products of CNGB1 gene suggests that CNGB1 gene is complex and its alternative splice products may have multiple roles in rod photoreceptors.

In order to better understand the GARP proteins, we did genomic analysis of the CNGB1 gene from multiple vertebrate species, and found CNGB1 orthologs in most vertebrate species including mammalians, birds and fishes. To examine GARP2 specificity, both human and bovine GARP2 mRNA sequences were used as a query to blast other database to characterize the occurrence of GARP2 in other species. However, no other species were found to have GARP2 gene products except for cow, human and mouse.

A multiple sequence alignment of CNGB1 proteins across vertebrate evolution was performed. A list of the species in which CNGB1 or GARP proteins were found is presented in Appendix I. The alignment suggests that mammals have longer CNGB1 amino acid sequences compared with amphibian and fish. In these organisms, the N-terminal one third of the mammalian amino acid sequence is missing. The multiple sequence alignment reveals that several conserved regions in the sequence of CNGB1, including the major portion of the calmodulin binding domain (567-599 a.a.), a part of transmembrane domain (655-769 a.a, 789-985 a.a.) and the whole region of cGMP binding domain (789-1083 a.a.). The phylogenetic tree of CNGB1 is shown in Fig. 2-17 suggesting that CNGB1 evolution follows expectations based on accepted evolutionary distance of various vertebrate families.
Because GARPI and GARPII are only observed in three species (human, cow and mouse), we repeated our multiple sequence alignment on the N-terminal region of CNGB1 (a.a.1-370 of human) to seek genomic evidence for the presence of GARPI-like proteins in other species. In mammals, the N-terminal region appears variable, with some
limited blocks of amino acid residues that appear more highly conserved. As seen in the Fig. 2-18, the first 20 amino acid are highly conserved and a.a. 267-272 is also conserved.

A phylogenetic tree of the N-terminal 370 a.a. is shown in Fig. 2-19. These results suggest that the N-terminal region of CNGB1 has some stretches of amino acids that are under selective pressure, whereas most of the region appears variable.

Fig. 2-18. The sequence alignment for GARP2 region of vertebrate CNGB1, using bovine sequence as the reference. A multiple sequence alignment of the N-terminal region of CNGB1 (a.a 1-371 of the human CNGB1 sequence) (shown in Appendix II). The accession numbers and source of the CNGB1 sequences used in this study are listed on Appendix I. Red represents unanimous sites, while blue reflects high sequence variation.
Summary

To summarize, using improved immunological methods with different primary antibodies, we are able to characterize and localize GARP2. We also improved several approaches to isolate PDE6 from its high-affinity bound GARP2 to prepare GARP2-free PDE6 and GARP2 lacking PDE6. In addition, recombinant GARP2 was constructed as a fusion protein with 6xHis or GST tag. Our initial work suggests that recombinant GARP2
behaves similarly to native GARP2. The multiple sequence aliments of CNGB1 and the N-terminal region of CNGB1 in vertebrate species also suggests that the GARP region may have been added to the CNGB1 gene during evolution to enhance rod photoreceptor function.
CHAPTER 3

REGULATION OF THE CATALYTIC AND ALLOSTERIC PROPERTIES OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BY THE GLUTAMIC ACID-RICH PROTEIN-2 (GARP2)

Abstract

As the central effector of the visual signaling pathway in photoreceptor cells, photoreceptor phosphodiesterase (PDE6) is precisely regulated to control the sensitivity, amplitude, and lifetime of the light response. The aim of this study is to define the regulatory significance of the Glutamic Acid-Rich Protein-2 (GARP2) on PDE6 during phototransduction. GARP2 is a splice variant of the rod photoreceptor cGMP-gated channel β-subunit which is abundant in the outer segment of rod photoreceptors and interacts with several photoreceptor proteins. Previous work demonstrated that GARP2 is an intrinsically disordered protein that binds PDE6 and lowers its catalytic activity when PDE6 is not light-activated. In this study, we identified the inhibitory ϒ-subunit (ϒ) of PDE6 as the primary site of interaction with GARP2. Using a series of ϒ truncation mutants, we localized the primary site of GARP2 interaction to the N-terminal region of ϒ. The C-terminal region of GARP2 had the greatest influence on suppressing PDE6 basal activity. Binding of GARP2 to PDE6 holoenzyme also lowered cGMP binding to allosteric binding sites on PDE6. Finally, we determined that GARP2 decreased the
maximum extent to which the photoreceptor G-protein, transducin, can activate PDE6. Together, these results are consistent with a role for GARP2 in both lowering PDE6 basal activity to reduce “dark noise” as well as in reducing the efficiency of transducin activation of PDE6 during photoreponse saturation of rod photoreceptors.

**Introduction**

Cyclic GMP (cGMP) is the primary intracellular messenger for the visual transduction cascade in retinal rod and cone photoreceptor cells. Light stimulation causes the photoisomerization of the visual pigment molecule (opsin) in the outer segment of rod and cone photoreceptor cells; each photoexcited opsin can activate over one hundred heterotrimeric G-proteins (transducin). Activated transducin, in turn, binds to and activates a cyclic nucleotide phosphodiesterase (PDE6) by displacing its γ-inhibitory subunit from the catalytic pocket and accelerating the hydrolysis of cGMP. As a result of PDE6 activation, cGMP levels in the photoreceptor fall rapidly, causing the closure of cGMP-gated ion channels in the plasma membrane of photoreceptor cells. The transient hyperpolarization of the cell membrane that results from this enzyme cascade generates the electrical response that is propagated to other retinal neurons (Arshavsky et al., 2012).

As the central effector enzyme of visual transduction, PDE6 must be precisely regulated to control the sensitivity, amplitude and kinetics of the photoresponse during excitation, termination and adaptation to light stimulation. The PDE6 holoenzyme consists of a catalytic heterodimer (Paβ) and two inhibitory γ-subunits (Py). Py is a 9.7 kDa protein which contains multiple sites of interaction with Paβ to allosterically regulate the activated lifetime of PDE6 during phototransduction (Cote, 2006; Guo et al., 2006).
2008). Previous studies have found that Py directly regulates the rate of cGMP hydrolysis
by binding of its C-terminal residues to the enzyme active site (Granovsky et al., 1997).
The central, polycationic region of Py serves an allosteric role in modulating cGMP
binding to noncatalytic regulatory sites located in the GAFα domain of each catalytic
subunit (Mou et al., 2001; Zhang et al., 2012). Additional sites of interaction of Py with
Paβ have been reported to allosterically inhibit cGMP hydrolysis by a mechanism which
is distinct from Py binding directly to the active site (Zhang et al., 2010a). Py also has
multiple sites of interaction with activated transducin α-subunit (Ta*-GTP) and the
regulator of G-protein signaling 9-1 (RGS9-1) that mediate both the excitation and
inactivation phases of PDE6 regulation during visual transduction (Slep et al., 2001; Guo
et al., 2010; Zhang et al., 2012). In spite of this knowledge, there are aspects of the
photoresponse that cannot be accounted for by established mechanisms, and hence other
regulatory mechanisms are likely to also contribute to modulation of PDE6 activation and
inactivation during phototransduction (Fain, 2011).

One candidate that may provide additional regulatory control of PDE6 is the
Glutamic Acid-Rich Protein-2 (GARP2), a 299 amino acid splice variant of the β-subunit
of the rod cyclic nucleotide-gated ion channel (CNGB1); a longer (590 a.a) splice variant
GARP1, has also been identified (Colville et al., 1996). GARP2 is expressed exclusively
in rod photoreceptor cells and localizes to the signal-transducing outer segment portion of
the cell (Korschen et al., 1999). GARP2 is considered a natively unfolded protein
exhibiting a large degree of intrinsic disorder (Batra-Safferling et al., 2006). GARP2
interacts with several phototransduction proteins including PDE6 and peripherin
(Korschen et al., 1999; Poetsch et al., 2001; Pentia et al., 2006). GARPs may also play a
role in buffering Ca$^{2+}$ concentration in rods (HaberPohlmeier et al., 2007) and act as a
gating inhibitor of CNG channels to reduce the current noise from the CNG channels in
the absence of cGMP (Michalakis et al., 2011).

