SPECIFIC AIMS

Photoreceptor phosphodiesterase (PDE6) is the central effector of visual transduction, where its precise regulation is essential for the speed, sensitivity, and recovery of rod and cone photoreceptors to illumination. Mutations and adverse environmental factors that impair PDE6 underlie many visual disorders and retinal diseases (e.g., retinitis pigmentosa). PDE6 is a multi-subunit enzyme consisting of a catalytic dimer to which regulatory γ-subunits bind to inhibit cGMP hydrolysis. PDE6 is activated by the heterotrimeric G-protein, transducin, and is allosterically regulated by cGMP binding to regulatory sites in the GAF domains of the enzyme. Deactivation of PDE6 results from inactivation of transducin through acceleration of its GTPase activity by RGS9-1 (Regulator of G-protein Signaling9-1), a process facilitated by the PDE6 γ-subunit. While these basic features of visual transduction in rod photoreceptors are generally accepted, the protein-protein interactions and allosteric regulation that occur during activation and deactivation of rod and cone PDE6 are poorly understood. Furthermore, the biochemical mechanisms underlying light adaptation of the mammalian photoresponse are not well delineated, though mounting evidence suggests a pivotal role for PDE6 in photoresponse desensitization. Until we define the key regulatory mechanisms in visual signaling at the molecular level, we will be unable to fully understand how alterations in PDE6 or its binding partners can lead to visual dysfunction and retinal disease.

Our ***long-term goal*** is to elucidate the multiple mechanisms controlling PDE6 activity during excitation, recovery, and adaptation of the photoresponse in retinal photoreceptors. The ***overall objective*** of this application, which is the next logical step toward attaining our long-term goal, is to define the biochemical and structural progression that starts with light-induced activation of PDE6 holoenzyme by transducin, followed by the events responsible for PDE6 recovery to the dark-adapted state and its desensitization during light adaptation. Our ***unifying hypothesis*** is that the inhibitory γ-subunit of PDE6 is responsible for mediating the allosteric interactions that occur between the PDE6 catalytic subunits and transducin as well as with other regulatory proteins (e.g., RGS9-1, GARP2). The experimental basis for this hypothesis relies on recent work from our lab showing that Pγ interacts with several structurally and functionally distinct domains of the PDE6 catalytic subunits. Furthermore, the linearly extended conformation of Pγ bound to the PDE6 catalytic dimer provides multiple sites of interaction with activated transducin, RGS9-1, as well as with another PDE6 binding partner, GARP2. The ***rationale*** for the proposed research is that once the sequence of steps in the activation, inactivation, and adaptation of the macromolecular complex of PDE6 and its interacting partners is known, new therapeutic treatments can be developed to intervene in retinal degenerative diseases and visual disorders that result from dysfunction of the PDE6 signaling complex in photoreceptor cells.

To accomplish the objective of this application, we will pursue the following ***specific aims:***

1. **Define the binding interactions required for activation of PDE6 holoenzyme following initial binding of transducin to Pγ.**
   Our working hypothesis is that transducin docks to the glycine-rich region of the γ-subunit, thereby relieving inhibition of the C-terminal region and resulting in PDE6 activation.
2. **Determine the allosteric mechanisms in the GAF domains regulating the active lifetime of PDE6.**
   We hypothesize that the interactions of RGS9-1 with transducin, which lead to acceleration of transducin GTPase activity and deactivation of PDE6, are facilitated by a decrease in binding affinity of the γ-subunit to the PDE6 GAF domains during photoresponse recovery and desensitization.
3. **Define the topological relationship of known PDE6-interacting proteins and the mechanism(s) by which they modulate PDE6 activity.**
   Our working hypothesis is that the γ-subunit serves as the core scaffold for the PDE6 signaling complex, which contains additional proteins of regulatory significance for the mechanism of light adaptation.

Understanding the molecular dynamics of PDE6 during visual transduction will bring a better understanding of the complex mechanisms regulating cGMP levels in photoreceptor cells. The outcomes of this research will advance the goals of the Retinal Diseases Program at NEI because a better understanding of the regulation of visual signaling by PDE6 and its binding partners will enhance our ability to predict and to develop therapeutic interventions for retinal diseases (e.g., retinitis pigmentosa, congenital stationary night blindness) resulting from genetic or environmental disruptions of the PDE6 signaling complex in rod and cone photoreceptor cells.

RESEARCH STRATEGY

SIGNIFICANCE

Many retinal diseases resulting from genomic mutations, such as retinitis pigmentosa (Ferrari et al., 2011), and congenital stationary night blindness (Lem and Fain, 2004), are caused by dysfunctional proteins involved in the cyclic GMP (cGMP) signaling pathway in rod and cone photoreceptors. Physiological or pharmacological changes in the environment of the retina (including aging) can also perturb normal functioning of the phototransduction pathway. The photoreceptor cGMP phosphodiesterase (PDE6) is tightly regulated during visual transduction: (1) under dark-adapted conditions, the PDE6 inhibitory γ-subunit strictly maintains a very low rate of cGMP hydrolysis; (2) upon light activation, the heterotrimeric G-protein (transducin) rapidly relieves the inhibitory constraint of the γ-subunit, and; (3) the transient elevation of PDE6 catalytic activity recovers to its nonactivated state by a termination mechanism in which Regulator of G-protein Signaling9-1 (RGS9-1) accelerates transducin GTPase activity (Cote, 2006; Guo and Ruoho, 2008; Burns and Pugh, 2010; Cote and Cahill, 2010). Disruption of the structure, function, or regulation of rod or cone PDE6 or of PDE6 binding proteins can impair vision, lead to retinal degeneration, and, ultimately, result in blindness. However, we currently lack sufficient knowledge of the structure and regulation of the PDE6 signaling complex to be able to develop therapeutic interventions for genetic or environmental disruptions of the phototransduction pathway.

