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Regulation of photoreceptor phosphodiesterase by prenyl binding protein and by other interacting proteins in photoreceptor cells

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Abstract
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Keywords
Chemistry, Biochemistry, Biology, Molecular

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REGULATION OF PHOTORECEPTOR PHOSPHODIESTERASE BY PRENYL BINDING PROTEIN AND BY OTHER INTERACTING PROTEINS IN PHOTORECEPTOR CELLS

BY

HANNAH J. GITSCHIER
B.A., Colgate University, 2007

THESIS

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

Master of Science
In
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This thesis has been examined and approved.

Thesis Director, Richard H. Cote, Professor of Biology and Biochemistry and Molecular Biology

Feixia Chu, Assistant Professor of Biochemistry and Molecular Biology

Thomas M. Laue, Professor of Biology and Biochemistry and Molecular Biology

12/08/16

Date
DEDICATION

This thesis is dedicated to my family and friends, especially my husband, Joshua Gitschier, and my parents, David & Peg Failing. I would like to thank them for their constant love, support and encouragement while my life was committed to this research.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Rick Cote for his guidance and for making this work possible. In the course of fulfilling the requirements for this degree he has challenged me to be a strong and skillful scientist, and encouraged me to do my best research.

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ABSTRACT

REGULATION OF PHOTORECEPTOR PHOSPHODIESTERASE BY PRENYL BINDING PROTEIN AND BY OTHER INTERACTING PROTEINS IN PHOTORECEPTOR CELLS

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Photoreceptor phosphodiesterase (PDE6) is a central component of the visual pathway. While initial PDE6 activation following light stimulation of photoreceptors is well understood, regulation of PDE6 during recovery and light adaptation may require additional components that interact with PDE6. Two approaches were taken to characterize these potential interactions. The first explored the interaction of PDE6 with prenyl binding protein (PrBP/δ) and demonstrated that changes in membrane localization of PDE6 may occur during light adaptation as a result of association with PrBP/δ. The second approach characterized the PDE6 "interactome" by a mass spectrometric identification of 75 proteins that co-purify with PDE6 upon release from photoreceptor membranes. This work advances our understanding of the role of PrBP/δ in photoreceptors and provides an initial characterization of the entire complement of PDE6 binding partners in photoreceptors.
INTRODUCTION

Photoreceptor Cells

Rod and cone photoreceptors of the vertebrate retina are highly organized, specialized cells consisting of two major components: the outer segment, containing phototransduction proteins on an extensive disk membrane system, and the inner segment, containing nuclei, other organelles, and the synapse. In rod cells, phototransduction proteins must travel from their site of synthesis in the inner segment to stacked disk membranes in the outer segment through the connecting cilium: the detailed mechanisms of this protein transport process are not clear (Karan et al., 2008). Whereas rod photoreceptor cells are cylindrical in shape, cones are smaller and conical in shape. These morphological differences facilitate rod cell function for peripheral vision under dim light conditions, with saturation occurring at moderate light intensities. In contrast, cone cells function over an enormous range of light intensities, allowing for visual acuity and color vision.

Visual Transduction

Visual excitation in rod photoreceptors takes place on the disk membranes in the specialized outer segment (ROS). Rhodopsin, a G protein-coupled receptor sensitive to light, activates the signaling trimeric G-protein transducin (Tαβγ) by catalyzing exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) at the α-subunit (Tα). GTP-bound Tα dissociates from Tβγ to activate the central effector enzyme of the photoresponse, photoreceptor cGMP phosphodiesterase (PDE6). Tα promotes hydrolysis
of the second messenger, cyclic guanosine 3'5'-monophosphate (cGMP), by relieving PDE6 of its inhibitory γ-subunits (Pγ) (Figure 1.0).

**Figure 1.0:** Schematic Diagram of the Light Activation Pathway in ROS

Upon absorption of light (hv), photoactivated rhodopsin (R*) activates transducin (T*) by catalyzing the exchange of GDP for GTP at Tα subunit. GTP-Tα activates PDE6 (P*) by displacing the PDE6 inhibitory gamma subunits (Pγ) at the catalytic site. This activation results in rapid hydrolysis of cGMP.

When photoreceptor cells are in the dark-adapted state, the cGMP-gated ion channels are open and allow for a steady influx of Na⁺ and Ca²⁺ into the ROS, which are circulated as they exit the cell through the sodium/potassium (Na⁺/K⁺)-ATPase pump in the inner segment. When activated, PDE6 lowers cGMP levels and the cGMP-gated ion channels on the plasma membranes close, preventing Ca²⁺ and Na⁺ from entering the cell. However, Ca²⁺ continues to exit the cell through the exchanger, adding to the decrease in positive ions in the cell, which results in plasma membrane hyperpolarization, inhibiting neurotransmitter release at the rod synaptic terminal (reviewed in Pugh and Lamb, 2000; Arshavsky et al., 2002; Cote, 2008; and Wensel, 2008).
In order to amplify visual excitation in rod cells, which are capable of detecting a single photon of light, rhodopsin remains active after catalyzing the activation of transducin. This allows a single activated rhodopsin to continue to activate ~100 transducin molecules, until it becomes deactivated. Additionally, activated PDE6 will hydrolyze up to thousands of cGMP molecules per second before transducin is deactivated and dissociates, allowing for re-inhibition of PDE6 by its Pγ subunits.

While the major components of the light activation pathway have been characterized and our current knowledge of the biochemical signaling cascade can account for electrophysiological responses observed in rod cells, the recovery and light adaptation pathways are less well-defined. The re-opening of cGMP-gated ion channels is dependent upon the restoration of cGMP levels in ROS, requiring PDE6 activity to be quenched, and cGMP regeneration by activation of guanylate cyclase. PDE6 will remain active until transducin in inactivated by hydrolysis of GTP to GDP, allowing Ta-GDP to dissociate. To achieve recovery to the dark state, photoactivated rhodopsin must also be deactivated and the pigment regenerated. The decrease in intracellular Ca²⁺ stimulates recoverin, a neuron-specific Ca²⁺-binding protein, to release rhodopsin kinase, which is associated to recoverin in the dark-state. Rhodopsin kinase then phosphorylates rhodopsin, increasing the affinity of rhodopsin for arrestin, which physically blocks the G-protein activation site of rhodopsin and prevents further transducin activation (reviewed in Pugh and Lamb, 2000).

While these events are essential for normal recovery, the complexes that regulate these alterations and the rate-limiting steps for photoreceptor recovery to dark state are not clearly defined (Sagoo and Lagnado, 1997; Laitko and Hofmann, 1998; Kennedy et
Further, our current understanding of light adaptation, which may include detachment of transducin from the disk membranes and diffusion into the inner segment, and translocation of PDE6 from the edges of the disk membranes towards the center of the disk membranes in ROS, is not conclusive (Calvert et al., 2006; Artemyev, 2008; Chen et al., 2008; Slepak and Hurley, 2008). Therefore, the identification and classification of ROS proteins and understanding of how these proteins regulate all aspects of visual transduction is necessary.

**Rod cGMP Phosphodiesterase Structure and Regulation**

The structure and regulation of PDE6 has been a major focus of research due to its central role in the visual transduction pathway. As rapid hydrolysis of cGMP results in the closure of cGMP-gated ion channels, the precise regulation of PDE6 is critical to control the speed and sensitivity of light activation. Rod photoreceptor PDE6 is comprised of three subunits: Pα and Pβ (catalytic subunits), and Pγ (inhibitory subunit) that form a Pαβγγ heterotetramer in a ratio of 1:1:2. Each catalytic subunit consists of a catalytic domain for cGMP hydrolysis, two regulatory GAF domains (GAF-A and GAF-B) with a non-catalytic high affinity cGMP binding site in the N-terminal GAF-A domain, and is post-translationally isoprenylated (Pα: farnesylated; Pβ: geranylgeranylated) at the carboxyl terminus (C-terminus). The attachment of isoprenyl groups results in a high affinity association of PDE6 with ROS disk membranes, which is necessary for transducin activation (reviewed by Cote, 2003).

During visual transduction, predominant regulation of PDE6 catalytic activity occurs through tight association of inhibitory Pγ subunits to block cGMP active sites, and the de-inhibition of PDE6 upon GTP-Tα association. PDE6 may also be regulated by
allosteric communication between the GAF-A domain, where cGMP-binding occurs at non-catalytic sites, and the catalytic domains of Paβ subunits (Zhang et al., 2008). While Tα activation and allosteric regulation of PDE6 during light activation have been documented, other regulatory mechanisms for PDE6 may be present in ROS and other proteins may regulate PDE6 activity.

In contrast to light activation, the rate-limiting steps that characterize the return to dark-state of photoreceptor cells (recovery) are also not as well-defined. Despite highly reproducible signals from single-photon responses in rod photoreceptors, individual rhodopsin molecule activity seems to vary widely from one experiment to the next (reviewed in Burns and Baylor, 2001). Further, rhodopsin can be deactivated by altering or removing phosphorylation sites (Chen et al., 1995; Mendez et al., 2000), altering adenosine triphosphate (ATP) levels (Nakatani and Yau, 1988), or in the absence of rhodopsin kinase (Chen et al., 1999). Alternatively, others have suggested the while the deactivation of rhodopsin may limit duration of the light response, photoreceptor recovery is dependent on the hydrolysis of GTP by transducin and re-inhibition of PDE6 (Sagoo and Lagnado, 1997; Burns and Baylor, 2001; Kennedy et al., 2003). Other proteins, such as the regulator of G-protein signaling-9-1 (RGS9-1), are necessary for deactivation to accelerate the GTPase activity of Tα (Angleson and Wensel, 1993; He et al., 1998; Chen et al., 2000). Further, RGS9-1 forms a complex with an isoform of the β subunit of a G-protein (Gβ5L) and an RGS9-1-anchor protein (R9AP) (reviewed in Wensel, 2008).

Additionally, PDE6 regulation during light adaptation is even less defined. Light adaptation is thought to occur in rod photoreceptors as a means of desensitizing the
photoresponse in ROS when rods are saturated by the presence of bleached pigment. Some studies have documented a calcium-dependent regulation of PDE6 activity, potentially mediated by recoverin, but observed changes in PDE6 activity were dependent on the timing and intensity of flash responses, as well as large changes in calcium levels (reviewed in Burns and Baylor, 2001). Further, regulation of the sensitivity of cGMP-gated ion channel may play a role in light adaptation (Sagoo and Lagnado, 1996), but others suggest this channel modulation effect is smaller than that of other calcium-dependent mechanisms (Koutalos et al., 1995). Therefore, it would be beneficial to examine PDE6 binding complexes that may regulate PDE6 during these as-yet unexplained aspects of the recovery and light adaptation pathways in rod photoreceptor cells.

**Other PDE6 Interacting Proteins**

In addition to regulation by Pγ inhibitory subunits and transducin, other photoreceptor proteins have been suggested to interact with PDE6 and affect visual transduction. Glutamic acid-rich protein-2 (GARP2) is an alternative splice variant of the β-subunit of rod cGMP-gated ion channel 1 (CNGB1), and is hypothesized to play a role in stabilizing the inactive PDE6 holoenzyme, thereby reducing background PDE6 activity in the dark, or preventing cGMP hydrolysis during light adaptation, when rod cells are saturated (Körschen et al., 1999; Pentia et al., 2006). Alternatively, a 17 kDa prenyl binding protein (PrBP/δ) has been suggested to play a role in photoreceptor desensitization through the solubilization of PDE6 from ROS disk membranes. PrBP/δ was originally identified upon co-purification with soluble PDE6 from bovine ROS, and was misleadingly named PDEδ (Gillespie et al., 1989; Florio et al., 1996). However,
studies of this 17 kDa protein have demonstrated its wide tissue distribution and assortment of prenylated and non-prenylated binding partners, and its assignment as a fourth subunit of PDE6 has been challenged (Florio et al., 1996; Marzesco et al., 1998; Cook et al., 2000; Norton et al., 2005; Zhang et al., 2005; Karan et al., 2008). The ability of PrBP/δ to interact with farnesylated and geranylgeranylated PDE6, farnesylated rhodopsin kinase, as well as several small prenylated GTPases, the N-terminal portion of retinitis pigmentosa GTPase regulator (RPGR) and Arl2/Arl3, which are not prenylated, suggest a more indirect role for PDE6 in visual transduction (Li and Baehr, 1998; Linari et al., 1999a, 1999b; Hillig et al., 2000; Hanzel-Bayer et al., 2002; Nancy et al., 2002; Zhang et al., 2004; Zhang et al., 2005). However, the potential role of PrBP/δ in regulation of PDE6 during light adaptation has not been fully analyzed. Therefore, one major goal of this thesis research is to further characterize the interaction between PDE6 and PrBP/δ in solution and in the context of ROS membranes.