The physiological role of GARP splice variants of the β-subunit of the cyclic
nucleotide-gated channel has been investigated using transgenic animals in which the
CNGB1 gene has been manipulated. Deletion of exon 26 of the mouse CNGB1 gene
(Cngb1-X26) resulted in loss of expression of the channel portion of CNGB1 without loss
of expression of the GARP1/2 region; Cngb1-X26 mice exhibited shortened rod outer
segments (ROS) but normal morphological organization of disk membranes (Huttl et al.,
2005). In contrast, complete ablation of the CNGB1 gene (Cngb1-X1) where neither the
GARP1/2 domain nor the channel domain was expressed resulted in shortened ROS and
misaligned and misshapen disks (Zhang et al., 2009). The light sensitivity and response
amplitude in rods of both Cngb1-X26 and Cngb1-X1 mice were greatly reduced or
abolished (Huttl et al., 2005; Zhang et al., 2009). In addition, overexpression of GARP2 in
transgenic mouse rods significantly increased phototransduction gain and delayed the
photoresponse recovery time (McKeon et al., 2012).

The role of GARP2 may reflect one or more of the physiological differences
between rods and cones. For example, rods exhibit much greater light sensitivity than
cones (Korenbrot, 2012), attributed in part to the lower level of “dark noise” that allows
rods to achieve single photon sensitivity (Baylor et al., 1980; Rieke et al., 1996; Rieke et
al., 2000). The smaller, continuous component of dark noise in rods has been attributed to
spontaneous activation of PDE6 independent of transducin (Rieke et al., 1996)]. A
second physiological difference between rods and cones is that the rod photoresponse
saturates in bright light, but cones do not (Korenbrot, 2012). Since the maintenance of the circulating ion current and the activity of phototransduction enzymes are major energy-requiring pathways in photoreceptors (Okawa et al., 2008), it is reasonable that rod-specific mechanisms exists to reduce cGMP metabolic flux to conserve metabolic energy during rod photoresponse saturation.

In this paper, we demonstrate that GARP2 primarily interacts with the inhibitory γ subunit of PDE6, with the N-terminal half of Py showing the high affinity for GARP2. We show that GARP2 influences PDE6 in three distinct ways: (1) suppresses basal activity of PDE6, an effect localized to the C-terminal portion of GARP2; (2) destabilizes cGMP binding to PDE6 GAF domains, and; (3) reduces the extent to which transducin activates PDE6. The ability of GARP2 to influence both the allosteric and catalytic properties of PDE6 suggests that GARP2 regulates PDE6 activity and lifetime through multiple interacting sites with the rod PDE6 holoenzyme. GARP2 may be responsible for maintaining lower levels of dark noise characteristic of rod photoreceptors, as well as reducing cGMP metabolic flux (and thus ATP consumption) under bright light conditions where the rod photoresponse is saturated.

**Materials and Methods**

*Reagents*—Mouse monoclonal anti-GARP2 antibody (8G8) to the first 15 amino acid of GARP2 was a generous gift from Dr. Robert Molday (University of British Columbia). Rabbit polyclonal anti-GARP antibodies to bovine sequences common to GARP1, GARP2 and the rod β-subunit CNGB1 was a kind gift of Dr. Benjamin Kaupp (Center of Advanced European Studies and Research, Bonn, Germany). The ROS1
mouse monoclonal antibody (Hurwitz et al., 1984a) directed against the PDE6 holoenzyme was kindly provided by Dr. Rich Hurwitz (Baylor College of Medicine). Affinity-purified anti-peptide rabbit polyclonal antibodies directed to the PDE6 GAFb domain (NC397-64), the C-terminus of the Py-subunit of PDE6 (CT-9710) and to the central region of Py-subunit of PDE6 (CENT-9712) were prepared in our laboratory. The Ni$^{2+}$-nitriloacetic acid (NTA) His-Bind resin was obtained from Novagen, and glutathione-agarose was from Thermo-Fisher/Pierce. Phospholipids were purchased from Avanti Polar Lipids, Inc. All other reagents were from Sigma Chemical Co. or from Thermo-Fisher.

Preparation of Bovine Rod Outer Segments (ROS)—ROS from frozen bovine retina (W. L. Lawson, Inc.) were prepared as described previously (Pentia et al., 2005). Briefly, ROS were isolated from frozen bovine retinas on a discontinuous sucrose gradient. ROS membranes were isolated by homogenizing ROS in an isotonic buffer (10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 2 mM MgCl$_2$, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride) using a glass, hand-held homogenizer, and the ROS membranes were separated from the soluble protein fraction by centrifugation.

Purification and Functional Assays of PDE6 Holoenzyme and Catalytic Dimer (Paβ)—PDE6 holoenzyme was purified by successive steps of protein extraction from ROS membranes, anion exchange chromatography, and gel filtration chromatography as described previously (Pentia et al., 2005). Paβ catalytic dimer lacking Py were prepared from purified PDE6 holoenzyme by limited trypsin proteolysis followed by Mono Q anion exchange chromatography (Pentia et al., 2005).
Two methods were used to purify PDE6 holoenzyme lacking bound GARP2. The first approach relied on the ability of 0.1% Triton X-100 to selectively extract GARP2 from ROS membranes. ROS membranes (depleted of soluble proteins) were resuspended in a solution containing 0.1% (v/v) Triton X-100, 5 mM Tris, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 0.3 mM PMSF at pH 7.5, and the detergent-solubilized proteins were separated by centrifugation; this process was repeated three times. Subsequently, GARP2-depleted PDE6 was extracted from the membranes in the same hypotonic buffer lacking MgCl₂ and Triton X-100. The second method used the prenyl binding protein/δ [PrBP/δ; (Goc et al., 2010)] to selectively extract PDE6 holoenzyme free of GARP2 from ROS membranes. Briefly, washed ROS membranes were resuspended in isotonic buffer containing a 20-fold molar excess of recombinant PrBP/δ per PDE6 holoenzyme. After 40 min incubation at 4 °C, the solubilized PrBP/δ-PDE6 complex was separated by centrifugation. PDE6 was subsequently purified by Mono Q and gel filtration chromatography.

The catalytic activity of PDE6 was measured using a colorimetric assay (Cote, 2000). The PDE6 concentration was estimated based on the rate of cGMP hydrolysis of trypsin-activated PDE6 and the knowledge of the $k_{\text{cat}}$ of the enzyme [5600 mol cGMP hydrolyzed per mol Paβ per second (Mou et al., 1999)].

The equilibrium and kinetic properties of [³H]cGMP binding to Paβ were determined with a filter binding assay (Cote, 2005). To minimize catalytic activity, Paβ was preincubated with 10 mM EDTA, 20 mM dipicolinic acid and 100 μM vardenafil in binding buffer (100 mM Tris, 2 mM MgCl₂, and 0.5 mg/ml bovine serum albumin) for 2 h at 22 °C. Paβ was then reconstituted with Py and incubated with the indicated
concentrations of \[^{3}\text{H}\]cGMP for 5 min at room temperature; portions were directly filtered on prewet nitrocellulose membranes (Millipore HA membrane, 0.45 mm) and quickly rinsed with three 1 ml portions of 100 mM Tris (pH 7.5). Nonspecific binding was determined as described previously.

*Expression and Purification of (His)$_6$-GARP2 and GST-GARP2*—Full-length bovine GARP2 (NP_001129113) and three fragments of GARP2 (amino acids 1-150, 75-225, and 151-299) were generated by sub-cloning the GARP2 gene into the pET47b vector which contains an N-terminal (His)$_6$ tag. Recombinant protein was expressed in *E. coli* (DE3) Rosetta cells (EMD Lifesciences) or Arctic Express cells (Agilent Technology) by induction with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) at 20 °C for 3 hours or at 10 °C for 18 h. After lysing the cells by sonication followed by centrifugation, proteins in the supernatant were purified using His-Bind affinity resin. The column was washed with 60 mM imidazole prior to elution of His-tagged GARP2 with 250 mM imidazole. Full-length GARP2 was also subcloned into the pGEX-6P-1 expression vector (containing a GST fusion partner) and expressed in Rosetta cells after IPTG induction for 3 h at 20 °C. GST-GARP2 was affinity purified using immobilized glutathione beads, and subsequently treated with HRV3C protease to remove the fusion tag. The concentrations of all recombinant proteins were determined with a colorimetric protein assay (Bradford, 1976).