The contribution of the proposed work is expected to be an integration of the biochemical signaling pathway with dynamic changes in the structure and topology of the central PDE6 signaling complex during excitation, recovery, and adaptation in rod and cone photoreceptor cells. *This contribution will be significant because this integration of biochemical pathway information with the structural alterations in the PDE6 signaling complex will bring a new level of understanding of normal phototransduction mechanisms and the PDE6 signaling complex. This knowledge is a prerequisite for designing effective treatments for those retinal diseases resulting from defects in the phototransduction pathway*.

The proposed research will contribute to attaining three goals of the strategic plan established by the NEI Retinal Diseases Program: (1) analyze the mechanisms underlying light adaptation and recovery in photoreceptors; (2) understand the phototransduction cascade in cones; and (3) determine the pathophysiological mechanisms underlying retinal degenerative disease gene mutations.

One benefit that will accrue from completion of this work will be improved prediction of the pathogenicity of mutations in PDE6 and its binding partners. Understanding the structure-function relationship of phototransduction proteins that are critical for catalysis, allosteric regulation, and protein-protein interactions will enhance our ability to interpret the genomic information that is increasingly available in the age of “personalized medicine.” In addition to direct benefits to treating retinal diseases, the work will also benefit those seeking therapeutic interventions for other diseases involving G-protein-coupled signaling pathways.

INNOVATION

Many major advances in understanding the visual signaling pathway in photoreceptors have resulted from correlating electrophysiological measurements with biochemical studies of the major phototransduction proteins, including PDE6 holoenzyme and the proteins with which it interacts [e.g., (Pugh and Lamb, 1993)]. Additional information about the various stages of phototransduction (i.e., excitation, recovery, desensitization) has been obtained using transgenic animals to manipulate the components of the visual signaling pathway (Burns and Pugh, 2010). *What we currently lack is mechanistic and structural knowledge of the role of the PDE6 inhibitory γ-subunit during activation, deactivation, and adaptation of the PDE6 signaling complex.*

In our opinion, this proposal is innovative because it departs from the *status quo* in the following ways:

- Re-conceptualizes Pγ as the central regulatory element and organizing scaffold for the PDE6 signaling complex.
- Applies mass spectrometric-based proteomics (and complementary structural methods) to the study of topology, dynamics, and complex formation of the central proteins in the phototransduction pathway.
- Utilizes analytical ultracentrifugation with a novel fluorescence detection system (AU-FDS) to obtain hydrodynamic (sedimentation velocity) and binding stoichiometry (sedimentation equilibrium) information for protein-protein interactions over a range of binding affinities that cannot be obtained by other methods.

Our recently published work and the preliminary data described in the proposal support the view that these innovations will lead to greater understanding at the molecular level of the regulation and structural organization of the PDE6 signaling complex. This increase in knowledge will have a positive impact on the development of therapeutic interventions for retinal degenerative diseases resulting from dysfunction of PDE6 and its interacting partners.

APPROACH

Progress Report

Period covered: October, 2009 (last competitive review) to March, 2013 (submission date).

Summary of previous period's specific aims: When the grant was awarded, the requested funds were reduced by ~25% and the duration was reduced to 3 years. Hence, the scope of work concentrated on Aim #1, with completion of selected activities in Aims #2 and #3. We feel that progress has been excellent given the reduced support and duration. We have published five papers in *J. Biol. Chem.,* two manuscripts are in preparation, and four review articles came out during the current period (see Progress Report Publication List).

Specific Aim #1: Determine the mechanism by which transducin interacts with the two non-identical PDE6 catalytic subunits and their inhibitory γ-subunits.

The majority of the activities in Aim #1 have been accomplished and published. The proposed experiments to chemically cross-link Pγ to the PDE6 catalytic dimer will be completed as part of this proposal.

In ***Liu et al. (2009)***, we used radiolabeled PDE5/6 inhibitors to demonstrate that *both the α- and β-subunits of rod PDE6 are able to bind ligands to the enzyme active site*, but only when Pγ is not blocking the drug binding pocket. This work also revealed a *biphasic mechanism of transducin activation* of PDE6 that is consistent with one of the PDE6 catalytic subunits having lower affinity for its Pγ subunit. ***Zhang et al. (2010)*** presented evidence for *two distinct mechanisms of Pγ inhibition* of PDE6. One mechanism involves *direct interaction of the C-terminal residues with the catalytic site*. However, relief of Pγ inhibition by activated transducin requires additional interactions with Pγ. A second, *allosteric mechanism of Pγ inhibition* can *reduce catalytic activity without involving the C-terminus of Pγ*. The mechanism of this regulation is unclear. In ***Zhang et al. (2012)***, we identified the *multiple sites of interaction of the Pγ subunit* with PDE6 catalytic subunits, with activated transducin subunit, and with the transducin-RGS9-1 complex. We identified the *glycine-rich region of Pγ as a potential "docking" site for PDE6-interacting proteins* involved in both the activation and inactivation pathways. ***Matte et al. (2012)*** provided the first demonstration of *large conformational changes in PDE6 catalytic subunits upon cGMP binding to the GAF domains* using analytical ultracentrifugation, which highlights the potential importance of the regulatory GAF domains for regulation of PDE6 activity.

Specific Aim #2: Identify the structural features of PDE6 responsible for drug discrimination, catalytic acceleration, and domain folding using a comparative analysis of PDE6 and PDE5 structures.