**Protein Identification by Mass Spectrometry**

In addition to studying the interaction between PDE6 and known photoreceptor interacting proteins (such as PrBP/δ), the search for potential PDE6 regulating proteins was undertaken with a proteomics-based approach of mass spectrometry identification. Mass spectrometry techniques have recently been used to identify the protein components in biochemical signaling pathways (reviewed in Charbonnier et al., 2008; Kaake et al., 2010), and have also been applied to ocular and retinal proteomics (McKay et al., 2004; Panfoli et al., 2008). The experiments in this thesis were performed in an attempt to catalog potential PDE6 interacting proteins in ROS and identify those binding partners subject to light regulation.
Relevance to Retinitis Pigmentosa and Visual Impairment

Genetic defects in the major components of the light activation pathway, including the subunits of PDE6 (rod catalytic subunits PDEA and PDEB, cone catalytic subunit PDEC, and inhibitory subunit PDEG), are detrimental to visual transduction, often resulting in retinal degeneration, rod/cone dystrophy, congenital stationary night blindness, and/or total blindness (reviewed in Daiger et al., 2007; Baehr and Frederick, 2009). PDE6 mutations have been identified with retinitis pigmentosa, which is classified as inherited progressive retinal degeneration and leads to irreversible vision loss (Tsang et al., 2008). Genetic mutations in other photoreceptor proteins, such as Tα, GARP2, aryl hydrocarbon receptor interacting protein-1 (AIPL1), regulator of G-protein signaling-9 (RGS9), and PrBP/δ, can also result in visual disorders and/or retinal degeneration (Muradov and Artemyev, 2000; Nishiguchi et al., 2004; Galvin et al., 2005; Zhang et al., 2007; Zhang et al., 2009). In order to better understand these retinal diseases and develop more effective treatments, a clear understanding of PDE6 interacting proteins during light activation, recovery, and light adaptation is needed.

Goals of Thesis Research

Based on the rationale that there are genetic mutations in many photoreceptor proteins that lead to visual impairment or retinal disorders, and there are unanswered questions regarding certain aspects of recovery and light adaptation, the overall goals of this thesis research are to: 1) Test the hypothesis that PrBP/δ may be responsible for PDE6 translocation on disk membranes as a mechanism of light adaptation; and 2) identify PDE6 interacting proteins that may account for as-yet unexplained aspects of the
photoresponse. Therefore, several experimental approaches have been taken to achieve these goals.

To characterize the interactions between PDE6 and PrBP/δ, recombinant bovine PrBP/δ was cloned, expressed, and purified in order to characterize its hydrodynamic properties and to examine its ability to solubilize PDE6 from ROS membranes. Furthermore, PrBP/δ was also used as a tool to solubilize PDE6 and its putative binding partners as a prelude to mass spectrometry protein identification.

In addition to extracting PDE6 from ROS membranes with PrBP/δ, two other methods for solubilizing PDE6 and its binding partners from ROS membranes were developed and compared: hypotonic extraction and detergent solubilization with 1% Triton X-100. These protocols were developed so that PDE6-containing photoreceptor protein complexes could be isolated by immunoprecipitation and then examined by mass spectrometry. Potential PDE6 interacting proteins were documented and bona fide PDE6 interacting proteins were elucidated with a cross-linking approach in tandem with immunoprecipitation and mass spectrometric analysis.

Finally, covalent cross-linking was optimized to semi-quantitatively evaluate PDE6 interacting proteins that are subject to light-induced changes in their binding, and also used on purified PDE6 holoenzyme in solution for initial studies that may eventually provide topographical information about the interaction sites between subunits of the PDE6 holoenzyme.
CHAPTER I

CHARACTERIZING THE INTERACTION OF PRENYL BINDING PROTEIN AND PHOTORECEPTOR PHOSPHODIESTERASE

Introduction

Prenyl binding protein (PrBP/δ) is a 17 kDa protein, originally defined as a photoreceptor cGMP phosphodiesterase (PDE6) subunit for its ability to co-purify with soluble PDE6 from retinal extracts (Gillespie et al., 1989; Florio et al., 1996). Binding of PrBP/δ has no direct effect on PDE6 catalytic activity, though exogenous addition of recombinant PrBP/δ prevents PDE6 activation by membrane-associated transducin (Norton et al., 2005). Further, PrBP/δ interaction with soluble PDE6 may enhance noncatalytic site cGMP exchange at the PDE6 GAF-A binding sites, which do not easily exchange ligand in membrane-associated PDE6 (Mou et al., 1999). Based on the ability of PrBP/δ to solubilize PDE6 from rod outer segment (ROS) disk membranes, it has been hypothesized to play a role in PDE6 desensitization during light adaptation (Figure 1.1; Florio et al., 1996; Cook et al., 2001). In a recent electron microscopy study, PrBP/δ was localized to PDE6 catalytic domains, suggesting two PrBP/δ molecules are capable of binding each PDE6 catalytic dimer in vitro (Goc et al., 2010).

While the exact sub-cellular localization of PrBP/δ in retina and the nature of PrBP/δ binding to PDE6 are not entirely clear, studies have shown there are sub-stoichiometric quantities of PrBP/δ compared to PDE6 in both amphibian and bovine
ROS (Norton et al., 2005; Zhang et al., 2005). In addition, PrBP/δ orthologs have been identified in many animals, including the eyeless Caenorhabditis elegans (Li and Baehr, 1998). Though PrBP/δ mRNA is present in highest levels in retina, unlike other photoreceptor proteins involved in vision transduction, PrBP/δ is also expressed in a wide variety of non-retinal tissues (Florio et al., 1996; Lorenz et al., 1998; Marzesco et al., 1998; Wang et al., 1999). Therefore, PrBP/δ is no longer considered a PDE6 subunit and in addition to interacting with the farnesylated and geranylgeranylated PDE6, may play a more global role in prenylated protein transport in photoreceptor cells (Karan et al., 2008).

**Figure 1.1: Proposed Light Adaptation Role for PrBP/δ**

Upon activation by photoexcited rhodopsin, membrane-associated transducin (T*) activates PDE6 by alleviating cGMP catalytic sites from the inhibitory Pγ subunits. However, if PDE6 is solubilized or translocated through association of PrBP/δ (δ), PDE6 may remain inactive (P). This presents a possible role for PrBP/δ in visual transduction during light adaptation.

In support of a role in protein transport, PrBP/δ has been shown to interact in vitro or through yeast two-hybrid screening with several prenylated and non-prenylated proteins: including members of the Ras and Rho GTPase family, Rab13, the retinitis pigmentosa GTPase regulator (RPGR), rhodopsin kinase (GRK1), and ARF-like proteins Arl2 and Arl3 (Li and Baehr, 1998; Linari et al., 1999a, 1999b; Hillig et al., 2000;
Hanzal-Bayer et al., 2002; Nancy et al., 2002; Zhang et al., 2004; Zhang et al., 2005).

Further, crystal structure analysis reveals PrBP/δ is composed of a series of β-sheets that form a hydrophobic pocket into which prenyl groups insert (PDB 1KSH; Hanzal-Bayer et al., 2002). Therefore, based on sequence alignment and crystal-structure comparison, PrBP/δ has been classified as a prenyl binding protein belonging to a super-family that includes Unc119 and RhoGDI, though the functional significance of PrBP/δ binding to non-prenylated proteins is unclear at present (Nancy et al., 2002; Zhang et al., 2005). Interestingly, despite PrBP/δ and Unc119 having common interaction partners, such as Arl2 (Kobayashi et al., 2003), and 23% overall sequence homology (Li et al., 1998), Unc119 is not able to solubilize PDE6 from ROS as PrBP/δ can (Li and Baehr, 1998).

Knockout of the PrBP/δ encoding gene in mice (Pde6d+/−) leads to reduced levels of G-protein coupled receptor kinase-1 (GRK-1) and cone phosphodiesterase α’ subunit (PDE6α’) expressed in rod and cone outer segments. Further, GRK-1 and PDE6 catalytic subunits normally present in rod and cone outer segments are mislocalized, and slow degeneration of photoreceptor cells is observed (Zhang et al., 2007). Therefore, based on the mislocalization of these prenylated proteins in PrBP/δ-deficient mice and the ability of PrBP/δ to bind prenylated proteins, it has been implicated in the transport of membrane-bound prenylated protein in photoreceptor cells (Karan et al., 2008).

There is circumstantial evidence that PrBP/δ may also assist in the translocation of PDE6 on rod disk membranes during light adaptation. In a recent transmission electron microscopy study, PDE6 migration from the edges of dark-adapted disk membranes towards the center was observed upon light exposure of rat ROS (Chen et al.,
Furthermore, light-induced changes in PDE6 distribution between detergent soluble and detergent-resistant membrane rafts in ROS has been reported (Seno et al., 2001; Liu et al., 2004), as well as changes in PDE6 affinity for certain phospholipids (Hessel et al. 2003). While the above mentioned studies do not directly implicate PrBP/δ, the fact that PrBP/δ can mobilize PDE6 makes it a candidate for facilitation PDE6 movement within ROS.

In order to further our understanding of the role of PrBP/δ binding to PDE6, recombinant bovine PrBP/δ was expressed, expression methods were optimized, and PrBP/δ and PDE6 interactions were studied in solution. Analytical ultracentrifugation is a common method for discerning the behavior of macromolecules in solution based on their size, shape and interaction with other macromolecules. Using a fluorescence detection system with analytical ultracentrifugation (AU-FDS) allows for much lower concentrations of fluorescently labeled or intrinsically fluorescent macromolecules to be studied (MacGregor et al., 2004). This AU-FDS approach was taken to characterize the hydrodynamic properties of recombinant PrBP/δ, and better understand the interaction between PrBP/δ and PDE6 in solution. Further, based on the hypothesis that PrBP/δ interacts with PDE6 in ROS to disrupt transducin-dependent activation of PDE6 during light adaptation, the ability of recombinant PrBP/δ to interact with PDE6 on ROS membranes was characterized.

**Methods**

*Bovine ROS and PDE6 Purification*
Bovine retinas (W. Lawson, Lincoln, NE) were used to obtain purified ROS and PDE6, performed as previously described (Pentia et al., 2005). Briefly, in a darkroom under infrared illumination, bovine retinas were mechanically disrupted with a magnetic stir-bar in 45% (w/v) sucrose in 20 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 2.0 mM MgCl₂, 60 mM KCl, 30 mM NaCl, 1 mM DTT, 0.3 mM PMSF, pH 7.2 to release ROS. ROS were purified by centrifugation on a discontinuous sucrose gradient. After depletion of soluble proteins and homogenization of ROS membranes, PDE6 was isolated by hypotonic extraction in 5 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.5 and purified by anion exchange chromatography on a Mono Q™ 5/50 GL pre-packed column (GE Healthcare) followed by gel filtration chromatography on a Superdex™ 200 10/300 GL column (GE Healthcare). Purified PDE6 was stored in 100 mM NaCl, 10 mM Tris, 2 mM MgCl₂, 2 mM DTT, pH 7.5 (PDE6 storage buffer), in 50% glycerol at -20°C. PDE6 catalytic activity was measured in 20 mM Tris, 10 mM MgCl₂, 0.5 mg/ml bovine serum albumin with a phosphate release microplate assay, performed as previously described (Cote, 2000).