*Expression and Purification of Py Mutants*—Wild-type and mutant Py were generated and purified by SP Sepharose chromatography, followed by C4 reverse-phase high pressure liquid chromatography as described previously (Zhang et al., 2012). The purity (> 95%) and molecular weight of these proteins were confirmed by sodium
dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined by the bicinchoninic acid protein assay using bovine γ-globulin as a standard.

**Pull Down Assays**—6xHis-tagged or GST-tagged proteins were pre-incubated overnight at 4 °C with Ni-NTA beads or immobilized glutathione beads, respectively. Excess protein was removed and the washed beads were incubated with potential binding partners for 2 h at 4 °C. Following centrifugation, the beads were washed three times and proteins bound to the beads were eluted in 2x gel loading buffer. To control for nonspecific binding, identical samples were incubated with Ni-NTA beads or immobilized glutathione beads lacking “bait.” The total loading material, bound proteins, and unbound proteins were subjected to SDS-PAGE followed by Western blotting to determine specific protein-protein interactions. For pull-down assays using Ni-NTA beads, the binding buffer contained 25 mM imidazole, 20 mM Tris and 100 mM NaCl, pH 8.0; the washing buffer contained 60 mM imidazole, 20 mM Tris, 300 mM NaCl with 0.015% Triton X-100, pH 8.0. For the pull-down assay using immobilized glutathione beads, the binding buffer contained 50 mM Tris, 150 mM NaCl, pH 7.5; the washing buffer contained 50 mM Tris, 300 mM NaCl and 0.015% Triton X-100, pH 7.5.

**Immunoprecipitation Assay of the PDE6-GARP2 Interaction with ROS1 Antibody**—The ROS1 antibody was coupled to AminoLink Plus coupling resin using Pierce Direct IP Kit (Thermo). For each reaction, 20 μl ROS1-coupled beads were incubated with recombinant GARP2 and GARP2-free PDE6 (500-fold molar excess GARP2 over PDE6 holoenzyme) in binding buffer (20 mM Tris, 100 mM NaCl, 2 mM MgCl₂, pH 7.5) for 2 hours at 4 °C. After washing the beads three times with binding buffer, the immunoprecipitate was eluted with sample buffer and subjected to SDS-PAGE.
PAGE and detected by immunoblot analysis using PDE6 NC antibody and GARP2 8G8 antibody.

**SDS-PAGE and Western Blotting and Protein Quantification** — SDS-PAGE was performed with NuPAGE 4-12% Bis-Tris gels. For the immunoblot procedure, proteins were transferred to a nitrocellulose membrane at 60 V for 2 h. The membranes were blocked in 5% non-fat milk in TBS buffer (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 7.5) for 2 hours followed by incubation in primary antibody solutions overnight at room temperature. After rinsing the membranes with TBS/0.05% Tween-20, the membranes were incubated with the appropriate IRDye secondary antibodies (Li-Cor) for 1 hour prior to scanning the membrane.

The intensity of individual bands of GARP2 and PDE6 on Coomassie-stained SDS-PAGE were determined using the Li-Cor imaging system software. To quantify GARP2 and PDE6, the intensity of GARP2 or PDE6 bands was referenced to a set of protein standards whose concentration was determined by either the Bradford protein assay (GARP2 and PDE6) or enzymatic activity (PDE6).

**Formation of Large Unilamellar Vesicles (LUVs)**—LUVs were utilized to improve the efficiency of transducin activation of PDE6 (Melia et al., 2000), following an established procedure (Wensel et al., 2005). Briefly, the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were mixed at molar ratio of 80:20 in chloroform, evaporated, and resuspended in a buffer containing 20 mM HEPES, 120 mM NaCl, 5 mM MgCl₂, pH 7.5. After five freeze-thaw cycles, the LUVs were formed by extruding the lipid solution ten times.
through a 0.1 μM polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids, Inc.).

Purification of Persistently Activated Transducin α-subunit—Transducin α-subunit was extracted from the PDE6-depleted ROS membranes by addition of 50 μM GTPγS in low salt buffer (5 mM Tris, 0.5 mM MgCl₂, pH 7.2). The extracted Ta*-GTPγS solution was purified by Blue-Sepharose column chromatography (Wensel et al., 2005) followed by gel filtration chromatography to completely remove PDE6.

Data Analysis—All experiments were repeated at least three times, and averages are reported as the mean ± S.D. Curve fitting was performed using Sigmaplot (SPSS, Inc.). Statistical analysis of cGMP binding experiments was evaluated using one-way ANOVA in conjunction with Tukey’s test. Significance was defined as $p < 0.05$.

Results and Discussion

GARP2 primarily binds to the PDE6 holoenzyme through interactions with the inhibitory subunit, Py. Previous work in our lab demonstrated that GARP2 binds PDE6 with high-affinity, as judged by co-purification of PDE6 with GARP2 through several stages of purification (Pentia et al., 2006). To better understand how GARP2 binds to and regulates PDE6, we developed protocols to isolate and purify PDE6 holoenzyme lacking bound GARP2 (see Methods). Using purified, GARP2-free PDE6 holoenzyme, we performed pull-down experiments with recombinant, (His)₆-GARP2 immobilized on Ni-NTA beads. We found that GARP-free PDE6 holoenzyme interacted with the immobilized GARP2 and was pulled down (Fig. 3-1A). We also confirmed the ability of exogenous GARP2 to be immunoprecipitated in a complex with PDE6 holoenzyme using
the ROS1 antibody which recognizes the PDE6 holoenzyme [(Hurwitz et al., 1984b); data not shown].

We next addressed the question of the binding stoichiometry of GARP2 to the purified, heterotetrameric PDE6 holoenzyme. To quantify GARP2 bound to PDE6, we used recombinant GARP2 as a protein standard on Coomassie-stained SDS-PAGE. PDE6

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**Fig. 3-1. Interaction of GARP2 with PDE6 subunits.** A. GARP2 interacts with PDE6 holoenzyme and Py but not Paβ. His-GARP2 (2 nmol) pre-adsorbed to 20 μl Ni-NTA beads were incubated with 5 pmol purified PDE6 holoenzyme (Paβγγ), 5 pmol catalytic dimer (Paβ), or 10 pmol Py. Equivalent amounts of initial protein sample (“T”), proteins bound to Ni-NTA beads (“B”), and proteins nonspecifically bound to control beads (lacking His-GARP2; “C”) were loaded to SDS-PAGE. Proteins were detected by immunoblot analysis using PDE6 catalytic subunit antibody (NC397-64), GARP2 antibody (8G8), or Py antibody (CT9710). B. GARP2 forms a stable complex with immobilized Py. His-Pγ (0.5 nmol) was bound to 20 μl Ni-NTA beads and then mixed with either GARP2 (1 nmol) or with known Py-interacting proteins: Paβγγ (5 pmol), Paβ (5 pmol) or Ta*-GTPγS (1 nmol). Samples bound to the Ni-NTA-Pγ beads (“B”) were separated from unbound protein (“U”), and control samples (lacking His-Pγ bait) were also tested (“C”).

was quantified by both activity measurements and the Bradford assay after correcting for the purity of the PDE6 (as determined with Coomassie-stained gels. In this way, the measured stoichiometry of GARP2 bound to purified PDE6 holoenzyme was 1.0 ± 0.2 mol GARP2/mol PDE6 (n = 3).

We next asked which subunits of the PDE6 holoenzyme are responsible for interacting with GARP2. Using (His)₆-GARP2 immobilized on Ni-NTA beads as “bait”, we performed pull down assays on purified PDE6 holoenzyme, catalytic dimer (Paβ),
and Py. As shown in Fig. 3-1A, GARP2 interacted with PDE6 holoenzyme and with Py, whereas no significant association could be detected with the Paβ catalytic dimer. We confirmed the specificity of GARP2 interaction with Py by using (His)₆-Pγ as the bait to pull down recombinant GARP2 (lacking the 6xHis tag) as well as other known Py-interacting proteins (i.e., PDE6 holoenzyme, Paβ, and activated transducin α-subunit, Ta*-GTPγS; Fig. 3-1B). We conclude that GARP2 forms high affinity interactions primarily with the inhibitory Py subunit, regardless of whether Py is associated with PDE6.