We accomplished a primary objective of Aim #2, which is reported in ***Cahill et al. (2012)***. Using a combination of evolutionary trace analysis, structural homology modeling, and site-directed mutagenesis of rationally selected PDE6 residues into the recombinant PDE5 catalytic domain, we identified the *molecular basis for drug discrimination in the PDE6 active site*. Further progress in Aim #2 was slowed by *difficulties in expression of properly folded PDE5 catalytic domain proteins containing multiple PDE6 amino acid substitutions*. Rather than continue efforts in Aim 2 to identify factors that contribute to catalytic domain misfolding during heterologous expression, we are re-focusing our efforts on the structure/function relationships of the regulatory GAFab domain, which can be expressed (new Aim #2B).

Specific Aim #3: Characterize the PDE6 interaction network to identify PDE6 binding proteins of regulatory significance for photoreceptor cell function.

Several activities in Aim #3 have been accomplished. ***Matte et al. (2012)*** characterized the hydrodynamic behavior and protein-protein interactions for several PDE6 binding proteins, including transducin and the prenyl binding protein (PrBP/δ). In addition, two manuscripts will be submitted within the next three months, preliminary versions of which will be presented in April, 2013 at the ASBMB meeting. The *Yao and Cote (2013)* manuscript reports on the regulation of PDE6 catalysis, cGMP binding affinity, and transducin activation by the Glutamic Acid-Rich Protein2 (GARP2). The *Zeng et al. (2013)* manuscript characterizes the topology of Pγ interactions with the PDE6 catalytic dimer using chemical cross-linking and MS-based proteomics. Because they document feasibility to carry out the work proposed in our new Aim #3, the key results from each manuscript are reported in the Approach section.

Work completed during the current period of support has added substantially to our knowledge of the complex regulation of PDE6 during visual transduction.

- We now better understand the central importance of Pγ, not only in binding to PDE6 catalytic subunits, but also its interactions with transducin, RGS9-1, GARP2, and their potential involvement in photoresponse recovery and light adaptation.
- The large cGMP-induced conformational change in PDE6 is the first demonstration of allosteric communication between the GAF domains and the catalytic domain of the PDE6 holoenzyme, and may

modulate PDE6 during light adaptation.

- Identification of amino acid residues in the catalytic site responsible for drug discrimination will advance efforts to develop PDE5-selective inhibitors with fewer adverse side effects on PDE6 and vision.

Aim 1. Define the binding interactions required for activation of PDE6 holoenzyme following initial binding of transducin to Pγ

It is well understood that PDE6 holoenzyme ($\alpha\beta\gamma\gamma$) is activated by the GTP-bound α-subunit of transducin (Tα*-GTP) during visual excitation [reviewed in (Cote and Cahill, 2010)]. However, the sequence of events leading to catalytic activation of PDE6 is unclear. In Aim 1, *we will test the hypothesis that Tα* interacts with a "docking region" of the γ-subunit (Pγ) that is required for relieving inhibition of PDE6*, *and that additional interactions with the C-terminal region of Pγ result in PDE6 activation* (Fig. 1A-1). We will also determine whether one subunit of Pαβ is preferentially activated by Tα*, and determine whether the sequence differences between rod and cone Pγ contribute to their marked differences in light responsiveness.

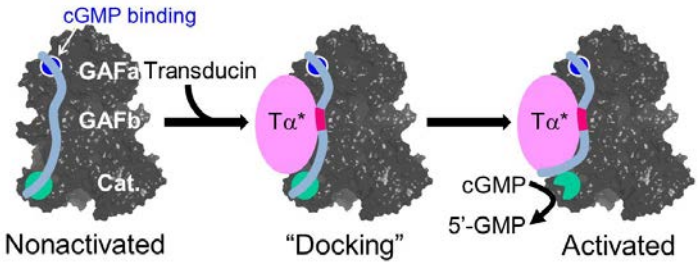

Fig. 2-1. Mechanisms regulating the lifetime of activated PDE6. **A.** Pγ regulation of RGS9-1 activity. **B.** Reciprocal allosteric regulation of Pγ and cGMP binding to PDE6.**Fig. 1A-1. Model for Mechanism of transducin activation of PDE6.** Binding of transducin (Tα*) to PDE6 holoenzyme at a

Aim 1A. Define the sites of interaction of activated transducin (Tα*-GTPγS) with Pγ that lead to the relief of PDE6 catalytic inhibition

Background: The C-terminal tail of Pγ (Fig. 1A-2) directly blocks the catalytic site of non-activated PDE6 and is displaced by transducin to relieve inhibition [reviewed in (Guo and Ruoho, 2008)]. Biochemical and physiological studies support the idea that transducin makes multiple contacts with Pγ in all regions except the extreme N-terminus (Tsang et al., 1998; Tsang et al., 2001; Tsang et al., 2007; Woodruff et al., 2008; Guo et al., 2010). *However, the full set of binding determinants of Tα* required for PDE6 activation is not known*.

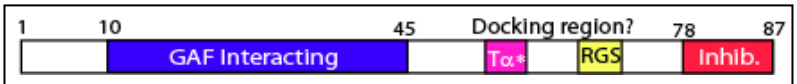

Fig. 1A-2. Domain organization of Pγ subunit. Sites of interaction of Pγ with Pαβ (including GAF region and C-terminal residues), with Tα* (including proposed "docking" region and C-terminal residues).