Cloning of Bovine PrBP/δ

The authentic 450 base pair (bp) coding region of bovine PrBP/δ was amplified from the pGEX2T plasmid, a generous gift from Drs. Terry Cook and Joe Beavo (University of Washington), by PCR using primers purchased from Invitrogen (BTAdelta100F: 5'-TTAGGGTACCCAGATGTCAGCCAAGGACG and BTAdelta101R: 5'-AATAAGCCGCCGCTGTCAAACGTAGAAAAAGCC) containing Kpn1 and Not1 restriction sites (underlined). The PCR product was inserted into the pET47b expression vector (Novagen) with T₄ DNA ligase in a 14°C ice bath for 20 minutes, followed by
overnight incubation at 4°C. The ligation product was transformed into DH5α-T1R<br>
*Escherichia coli* cells for optimal amplification of plasmid DNA. Sequencing of both<br>strands with primers specific to the T7 promoter region in the pET47b plasmid, as well as<br>with primers, specific to the bovine PrBP/δ sequence (Delta Forward: 5'-<br>TTCGAGTTTGGC and Delta Reverse: 5'-CAACCATTCTTC), confirmed the authentic<br>bovine PrBP/δ was properly inserted into the pET47b plasmid.<br><br>For the purpose of generating PrBP/δ with a GST fusion tag, the 450 bp insert of<br>bovine PrBP/δ was amplified from the pET47b plasmid by PCR, using a different<br>forward primer (BTAdelta102F: 5'-ATAGGATCCATGTCAAGCCAAGGACGAGC)<br>that contained the BamH1 restriction site (underlined), and the BTAdelta101R reverse<br>primer (described above) that contained the NotI restriction site. The PCR product was<br>inserted into the pGEX6p1 expression vector (GE Healthcare) by ligation with T4 DNA<br>ligase at 16°C overnight. Sequencing of both strands with primers specific to the<br>pGEX6p1 plasmid and within the bovine PrBP/δ gene of interest (described above),<br>confirmed the coding region contained the authentic nucleotide sequence.<br><br>*Expression and Affinity Purification of Recombinant PrBP/δ*<br><br>After sequence confirmation, pET47b-PrBP/δ and pGEX6p1-PrBP/δ constructs<br>were transformed into the BL21 (DE3) strain of *E. coli* for protein expression. For cells<br>harboring the pET47b-PrBP/δ construct, growth was carried out in Luria Broth (LB)<br>medium containing 50 µg/mL kanamycin at 37°C with shaking to an optical density of<br>0.6 – 0.8, measured at 600 nm (OD_{600}). Isopropyl β-D-1-thiogalactopyranoside (IPTG)<br>was added for a final concentration of 0.5 mM, and His-PrBP/δ expression proceeded at
37°C for 3 hours. Cells were harvested by centrifugation (10,000 x g, 30 min, 4°C), re-suspended in 20 mM Tris, 100 mM NaCl, pH 8.00 (Buffer A) with Bacterial Protease Inhibitor Cocktail (Sigma), and sonicated (50 Sonic Dismembrator, Fischer-Scientific, 70-80% of full power) on ice for a 20 second duration and 40 second intervals for a total of 11 cycles. The supernatant was collected by centrifugation of the lysate (10,000 x g, 35 min, 4°C).

His-PrBP/Δ was purified from the soluble lysate by metal chelate affinity chromatography using Ni-nitrilo acetic acid (NTA) resin (Novagen) and a gradient of 0-1M imidazole. The supernatant was incubated with the resin in a batch-bind step at 4°C for 2 hours prior to loading into a column for purification. The beads were washed in 20 mL Buffer A, and proteins were eluted with a 0-10% gradient of 20 mM Tris, 100 mM NaCl, 1 M Imidazole, pH 8.00 (Buffer B) for 20 mL at 0.30 mL/min, followed by a 10-100% gradient of Buffer B for 20 mL at 0.30 mL/min. Individual fractions were collected in 1 mL aliquots and analyzed for protein content at 280 nm. Throughout the purification, absorbance at 280 nm and conductivity measurements were obtained.

Samples of the total lysate, soluble, insoluble, and unbound proteins, as well as individual fractions were mixed with 125 mM Tris, 20% glycerol, 4% SDS, 0.01% bromophenol blue, pH 6.8 (gel sample buffer) and analyzed by SDS-PAGE on 4-12% Bis-Tris NuPAGE gels (Invitrogen), run at 160 V in 50 mM Tris, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.1% SDS, 0.8 mM EDTA (MES buffer) and stained with colloidal Coomassie overnight. The quantity of purified protein was determined by Bradford assay, using bovine gamma globulin (BGG) as the protein standard.
For the pGEX6p1-PrBP/δ construct, recombinant PrBP/δ was expressed in a culture of BL21-DE3 E. coli cells at 37°C for 3 hours after induction with 0.5 mM IPTG in LB medium containing 100 µg/mL ampicillin. Cells were harvested by centrifugation and soluble proteins obtained as previously described for the pET47b-PrBP/δ construct. GST-PrBP/δ was purified from the soluble lysate by affinity chromatography using Pierce immobilized glutathione resin (Thermo Scientific) and eluted with 10 mM reduced glutathione. The supernatant was loaded directly onto a column containing 1.5 mL resin with 50 mM Tris, 150 mM NaCl, pH 8.0 (Buffer A) at 0.2 mL/min. After washing with Buffer A until the absorbance at 280 nm returned to baseline, Buffer A was run for an additional 20 mL at 0.3 mL/min, followed by 30 mL Buffer A containing 10 mM glutathione at 0.30 mL/min. Individual fractions were analyzed for protein content at 280 nm and by SDS-PAGE, as previously described.

When desired, the 6-His or GST fusion tags were removed by incubation with HRV3c Protease (Qiagen) in a 1 Unit protease to 50 µg recombinant protein ratio, for 16 hours at 4°C in 150 mM Tris, 50 mM NaCl, pH 7.5. To separate the HRV3c Protease, un-cleaved protein, and cleaved fusion tags from cleaved recombinant protein of interest, Ni-NTA resin or a mixture of Ni-NTA and immobilized glutathione resin were used for cleavage of His-PrBP/δ and GST-PrBP/δ, respectively. Storage of His-PrBP/δ, GST-PrBP/δ, and PrBP/δ with fusion tags removed (cleaved PrBP/δ) was carried out at 4°C in 1X PDE6 storage buffer in the absence of DTT.

*Analytical Ultracentrifugation-Fluorescence Detection System Analysis*

Recombinant bovine 6-His-PrBP/δ and PrBP/δ without the 6-His fusion tag were exposed to a 10-fold molar excess of Tris-(2-carboxyethyl) phosphine-HCl (TCEP) at
room temperature for 30 minutes in 10 mM Tris, 100 mM NaCl, 2 mM MgCl₂, pH 7.5 to reduce disulfide bonds. After the pH was adjusted to 7.5-8.0 with 100 mM Tris base, proteins were labeled with a 20-fold molar excess of 5'-iodoacetamidofluorescein (5'-IAF) dissolved in DMSO overnight at 4°C in the dark. Dialysis overnight in 1X PDE6 storage buffer with 100 mM DTT or buffer exchange with a 10,000 molecular weight cut off (MWCO) Centricon ultrafiltration device was used to remove free 5'-IAF from solution. For examination of the PrBP/δ-PDE6 interaction, labeled His-PrBP/δ and PrBP/δ were incubated with purified unlabeled PDE6 overnight prior to AU-FDS analysis.

Sedimentation velocity studies were performed with the assistance of Sue Matte (Cote Lab) in an Optima XL-I analytical ultracentrifuge (Beckman-Coulter) with a fluorescence detection system (AVIV Biomedical). Samples were prepared in double-sector cells (Spin Analytical) with sapphire windows and run at 50,000 RPM at 20°C until 500 scans were collected (fluorescence data acquired every minute). Data analysis was kindly performed by Sue Matte (Cote Lab) according to a c(s) distribution model (reviewed in Lebowitz et al., 2002) using the SEDFIT program (Schuck et al., 2002) to determine sedimentation coefficients.

**PrBP/δ Solubilization of PDE6 Assays**

For time-course analysis, ROS membranes prepared as previously described were re-suspended in 20 mM MOPS, 2.0 mM MgCl₂, 60 mM KCl, 30 mM NaCl, 1 mM DTT, pH 7.2 (isotonic buffer), disrupted 20 times with an insulin syringe, and incubated with a 20-fold molar excess of His-PrBP/δ, GST-PrBP/δ, or PrBP/δ with the fusion tag removed. Solubilized proteins were recovered by centrifugation in an Airfuge
(Beckman) for 5 minutes at 28 psi (180,000 x g), and analyzed for PDE6 content or by SDS-PAGE analysis. Likewise, insoluble proteins and membranes were re-suspended in an equal volume of isotonic buffer and analyzed for PDE6 content. PDE6 activity was determined in soluble and insoluble fractions and compared to total PDE6 activity in the ROS homogenate. Samples also were analyzed for PDE6 and PrBP/δ content by immunoblot analysis. For isolation of PDE6-PrBP/δ complexes, the solubilized proteins were incubated with Ni-NTA resin for 1 hour at 4°C and the beads were washed with isotonic buffer prior to re-suspension in gel sample buffer for SDS-PAGE and immunoblot analysis.

For immunoblot analysis, proteins separated by SDS-PAGE were transferred to Whatman nitrocellulose membranes (Novagen) at constant 400 mA in 1X transfer buffer with 20% methanol at 4°C for 2 hours. Membranes were blocked with a 1:4 dilution of Odyssey blocking buffer (Li-Cor Biosciences) in 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 7.5 (1X TBS buffer) for 1 hour and incubated with primary antibodies diluted in 1X TBS buffer overnight. Primary antibodies used for immunoblot analysis include: PDE6 catalytic subunits (rabbit polyclonal NC 63F), Mouse anti His mAb (mouse monoclonal, GenScript Corp.) and PrBP/δ (rabbit polyclonal FLδ). Odyssey Infrared Imaging System secondary antibodies [donkey anti-rabbit IRDye 680 and goat anti-mouse IRDye 800 CW; (Li-Cor Biosciences)] were used for visualization.

Assay for Re-Binding of His-PrBP/δ-PDE6 Complex to ROS Membranes

ROS membranes prepared as previously described were re-suspended in isotonic buffer, disrupted 20 times with an insulin syringe, and then incubated with a 20-fold
molar excess of His-PrBP/δ for 1 hour on ice. Solubilized proteins were obtained by centrifugation in the Airfuge (5 min, 28 psi, 180,000 x g). Excess His-PrBP/δ was removed by centrifugation in a 30,000 MWCO Centricon ultrafiltration device and the His-PrBP/δ-PDE6 protein complex was added back to the depleted ROS membranes for various times. At each time-point, supernatant and pelleted fractions were separated by centrifugation in the Airfuge. Supernatants, and pelleted fractions re-suspended in equal volume of isotonic buffer, were examined for PDE6 activity.

Results and Discussion

Bovine PrBP/δ Cloning and Recombinant Expression

The authentic bovine PrBP/δ 450 bp gene was successfully ligated into both the pET47b (His-tagged) and pGEX6p1 (GST-tagged) plasmids, and these two expression vectors were transformed into BL21-DE3 E. coli cells. For pET47b, conditions for expressing recombinant PrBP/δ were optimized in LB medium containing 50 µg/mL kanamycin when growth was carried out at 37°C. Recombinant PrBP/δ was purified from the soluble lysate by affinity chromatography using Ni-NTA His-Bind resin and a gradient of 1M imidazole (Figure 1.2A). SDS-PAGE analysis of the total cell lysate, insoluble, soluble, and unbound proteins, as well as individual fractions and the pool of fractions with the highest protein content, confirms the successful isolation and relative purity of His-PrBP/δ (Figure 1.2B). Typical yield of His-PrBP/δ was 2-6 mg of affinity purified protein per 500 mL bacterial culture. For the pGEX6p1-PrBP/δ construct, growth was also carried out at 37°C and protein expression induced with 0.5 mM IPTG. Unlike expression of His-PrBP/δ, the yield of purification of GST-PrBP/δ was poor.
generating only ~0.8 mg of GST-tagged recombinant protein for a 500 mL culture. However, the purity of GST-PrBP/δ, as assessed by SDS-PAGE analysis, was nearly 100% (data not shown).

Upon incubation with HRV3c Protease, the 6-His fusion tags or the GST fusion tags were removed from recombinant proteins. Cleaved PrBP/δ was successfully separated from the protease, un-cleaved PrBP/δ, and GST or 6-His fusion tags by incubation with Ni-NTA resin or immobilized glutathione resin (data not shown). As PrBP/δ is not known to have catalytic activity, the effects of storing recombinant PrBP/δ at 4°C for several months on its ability to bind prenylated proteins was not elucidated in detail. However, storing PrBP/δ at this temperature in the absence of DTT and glycerol did not regularly lead to precipitation or aggregation of the protein complexes in solution, and was therefore considered a reliable method of storage. These protocols for His-PrBP/δ and GST-PrBP/δ expression, purification, and storage were determined so that large quantities of recombinant PrBP/δ protein could be generated and used for further experiments to better understand the role of PrBP/δ in PDE6 binding and photoresponse regulation.
His-PrBP/δ expressed in BL21-DE3 cells was affinity purified with 2.5 mL Ni-NTA resin at 0.3 mL/min. and individual fractions were collected. Conductivity and absorbance measurements at 280 nm were made during purification to obtain the chromatogram (panel A). Samples of the total lysate (TL), insoluble pellet (P), soluble lysate (S), unbound proteins (UB), individual fractions (#30-35), pool of fractions 33-35 (Pool), and concentrated pool (UF-P) were mixed with gel sample buffer, separated on a 4-12% SDS-PAGE gel, and stained with colloidal Coomassie for visualization (panel B).
Characterization of Prenyl Binding Protein by Analytical-Ultracentrifuge

AU-FDS allows for measurement of the hydrodynamic properties of a molecule, as well as detection of intermolecular interactions at sub-nanomolal concentrations. Additionally, this method allows for macromolecule interactions to be studied where one protein is fluorescently labeled and the other is essentially invisible. This approach was used to study recombinant PrBP/δ, as well as to characterize the interaction between PrBP/δ and PDE6 in solution. Previously, Sue Matte (Cote Lab) used AU-FDS to study a previously cloned GST-PrBP/δ in a pGEX2T plasmid. However, a nucleotide deletion was discovered in the PrBP/δ cloning region of the pGEX2T-PrBP/δ construct. Furthermore, the GST fusion tag is relatively large (23 kDa) compared to bovine PrBP/δ (17 kDa), and may have significantly affected the results. Thus, recombinant His-PrBP/δ and cleaved PrBP/δ were used for these AU-FDS studies.