The N-terminal half of Py is the major interacting region between Py and GARP2. Having identified Py as the major interacting subunit for GARP2 binding to PDE6, we next sought to define the region(s) of Py responsible for stabilizing GARP2 binding. The 87-amino acid Py of rod photoreceptors consists of multiple sites of interaction along its entire length with PDE6 catalytic subunits, transducin α-subunit, RGS9-1, as well as serving as a substrate for post-translational modifications, all of which are proposed to regulate PDE6 activity during various stages of phototransduction (Guo et al., 2008). Knowing the sites of interaction of GARP2 with Py may provide insight into the regulatory role of GARP2 binding to PDE6.

To localize the regions on Py that bind to GARP2, we created a set of GST-tagged truncation mutants of Py to determine which mutants could stably interact with GARP2 in pull-down assays. Following addition of GARP2 to various Py truncation mutants that were immobilized on glutathione-agarose beads, we assayed the ability of the Py mutants to stably bind GARP2 in pull-down assays. As seen in Fig. 3-2A, the Py1-45 truncation mutant was able to bind GARP2, whereas Py46-87 or Py67-87 truncation mutants did not
detectibly interact with GARP2 in this assay. We also tested the ability of soluble Py fragments to compete with immobilized full-length (His)_6-Pγ for binding to GARP2; as shown in Fig. 3-2B, two truncation mutants, Pγ1-45 or Pγ1-66, were as effective as full-length Pγ in blocking binding of GARP2 to immobilized Pγ, whereas Pγ truncation mutants lacking the N-terminal half of the sequence were ineffective (Fig.3-2B). We conclude that the N-terminal half of Pγ represents the primary sites of interaction with GARP2.

Fig. 3-2. GARP2 has high-affinity interactions with the N-terminal half of Pγ. A. The N-terminal half of Pγ interacts with GARP2. GST-tagged wild-type or truncation mutants of Pγ (20 nmol) were immobilized on 20 μl glutathione beads and incubated with His_6-GARP2 (5 nmol). Proteins bound to the beads were separated by SDS-PAGE and detected with GARP2 antibody. T: starting sample, followed by bound fractions of GST-Pγ1-87, Pγ1-45, Pγ46-87, Pγ67-87-45, and control glutathione beads (lacking GST-Pγ; “C”). B. Competition of immobilized Pγ with exogenous Pγ or Pγ truncation mutants. His-Pγ (20 nM) premixed with GARP2 (100 nM) and a 1000-fold excess of either wild-type Pγ “1-87” or the indicated Pγ truncation mutant (20 μM) or no exogenous Pγ (“C”) was pulled down using Ni-NTA beads (20 μl). Proteins bound to the beads were analyzed for the ability to bind GARP2 using immunoblot detection with the GARP antibody.

The C-terminal region of GARP2 most effectively reduces the basal activity of non-activated PDE6. Previous work demonstrated that GARP2 isolated from ROS was able to reduce the basal catalytic activity of PDE6 (containing endogenous, bound GARP2) when exogenous GARP2 was added to ROS membranes containing non-activated PDE6 (Pentia et al., 2006). We re-evaluated the ability of GARP2 to suppress PDE6 basal activity in a reconstituted system containing recombinant GARP2 and
purified GARP2-free PDE6 holoenzyme. As shown in Fig. 3-3. (filled circles),
exogenous GARP2 suppressed up to 90% of the basal activity of GARP2-free PDE6 in a
dose-dependent manner, with half-maximal suppression requiring a large excess of
exogenous GARP2 (IC$_{50}$ = 240 ± 40, n=5). In contrast to the effects of GARP2 on PDE6
holoenzyme, we observed that incubation of GARP2 with Paβ resulted in less than a 10%
change in catalytic activity at concentrations where the activity of PDE6 holoenzyme was
reduced by 90% (data not shown).

![Graph showing suppression of PDE6 activity by GARP2 fragments](image)

**Fig. 3-3.** The C-terminal half of GARP2 is most effective in suppressing the
basal activity of GARP2-free PDE6. Three different fragments of GARP2 (N-
terminal half (a.a. 1-150), the central region (a.a. 75-225), and the C-terminal half
(a.a., 151-299) as well as full-length GARP2 (FL-GARP2) were expressed and
purified (see Methods). 5 nM GARP2-free PDE6 holoenzyme (obtained by PrBP/δ
solubilization of PDE6; see Experimental Procedures) was incubated for 15 min
with increasing concentration of GARP2 or GARP2 fragments, and then PDE
activity measured. The data are representative of at least 3 independent experiments
for each condition. Similar results were also obtained with GARP2-free PDE6
obtained by Triton X-100 treatment.

To identify the region of GARP2 responsible for suppressing the basal activity of
PDE6, we generated three fragments of GARP2 (N-terminal half, C-terminal half, and
central region) and tested their ability to reduce PDE6 basal activity. As shown in Fig. 3-
3, the C-terminal half of GARP2 (a.a. 151-299) was similar to full-length GARP2 (299
a.a.) in its ability to suppress PDE6 basal activity. In contrast, both the N-terminal half (a.a. 1-150) and the central region of GARP2 (a.a. 75-225) suppress PDE6 activity to a lesser extent (~60% maximal decrease) and require higher concentrations of GARP2 compared to the C-terminal half of GARP2 (Fig. 3-3). We conclude that the last ~75 amino acids at the C-terminus of GARP2 is most effective in causing suppression of the basal catalytic activity of non-activated PDE6, presumably by enhancing the interactions of Pγ with Paβ to more effectively block substrate access to the enzyme active site.

**GARP2 reduces cGMP binding to PDE6.** In addition to regulation by its Pγ subunit, PDE6 is also allosterically regulated by cGMP binding to sites located in the regulatory GAFα domains of PDE6 [reviewed in (Cote, 2006)]. Furthermore, the ability of Pγ to alter cGMP binding to the regulatory GAFα domain has been shown to reside within the N-terminal half of Pγ (Zhang et al., 2012), the same region of Pγ that binds to GARP2 with highest affinity (Fig. 3-1B).

We therefore asked whether GARP2 altered the cGMP binding properties of PDE6 by measuring cGMP binding to PDE6 GAF domains in the presence of increasing concentrations of GARP2. As shown in Fig. 3-4A, addition of increasing concentrations of GARP2 resulted in dose-dependent reductions in the amount of cGMP bound to either PDE6 holoenzyme or Paβ. The observation that a large molar excess of added GARP2 is needed to induce cGMP dissociation (Fig. 3-4A) or to suppress basal catalytic activity (Fig. 3-3) may arise from the intrinsically disordered structure of GARP2 in solution (Batra-Safferling et al., 2006) resulting in only a small fraction of the purified GARP2 molecules being in a suitable conformation for high-affinity interactions with PDE6. The ability of GARP2 to alter cGMP binding to Paβ was unexpected based on the failure to
detect direct binding of GARP2 to Paβ in pull-down experiments (Fig. 3-1A); this suggests that GARP2 does directly interact with Paβ but its binding affinity is lower when the stabilizing influence of Pγ is absent.

**Fig. 3-4. GARP2 decreases the extent of cGMP binding to noncatalytic sites on PDE6.** A. PDE6 catalytic dimer (Paβ; 20 nM) or reconstituted holoenzyme (Paβγγ; 20 nM) was pre-incubated for 15 min with the indicated amount of GARP2 and then mixed with 1 μM [3H]cGMP for 10 min prior to determining the amount of [3H]cGMP bound; see Experimental Procedures. Binding data compiled from four experiments was normalized to the amount bound in the absence of GARP2 and fit to a hyperbolic relationship

\[ IC_{50} = 373 \pm 104 \text{ GARP2/Paβ, maximum decrease = 88% \pm 7\%; PDE6 holoenzyme (triangles): } IC_{50} = 264 \pm 67 \text{ GARP2/PDE6, maximum decrease = 98% \pm 7\%;} \]

B. Full-length GARP2 (black) or N-terminal (gray), central (ladder), and C-terminal (hatched) fragments of GARP2 (all 20 pM final concentration) were incubated with Paβ or Paβγγ (20 nM) for 15 min, and then the extent of binding of 1 μM [3H]cGMP was determined. Controls lacking GARP2 (white) were also measured, with the extent of binding normalized to the amount of cGMP bound to PDE6 holoenzyme in the absence of GARP2 (n=4). One-way ANOVA analysis with Tukey test identified statistically significant differences between control and all GARP2-containing samples (*, p < 0.001), as well between certain GARP2-containing samples (**, p < 0.001; and ***, p < 0.005).