1A-1. Define the high affinity ("docking") transducin interacting region on Pγ (Fig. 1A-2)

One model for activation of PDE6 by Tα*-GTPγS proposes that transducin initially binds to Pγ at L76, which induces a hinge-like conformational change that dissociates the C-terminal residues of Pγ from the PDE6 active site (Granovsky and Artemyev, 2001). Recently, we demonstrated that the minimum Pγ sequence needed for Tα*-GTPγS activation is Pγ54-87 (Zhang et al., 2012). *We hypothesize that Pγ residues in the vicinity of I54 may constitute an initial high affinity binding or "docking" site that is critical for transducin activation.*

Studies of Pγ-(Tα*-GTPγS) interactions often fail to consider the fact that in solution Pγ assumes a primarily disordered structure (Song et al., 2008) that radically differs from the extended, linear conformation Pγ assumes upon binding to the PDE6 catalytic dimer (Berger et al., 1997; Guo et al., 2006). Because of this, we question whether reported interactions between Tα*-GTPγS and purified Pγ in solution occur when Pγ is already complexed with PDE6. To circumvent this potential issue, we will use a *peptide disruption strategy* in which a set of overlapping, short (~15 a.a.) Pγ synthetic peptides will be incubated with purified PDE6 holoenzyme and purified Tα*-GTPγS reconstituted on Large Unilamellar Vesicles [LUVs enhance transducin activation efficiency >10-fold; (Wensel et al., 2005)]. The concentration needed to block transducin activation of PDE6 will be determined. This approach will identify the high affinity "docking" region of Pγ that allows Tα*-GTPγS to stably bind to PDE6 holoenzyme. A potential problem with disrupting peptides is that the affinity for these short peptides might be too low to disrupt transducin activation of PDE6 holoenzyme, in which case we would use longer peptides. Monitoring nonactivated PDE6 activity in the presence of the peptides will ascertain whether the peptides are displacing endogenous Pγ bound to the holoenzyme.

Alternatively, we will use *Pγ fluorescently labeled at sites sensitive to transducin binding* (Granovsky et al., 1998a) that has been reconstituted with Pαβ prior to transducin addition. The ability of the disrupting peptides to block fluorescent changes at regions distinct from the site of the label will identify high affinity binding sites of Pγ for Tα*. To pinpoint the major sites of interaction, site-directed mutagenesis within the identified "docking" region will be used to map the major stabilizing interactions of Tα* and Pγ.

1A-2. Identify the Pγ residues that discriminate active and inactive transducin

The activated state of PDE6 requires that the transducin α-subunit bind to Pγ in its GTP-bound form. Upon GTP hydrolysis, favorable binding interactions between Pγ and Tα must be lost, as transducin assumes a "ready to leave" conformation. The observation that Tα-GDP is capable of activating PDE6 catalysis [but with ~100-fold lower affinity than Tα*-GTPγS or Tα*-GDP-AlF_3 (Kutuzov and Pfister, 1994; Erickson et al., 1995)] suggests that Tα-GDP can bind to the C-terminal inhibitory region of Pγ, but lacks additional interaction sites needed for high affinity binding.

To test the hypothesis that the "docking" region serves to discriminate Tα-GDP from Tα*-GTPγS binding, we will use fluorescently labeled Pγ reconstituted with Pαβ (as above) to compare the *relative binding affinities of Tα-GDP with Tα*-GTPγS*. We will use Pγ disrupting peptides to evaluate which regions of Pγ can bind only the GTPγS-activated form of transducin and which bind active and inactive equally well (e.g., C-terminal inhibitory region).

Aim 1B. Determine whether the α- or β-subunit of rod PDE6 is preferentially activated by transducin and the structural basis for two non-identical Pγ binding sites to Pαβ

[This sub-aim was reorganized and new preliminary data was added.]

Background: The rod PDE6 holoenzyme contains two non-identical α- and β-subunits that bind two Pγ subunits with affinities that differ ≥ 10-fold (Mou and Cote, 2001). Transducin activation of PDE6 is biphasic [reviewed in (Cote, 2006)], with activation of the first 50% of the maximum catalytic rate achieved more easily (Liu et al., 2009). This may result from different intrinsic affinities of the Pγ subunit for the α- and β-subunit and/or different affinities of activated transducin for the two Pγ subunits bound to the catalytic dimer.

1B-1. Determine the structural basis for differences in Pγ interactions with the α- and β-subunits

Considerable biochemical evidence supports the existence of two classes of Pγ binding sites and two non-identical classes of cGMP binding sites on rod Pαβ [reviewed in (Cote, 2006)]. There is some structural evidence favoring the idea that the heterogeneity in Pγ binding is conferred by the GAF domains, not the catalytic domains of Pαβ (Guo et al., 2005). Furthermore, it has been proposed that a single Pγ molecule may interact with the GAF domains of one catalytic subunit and cross over to interact with the catalytic domain of the other subunit (Guo et al., 2006).