Upon analysis of a concentration series of 5'-IAF labeled PrBP/δ and 5'-IAF labeled His-PrBP/δ, the sedimentation coefficient (S) was determined to be 2.0 ± 0.6 and 2.0 ± 0.7, respectively (Figure 1.3A and 1.3B). These results are consistent with the S-value of 2.0 ± 0.04 determined by Sue Matte for PrBP/δ expressed from the pGEX2T-PrBP/δ plasmid containing the sequence error. Upon incubation of 100 nM 5'-IAF-PrBP/δ with 100 nM PDE6 (unlabeled) overnight at 4°C, the sedimentation coefficient shifts to ~8.0 (Figure 1.3). This shift is likely the result of PrBP/δ binding to PDE6, as only labeled species are detected by AU-FDS. These results also confirm that PrBP/δ binds tightly to PDE6 in solution, with or without the 6-His tag at the N-terminus of recombinantly expressed PrBP/δ.
Figure 1.3: Analytical Ultracentrifugation Fluorescence Detection of Recombinant PrBP/δ Association with PDE6 in Solution

A.

B.

Concentration series of 5'-IAF labeled His-PrBP/δ (panel A) and PrBP/δ (panel B) were prepared ranging from 1-100 nM. 100 nM unlabeled PDE6 (nPDE) was incubated with 100 nM 5'-IAF labeled His-PrBP/δ or PrBP/δ overnight at 4°C. Sedimentation velocity studies were run and average sedimentation coefficients of His-PrBP/δ and PrBP/δ alone were calculated, as well as for both complexes with nPDE.
Solubilization of PDE6 by PrBP/δ from ROS Disk Membranes

As recombinant His-PrBP/δ was shown to interact with PDE6 in solution, the ability of His-PrBP/δ to solubilize PDE6 from ROS disk membranes was analyzed. In previous studies using frog ROS and recombinant frog PrBP/δ, the ability of GST-PrBP/δ to solubilize PDE6 appeared to occur on a time scale too slow (~hours) to support a role for PrBP/δ in PDE6 regulation during visual transduction (Angie Norton, unpublished data). Further, association of GST-PrBP/δ into the membrane fraction of the ROS homogenate was not observed (Norton et al., 2005).

In this study, bovine PrBP/δ with a 6-His fusion tag was incubated with bovine ROS homogenate. Upon incubation of homogenized ROS membranes with His-PrBP/δ, PDE6 is rapidly released into the soluble fraction with more than 50% PDE6 released from ROS membranes after only 10 seconds of His-PrBP/δ incubation (Figure 1.4A). This fast response could be due to the difference in size between the fusion tags on His-PrBP/δ and GST-PrBP/δ, or may be due to the species difference between recombinant frog PrBP/δ and its interaction with frog ROS versus recombinant bovine PrBP/δ and bovine ROS.

To further elucidate the reason for this discrepancy, the ability of His-PrBP/δ, GST-PrBP/δ, and PrBP/δ (lacking the fusion tag), to solubilize PDE6 from ROS were compared. Using a 20-fold molar excess of each form of GST-PrBP/δ and cleaved PrBP/δ, the amount of solubilized PDE6 was quantified after incubation time-points ranging from 5 seconds to 1 hour, with a phosphate release microplate assay (see Figure 1.4B and 1.4C). Based on this comparison, PrBP/δ in the absence of a fusion tag, and
His-PrBP/δ appear to act faster to remove PDE6 from ROS membranes than GST-PrBP/δ. Therefore, the GST fusion tag may interfere with the ability of PrBP/δ to solubilize PDE6 when associated with ROS membranes. This may be due to GST-PrBP/δ having limited access to the farnesyl and geranylgeranyl moieties on the C-terminus of PDE6 catalytic domains in this membrane-associated state.
Figure 1.4: Time-Course of Bovine PrBP/δ Solubilization of PDE6 from ROS.

A. PDE6 Released from ROS Membranes by His-PrBP/δ

B. PDE6 Released from ROS Membranes by GST-PrBP/δ
Bovine ROS homogenates (100-120 nM PDE6) were incubated with a 20-fold molar excess of recombinant His-PrBP/δ (panel A), GST-PrBP/δ (panel B), or PrBP/δ (panel C) for incubation times ranging from 10 sec to 60 min on ice. Samples were centrifuged to separate soluble and insoluble proteins. The amount of PDE6 solubilized from ROS was determined by PDE6 activity relative to the maximum activity of PDE6 in total ROS homogenate. Graphs show average ± SEM for (n= 3 for panel A, n=2 for panels B and C) and are curve fit with an exponential rise to maximum. Immunoblot analysis was performed with primary antibodies against PDE6 (NC 63F) and PrBP/δ (FLδ).

The faster time-course of PDE6 solubilization from bovine ROS with His-PrBP/δ or cleaved PrBP/δ may indicate a role for PrBP/δ transporting PDE6 during light adaptation. The translocation of PDE6 on rod disk membranes has been previously observed using electron microscopy, where approximately 19% of PDE6 was localized near the disk rim edge during dark state, but within 1 minute of light exposure approximately 50% of the disk rim-localized PDE6 had relocated towards the center of the disk (Chen et al., 2008). This time course during which the concentration of PDE6 relocates on rod disk membranes is comparable to the time course of PDE6 solubilization.
from ROS membranes by the exogenous addition of His-PrBP/δ or cleaved PrBP/δ (Figure 1.4A and 1.4C).

To determine if the PrBP/δ remains bound to PDE6 after solubilization, Ni-NTA resin was incubated with the solubilized PrBP/δ-PDE6 complex after His-PrBP/δ was incubated with ROS homogenate for 1 or 5 minutes on ice. SDS-PAGE analysis confirms that His-PrBP/δ is binding to and solubilizing PDE6 from the disk membranes, forming a stable complex that can be isolated (Figure 1.5A, Lanes T=1' and T=5'). Immunoblot analysis confirms the presence of His-PrBP/δ and PDE6 catalytic subunits in the immunoprecipitated samples (Figure 1.5B). Further, the endogenous soluble PDE6 does not bind to the Ni-NTA resin when exogenous His-PrBP/δ is not present, eliminating the possibility of non-specific interaction between PDE6 and Ni-NTA beads (Figure 1.5B, Lanes T=0'). In addition to providing support for stable PrBP/δ-PDE6 interaction, these results also support a role for PrBP/δ as a tool to purify and isolate PDE6 from ROS membranes.
Figure 1.5: Bovine PrBP/δ Binds to PDE6 upon Release from ROS Disk Membranes

Bovine ROS homogenate (100-120 nM PDE6) was incubated with a 20-fold excess of recombinant His-PrBP/δ relative to PDE6 for 1 min or 5 min on ice. Solubilized fractions were collected by centrifugation and incubated with Ni-NTA beads for 1 hour at 4°C. The solubilized proteins (S) and proteins bound to the beads (B) were analyzed by SDS-PAGE (panel A) or immunoblot (panel B). The total ROS homogenate (T), proteins that did not bind to the beads (UB) and a bead wash sample (W) were also collected and analyzed by immunoblot with antibodies to the 6-His tag (anti-His) and PDE6 (NC 63F).

Rebinding of PDE6 to ROS Membranes after PrBP/δ Solubilization

If PrBP/δ has a role in dynamically translocating PDE6 during light adaptation, PrBP/δ ought to have the ability to release PDE6 for rebinding to the ROS membranes to restore the dark-adapted state. To examine the competition between ROS membranes and His-PrBP/δ for PDE6 binding, a ROS homogenate was depleted of ~90% of its PDE6 by incubation with a 20-fold molar excess of His-PrBP/δ relative to PDE6 for 1 hour on ice. The solubilized PDE6-containing fraction was isolated by centrifugation, the free His-PrBP/δ removed by ultrafiltration, and then the PDE6 sample was mixed with
ROS membranes for various times. The ROS membrane-associated PDE6 and the unbound PDE6 were analyzed with the standard enzyme assay. While the initial re-binding of PDE6 seemed to occur on a time scale of minutes, PDE6 activity in the solubilized fractions began to increase again after several hours of incubation with PrBP/δ (Figure 1.6). It is not clear why this fluctuation in PDE6 between the membrane-associated state and the PrBP/δ-bound soluble state would occur, but the ability of some PDE6 to reassociate with ROS disk membranes having previously been bound to PrBP/δ does suggest that PrBP/δ can dissociate from the isoprenyl groups of PDE6 to allow rebinding of PDE6 to the disk membranes.
Figure 1.6: PDE6 Re-Associates with ROS Membranes After-Solubilization with PrBP/δ

Bovine ROS homogenates (100-120 nM) were incubated with a 20-fold excess of His-PrBP/δ (relative to PDE6). Solubilized proteins were collected by centrifugation and excess His-PrBP/δ was removed. His-PrBP/δ-PDE6 complex in solution was added back to ROS membranes for 10 to 1200 min. The soluble (triangles) and membrane-associated fractions (circles) were analyzed for PDE6 activity and compared to total activity in the ROS membrane and unbound supernatant fractions after excess His-PrBP/δ incubation.

Conclusions

Protocols for the expression and purification of recombinant bovine PrBP/δ, expressed from the authentic nucleotide sequence of the 450 bp bovine PrBP/δ insert, have been developed and optimized. This has allowed for production of milligram quantities of His-PrBP/δ and GST-PrBP/δ for use in characterizing stable interactions with PDE6 in solution, and the reversible interaction with PDE6 in the presence of ROS membranes. PDE6 and recombinant PrBP/δ show high affinity interactions in solution,
as detected by AU-FDS analysis, and this interaction remains stable in the absence of ROS membranes. Upon characterization of PrBP/δ and PDE6 interactions in ROS homogenates, His-PrBP/δ and PrBP/δ without a fusion tag appear to act on a time-scale faster than GST-PrBP/δ, suggesting the larger fusion tag provides steric hindrance that interferes with the ability of the hydrophobic pocket of PrBP/δ to access the farnesyl and geranylgeranyl groups at the C-terminus of PDE6. Finally, PrBP/δ and disk membranes may compete for the binding of PDE6 in ROS, though the binding constants of PDE6 to each have not been elucidated in detail here.

This work supports the ability of PrBP/δ to solubilize PDE6 from ROS membranes on a time-scale relevant to some aspects of light adaptation. Preliminary results also suggest PDE6 can re-associate with ROS membranes in a reversible manner. However, while the exact concentration and localization of PrBP/δ in bovine ROS is not clear, it is possible that there is not enough PrBP/δ in ROS to solubilize a significant amount of PDE6 to play a role in light adaptation. However, as it is also not clear whether 1 or 2 PrBP/δ molecules bind per PDE6 molecule in vivo, this model cannot be ruled out. While PrBP/δ may still serve to function in prenylated protein transport in photoreceptor cells, this protein may also serve a role in desensitizing PDE6 during light adaptation of rod photoreceptors. Therefore, while the role of PrBP/δ in transport of prenylated proteins from the inner segment to the outer segment of photoreceptor cells is not contradicted, the potential role for PrBP/δ in PDE6 regulation during light adaptation is supported.