To determine which region of GARP2 may be responsible for altering cGMP binding to PDE6, we repeated cGMP binding experiments using the three recombinant GARP2 fragments described above. As shown in Fig. 3-4B, cGMP binding to Paβ or
PDE6 holoenzyme was reduced following incubation with any of the three GARP2 fragments (N-terminal, central and C-terminal region of GARP2). The central GARP2 fragment (a.a. 75-225) reduced cGMP binding to the same extent as full-length GARP2 (~60% decrease), whereas the N-terminal region (a.a. 1-150) was only half as effective in reducing cGMP binding (Fig. 3-4B). We conclude from these experiments that the central region of GARP2 is primarily responsible for interacting with PDE6 to destabilize cGMP binding to the GAF domains of the catalytic subunits.

**GARP2 decreases the efficiency of transducin activation of PDE6.** Previous work demonstrated that addition of GARP2 to transducin-activated PDE6 on rod outer segment membranes had little effect on the catalytic activity of PDE6 (Pentia et al., 2006), but did not address whether GARP2 could alter the extent to which transducin can activate PDE6. Because GARP2 binding to nonactivated PDE6 alters both catalytic activity and cGMP binding properties, we sought to determine the ability of GARP2 to alter the mechanism of transducin activation of PDE6. Using a reconstituted system consisting of purified, activated transducin α-subunit (Tα*-GTPγS), PDE6 holoenzyme, and large unilamellar membranes to enhance transducin activation efficiency; (Melia et al., 2000), we tested whether addition of GARP2 prior to transducin could alter the efficacy of transducin to activate PDE6. As shown in Fig. 3-5, the presence of GARP2 was able to reduce by 30% the maximum extent to which PDE6 could be activated; no significant change in concentration dependence (IC₅₀) was observed. Similar results were obtained regardless of whether the PDE6 holoenzyme preparations contained or lacked endogenous, bound GARP2 (*data not shown*). We conclude from these results that
GARP2 is able to interfere with the ability of transducin to fully activity PDE6 under these experimental conditions.

To probe the underlying mechanism by which GARP2 lowers the efficiency of transducin activation of PDE6, we evaluated whether GARP2 was able to directly bind to transducin. When (His)$_6$-GARP2 was pre-bound to beads and then incubated with increasing concentrations of Tα*, no significant interaction of Tα*-GTPγS with GARP2 was observed (Fig. 3-6). This result suggests that there are no direct, high-affinity interactions between GARP2 and Tα*-GTPγS. This led us to hypothesize that the effect of GARP2 to suppress the extent to which transducin activates PDE6 may be mediated through Py which as we showed above (Fig. 3-1B) is capable of binding both GARP2 and Tα*-GTPγS. To test this, we added Py to the GARP2-immobilized beads prior to addition of Tα*-GTPγS and found that Py enabled transducin to bind to the beads when Py was also present (Fig. 3-6). The simplest explanation of these results is that GARP2 exerts a
desensitizing effect on transducin activation of PDE6 through direct interactions with Pγ, not with Ta*-GTPγS or Pαβ.

**Summary**

The experiments reported above identify several regulatory interactions of GARP2 with the PDE6 holoenzyme that may underlie some of the physiological properties that distinguish rod and cone responsiveness to light stimulation. First, GARP2 may serve to lower the basal rate of cGMP hydrolysis as a result of direct interactions with the inhibitory Pγ subunit when the PDE6 holoenzyme is in its dark-adapted (i.e., nonactivated) state. This regulatory feature may reflect an allosteric change in Pγ conformation upon binding of the C-terminal region of GARP2 (Fig. 3-3) to the N-terminal half of Pγ (Fig. 3-2) that enhances direct blockage of the active site of the enzyme (Granovsky et al., 1997). Reducing PDE6 basal activity would serve to reduce the component of "dark-noise" attributed to spontaneous activation dark-adapted PDE6 (Rieke et al., 1996), thereby enhancing the signal-to-noise ratio for detection of single photons by rod photoreceptors in their dark-adapted state.

The ability of GARP2 to destabilize cGMP binding to noncatalytic regulatory sites on the PDE6 catalytic dimer (Fig. 3-4) and to reduce the extent of transducin
activation (Fig. 3-5) may together serve a different function, namely to reduce the fraction of rod PDE6 that is light-activated during bright, continuous illumination that causes rod photoresponse saturation. It has been previously shown that when PDE6 lacks bound cGMP at its noncatalytic sites, transducin inactivation is accelerated (Arshavsky et al., 1991) by a mechanism requiring RGS9-1 (He et al., 1998)] and modulated by Pγ (Arshavsky et al., 1992a). By reducing the extent of cGMP binding to PDE6 catalytic subunits, GARP2 may contribute to accelerating transducin GTPase activity that leads to PDE6 inactivation. Likewise, the ability of GARP2 binding to PDE6 to reduce the extent to which PDE6 is activated by transducin also serves to reduce the efficacy of the phototransduction activation pathway. We hypothesize that these two effects of GARP2 on the phototransduction pathway become relevant only when rod photoreceptors are exposed to bright continuous light, and may serve to reduce energy expenditure under conditions where cGMP metabolic flux represents a “futile cycle.”
CHAPTER 4

THE EFFECTS OF ZINC ON PDE6 CATALYTIC ACTIVITY

Abstract

In the PDE superfamily, divalent cations, including zinc, magnesium and manganese, are required for the normal catalytic function of most PDEs. A previous study has identified the specific conserved residues within the catalytic domain of PDE5 that contribute to the interaction with magnesium or manganese in supporting catalysis; and it also suggested that zinc is the most potent divalent cation supporting catalysis of PDE5 (Francis et al., 1994). Although it has been found that the removal of tightly bound zinc from PDE6 causes a loss of the activity (He et al., 2000a), the potential regulation of zinc content as it related to function of PDE6 is not totally clear.

We hypothesize that zinc is required for the PDE6 catalytic mechanism and structural stability of PDE6 and that distinct binding sites where zinc can rapidly exchange are likely occupied by magnesium under physiological conditions. In this study, different zinc-chelator systems were used to make solutions with defined concentrations of free zinc using calculations from the Hyperquad Simulation and Speciation (HySS) program. We demonstrated that low concentrations of zinc are able to activate PDE6 catalytic activity, while high concentrations cause the loss of PDE6 activity.
Introduction

It is generally accepted that divalent cations are critical to the catalytic function and structure of PDEs. Magnesium is used as the divalent cation in PDE activity assays (Butcher et al., 1962) and previous studies indicate that the coordination of magnesium in enzymes typically requires a site rich in oxygen atoms derived from residues such as Asp, Glu, Thr, Ser, and Tyr (Francis et al., 2000). Although it has been widely accepted that magnesium is required for PDE catalytic function, other metals including zinc, manganese and cobalt were also found to support catalytic activity of most PDEs and in certain instances, they are more effective than magnesium (Francis et al., 1994). Several studies have reported that the metal-binding sites in several Zn$^{2+}$ and/or Mn$^{2+}$-binding enzymes resemble one another by having multiple histidines and acidic residues (Christianson, 1997; Egloff et al., 1995). For example, the metal-binding sites in the active site of thermolysin uses a nitrogen-rich HX$_3$HX$_n$E motif to coordinate a catalytic Zn$^{2+}$, which can also bind Mn$^{2+}$ (Matthews et al., 1972).