To test the differences in Pγ binding to the α- and β-subunits of PDE6, we will use *Pγ mutants with cross-linking probes* to covalently react with PDE6 catalytic subunits. Since the previous submission, we have constructed a number of Pγ mutants (already containing the C68S substitution) in which a cysteine is substituted for the natural amino acid at individual positions along the length of Pγ (Fig. 1B-1A). The Pγ mutants are then labeled with 4-(N-maleimido) benzophenone (MBP), reconstituted with Pαβ, and photoactivated with 365 nm UV light. The shift in migration of the catalytic subunit band on SDS-PAGE from 100 to 110 kDa is indicative of the formation of an α-γ or β-γ cross-link (Fig. 1B-1C). Mass spectrometric analysis of the proteolytic digests of the 100 and 110 kDa protein samples and semi-quantitative analysis through spectra-counting (Zybailov et al., 2005; Brickner et al., 2012) identifies which catalytic subunit preferentially interacts with Pγ at various regions of the GAF and catalytic domains. Our initial results show that PγF30C-MBP preferentially cross-links to the β-subunit (β/α = 1.54 ± 0.07; n=3) whereas PγP23Bpa [created by biosynthetic incorporation with the unnatural, photoactivatable amino acid, benzoylphenylalanine [Bpa; (Young et al., 2010)] shows preferential cross-linking to the α-subunit (β/α = 0.48 ± 0.03; n=4) (Kozacka et al., 2013). Identification of cross-linked sites for these mutants are consistent with catalytic subunit preference derived from analysis of cross-linking data using bifunctional cross-linkers (see Aim 3A). These initial results will be repeated over a range of Pγ concentrations from sub-stoichiometric ratios of Pγ per Pαβ to excess Pγ per Pαβ before drawing conclusions about preferential interactions of Pγ with one of the rod catalytic subunits.

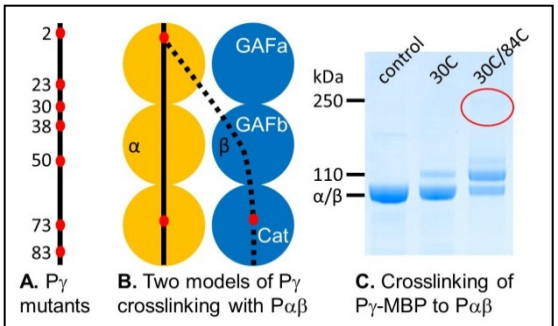

Fig. 1B-1: A. Pγ site-directed mutants (*red*: positions where cross-linking of single mutants to α and β subunits have been confirmed. **B.** Two-site crosslinking of Pγ to Pαβ will crosslink within a subunit only (*solid line*) or covalently link both subunits (*dashed line).***C.*** Cross-linking gel for single and dual cross-linking.

To evaluate whether a single Pγ molecule can interact with one or both catalytic subunits [Fig. 1B-1B; (Guo et al., 2006)], we will incorporate two photoprobes at different positions on Pγ, and examine whether chemical cross-linking results in formation of only 110 kDa bands or ~210 kDa bands as well. Thus far, we have not observed tethering of α- and β-subunits (i.e., no 210 kDa band; see *oval* in Fig. 1B-1C) with the Pγ

mutants tested to date.

A potential pitfall is that the very high affinity (~pM) of wild-type Pγ could obscure detection of preferential catalytic subunit binding. If we were to suspect this is occurring, we would test truncated Pγ peptides with reduced affinity that can stably interact with either the GAF domains (Pγ1-45/P23Bpa) or the catalytic domain (Pγ46-84C-MBP). These probes would also be useful in the next section to understand the structural basis of transducin activation of PDE6.

1B-2. Identify which catalytic domain (α or β) is preferentially activated by transducin using a photoprobe-labeled Pγ C-terminal peptide

PDE6 activated by transducin can be re-inhibited by excess, exogenous Pγ, both *in vitro* (Norton et al., 2000) and *in vivo* (Tsang et al., 2006). Because the C-terminal region alone can fully inhibit PDE catalysis (Zhang et al., 2010), and this region can be labeled with chemical cross-linkers (Artemyev et al., 1996), we will use photoactivatable cross-linkers attached to C-terminal Pγ peptides to identify which subunit is preferentially activated by transducin.

We have recently constructed the Pγ peptide Pγ46-84 with a tyrosine to cysteine replacement (Y84C), labeled it with MBP (Pγ46-84C-MBP), and performed preliminary experiments to determine that addition of this probe to Pαβ results in efficient cross-linking. Using this reagent as a probe, PDE6 holoenzyme will be activated from 0 to >80% of its maximum activity by varying the Tα*GTPγS concentration (Liu et al., 2009). Accessible PDE6 catalytic sites will be cross-linked with Pγ46-84C-MBP (nonactivated PDE6 will serve as a control), the cross-linked proteins will be separated by SDS-PAGE, and the spectral ratio and sequence of the cross-linked bands will be determined by mass spectrometry. The PDE6 catalytic subunit sequence attached to Pγ photo-probe (after accounting for any intrinsic subunit preference for photolabeling of nonactivated PDE6 catalytic subunits) will reveal whether the α- or β-subunit is preferentially activated by transducin.

If equal amounts of α- and β-subunit cross-linking occur even when only a small fraction (<<50%) of the total PDE is activated, we will conclude that binding of the first Tα*-GTPγS occurs randomly to α or β, but that steric hindrance or negative cooperativity reduce the ability of the second catalytic site to be transducin-activated. We also plan to take a similar approach with labeled N-terminal Pγ peptides [we already have designed Pγ1-45/P23Bpa based on earlier work (Muradov et al., 2002)] to evaluate whether the N-terminal region of Pγ dissociates from α and/or β upon transducin activation.

Aim 1C. What is the structural basis for differential activation of rod and cone PDE by transducin?