**Future Directions**
While this work suggests PrBP/δ interacts with PDE6 with high affinity in solution and in the context of ROS membranes, future in vivo or ex vivo studies are needed to conclusively support a role for PrBP/δ in PDE6 translocation during light adaptation. Further, the mechanism by which PrBP/δ is able to access the prenyl groups on PDE6, associated with the ROS disk membranes, is unknown at present. In cone photoreceptors, the α' subunits of PDE6 are geranylgeranylated and isolated soluble from retinal extracts. In contrast, the PDE6 αβ heterodimer in rod photoreceptors is farnesylated and geranylgeranylated, and the majority is isolated on the membrane. Therefore, there are likely differences in the affinity of PrBP/δ for the different isoprenyl groups and differences in the affinity of the farnesyl versus geranylgeranyl moieties for the ROS disk membrane under various conditions. Further, as the exact sub-cellular localization of PrBP/δ in photoreceptor cells is not clear, PrBP/δ may exhibit a light dependent translocation, like other photoreceptor proteins, that allows for PDE6 interaction only in the context of light adaptation in rod cells. It is suspected that other binding proteins may be involved that regulate the interaction between PrBP/δ and PDE6 in ROS. Future studies examining the proteins that interact with PDE6 and PrBP/δ in dark-adapted and light-activated states of ROS may provide additional information about the regulation of their interaction. Further, the role of the GAF domains in regulating PDE6 binding to PrBP/δ have not been investigated here. Since effects of PrBP/δ on cGMP binding to the noncatalytic sites in the GAF-A domains of PDE6 have been documented in vitro (Mou et al., 1999), it would be helpful to clarify this interaction in the context of the ROS membranes in the presence or absence of PrBP/δ.
CHAPTER II

A PROTEOMICS-BASED APPROACH FOR THE IDENTIFICATION OF
PHOTORECEPTOR PHOSPHODIESTERASE BINDING PARTNERS IN BOVINE
ROD OUTER SEGMENTS

Introduction

Several studies have identified potential photoreceptor phosphodiesterase (PDE6) binding partners in the specialized disk membranes of rod outer segment (ROS) that are important to normal photoreceptor cell physiology and function. As discussed in the previous chapter, prenyl binding protein (PrBP/δ) may play a role in the localization of prenylated photoreceptor proteins, such as rhodopsin kinase (GRK-1) and the catalytic subunits of PDE6, in rod and cone cells (Zhang et al., 2007; Karan et al., 2008). Additionally, glutamic acid-rich protein-2 (GARP2), an alternative splice variant of the β-subunit of rod cGMP-gated ion channel 1 (CNGB1), is important for ROS morphology of disk membranes and may assist in keeping PDE6 inactive during the dark state (Körschen et al., 1999; Pentia et al., 2006; Zhang et al., 2009). Further, in human studies and animal models, genetic mutations in any of the PDE6 subunits or main visual excitation components, including rhodopsin and transducin, can lead to retinal dystrophy, retinitis pigmentosa and/or complete blindness (reviewed in Baehr and Frederick, 2009; Daiger et al., 2007).

Additionally, in order to completely understand PDE6 structure and function, a better understanding of how PDE6 is expressed and folded in vivo is necessary. Progress
in the recombinant expression of PDE6 has been challenging, as a soluble and active form of recombinant PDE6 has not been reported to date. It has been suggested that a chaperone protein, aryl hydrocarbon receptor-interacting protein-like (AIPL1), may play a role in the stability of PDE6 as it is expressed in rod photoreceptors. This is based on studies of mice with reduced levels of or lacking AIPL1, where progressive retinal degeneration is observed and there is reduced expression of PDE6 (Liu et al., 2004; Ramamurthy et al., 2004). Additionally, humans with genetic mutations in AIPL1 develop Leber congenital amaurosis, characterized by the severe early onset of retinal degeneration, due to low levels of PDE6 expression (Sohocki et al., 2000). Therefore, the identification of potential PDE6 binding partners, such as AIPL1, may provide additional information about photoreceptor specific chaperones that are necessary to generate functional PDE6.

There is also evidence for light-induced changes in PDE6 binding partners, as studies with detergent solubilized and detergent-resistant membrane rafts show the distribution of PDE6 and transducin on membranes can change under various conditions (Seno et al., 2001; Nair et al., 2002; Liu et al., 2003). Therefore, in order to better understand the role of these potential PDE6 interacting proteins, identify novel PDE6 interacting proteins, and evaluate which PDE6 binding partners are subject to light regulation, a proteomics-based approach utilizing mass spectrometry was undertaken.

A proteomics approach to identify proteins is an important analytical tool for understanding biochemical signaling cascades. Typically, the protein under study and its binding partners are purified and separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Separated proteins are individually treated with trypsin,
and peptides are analyzed by either matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (MS) or liquid chromatography-electrospray ionization MS (LC-MS/MS). Due to the hydrophobic nature of membrane-bound or membrane-associated proteins, 2D-PAGE may not be optimal, so one-dimensional PAGE (1D-PAGE) separation of proteins can be used, resulting in a mixture of proteins in each gel band that are digested with trypsin and analyzed by LC-MS/MS. Here, we have used this proteomics-based approach of 1D-PAGE followed by LC-MS/MS to study PDE6 interacting partners in ROS membranes and identify those proteins that are subject to light-induced changes in their association (Figure 2.1).

**Figure 2.1** Schematic Diagram of Proteomics-Based Approach for Photoreceptor Protein Identification by Mass Spectrometry

In addition, chemical cross-linking can be used in conjunction with the above mentioned proteomic strategy to analyze *bona fide* PDE6 binding partners and gain insight into the structure of PDE6 interactome. This approach also allows us to carry out detailed topological analysis of the interactions that occur within the PDE6 holoenzyme. By optimizing cross-linking conditions so that the PDE6 α and β catalytic subunits are covalently linked to the inhibitory Py subunits (which can be confirmed by observation of

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catalytic and inhibitory subunit immunoreactivity in higher molecular weight complexes after SDS-PAGE separation), protein bands can be isolated and fragmented into peptides with trypsin treatment. Upon LC-MS/MS analysis, peptides covalently attached to each other can be traced back to their originating protein by in silico comparison to a protein database, and residue pairs in close proximity to one another can be identified.

Therefore, in order to develop a better understanding of the as-yet unexplained aspects of phototransduction and identify potential PDE6 regulatory proteins, the goals of this portion of thesis research include: 1) to optimize solubilization of PDE6 and binding partners from ROS membranes, 2) catalog PDE6 interacting proteins, 3) identify those PDE6 interacting proteins subject to light-induced changes in their binding, and 4) develop a mass spectrometry approach for the initial mapping of PDE6 holoenzyme topology as it exists in solution.

Methods

Bovine ROS and PDE6 Purification

Bovine ROS were prepared and PDE6 was purified from ROS membranes by methods previously described (Pentia et al., 2005), and briefly outlined in Chapter 1.

Extraction of PDE6 and ROS Membrane Proteins

For hypotonic and PrBP/δ extraction procedures, bovine ROS were centrifuged to remove soluble proteins, then re-suspended in 20 mM MOPS, 2.0 mM MgCl₂, 60 mM KCl, 30 mM NaCl, 1 mM DTT, pH 7.2 (isotonic buffer) for a final concentration of PDE6 ranging from 200-400 nM. For detergent solubilization, bovine ROS with soluble proteins removed were re-suspended in 100 mM HEPES, 2 mM MgCl₂, pH 7.5 to a similar concentration of PDE6. For hypotonic extraction, the membranes were washed
with a high magnesium hypotonic buffer (5 mM Tris, 10 mM MgCl₂, 10 mM DTT, pH 7.5) followed by three sequential washes with hypotonic buffer (5 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.5). For PrBP/δ solubilization of prenylated ROS proteins, a 20-fold molar excess of recombinant bovine His-PrBP/δ (see Chapter 1) relative to PDE6 was added to the ROS re-suspension and allowed to incubate overnight at 4°C. For detergent solubilization, 15 mM Tris, 1% Triton X-100, pH 7.5 was added to the ROS membrane re-suspension (final concentrations) to incubate on ice for 30 minutes. In each extraction condition, solubilized proteins were separated from membranes by centrifugation in an Airfuge (Beckman) for 5-15 minutes at 26-28 psi (180,000 x g). Solubilized extracts were analyzed for PDE6 content by SDS-PAGE and immunoblot analysis (as described in Chapter 1), or used for immunoprecipitation assays with ROS-1 antibody conjugated resin to enrich for PDE6-interacting proteins. Primary antibodies used for immunoblot analysis include: PDE6 catalytic subunits (rabbit polyclonal NC 63F); Py inhibitory subunits (rabbit polyclonal Py CT 9710); transducin [rabbit polyclonal Gα t1 (Santa Cruz Biotechnology)]; and rhodopsin [mouse monoclonal rhodopsin ID4 (Chemicon International)]. Goat anti-mouse IRDye 800CW and Donkey anti-rabbit IRdye 680 secondary antibodies (Li-Cor Biosciences) were used for visualization.

Cross-linking of Proteins on ROS Membranes

Bovine ROS membranes were re-suspended in 100 mM HEPES, 2 mM MgCl₂, pH 7.5, and exposed to homobifunctional N-hydroxysuccinimide esters (Pierce Biotechnology), either the water-insoluble disuccinimidyl suberate (DSS) dissolved in DMSO, or its water-soluble analog bis(sulfo succinimidyl) suberate (BS²), dissolved in 100 mM HEPES, 2 mM MgCl₂, pH 7.5. For detecting light-dark differences in PDE6
binding partners, a 10-fold molar excess of cross-linker relative to rhodopsin was used. For dark samples, all procedures were performed in a darkroom under infrared illumination until solubilized cross-linked proteins had been separated from ROS membranes by detergent extraction. For light samples, a 10-fold excess (relative to rhodopsin) of the non-hydrolysable GTP analog, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), was incubated with re-suspended ROS membranes, and all procedures were performed under ambient room light. Cross-linking reagents were allowed to incubate with re-suspended ROS membranes for 10-15 minutes on ice, and quenched with 15 mM Tris, 1% Triton X-100 (final concentrations) for 30 minutes on ice. Triton X-100 was used to extract cross-linked proteins from ROS membranes, and soluble extracts were collected by centrifugation in the Airfuge. Confirmation of PDE6 cross-linking and extraction from ROS membranes was analyzed by SDS-PAGE with pre-cast 4-12% Bis-Tris NuPAGE gels run at 160 V in 1X MES buffer and stained with colloidal Coomassie overnight. The 1% Triton X-100 solubilized extracts were used for immunoprecipitation assays to enrich for PDE6 and PDE6 binding partners, or processed for immunoblot analysis as described previously.

*ROS-1 Immunoprecipitation*

The high-affinity PDE6 mouse monoclonal ROS-1 antibody (Hurwitz and Beavo, 1984) was coupled to an agarose support of AminoLink Resin using the commercially available Pierce Direct IP kit (Thermo Scientific). The extracts from hypotonic, PrBP/δ, or 1% Triton X-100 solubilization of ROS membranes, or the cross-linked proteins solubilized from ROS membranes, were incubated with ROS-1-coupled resin for 2 hours at 4°C with end-over-end mixing. Unbound proteins were removed with three sequential
washes of 1X TBS buffer, and the resin/bound proteins were prepared for SDS-PAGE analysis by re-suspension in gel sample buffer. Immunoprecipitated proteins were separated on a 4-12% Bis-Tris NuPAGE gel, run at 160 V in 1X MES buffer, and stained with colloidal Coomassie overnight, or prepared for immunoblot analysis.

Cross-linking of purified PDE6 holoenzyme in solution

PDE6 holoenzyme cross-linking in aqueous solution was optimized with BS³ or DSS dissolved in DMSO, in 100 mM HEPES, 5 mM MgCl₂, pH 7.5. Cross-linking was allowed to proceed for 45 minutes at room temperature prior to quenching with 8 M ammonium hydroxide (NH₄OH). Quenched samples were mixed with gel sample buffer for SDS-PAGE and immunoblot analysis of PDE6 catalytic and inhibitory subunit migration to higher molecular weight complexes.

Protein separation and mass spectrometry identification

Individual bands of interest were manually excised from SDS-PAGE gels, reduced with 10 mM DTT for 1 hour at 56°C, and alkylated with 55 mM iodoacetimide for 45 minutes at room temperature in the dark. The gel pieces were dried down to completeness, rehydrated in 120-280 ng sequencing grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate, and digested for 4 hours at 37°C. The peptides were isolated from the gel pieces by 2-3 repeated washes in 50% (v/v) acetonitrile and 3% (v/v) acetic acid. The aqueous extracts were concentrated to a final volume of approximately 5-7 µL, and injected into a capillary reverse-phase liquid chromatography system. Peptide separation occurred on a 75 µm x 15 cm reverse-phase capillary column at a flow rate of approximately 300 nL/min, with mobile phases of water with 0.1% (v/v) formic acid (buffer A), and acetonitrile with 0.1% (v/v) formic acid
(buffer B) delivered by a Thermo Surveyor MS Pump, running a 35 minute gradient from 2-35 % buffer B. Eluting peptides were introduced directly to an ion electrospray source of a tandem Linear Trap Quadrupole (LTQ) Orbitrap mass spectrometer (Thermo Fisher). LC-MS/MS data were acquired in positive ion mode, cycling between one precursor ion scan of peptides measured in Orbitrap and six collision-induced dissociation (CID) scans measured in LTQ. Mass spectra ranging from m/z 310 to 2000 were collected, and for each spectrum, the three most intense ion peaks were selected for fragmentation. LC-MS/MS data files from each chromatographic run were analyzed using Protein Prospector (UCSF version 5.3.2) or with Mascot (Matrix Science Ltd., 2000) and searched against the mammalian SwissProt (2009.11.24) database. Mass tolerance was set to 50 ppm for precursor ions and 0.7 Da for fragment ions during the database search. The threshold for positive protein identification was at least one unique peptide with a Protein Prospector peptide score of 20 or a Mascot value of 30. Searches were performed with trypsin specificity, and common modifications were considered, including alkylation of cysteine residues by carbamidomethylation, N-terminal acetylation, and oxidation of methionine. For analysis of cross-linked proteins, mass additions on lysine residues and the N-terminus were also considered. The number of unique peptides and spectral count for each protein identified were documented for semi-quantitative analysis of proteins.