In previous research, Francis et al. (1994) showed that the catalytic domains of several PDEs (PDE5, PDE6 and PDE3) contained two metal-binding motifs (HX$_3$HX$_n$E) arranged in tandem that were separated by 10 amino acids (Francis et al., 1994). These motifs were similar to the catalytic Zn$^{2+}$-binding motif found in some metalloendopeptidases (Vallee et al., 1990a; Vallee et al., 1990b). The stoichiometry of Zn$^{2+}$ binding to PDE5 was determined to be three zinc ions per PDE5 monomer (Francis et al., 1994). Furthermore, single-site mutation of five of the six residues in the two motifs of PDE5 (His-603, His-607, His-643, His-647, and Glu-672) caused significant loss of catalytic activity measured in the presence of Mg$^{2+}$, which suggested that both of these...
metal-binding motifs are required to support normal catalysis in PDEs (Francis et al., 2000).

Zinc has also been visualized to bind to a metal-ion binding site (Me-1 site) in the initial x-ray crystallographic structure of the PDE4 catalytic domain (Xu et al., 2000). The atomic-level structure of the PDE9 catalytic domain revealed that the Me-1 site is occupied by zinc and the Me-2 site is occupied by magnesium (Huai et al., 2004). All other x-ray crystallographic structures of isolated PDE catalytic domains are reported to have zinc bound, with the exception of PDE3 (Ke et al., 2007) (Scapin et al., 2004).

Although zinc shows a similar coordination with active site residues in the x-ray crystal structures, the measured affinity for zinc varies among these PDEs. The crystal structure with zinc binding at Me-1 in the PDE4D catalytic domain was obtained in the presence of EDTA, but the zinc can be removed from the PDE10 isolated catalytic domain after incubation with EDTA for 30 min (Wang et al., 2007). However, the incubation of PDE6 with EDTA for several days is relatively ineffective at removing zinc from PDE6 holoenzyme, whereas dipicolinic acid (DPA), a higher-affinity zinc chelator, is required to remove the tightly bound zinc (He et al., 2000a). The presence of zinc in structures of almost all mammalian PDEs along with the biochemical evidence for the tight binding of this zinc indicate that it is possible that all mammalian PDEs, except PDE3, may constitutively contain zinc in the Me-1 site under physiological conditions. Magnesium has been widely considered to occupy the Me-2 binding site, but many PDEs have higher affinity for manganese or cobalt than for magnesium in supporting catalytic activity, such as in PDE9, in which the activity of manganese is twice that for magnesium (Huai et al., 2004).
The Me-1 and Me-2 metal-binding sites in mammalian PDE catalytic domains are dissimilar. The coordination of zinc in Me-1 utilizes two invariant histidines and two invariant aspartic acid residues and three of these (two histidines and one aspartic acid) localize to the “histidine-rich” segment of the PDE catalytic domain sequence. However, the other aspartic acid is located near the C terminus of this region. The binding of the metal ion in the Me-2 metal-binding site involves one of the aspartic acids, that is also found in the Me-1 site along with five water molecules (Ke et al., 2007). Only one amino acid in Me-2 directly contacts with the metal ion likely accounting for the lower binding affinity for metal at this site. In the study of PDE9, it has been found that the catalytic domain of PDE9 can be activated by different combinations of divalent metal ions in Me-1 versus Me-2 sites, but the activity and structure of PDE9 differ under various combinations of metal ions (Liu et al., 2008). Another study of the structure of PDE4, PDE5 and PDE9 found that a hydroxyl ion is the bridging nucleophile for the metals between Me-1 and Me-2, with Me-1 believed to be occupied by zinc, and Me-2 occupied by either magnesium, manganese, or zinc (Xiong et al., 2006; Liu et al., 2008).

The regulatory significance of metal-ion content in the active sites of PDEs is not totally clear. In the core of PDE4, it was found that the phosphorylation of PDE4 by PKA increased binding affinity for magnesium (Tollefson et al., 2010). The differing affinities for magnesium among different states of the same PDE suggests that changes in divalent metal ion concentration within cellular compartments could alter PDE activity and serve to regulate PDE activity under physiological conditions.

Zinc plays an important role in the catalytic function of PDEs, but it is unclear whether it is critical for stabilizing the subdomain structure of the catalytic domain.
study with PDE10A2, the residues His-529, His-563, Asp-564 and Asp-674 were identified to directly interact with zinc and contribute to the high-affinity binding of zinc. Two mutants (D564A and D674A) were generated that lowered the catalytic activity 1000–10,000-fold compared to the wild type enzyme with no significant difference in the crystal structures (Wang et al., 2006). In the case of PDE5, mutations of His-617 or His-653 that directly coordinated with zinc showed the loss of catalytic activity, but the activity can be recovered in the presence of a high concentration of manganese (not magnesium) (Francis et al., 2000), which suggests that high manganese can fill the catalytic role of zinc in the Me-1 site. The removal of metals can also abolish the activity of PDE5, but the nucleotide analogs or inhibitors were still able to interact with its catalytic pocket (Corbin et al., 2003). The mutation of the downstream aspartic acid in PDE5 caused the loss of catalytic activity as well (Turko et al., 1998), which emphasize the important role of the downstream aspartic acid in PDE catalytic function.

Previous studies with rod PDE6 have shown that Mg$^{2+}$ is required for the catalytic activity of PDE6 (Pannbacker et al., 1972) and that the concentration of Mg$^{2+}$ affects the apparent $K_m$ for cGMP (Srivastava et al., 1995). Rod PDE6 also requires tightly-bound zinc for its catalytic activity, with purified PDE6 containing 3–4 g atoms of endogenous zinc per mole PDE6 (He et al., 2000a). This is consistent with two tightly bound zinCs per catalytic subunit. Loss of PDE6 activity occurs when free divalent cations are absent; this loss of activity is reversible upon addition of magnesium, manganese, cobalt or zinc. DPA and EDTA can remove the majority of the tightly-bound zinc at pH 6.0, which causes the complete loss of activity of PDE6 even in the presence of magnesium, but the lost activity could be restored by the addition of zinc (He et al., 2000a).
The efforts to fully understand the role of divalent cations in the structure, catalytic mechanism, and the potential regulation of PDE6 are hampered by difficulties in manipulating the identity and stoichiometry of bound metals, as well as controlling the free concentration of divalent cations. A better understanding of divalent cations in PDE6 is important because of evidence that heavy metals may cause loss of visual function of the rods and cones of the retina. Therefore, we developed methods to evaluate the role of divalent cations in PDE6 activity.

**Materials and Methods**

*Materials*—All chelators and other chemicals were obtained from Sigma Chemical Co. or from Thermo-Fisher.

*Purification of PDE6 Holoenzyme (Paβγγ) and PDE6 Catalytic dimer (Paβ)*—Rod PDE6 holoenzyme was purified as described in detail in Chapter 2. The PDE6 catalytic dimer (Paβ) was prepared from the PDE6 holoenzyme by digesting the inhibitory Py subunits by limited trypsin proteolysis as described in Chapter 2. The purified Paβ was buffer-exchanged into 150 mM NaCl in 20 mM HEPES, pH 7.5 prepared with 50% glycerol and stored at -20 °C. The protein (PDE6 or Paβ) was buffer exchanged into HEPES buffer to remove the glycerol before use.

*Preparation of Zinc-containing Buffers*—First, the metal-free HEPES buffers at pH 7.4 or pH 7.0 were made by dissolving the powder-formed reagents of the highest purity in commercially available metal-free distilled water or distilled water purified to 18 megohms of resistance with a Milli-Q reverse osmosis system. The metal-free solutions were stored in the containers that had been extensively washed with Milli-Q.
water and handled carefully. Second, the free zinc-containing buffers were prepared by adding zinc salt to the metal-free solutions based on calculations performed by the HySS program (see below). The pH of the zinc-containing buffers was finally adjusted by adding HCl or NaOH solution. All the zinc-containing solutions were stored at 20~25 °C, because low temperature sometimes caused precipitation, especially at high zinc concentrations.