Background: The speed and sensitivity of the electrical response of rod and cone photoreceptors are quite different, yet the overall phototransduction machinery is believed to be very similar (Arshavsky and Burns, 2012; Korenbrot, 2012). Two recent transgenic mouse studies in which the endogenous rod Tα subunit was replaced with cone Tα arrived at different conclusions about the functional equivalence of rod and cone Tα (Chen et al., 2010a; Mao et al., 2013). However, direct comparison of the biochemical properties of rod and cone Tα subunits failed to reveal significant functional differences (Gopalakrishna et al., 2012). Likewise, transgenic expression in *Xenopus laevis* of rod or cone catalytic domains linked to the same GAF domains failed to reveal differences in the catalytic mechanism of rod and cone PDE6 (Muradov et al., 2010). Importantly, both rod and cone transducin are much less effective in activating rod PDE6 than cone PDE6 (Gillespie and Beavo, 1988; Muradov et al., 2010). *We hypothesize that differences between rod and cone Pγ and/or rod and cone PDE6 GAF domains are responsible for the different properties of rod and cone PDE6.*

1C-1. Can differences in transducin activation of rod and cone PDE6 be ascribed to structural differences in rod and cone Pγ?

To identify differences in the structure of rod (PDE6G) and cone (PDE6H) Pγ, we performed a multiple sequence alignment of available mammalian rod and cone Pγ sequences. Six conserved differences were found, including a deletion of amino acids 6-9 of the rod Pγ sequence in cone Pγ, plus 5 conserved amino acid substitutions (each of which occurs in a functionally important region of the Pγ molecule).

Each of these differences will be evaluated by *site-directed mutagenesis of rod Pγ to replace a rod amino acid residue with its cone Pγ counterpart* (including the deletion). Once reconstituted with rod Pαβ, the following properties will be evaluated: (1) K_i for inhibition of Pαβ; (2) $K_{1/2}$ for rod transducin activation of Pαβ reconstituted with mutant Pγ; (3) extent to which the Pγ mutant can potentiate RGS9-1 stimulation of transducin GTPase activity; (4) extent to which Tα*-GTPγS accelerates cGMP dissociation from the GAF domain; (5) cGMP-induced changes in PDE6 conformation. All of these assays have been recently described (Zhang et al., 2012; Matte et al., 2012).

If single substitutions of Pγ lack an effect on Pγ properties, cumulative mutations will be introduced to

progressively convert rod Pγ into cone Pγ to identify whether combinations of amino acid substitutions reveal the structural basis of Pγ rod/cone differences when tested with rod Pαβ. Anticipating the possibility that rod and cone Pγ are functionally identical, we will simultaneously examine possible rod/cone differences that reside in structural differences in the rod versus cone PDE6 tandem GAF domains (*see next section*).

1C-2. Do structural differences between rod and cone tandem GAF regions account for more efficient transducin activation of cone PDE6 compared to rod PDE6?

[This sub-aim has been revised to address reviewers' comments.]

To test the hypothesis that differences in the GAF domains of rod and cone PDE6 account for the greater difficulty of transducin to activate rod PDE6, we will *compare the properties of recombinantly expressed rod and cone GAFa-GAFb ("GAFab") dimers*. New preliminary data described in Aim 2B demonstrates our ability to express recombinant cone PDE6 GAFab homodimers that can bind both Pγ and cGMP. While we have not yet attempted to co-express in bacteria the GAFab constructs for rod α- and β-subunits to form GAFab heterodimers, we will use the same optimization strategy that led to functional expression of cone PDE6 GAFab homodimers (see Aim 2B). If rod GAFab heterodimers cannot be expressed in bacteria, we would switch to an insect cell expression system that has been successfully used to create PDE5/6 chimeras consisting of the cone GAFab [Chi4; (Granovsky et al., 1998b)] or the rod GAFab heterodimer (Muradov et al., 2003), both of which were linked to the PDE5 catalytic domain. Since the primary dimerization determinants of PDE6 reside within the GAFab domains (Muradov et al., 2003; Muradov et al., 2005), we anticipate being successful in expressing (either in bacteria or in insect cells) both rod GAFab heterodimers and cone GAFab homodimers to carry out this work.

To determine whether rod/cone differences in PDE6 are intrinsic to the isolated GAFab proteins*, cGMP and Pγ binding affinity to the recombinant GAFab dimers* will be compared. [^{3}H]cGMP binding affinity will be measured according to (Cote, 2005). The binding affinity of fluorescently labeled Pγ1-45C will be performed as described previously (Granovsky et al., 1998a; Mou and Cote, 2001). We will also evaluate whether rod Tα* can interact equally well with the rod and cone GAFab dimers to alter cGMP and/or Pγ affinity.

Expected Outcomes: Upon completion of Aim 1 we expect the following outcomes: (1) a map of the interaction surface of various inactive and active forms of transducin with the Pγ molecule in its extended linear conformation (i.e., bound to Pαβ); (2) greater understanding of the binding determinants required for transducin to activate catalysis of PDE6 during visual excitation; (3) identification of the structural basis for the functional heterogeneity of Pγ binding to Pαβ and transducin activation of the rod heterodimeric PDE6 holoenzyme; (4) a better understanding of rod/cone PDE6 differences in the transducin activation mechanism.

Aim 2. Determine the allosteric mechanisms in the GAF domains regulating the active lifetime of PDE6

Controlling the duration of the transducin-activated state of PDE6 during visual transduction is as critical as the initial events leading to PDE6 activation. It is believed that PDE6 returns to its basal rate of cGMP hydrolysis immediately after activated transducin (Tα*-GTP) undergoes nucleotide hydrolysis to Tα-GDP, thereby releasing Pγ and allowing the C-terminal region of Pγ to re-inhibit the active site of PDE6 (Arshavsky et al., 2002). Hence, the key element controlling the lifetime of activated PDE6 is thought to reside in the kinetics of the transducin GTPase reaction.