**Results and Discussion**

*Solubilizing the PDE6 Interactome from ROS membranes*

In order to identify putative binding partners of PDE6, the membrane-associated PDE6 must be separated from ROS, while keeping binding partners intact. Therefore, three different approaches were developed to extract PDE6 and binding partners from
ROS membranes. Traditionally, hypotonic buffer has been used to extract membrane-associated proteins non-specifically, as is commonly done in PDE6 purification from ROS membranes. Here, hypotonic extracts were collected to obtain PDE6 and potential binding partners from ROS membranes. The pooled hypotonic extract after three sequential washes was enriched for PDE6 binding partners using ROS-1 antibody conjugated to agarose beads, and isolated proteins were separated by SDS-PAGE (Figure 2.2, Lanes H). This method of extraction yields a high number of bands, most likely due to the non-specific release of soluble and membrane-associated proteins from ROS.

Because recombinant PrBP/d is able to solubilize PDE6 from ROS membranes (as described in Chapter 1), it was used as a second approach to selectively solubilize prenylated proteins, including PDE6, from ROS membranes. By using a 20-fold molar excess of His-PrBP/d, the farnesylated and geranylgeranylated PDE6 was selectively solubilized, as well as additional photoreceptor proteins (Figure 2.2, Lanes δ).

Finally, a third method of extraction was optimized using 1% Triton X-100 in 15 mM Tris buffer, pH 7.5, to more thoroughly extract the membrane-associated proteins from ROS (Figure 2.2, Lanes 1%T). Comparison of these three methods of extraction resulted in the solubilization of many membrane-associated photoreceptor proteins from ROS, which were then selected for their ability to bind PDE6 by immunoprecipitation with the ROS-1 monoclonal PDE6 antibody.
Figure 2.2: Comparison of ROS Proteins Extracted by Hypotonic, His-PrBP/δ or Detergent Solubilization and Immunopurified with a PDE6 Antibody

Photoreceptor proteins were extracted after three repeated hypotonic extractions (H) of ROS membranes, incubation of His-PrBP/δ with ROS homogenates overnight (δ), or detergent solubilization of ROS membranes with 1% Triton X-100 (1% T) for 30 minutes on ice (panel A). After immunoprecipitation of the solubilized extracts with ROS-1 antibody-coupled beads, immunopurified proteins were isolated (panel B). Hypotonic extracts were also incubated with empty beads (CON) to control for non-specific binding to the agarose resin. Samples were run on a 4-12% SDS-PAGE gel and stained with colloidal Coomassie overnight. Brackets indicate molecular weight regions that were manually excised for LC-MS/MS analysis.

All three methods of extraction show strong bands at ~98 kDa after solubilization (Figure 2.2A) and after immunoprecipitation (Figure 2.2B), confirming the isolation of PDE6 α and β catalytic subunits (Pα and Pβ) from ROS membranes. Further, the PDE6 inhibitory subunits (Py) are observed in the immunoprecipitated samples as faint bands at
~12 kDa on Coomassie-stained SDS-PAGE gels. The strong bands apparent at ~27 kDa from all three methods of extraction after immunoprecipitation contains the light chain of the ROS-1 antibody, which was also separated by SDS-PAGE after gel sample buffer and 95°C preparation of the agarose beads for analysis. The heavy and light chains of ROS-1 antibody also display immunoreactivity with the Odyssey goat anti-mouse IR dye 800 secondary antibody used for later analysis of immunoprecipitated proteins (Figure 2.3B).

While hypotonic extraction yielded the highest number of bands after immunoprecipitation and SDS-PAGE separation, 1% Triton X-100 extraction resulted in the fewest (Figure 2.2B). These results were surprising, as the detergent extraction released more proteins than either the hypotonic extraction or the PrBP/δ solubilization prior to immunoprecipitation enrichment (Figure 2.2A). However, the amount of PDE6 catalytic subunits pulled down is similar in all three assays, so the fewer number of other bands visible after immunoprecipitation in the 1% Triton extraction may be the result of detergent disrupting weak or transient interactions between PDE6 and binding partners, or between binding partners and non-specific interactions with the ROS-1-conjugated beads. As indicated by the brackets, several band regions were identified on the SDS-PAGE gel as candidates for analysis by mass spectrometry (Figure 2.2B).

Cataloging the Photoreceptor Proteins that Interact with PDE6

To identify proteins that interact with PDE6 in ROS, three solubilization methods were developed to remove PDE6 and binding partners from ROS membranes. ROS-1 antibody-conjugated beads were used to enrich for PDE6 and interacting proteins, and immunoprecipitated proteins were separated by 1D-PAGE. Individual bands were excised, the proteins were digested with trypsin for 4 hours at 37°C, and tryptic peptides
were prepared for LC-MS/MS. An overview of the identified proteins from all three methods of extraction is shown in Table 2.1.

Proteins were classified into different groups according to their biological function or cellular role, as defined in the Swiss-Prot database. Out of 13 excised bands from the hypotonic extraction, 66 proteins were identified. Seventeen proteins were identified from the 10 excised bands after PrBP/δ solubilization. Out of the 6 excised bands from the 1% Triton X-100 extraction, 24 proteins were identified. Of the total 75 proteins identified, 18 can be classified as proteins related to vision in rod photoreceptor cells, including PDE6 subunits and previously identified binding partners: PDE6 α, β, and γ, transducin α, PrBP/δ and GARP2.

The identification of GARP2 as a potential PDE6 binding partner is not surprising, as the high-affinity association of GARP2 with PDE6 has been documented before (Körschen et al., 1999; Pentia et al., 2006). Further, PrBP/δ was identified after both hypotonic and His-PrBP/δ extractions, which was expected as PrBP/δ co-purifies with soluble PDE6 from bovine retinal extracts (Gillespie et al., 1989). Likewise, interactions between transducin α-subunit and PDE6 are well characterized, and previous cross-linking studies demonstrating their interaction have been reported (Hingorani et al., 1988; Clerc et al., 1992; Artemyev et al., 1993; Grant et al., 2006).

In addition to photoreceptor proteins that have been documented as high-affinity PDE6 binding partners, other proteins related to vision were identified from immunoprecipitated samples including: arrestin, CNGB1, rhodopsin, transducin β1 subunit, and rhodopsin kinase (GRK1). While these proteins have not been recognized
for their ability to directly interact with PDE6 holoenzyme during light activation, some previous studies suggest that transient or *in vitro* interactions are possible.

Arrestin was identified by mass spectrometry after immunoprecipitation under all 3 solubilization regimes. Arrestin primarily resides in the inner segment protein in dark-adapted photoreceptors and exhibits translocation to the outer segment to quench rhodopsin activation during light adaptation (Whelan *et al*., 1988; Peterson *et al*., 2003; Zhang *et al*., 2003; Strissel *et al*., 2006). Further, recombinant Pγ inhibitory subunits have been shown to interact with both transducin α subunit and arrestin from ROS extracts *in vitro* (Qin and Baehr, 1993). In addition, a shorter splice variant of arrestin (p44) previously demonstrated light-dependent translocation into detergent-resistant membranes that also contain PDE6 and transducin (Nair *et al*., 2002), consistent with a possible association of the p44 isozyme with PDE6.

CNGB1 and GARPI were characterized as PDE6 interacting proteins in this study based on mass spectrometry identification of peptides that fall within the first 292 amino acids of the CNGB1 sequence. As GARP1 and GARP2 are splice variants of this sequence, the possibility that CNGB1 or GARP1 are present cannot be excluded. However, GARP2 is the most likely assignment for these peptide sequences as it lacks amino acid residues 300-1394, and contains a unique C-terminus that begins with an argenine at amino acid residue 292, which some of the identified peptides contained.
Table 2.1: Functional Classification of Proteins Isolated from SDS-PAGE gel of PDE6 Immunoprecipitated Complexes from ROS, Identified by MS

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Chaperone & Protein Transport

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Abbreviations used: MW: molecular weight; Acc. ID: Swiss Prot accession identification number; Ext Met: Extraction method; H: hypotonic extraction; δ: His-PrBP/δ solubilization; T: 1% Triton solubilization; PDE: Photoreceptor phosphodiesterase; GARP: Glutamic acid rich protein; PrBP/δ: Prenyl binding protein
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Rhodopsin is an integral membrane protein that represents ~80-85% of the total protein (w/w) in ROS membranes (reviewed in Giusto et al., 2010). There is no evidence to date for direct interaction of rhodopsin and PDE6. However, since transducin activation is dependent on rhodopsin, these proteins most likely exist in very close proximity to one another and to PDE6 in ROS disk membranes. Likewise, while interactions between PDE6 and Tα have been well-documented (see previous section), PDE6 has not been shown to interact directly with transducin β1. Immunoprecipitation of rhodopsin and transducin β1 subunits with the ROS-1 antibody may reflect the formation of a larger complex where PDE6 interacts with the ROS-1 antibody-conjugated beads, Tα binds to PDE6, transducin β1 then binds to Tα, and finally rhodopsin binds to transducin, forming a large immunoprecipitated complex that is separated upon denaturing for SDS-PAGE analysis. Alternatively, rhodopsin and transducin β1 may be interacting with the ROS-1 antibody non-specifically.

The G-protein α0 subunit (Gα0) has previously been localized to the photoreceptor synapse, horizontal bipolar amacrine, and ganglion regions of bipolar retinal neurons by serial tangential sectioning (Song and Sokolov, 2009). This protein is better known for its interaction with a metabotropic glutamate receptor on bipolar cells that senses the glutamate release decrease by photoreceptors upon hyperpolarization in response to light (Nawy, 1999; Ghingra et al., 2000; Vardi et al., 2000). Thus, the identification of Gα0 subunits is not easily explained.

Farnesylated GRK-1 has been shown to interact with PrBP/δ in vitro (Zhang et al., 2004; Norton et al., 2005), but a direct interaction of this kinase with PDE6 in ROS
has not been documented. However, in a reconstituted system of rod disk membranes containing rhodopsin, transducin, and PDE6, the addition of GRK-1 was able to alter the life-time of light-driven PDE6 activation (Sitaramayya, 1986). Additionally, Pγ inhibitory subunits contain two threonine residues that may be candidates for phosphorylation, and when altered by site-directed mutagenesis, have profound effects on the rise and decay of light response (Tsang et al., 2007).

Peripherin-2 was detected after 1% Triton solubilization of membrane complexes from ROS. This protein has been detected in proteomics analysis of ROS before (Panfoli et al., 2008), and has been shown to localize exclusively to rim region of ROS disk membranes (Song and Sokolov, 2009). Further, this protein has been implicated in retinal diseases and is necessary for proper ROS disk membrane structure (Molday et al., 1987; Travis et al., 1989; Connell et al., 1991). While there is no evidence to date for direct interaction of peripherin-2 with PDE6, peripherin-2 has been shown to interact with GARP2, a known high affinity PDE6 binding partner (Poetsch et al., 2001; Pentia et al., 2006).

Retinaldehyde-binding protein 1 (RBP1) was detected as a PDE6 interacting protein under both hypotonic and 1% Triton X-100 solubilization regimes. PDE6 interactions with RBP1 have not been previously reported, though this protein is present in ROS and in the retinal pigment epithelium, where it assists in the re-generation of 11-cis retinal (reviewed in Saari, 2000). Recently, RBP1 was also shown to play an important role in ROS membranes organization (Wang et al., 2010).

Although some soluble proteins, such as arrestin, GRK1, and RBP1 may interact with PDE6 directly or indirectly through a binding complex in ROS, the presence of rod
inner segment-specific proteins is likely due to contamination of rod inner segment fractions in the rod outer segment preparation of bovine retinas. PDE6 is most densely concentrated in the outer segment of photoreceptor cells, but some studies using immunofluorescence or serial tangential sectioning of the retina also show lesser amounts of PDE6 present in the inner segment as well (Muradov, 2009; Song and Sokolov, 2009). Thus, the observation of PDE6 interacting with proteins believed to be confined to the inner segment is plausible, especially considering that some of the isolated rod photoreceptor fragments might include ROS to which a portion of the inner segment is still attached (Spencer et al., 1988). In addition, the most likely explanation for the presence of cone-specific proteins, such as cone phosphodiesterase subunits $\alpha'$ and $\gamma'$, is a result of contamination of cone cells in the rod photoreceptor preparation from bovine retina, and the ability of the ROS-1 antibody to also interact with cone PDE6$\alpha'$ subunits, which has been demonstrated before (Hurwitz et al., 1985).