**Determination of the Activity of PDE6 in Zinc Solutions**—All solutions used in the PDE6 activity assay were prepared in 20 mM HEPES buffer at pH 7.4. Purified Paβ was buffer exchanged into 150 mM NaCl in 20 mM HEPES, pH 7.4 to remove glycerol. Typically, 30 μl free zinc buffer was incubated with 10 ul PDE6 solution in presence of 0.5 mg/ml bovine γ-globulin (BGG) at room temperature for 15 min before adding cGMP. The PDE6 concentration was estimated based on the rate of cGMP hydrolysis of trypsin-activated PDE6 and the knowledge of the kcat of the enzyme [5600 mol cGMP hydrolyzed per mol Paβ per s (Mou et al., 1999)].

**Data Analysis**—The free zinc concentration in this study was calculated by HySS program (HySS2009, Ink.) as described by Alderghi. *et al.* (Alderighi et al., 1999).

**Results and Discussion**

**Optimize the conditions for measuring PDE6 activity in zinc-containing solutions.** In order to determine the effects of varying the free zinc concentration on the catalytic properties of PDE6, the standard PDE6 activity assay method had to be modified.
In the standard PDE6 activity assay method, 20 mM Tris (pH 7.5) is used to buffer the pH, 0.5 mg/ml bovine serum albumin (BSA) is used as a protein carrier or "stabilizer", and 10 mM Mg\(^{2+}\) is added to maximally activate PDE6. As Tris can interact with zinc, it is not an ideal buffer. Therefore, HEPES and MOPS buffering systems were tested as a replacement for Tris. The maximal activity of PDE6 in HEPES buffer at pH 7.5 (10 mM, 50 mM and 100 mM) or MOPS buffer at pH 7.5 (10 mM, 50 mM and 100 mM) were compared with the Tris buffering system and the results demonstrated that HEPES and MOPS buffers are equally good at buffering pH without altering the maximal activity of PDE6. We therefore used one of these Good’s buffers for subsequent experiments. Inclusion of BSA in the PDE6 assay buffer serves to reduce the loss of PDE6 to due surface effects. For example, in the absence of BSA, the maximal activity of PDE6 was reduced greater than 90% compared to including BSA in the assay, supporting the idea that a protein carrier or stabilizer is necessary to measure PDE6 activity accurately. However, previous studies indicate that BSA can bind zinc with high affinity (Ohyoshi et al., 1999). We therefore tested other proteins that do not bind zinc for their abilities to stabilize PDE6 in solutions. We tested bovine γ-globulin (BGG), ovalbumin, lysozyme, and dextran as the candidates to replace BSA. As shown in Fig. 4-1, the presence of BGG preserved more than 98% of PDE activity, ovalbumin recovered about 96% of the activity of the control, and lysozyme or dextran presented about 80% activity (all compared to BSA as control). This result shows that BGG can substitute for BSA in PDE6 activity assays in studying the effect of zinc on PDE6 activity.
Fig. 4-1. Characterization of stabilizers for PDE6 assay buffer.
The maximal activity of PDE6 was measured in the PDE6 assay buffer supplemented with different protein stabilizers (ovalbumin, lysozyme, BGG or dextran). The PDE6 assay buffer contained 20 mM Tris, 10 mM MgCl₂ with 0.5 mg/ml of each stabilizer at pH 7.5 (dextran at 1 mg/ml). The maximal activity measured with 0.5 mg/ml BSA-containing assay buffer was used as the control.

**Evaluate the effects of the chelators on PDE6 activity.** In order to prepare solutions with defined free zinc concentration in solutions, chelators with high affinity to zinc must be utilized. The effects of three zinc chelators (NTA, EDTA, and citrate) on PDE6 activity were screened. In Fig. 4-2, 10 mM NTA, 10 mM citrate and 10 mM EDTA were incubated with PDE6 for 30 min to remove the endogenous divalent cations from PDE6, and then increasing concentrations of Mg²⁺ were added. The activity of PDE6 was measured and normalized to the maximum activity in the absence of chelator. All three

Fig. 4-2. Comparison of the effects of different chelators on PDE6 activity.
The PDE6 activity was measured by adding 10 mM of each individual chelator to the PDE6 assay buffer for 30 min and then adding increasing concentrations of Mg²⁺ at pH 7.5. The chelators used were NTA (circles), citrate (squares) and EDTA (triangles). PDE6 activities were normalized to the value in the absence of chelator.
chelators were able to block greater than 95% of total PDE6 activity, and in the presence of sufficient Mg$^{2+}$ the maximum activity was restored. Since EDTA has a higher affinity for Mg$^{2+}$ than NTA or citrate, it required more Mg$^{2+}$ to fully restore activity.

A time course (from 30 min to 18 hours) of PDE6 exposure to chelator (NTA or EDTA) was conducted to determine the loss of PDE6 activity during incubation in the absence of free divalent cations. After adding back Mg$^{2+}$, it was found that short term incubation (less than 10 hours) of PDE6 with EDTA or NTA had no effect on the recovery of PDE6 activity. Even after an 18 hour incubation 90% of activity could be restored (data not shown). These results indicate that the removal of endogenous cations of PDE6 causes the loss of catalytic activity, while addition of excess Mg$^{2+}$ is able to restore PDE6 activity.

Use of different zinc chelators to study the effects of zinc on the catalysis of PDE6. Previous work suggests that in the absence of other divalent cations, lower concentrations of zinc (< 1 μM) are able to activate PDE6, while higher concentrations (> 1 μM) cause the loss of activity (He et al., 2000a). However, further work is needed to better understand the mechanism by which zinc can both activate and inhibit PDE6. In this section, several chelators were tested using Hyperquad Simulation and Speciation (HySS) program to calculate free zinc concentration in solution containing chelators. HySS program is a computer program which is able to provide a speciation diagram using the given equilibrium constants (β) and calculate concentrations of each species in the solution system (Alderighi et al., 1999).
The first chelator examined was nitrilotriacetic acid (NTA) in HEPES buffer at pH 7.4; the equilibrium constants of each species in the solution were provided by Dr. Roy Planalp and Kyle Burton (UNH Chemistry Department). In this experiment, zinc-containing solutions (with and without added Mg\(^{2+}\)) were prepared based on the calculations from the HySS program, and PDE6 incubated with each zinc solution for 15 min prior to measuring catalytic activity. The results suggest that low concentrations (< 1 \mu M) of free Zn\(^{2+}\) activate PDE6 and the highest activity of PDE6 (~90% activity of the control) is achieved under the free zinc concentration of ~100 nM. However, concentrations of zinc greater than 100 nM showed inhibitory effects on the activity of PDE6.

**Fig. 4-3. PDE6 activity in NTA-Zn HEPES solutions at pH 7.4.**
Solutions containing NTA and various concentrations of free Zn\(^{2+}\) in the presence or absence of Mg\(^{2+}\) were prepared based on the calculation from HySS program (See Methods) and summarized in Tables 4-1 and 4-2. PDE6 (1 nM) was incubated with zinc-containing HEPES buffer (pH 7.4) for 15 min before adding cGMP. The maximal activity of PDE6 in 10 mM Mg\(^{2+}\) with 0.2 mg/ml BGG in absence of zinc or NTA was used as the control. A. PDE6 activity as a function of free Zn\(^{2+}\) concentrations in absence of Mg\(^{2+}\). No BGG added (circles) and with 0.5 mg/ml BGG added (diamonds). B. The effect of zinc on PDE6 activity in the presence of 1 mM Mg\(^{2+}\). No BGG added (triangles) and with 0.5 mg/ml BGG added (squares).
PDE6 (Fig. 4-3A). It was found that the addition of 1 mM Mg$^{2+}$ was able to improve the basal activity of PDE6, but it did not change the concentration dependence of Zn$^{2+}$ on PDE6 activity (Fig. 4-3B).

In order to further define the range of zinc concentrations that activate PDE6 to inhibit PDE6, we lowered the pH to 7.0 to improve our ability to make a set of solutions with free zinc concentration between 100 nM to 1000 nM (Table 4-3). As shown in Fig. 4-4, the pH of the solutions was lowered from 7.4 to 7.0 and we observed a change of PDE6 activity from activation to inhibition over the range of 200–400 nM free zinc. These results are in general agreement with the observations at pH 7.4, but with improved ability to define the free zinc concentrations promoting catalytic activity. Note that the highest activity of PDE6 in Fig. 4-4 is 68% of the control at pH 7.0, whereas at pH 7.4, zinc was able to stimulate to 90% of the control.