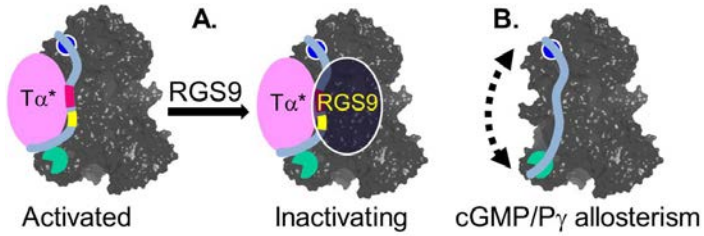

Fig. 2-1. Mechanisms regulating the lifetime of activated PDE6. A. Pγ regulation of RGS9-1 activity. **B.** Reciprocal allosteric regulation of Pγ and cGMP binding to PDE6.

The Pγ subunit plays an important role in controlling the GTPase turnoff mechanism via its ability to potentiate the action of RGS9-1, the GTPase accelerating protein (GAP) in photoreceptor cells [Fig. 2-1; (He et al., 1998)]. Little is known about the mechanism by which Pγ becomes involved in the GTPase accelerating mechanism, but one possibility is that cGMP binding to the PDE6 GAF domains may alter Pγ interactions with its binding partners. Furthermore, physiological studies suggest that additional mechanisms of PDE6 regulation may exist that directly modulate its active lifetime during light adaptation (Fain, 2011). *We hypothesize that the interactions of RGS9-1 with transducin, which lead to acceleration of transducin GTPase activity and deactivation of PDE6, are facilitated by a decrease in binding affinity of the γ-subunit to the PDE6 GAF domains during photoresponse recovery and desensitization.*

Aim 2A. Elucidate the mechanism by which RGS9-1 is recruited to the PDE6 signaling complex and interacts with Pγ to accelerate the GTPase activity of transducin during photoresponse recovery and adaptation

Background: The C-terminal portion of Pγ binds to Pαβ to inhibit catalysis. When activated transducin binds to this complex it relieves this inhibition, thereby activating PDE6. However, for transducin to fully activate Pαβ, it requires additional interactions between itself and Pγ (Zhang et al., 2012). Transducin is also reported to interact with the region of Pγ that stabilizes cGMP binding (Artemyev et al., 1993). Previous studies have shown that Pγ accelerates the GTPase activity of transducin, but not when cGMP is bound to the GAF domains (Arshavsky and Bownds, 1992). RGS9-1, an integral member of the GAP complex, also interacts with the N-terminal half of Pγ (Guo and Ruoho, 2011). Transgenic mice expressing a phosphorylation mutant of rod Pγ (T35A) suggest that phosphorylation of this residue affects Pγ conformation and impedes the modulation of the GAP-dependent hydrolysis of Tα*-GTP during light adaptation (Woodruff et al., 2008). *We predict that an interplay of interactions between cGMP binding in the GAF domain and/or the phosphorylation of Pγ at sites in the GAF interacting region enhance the ability of Pγ to potentiate the GTPase activity of the GAP complex.*

2A-1. Determine the requirements for the recruitment of RGS9-1 to the PDE6 signaling complex

Little is understood about the nature of the interactions of RGS9-1 with the PDE6 holoenzyme. A recent report suggested that the N-terminal region of Pγ specifically interacts with RGS9-1 (Guo and Ruoho, 2011), but these assays were performed with Pγ in its intrinsically disordered conformation. Studying Pγ bound in its native conformation to Pαβ will better define the interactions that occur *in situ*.

We will express, affinity purify (via His-tag), and use the RGS domain of RGS9-1 as bait for pull-down assays (Skiba et al., 2001) to *test the hypothesis that the RGS domain cannot interact with PDE6 holoenzyme (containing bound Pγ) unless activated transducin (Tα*-GTPγS) is also present*. Other samples containing Tα-GDP (inactive) or Tα*-GDP-AlF_4^- (mimics the transition state) will determine the requirement for the state of activation of transducin. To confirm the specificity of the affinity pull-down assays, we will perform immunoprecipitation assays with PDE6 antibody (ROS1) or transducin antibody (sc-389). *Alternative approaches* to detect lower affinity interactions will include the use of full length, co-expressed RGS9-1 and the atypical G-protein β-subunit, Gβ5L (Martemyanov and Arshavsky, 2004), along with chemical cross-linking of protein complexes prior to pull-down assays. *These results will determine conditions under which RGS9-1 is recruited to the PDE6 signaling complex in a reconstituted system.*

[*Sub-aim 2A-2 of the original submission has been deleted (see Introduction).*]

2A-2 (previous 2A-3). Test the hypotheses that (1) the association of Pγ with the GAF domains lacking bound cGMP or (2) the phosphorylation of Pγ enhances the effectiveness of Pγ to potentiate GTPase activity of the transducin- RGS9-1 complex

cGMP dependence of Pγ potentiating GTPase mechanism: Previous studies of RGS9-1 acceleration of transducin GTPase activity implicated cGMP binding to the PDE6 GAFa domains as regulating whether Pγ can potentiate the GTPase acceleration caused by RGS9-1 (Arshavsky and Bownds, 1992; Arshavsky et al., 1992; Angleson and Wensel, 1993; Angleson and Wensel, 1994). While Pγ sites required for *potentiation* of RGS9-1 have been identified (Slepak et al., 1995), the region(s) of Pγ that cause the cGMP-dependent acceleration of GTPase activity have not. Thus, we will test the ability of Pγ (full length and N-terminal truncation constructs) complexed with Pαβ to regulate the lifetime of transducin GTPase in a cGMP-dependent manner (Arshavsky and Bownds, 1992). Purified, activated transducin, recombinant RGS domain, and PDE6 holoenzyme [+/- cGMP occupying the GAFa domains; (Mou and Cote, 2001)] will be reconstituted to determine the conditions under which cGMP binding to the PDE6 GAF domains can prevent Pγ potentiating the RGS9-catalyzed acceleration of transducin GTPase activity. If we learn that the entire GAP complex (RGS9-1, Gβ5L and R9AP) is necessary to obtain optimal Pγ-induced GTPase acceleration, we will instead use a ROS membrane preparation depleted of PDE6 and transducin, but containing the GAP complex, for this work (Skiba et al., 2000). *These experiments will evaluate the important question of whether sustained decreases in cGMP levels in the rod outer segment alter Pγ binding interactions with the PDE6 GAF domain, thereby generating the physiological signal for RGS9-1 to catalyze the acceleration of GTPase activity during light adaptation.*