Further, as a large number of cytosolic proteins were also identified, and PDE6 is known to be a membrane-associated protein, these interactions may have occurred post-solubilization, and were enriched during immunoprecipitation due to non-specific binding of these abundant soluble proteins to PDE6 or a PDE6 binding partner. Non-specific interactions of proteins with the agarose resin is not likely, as control beads lacking conjugated antibodies were used and did not pull down any significant amount of protein (Figure 2.2, Lane CON).

Considering that inner segment proteins may be contamination in the ROS preparations and that the inner segment is the site of biosynthesis, it is not surprising to see several chaperone proteins that may be present in photoreceptor cells for assisting in
the folding of recently synthesized peptides (reviewed by Frydman, 2001). Further, heat shock protein 90 (Hsp90) subunits were also identified in the previous proteomics study of ROS (Panfoli et al., 2008), and have been localized to all retinal cell layers, including the outer segment of photoreceptors (Song and Sokolov, 2009). In addition, the ubiquitous 14-3-3 proteins have been detected in photoreceptor cells and exhibit light-dependent changes of their interacting proteins (Pozdeyev et al., 2006).

The presence of several proteins involved in anaerobic glycolysis in ROS has been previously reported in metabolic and proteomics studies of intact rod cells or ROS (Hsu and Molday, 1991; Panfoli et al., 2008). As some of these proteins have been hypothesized to be located near ROS plasma membranes, but not necessarily associated with disk membranes, the association of these proteins with PDE6 may also be an artifact of the hypotonic extraction method. Proteins involved in aerobic metabolism, normally located in mitochondria in the inner segment, were also identified, including components of ATP synthase (α and β subunits). This was unexpected and could be due to rod inner segment contamination of isolated ROS membranes. However, the previous proteomics study performed on ROS, but not specific to PDE6 interacting partners, also identified many proteins specific to aerobic metabolism, and followed up on this unexpected observation with an immunological approach that localized the presence of F1F0-ATP synthase on disk membranes in purified ROS (Panfoli et al., 2008). While this analysis provides a possible explanation for the detection of several mitochondria-derived proteins in ROS disk membranes, and has implications for phototransduction energy supply, further studies are necessary to determine the localization and function of these proteins on ROS disk membranes in vivo and the likelihood that they interact with PDE6.
In addition, several subunits of cytoskeleton proteins that commonly act in cell growth and/or maintenance were also identified by this PDE6 immunoprecipitation and mass spectrometry method. These proteins are expected in ROS (Woodford and Blanks, 1989; Song and Sokolov, 2009), as they are components of the ciliary axoneme and may influence the organization of ROS disk membranes or play a role in photoreceptor morphology, but their interaction with PDE6 was not expected.

Comparison of Dark-Adapted and Light-Activated Phosphodiesterase Binding Complexes in Rod Outer Segment

To reduce the likelihood of misidentifying interactions between photoreceptor proteins and PDE6 that represent non-specific associations of proteins following solubilization, a cross-linking approach was taken to covalently link PDE6 to its immediate binding partners while still attached to ROS membranes. The cross-linkers BS³ or DSS, each with an 11.4 Å spacer arm, were used for these mass spectrometry and immunoblot studies, and optimization of cross-linking conditions was determined based on shifts of PDE6 catalytic and inhibitory subunit immunoreactivity migration to higher molecular weight complexes after SDS-PAGE separation.

In addition, differences in PDE6 interacting proteins under dark-adapted versus illuminated conditions were semi-quantitatively evaluated by cross-linking ROS proteins in the dark, and in the light in the presence of GTPγS (to persistently activate transducin). Cross-linked complexes were solubilized from ROS membranes with 1% Triton X-100 and enriched for PDE6 by ROS-1 immunoprecipitation. PDE6 was successfully cross-linked to other proteins, as evidenced by shifts in molecular weight from ~98 kDa to ~110, 250, and >250 kDa upon SDS-PAGE and immunoblot analysis following
immunoprecipitation of PDE6 (Figure 2.3A and 2.3B). Bands containing PDE6 catalytic domains were excised from the SDS-PAGE gel and analyzed by LC-MS/MS for peptide identification. A comparison of proteins identified from the immunoprecipitated samples in an un-cross-linked control, in the dark sample, and in the light with GTPγS, is displayed in Table 2.2. These proteins are classified by their relative abundance as identified in the >250 kDa region, 250 kDa band, 110 kDa band, and 98 kDa band excised from the gel.
Figure 2.3 Cross Linking and Immunoprecipitation of Dark and Light-Activated PDE6 Complexes in ROS

A. Bovine ROS were cross-linked with a 10-fold molar excess of BS$_3$ (relative to rhodopsin) in the dark (D), or upon light exposure (L) in the presence of GTPyS. The addition of 15 mM Tris with 1% Triton X-100 quenched the reaction and solubilized proteins. A sample exposed to the same Tris and Triton X-100 treatment without BS$_3$ was used (C). ROS-1 immunoprecipitated (IP) proteins were separated on a 4-12% SDS-PAGE gel, and stained with colloidal Coomassie (panel A), or immunoblot analysis (WB) was performed by probing for Pγ inhibitory subunits (Pγ CT) or PDE6 catalytic subunits (NC 63F) (panel B). The immunoreactivity present at ~25 and ~55 kDa is the ROS-1 antibody, which shows cross-reactivity with goat anti-mouse IR dye (800 nm) secondary antibody and confirms equal loading of immunoprecipitated proteins onto the SDS-PAGE gel. PDE6 activation in ROS samples exposed to light in the presence of GTPyS (Light + GTPyS) was confirmed by enzyme activity measurements (panel C). Activity is expressed as a percentage of the fully activated enzyme (% relative to trypsin activation).
Table 2.2: Comparison of Unique Peptides of ROS-1 Immunoprecipitated Proteins Isolated from SDS-PAGE gels of Cross-linked PDE6 Complexes from ROS Homogenate, Identified by MS

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Abbreviations used: ID: Identification; UP: Number of unique peptides; SC: Spectra count; Con: Un-cross-linked control; DK: Dark cross-linked sample; LT: Light and GTPγS exposed cross-linked sample; PDE: Phosphodiesterase 6
To ensure that GTPγS addition and light exposure induced PDE6 activation, measurements of PDE6 hydrolytic activity revealed a 16-fold activation of PDE6 upon light activation: PDE6 activity in the dark samples was 1.1% ± 0.3% of the maximum activity, whereas 17.3% ± 4.8% PDE6 activation was detected in the GTPγS and light exposed samples (Figure 2.3C). Comparison of the number of unique peptides (UP) and spectra count (SC) for peptides corresponding to PDE6 catalytic subunits (PDE6α, PDE6β) in the un-cross-linked, and dark and light cross-linked samples provides information about the consistency of PDE6 loaded onto the SDS-PAGE gel, the efficiency of peptide release from the gel matrix, and the extent of cross-linking that took place within the light and dark samples (Table 2.2).

As expected, the un-cross-linked samples show the majority of PDE6α and PDE6β peptides detected at ~98 kDa, with 56 and 60 unique peptides identified, and 104 and 117 spectra counts for PDE6α and PDE6β, respectively. At high molecular weight bands PDE6 α and β catalytic subunits were much less abundant, as expected. The Pγ inhibitory subunit, which typically runs at 10-12 kDa, was not detected in these higher molecular weight bands of the un-cross-linked control sample, as expected. Interestingly, Pγ inhibitory subunits seemed difficult to detect overall in most of the conditions and bands at various molecular weights. One reason for the low number of unique peptides and spectra count for Pγ subunits is due to the small size of this protein, the few number of trypsin cleavage sites in the second half of the protein, and high number of acidic residues in the C-terminus that are difficult to detect in mass spectrometry studies.
Unexpectedly, rhodopsin was detected in all of the bands examined and in both un-cross-linked and cross-linked samples. One likely possibility is that rhodopsin is non-specifically immunoprecipitated due to its high abundance on ROS disk membranes and therefore contaminates all three conditions (un-cross-linked, dark, and light cross-linked) equally. This is supported by the relative even distribution of rhodopsin peptides and spectra count among the three conditions. Further, rhodopsin is a very hydrophobic protein that tends to aggregate, even forming dimers in vivo (Fotiadis et al., 2003), and these peptide identifications may be a result of those oligomers running at higher molecular weight bands during SDS-PAGE separation.

Cone PDE6α' subunit was also seen in most of the samples analyzed. A reasonable explanation is that the rod outer segment preparation from bovine retina was not pure, as discussed previously. However, unlike the solubilization studies, in these cross-linking studies the cone Py' subunit was not detected in any of the bands examined.

Finally, the presence of peptides comprising Na⁺/K⁺-transporting ATPase subunits is concerning as they appear in relative high abundance in the 250, 110 and 98 kDa bands excised from all three conditions. Like rhodopsin, these peptides also show a relatively even distribution, but the cross-linked samples in both dark and light contain more unique peptides and spectra counts for Na⁺/K⁺-transporting ATPase subunit α-1, than does the un-cross-linked control. Peptides of the Na⁺/K⁺-transporting ATPase subunit α-1 were also detected in a previous study examining the proteome of ROS disk membranes (Panfoli et al., 2008)

Upon cross-linking in the dark with a 10-fold molar excess of BS³ to rhodopsin, the number of unique peptides at ~98 kDa band decreases to 43 and 46 and the number of
spectra counts decreases proportionally, for PDE6α and PDE6β, respectively. For the light and GTPγS-induced sample, these numbers decreased in a similar manner with 53 unique peptides for both PDE6α and PDE6β, and 57 and 61 spectra counts, respectively. Therefore, the extent of cross-linking was similar in both the light and the dark, and the decrease in detected peptides for PDE6α and PDE6β at ~98 kDa confirms the migration of these peptides to higher molecular weight bands, where they are cross-linked to other proteins. Further, the number of unique peptides and spectra counts representing PDE6 α and β subunits is nearly identical for the dark and light-induced cross-linked samples at 110 and 250 kDa, supporting the relative equal intensities of catalytic subunit immunoreactivity (NC 63F) represented on the immunoblot (Figure 2.3 B).

However, the shifts in PDE6 inhibitory subunit immunoreactivity (Pγ CT) observed by western blot analysis are not as robustly supported by the mass spectrometry data. Where immunoreactivity for Pγ subunits is clearly observed at 110 and 250 kDa in the dark cross-linked sample, and perhaps to a lesser extent in the light, unique peptides and spectra count for Pγ subunits are only detected in dark cross-linked sample at 250 kDa, and in both the dark and light cross-linked samples at 110 kDa in relative low abundance. While Pγ subunit immunoreactivity at 250 kDa in the light-induced cross-linked sample is not as intense as the dark, the limitations for detecting peptides from the Pγ subunits may be a result of the small size (87 amino acid residues) of Pγ, relatively acidic C-terminus with few basic residues to carry the positive charges required for mass spectrometry detection, and the inability of trypsin to cleave the protein into smaller peptides past lysine at amino acid residue 45, generating only a small number of unique
peptides to be identified. However, the detection of even a single unique peptide for Py subunits is considered a success, as a previous study examining the protein composition of ROS disk membranes failed to identify any Py subunits despite the successful identification of PDE6 α and β subunits and all other main proteins involved in visual transduction (Panfoli et al., 2008).

Light-induced changes were observed for transducin (Tα), which was only detected in dark cross-linked samples also containing PDE6 catalytic domains at >250, 250, and 110 kDa. This observation was seemingly contradictory with our current understanding of the visual excitation pathway where Tα–GTP associates with PDE6 holoenzyme upon exposure of rod cells to light in order to relieve inhibition by the Py subunits and allow for cGMP hydrolysis at the PDE6 catalytic domains. However, transducin and other photoreceptor proteins have been shown to exhibit light-induced translocation from ROS membranes to the inner segment upon persistent light activation in as few as 30 seconds after exposure (reviewed in Burns and Arshavsky, 2005; Calvert et al., 2006; Artemyev, 2008; Slepak and Hurley, 2008). This dissociation of Tα from ROS disk membranes is most likely due to the weakened interaction of Tα-GTP, which has N-terminal acylation, compared to the dual interaction of Tα and farnesylated Ty with the ROS membranes in the inactive Tαβγ trimeric G-protein complex (Bigay et al., 1994; Kosloff et al., 2008; Wang et al., 2008). As cross-linking on ROS membranes took place over a period of 10-15 minutes, Tα-GTPγS initially interacting with PDE6 upon light activation may have dissociated from the membrane over the period of time required
for cross-linking, removing the possibility for BS\textsuperscript{3} to form stable amide bonds with primary amine groups on both T\alpha and PDE6.