We also tested the chelator N-(2-acetamido)-iminodiacetic acid (ADA) to prepare solutions with low free zinc concentration, because ADA has lower affinity for zinc than NTA. As seen in Fig. 4-5, free Zn$^{2+}$ activates PDE6 over the range of 50 to 1000 nM free Zn$^{2+}$ concentration with no inhibition of activity observed. 1 μM free Zn$^{2+}$ is the highest
concentration can be obtained in this ADA-chelating system because of the low solubility of the zinc salt under these conditions. Sodium salts including NaCl and NaNO₃ at different concentrations were added to increase the ionic strength which may improve the solubility of zinc, but no significant change was observed (data not shown). Further work is required to optimize the Zn-ADA system.

A third chelator of zinc, histidine (His), was also used. Initially, the effect of histidine itself on PDE6 activity in the presence of Mg²⁺ was evaluated and it was determined that the chelator has no adverse effect on PDE6 activity. In Fig. 4-6, the activity curve of PDE6 in Zn-His solutions indicates that this Zn-His system presents similar behavior as NTA, in that lower concentrations of free Zn²⁺ activates PDE6 and higher concentrations cause inhibition of PDE6 activity. However, the maximal activity is observed at 1000 nM of free Zn²⁺ using histidine as a chelator compared to ~ 200 nM free Zn²⁺ for the Zn-NTA buffering system. Also noteworthy is the fact that the highest activity of PDE6 only reaches 60% of the control.
Fig. 4-6. PDE6 activity in His-Zn buffers at pH 7.4.
Zinc solutions with His were prepared as described in Methods (Table 4-5.). PDE6 (1 nM) was incubated for 15 min in zinc buffers in the presence of 0.2 mg/ml BGG before cGMP was added. The maximal activity of PDE6 measured in 10 mM Mg$^{2+}$ in absence of zinc or His is used as the control. A. Free zinc concentration from submicromolar to µM range. B. Free zinc concentration up to mM level.

Table 4-1. The equilibrium constants of individual species in NTA-chelating zinc solutions at pH 7.4 (in absence of Mg$^{2+}$).

<table>
<thead>
<tr>
<th>log $\beta$</th>
<th>IC (M)</th>
<th>FC (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnNTA</td>
<td>10.66</td>
<td>Zn</td>
</tr>
<tr>
<td>ZnNTA2</td>
<td>14.24</td>
<td>NTA</td>
</tr>
<tr>
<td>NTAH</td>
<td>9.73</td>
<td>HEPES</td>
</tr>
<tr>
<td>NTAH2</td>
<td>12.22</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>NTAH3</td>
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<td>H</td>
</tr>
<tr>
<td>HEPESH</td>
<td>7.52</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>H-1</td>
<td>-13.77</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-2. The equilibrium constants of individual species in NTA-chelating zinc solutions at pH 7.4 (in presence of 1 mM Mg$^{2+}$). IC: initial concentration; FC: final concentration.
### Table 4-3. The equilibrium constants of individual species in NTA-chelating zinc solutions at pH 7.0.

<table>
<thead>
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<th></th>
<th>log beta</th>
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<th>FC (M)</th>
</tr>
</thead>
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<td>ZnNTA</td>
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<td>0.01</td>
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<tr>
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<td>HEPES 0.1</td>
<td>0.1</td>
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<td>NTAH2</td>
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<td>7.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HEPESH</td>
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</tr>
<tr>
<td>H-1</td>
<td>-13.77</td>
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</tr>
</tbody>
</table>

### Table 4-4. The equilibrium constants of individual species in ADA-chelating zinc solutions at pH 7.4.

<table>
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<th>log beta</th>
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<th>FC (M)</th>
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### Table 4-5. The equilibrium constants of individual species in His-chelating zinc solutions at pH 7.4.

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IC: initial concentration; FC: final concentration
Summary

The conditions for the PDE6 activity assay were optimized in this study in order to study the effects of zinc on the catalytic mechanism of PDE6. The conditions include using HEPES to buffer the pH instead of Tris, and replacing BSA with BGG as the protein carrier for stabilizing PDE6. Using these optimized conditions, the effects of zinc on PDE6 activity was determined using three different chelators including NTA, ADA and histidine. Although more work is needed to fully characterize divalent cation requirements for PDE6 activity, our preliminary results suggest that sub-micromolar concentrations of free Zn\(^{2+}\) activate PDE6 while higher concentrations are inhibitory. In one instance, inclusion of Mg\(^{2+}\) with Zn\(^{2+}\) did not shift the activation and inhibition profile. In the future, independent methods for validating the free zinc concentration need to be developed. We hypothesize that the binding of Zn\(^{2+}\) to the high-affinity binding site is necessary for the normal catalytic function of PDE6. However, Zn\(^{2+}\) at a high concentration may exchange Mg\(^{2+}\) from the low-affinity Zn\(^{2+}\) binding site and cause an inhibitory effect on PDE6 activity.
In order to better understand the regulatory mechanism of photoreceptor phosphodiesterase (PDE6) during dark- and light-adaptation, the interaction between PDE6 and its high-affinity binding partner, glutamic acid-rich protein (GARP2) was determined in this study and the regulatory function of GARP2 on PDE6 in phototransduction was also examined here.

First, we optimized the conditions of the immunological methods with different primary antibodies to characterize and localize GARP2 since GARP2 lacks enzymatic activity and presents abnormal migration on SDS-PAGE. We also improved several approaches to isolate PDE6 from its high-affinity bound GARP2 from rod outer segment membranes to achieve PDE6 lacking GARP2 as well as PDE6-free GARP2 used in subsequent study. In addition, we generated, expressed and purified 6xHis and GST tagged full-length bovine GARP2, as well as three fragments of GARP2 with 6xHis tag to overcome the drawbacks of native GARP2. The quantity and purity of recombinant GARP2 were greatly improved compared with native GARP2, and the biochemical and biophysical studies on recombinant GARP2 suggests that it behaves similarly to native GARP2 in its ability to suppress of the basal activity of PDE6, its high-affinity interaction with PDE6, as well as its hydrodynamic properties. Using recombinant GARP2 has allowed us to advance our knowledge of the regulatory functions of GARP2 on PDE6 activation and inactivation.
The primary objective of this thesis was to evaluate the high-affinity interactions between GARP2 and PDE6, and examine the regulatory functions of GARP2 on PDE6. Using immunoprecipitation and pull-down assays, we identified the inhibitory γ subunit as the primary interacting subunit with GARP2. Furthermore, we also found that the N-terminal region of Pγ is the major interacting sites between GARP2 and Pγ. We further discovered that the C-terminal portion of GARP2 is the most effective region to suppress the basal activity of PDE6. In addition to studying the effects of GARP2 on PDE6 catalytic properties, we also demonstrated that the binding of GARP2 reduces cGMP binding to the noncatalytic binding sites on PDE6 GAF domains. This effect may be related to the ability of GARP2 to decrease the extent to which transducin activates PDE6 (through its interaction with Pγ). Overall, these results indicate that GARP2 alters not only the catalytic but also the allosteric properties of PDE6 to regulate PDE6 activity and lifetime through multiple interacting sites with the PDE6 holoenzyme. We conclude that GARP2 may play an important role in lowering the level of “dark noise” by suppressing the basal activity of PDE6, as well as reducing cGMP metabolic flux. As a consequence of its effects on cGMP binding and transducin activation, GARP2 may play a second, distinct role in regulating PDE6 lifetime during bright light conditions where the rod photoresponse is saturated.

We also investigated the effects of zinc on the catalytic mechanism and structural stability of PDE6. In this research, we utilized several different zinc-chelator systems to prepare the solutions with defined concentrations of free zinc using calculations from the Hyperquad Simulation and Speciation (HySS) program. We demonstrated that low
concentrations of zinc (100~1000 nM) are able to activate PDE6 catalytic activity, while high concentrations (> 1 μM) cause the loss of PDE6 activity.
LIST OF REFERENCES


segments. Light-induced cGMP decreases as a putative feedback mechanism of the photoreponse. J. Biol. Chem. 266, 18530-18537.


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## Appendix I. List of vertebrate CNGB1 and GARP sequences

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**Total:** 402