PDE6 and T\alpha are very likely located in close proximity on dark-adapted rod disk membranes to maximize the speed and sensitivity of light activation. This could explain the observation of cross-linking of the two proteins together in the dark. However, although T\alpha is detected as a PDE6 interacting protein in the dark, it is not binding to PDE6 in a manner to cause displacement of the inhibitory P\gamma subunit to allow activation and cGMP hydrolysis. In the light and in the presence of GTP\gamma S, PDE6 becomes activated and T\alpha is no longer detected as a binding partner, suggesting activation of PDE6 by association of the C-terminus of P\gamma with T\alpha is transitory and the T\alpha-P\gamma complex may dissociate from the PDE6 catalytic dimer on a time-scale that is faster than the time-scale of stable cross-linker bond formation between T\alpha and PDE6 subunits. As the only samples that were analyzed by mass spectrometry were those that contained PDE6 catalytic subunit immunoreactivity after cross-linking and SDS-PAGE separation of immunoprecipitated proteins, a potential P\gamma-T\alpha complex cross-linked at \(~45\) kDa was not analyzed by this proteomics approach.

For the \(>250\) kDa SDS-PAGE gel band, the greatest number of unique peptides and spectra count for PDE6\alpha and PDE6\beta are found in the dark cross-linked sample. A small number of unique peptides were detected in the cross-linked light sample. This difference may be due to the close proximity of PDE6 molecules in the dark, where there is support for localization of PDE6 to the edges of ROS disk membranes, compared to after light exposure, where PDE6 molecules may not be as concentrated to the rim region.
of the disk membranes (Chen et al., 2008). Other possibilities include the formation of a rhodopsin, transducin, and PDE6 holoenzyme complex that would run >250 kDa on an SDS-PAGE gel.

To examine this further, immunoblot analysis was performed on ROS membranes and on 1% Triton X-100 solubilized proteins in the presence or absence of cross-linking in both dark and light-activated conditions. Detergent-resistant membranes and solubilized proteins were probed for the presence of PDE6 catalytic (NC 63F) and inhibitory subunits (Py CT), rhodopsin and Tα subunit (Figure 2.4).
Figure 2.4: PDE6 Moves to Detergent-Resistant Membranes upon Cross-linking in Light-activated ROS

Bovine ROS proteins were solubilized with 1% Triton X-100 in the dark, or after illumination in the presence of GTPγS (Light) before (-) or after (+) exposure to a 10-fold molar excess DSS (relative to rhodopsin). Soluble (sol.) and insoluble (insol.) fractions were collected by Airfuge centrifugation. Immunoblots were analyzed for PDE6 catalytic subunit (NC PDE6, panel A), Pγ inhibitory subunit (Pγ, panel B), rhodopsin (Rho, panel C), and transducin α subunit content (Tα, panel D).

Based on the relative intensities of immunoreactivity for PDE6 α, β, and γ subunits, as well as Tα subunit, several light-dark differences in the partitioning of these proteins in bovine ROS is observed. For PDE6 catalytic subunits, both dark and light-
activated conditions show PDE6 migration to higher molecular weight bands of 110, 250, and >250 kDa after cross-linking, but the cross-linked subunits seem to exhibit more resistance to solubilization by 1% Triton X-100 after illumination and GTPγS exposure (Figure 2.4A). This may be a result of PDE6 catalytic subunit interaction with detergent resistant membranes during light adaptation, which is plausible considering the affinity of PDE6 for specific phospholipids may be enhanced upon activation with Tα-GTPγS (Hessel et al., 2003). Further, the lower amount of >250 kDa cross-linked PDE6 catalytic subunit-containing complexes released from the membranes with 1% Triton X-100 in the light supports the smaller number of unique peptides and spectra count detected for PDE6 catalytic subunits after immunoprecipitation and mass spectrometry.

The migration of Pγ subunits to higher molecular weight complexes also reflects the mass spectrometry data, as there are considerably more cross-linked Pγ subunit-containing complexes identified in the dark samples at 110, 250 and >250 kDa (Figure 2.4B). Interestingly, the majority of Pγ immunoreactivity in the cross-linked light-activated sample shifts to an apparent molecular weight of 30-35 kDa and is detected only in the insoluble fraction. This immunoreactivity could represent a cross-linked Pγ-Tα complex that migrates to detergent-resistant membranes, as transducin α immunoreactivity is also present at ~35 kDa (Figure 2.4D). However, as the molecular weight of Tα is 40 kDa, it is surprising to see this intense immunoreactivity present at ~35 kDa in all sample of the light-activated ROS homogenate.

Further, the presence of Tα immunoreactivity as a smear in higher molecular weight complexes is observed for 1% Triton X-100 soluble fractions for both cross-
linked and un-cross-linked samples in the dark and in the light, but is not present in the insoluble fraction in un-cross-linked samples (Figure 2.4D). Even though this immunoreactivity is detected in un-cross-linked soluble fractions and light-activated soluble factions, Tα peptides were not identified by mass spectrometry under these conditions. Therefore, this immunoreactivity is not necessarily specific to the population of Tα that interacts with PDE6, and in the light and un-cross-linked samples was not isolated after immunoprecipitation.

Rhodopsin immunoreactivity is clearly spread across a range of higher molecular weight bands and exhibits the strongest immunoreactivity at ~120 kDa in un-cross-linked samples (Figure 2.4C), supporting the equal identification of rhodopsin peptides in higher molecular weight complexes of 110, 250 and >250 kDa by mass spectrometry, and the oligomerization of this high abundance protein.

Initial Topographical Mapping of Phosphodiesterase Holoenzyme

To elucidate sites of interaction between subunits of the holoenzyme, PDE6 was extracted and purified from bovine ROS and exposed to cross-linker DSS in solution. Optimization of the extent of cross-linking was analyzed by immunoblot analysis with primary antibodies directed against PDE6 catalytic subunits and Pγ inhibitory subunits. Un-cross-linked PDE6 holoenzyme (0x) shows a strong band at ~98 kDa upon SDS-PAGE analysis, representing the Pα or Pβ catalytic subunits (Figure 2.5A). Upon cross-linking with a 10-, 50-, or 100-fold excess of DSS to PDE6, bands with catalytic subunit and Pγ immunoreactivity appear in the higher molecular weight bands at ~110 kDa and 250 kDa (Figure 2.5B). These higher molecular weight bands may contain a single catalytic subunit cross-linked to a single inhibitory subunit (Pαγ or Pβγ), or the entire
holoenzyme cross-linked together (Pαβγγ). Bands containing PDE6 at 98, 110, 250, and >250 kDa were excised from SDS-PAGE gels and prepared for LC-MS/MS analysis. The peptides identified were analyzed for unanticipated mass additions, which correspond to the addition of a cross-linked peptide fragment by interaction with another internal lysine residue, or a lysine residue from a different PDE6 subunit.

Unfortunately, due to the highly negative nature of the Pγ C-terminus, as well as the limited number of trypsin cleavage sites available in this region, there were no inter-subunit interaction sites identified from these cross-linked complexes, despite the immunological confirmation that both subunits exist in these higher molecular weight complexes. Where the extent of cross-linking has been optimized by an immunoblot perspective, identifying high molecular weight smears of PDE6 catalytic and inhibitory subunit reactivity, the extent of cross-linking for mass spectrometry identification may need further optimization. A few peptides with cross-linker mass additions that represented catalytic subunits were identified, but the nature of the cross-linking was determined to be intra-peptide or intra-subunit and did not correspond to interaction sites between Pα, Pβ and Pγ subunits, as desired (data not shown).
Figure 2.5 Cross-linking of PDE6 Holoenzyme in Solution

A. 0x 10x 50x 100x

250— 150— 100— 75—

Pα or Pβ

B. 0x 10x 50x 100x

250— 150— 100— 75—

15—

10—

PDE6 NC 63F

PγCT

Gel filtration-purified PDE6 holoenzyme (Pαβγγ) was exposed to a 10-, 50-, or 100-fold molar excess of DSS for 45 minutes at room temperature. The cross-linking reaction was quenched with 8 M NH₄OH and samples were prepared for SDS-PAGE analysis with gel sample buffer. Samples were run on a 4-12% Bis-Tris gel and stained with colloidal Coomassie (panel A) or processed for immunoblot analysis and probed for PDE6 catalytic (PDE6 NC 63F) or inhibitory subunit (PγCT) content (panel B).

Conclusions

The identification of PDE6 interacting proteins is important for furthering our knowledge of as-yet unexplained aspects of visual transduction, for gaining insight into PDE6 structure and novel PDE6 regulatory mechanisms in photoreceptor cells, and for better understanding photoreceptor proteins that may influence retinal degeneration and diseases. In this study, a proteomics approach was used to optimize the extraction of PDE6 and binding partners from ROS membranes, to catalog PDE6 interacting proteins as isolated by immunoprecipitation assays, and to document those PDE6 interacting proteins that are subject to light regulation in their binding.

To achieve the goals set-forth, three separate methods have been developed to release PDE6 and photoreceptor proteins from ROS. First, hypotonic extraction was used
to non-specifically release PDE6 and membrane-associated proteins from ROS membranes. Second, His-PrBP/δ was used as a tool to selectively solubilize PDE6, and other prenylated proteins from ROS membranes. Third, treatment with a mild detergent, 1% Triton X-100, was used to extract a larger population of photoreceptor proteins from ROS membranes. These methods have been used in series with immunoprecipitation assays to isolate PDE6 and binding partners from other solubilized photoreceptor proteins.

Immunoprecipitation assays, using the PDE6-catalytic domain specific ROS-1 monoclonal mouse antibody, were optimized to enrich solubilized photoreceptor proteins for PDE6 and PDE6 interacting proteins. Immunoprecipitated proteins were then separated by SDS-PAGE and excised from the gels for mass spectrometry analysis of protein content. This proteomics-based approach has allowed for the documentation of putative PDE6 binding partners in rod photoreceptor cells. Known PDE6-interacting partners, such as the inhibitory γ subunit of PDE6, PrBP/δ, and GARP2, were successfully identified by mass spectrometry under various solubilization regimes. In addition, other photoreceptor proteins related to vision were also identified as PDE6 interacting proteins, such as arrestin, GRK-1, transducin, and rhodopsin. While direct interactions of these proteins with PDE6 are not characterized, they may be located in close proximity in ROS under certain conditions and form a PDE6 interacting complex.

Covalent cross-linking of PDE6 in ROS was also optimized to identify a set of bona fide PDE6 binding partners that may be subject to light regulation in their binding. The identification transducin α subunit exhibiting changes in its association with PDE6 is consistent with current literature that documents photoreceptor protein translocation
within rod cells and altering distribution of PDE6 on ROS disk membranes. It is thought that there were fewer proteins identified from this cross-linking approach, when compared to the initial immunoprecipitation studies under various solubilization regimes, because for proteins to be cross-linked they must be in direct contact or within close enough proximity of each other for the cross-linker with an 11.4 Å spacer arm to covalently interact with both proteins. As these mass spectrometry studies had many internal controls in the comparison of un-cross-linked and cross-linked proteins, there are implications for studying PDE6 interacting complexes on whole retina. Further, many of the observations made from the mass spectrometry analysis were validated with immunoblot analysis for a select group of proteins.

Finally, initial steps have been taken to optimize PDE6 holoenzyme cross-linking in solution with the intention of mapping the topography of this elusive protein that has not yet been successfully expressed as a soluble recombinant protein and purified in its active form to date. These experiments lay a solid foundation for performing cross-linking studies on purified PDE6 or membrane-associated PDE6 that can be optimized for detecting interaction sites between the PDE6 holoenzyme subunits.

**Future Directions**

As this work was done with ROS homogenate, in ROS preparations that were not 100% pure, optimization for detecting PDE6-interacting proteins on whole bovine retina is highly recommended. These studies provide a good start to understanding potential PDE6 binding partners in ROS, but ultimately it would be beneficial to examine these protein-protein interactions in a more physiological state, such as using ex vivo retina. Alternatively, if optimization of ROS preparation steps were made, the mass
spectrometry detection of cone and inner segment contaminating proteins could be reduced, and clearer insight into *bona fide* PDE6 interacting proteins in ROS elucidated.

In addition, the cross-linking conditions can be optimized so that a greater portion of PDE6 may be solubilized from ROS membranes and provide more information about the differences in PDE6 interacting proteins detected in the dark and in the light. This approach can also be applied to entire retina samples, where PDE6 interacting proteins can be compared as they are detected in the dark, upon light activation, and under steady-state conditions. Further, the mass spectrometry data generated here may be followed by a more rigorous immunological approach to verify interactions between PDE6 and potential binding proteins *in vivo*.

Finally, as the initial topology experiments provide a good start for the optimization of PDE6 cross-linking in solution, further studies can be performed to optimize the ability to detect interacting regions between subunits of the PDE6 holoenzyme, as they exist in solution, or even as they exist in ROS or *ex vivo* retina preparations.
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