Effects of Pγ phosphorylation on Pγ-stimulated GTPase mechanism: Phosphorylation of Pγ at T22 or T35 causes only minor changes in its affinity for Pαβ, diminishes its interaction with activated transducin, and (in the case of phosphorylation at T22) also enhances cGMP exchange to the GAF domain of PDE6 (Paglia et al., 2002). Transgenic mice expressing Pγ T22A and T35A single or double mutants provide evidence that regulation of PDE6 activation and deactivation are influenced by phosphorylation at these sites (Tsang et al., 2007; Woodruff et al., 2008). We hypothesize that PDE6 is not directly regulated by phosphorylated Pγ, but that the deactivation step in the signaling pathway is susceptible to this regulation. Pγ site-directed mutants

replacing Thr with a Glu residue (mimicking phosphorylated threonine) at one or both sites will be evaluated for their Pαβ binding affinities, changes in $K_{1/2}$ upon transducin activation, and their ability to potentiate RGS9-1 induced GTPase acceleration. *If Pγ T22E or T35E effectively increases the GTPase activity, this would support the hypothesis that Pγ phosphorylation is a novel mechanism of light adaptation of the photoresponse.*

Aim 2B. Determine the structural basis for the allosteric regulation of PDE6 induced by binding of cGMP and Pγ to the regulatory GAF domains

[Aim 2B has been revised to include results demonstrating feasibility, as requested by the reviewers.]

Background: The regulatory GAF domain occurs in tandem (GAFa linked to GAFb) in cyclic nucleotide PDEs (PDE2, 5, 6, 10 and 11) (Zoraghi et al., 2004), which are allosterically regulated by cyclic nucleotide binding to this tandem GAF domain (Heikaus et al., 2009). Amazingly, even chimeric proteins in which the tandem GAFab domains are swapped with the catalytic domain of another protein typically retain the ability to allosterically communicate (Schultz, 2009), implying the functional integrity of these GAFab-containing PDEs.

For PDE6, cGMP binding to the GAFa domain has several effects on the rod PDE6 holoenzyme: (1) enhances the overall affinity of Pγ for Pαβ (Mou and Cote, 2001); (2) alters the binding affinity of inhibitors to the PDE6 active site (Zhang et al., 2008); (3) induces a conformational change in Pαβ reconstituted with Pγ truncation mutants (Matte et al., 2012), and; (4) reduces the effectiveness of Pγ to stimulate transducin GTPase activity (see Aim 2A-2). Pγ acts reciprocally on the PDE6 holoenzyme to enhance the binding affinity of cGMP (Yamazaki et al., 1982; Cote et al., 1994), an effect localized to the N-terminal half of Pγ [Fig. 1A-1; (Zhang et al., 2012)]. Transducin activation of PDE6 in rod outer segment (ROS) membranes induces increased cGMP dissociation from the GAF domains (Cote et al., 1994; Norton et al., 2000). This effect can also be observed with purified Tα*-GTPγS and Pαβ reconstituted with Pγ or certain Pγ truncation mutants (Zhang et al., 2012). Interaction sites located within the first 40 amino acids of Pγ stabilize its binding to Pαβ and are believed to interact with the GAFab domains of PDE6 [reviewed in (Guo and Ruoho, 2008)] and with Tα (Artemyev et al., 1992) (Brown, 1992). *Structural studies of the PDE6 GAFab domains—with and without bound cGMP and Pγ—are needed to understand these complex allosteric regulatory mechanisms of PDE6 that sharply contrast the simpler, direct allosteric activation models for PDE2 and PDE5 (Heikaus et al., 2009)].*

2B-1. Expression and characterization of the allosteric properties of the PDE6 GAFab domain

Expression of PDE6 GAFab domains: Since the original submission, we have successfully expressed functional cone GAFab homodimer ("C-GAFab"). The coding sequence for human PDE6C (a.a. 1-445 as well as several N- and C-terminal truncations of GAFab) has been cloned into the pGEX6P-1 vector (GST fusion tag) or pET47b ($(His)_6$ fusion tag) and expressed in *E. coli* Arctic Express (Agilent) cells at 10 ºC, yielding >0.25 mg soluble protein per liter culture of the predicted molecular weight (77 kDa; Fig. 2B-1) and immunoreactive with a PDE6 GAF antibody (*not shown*). Affinity purification and gel filtration chromatography resulted in a peak that eluted at a MW consistent with formation of the C-GAFab dimer, and which could be separated from higher MW aggregates and low MW impurities. Pγ immobilized on agarose beads was able to interact with (i.e., pull down) the expressed C-GAFab protein (Fig. 2B-1, lane "B"). The purified C-GAFab protein also bound [^{3}H]cGMP when assayed with a membrane filtration assay (*data not shown*). *These results demonstrate the feasibility of expressing sufficient quantities of recombinant GAFab dimeric proteins for the biochemical experiments proposed here and in Aim 1C-2.*

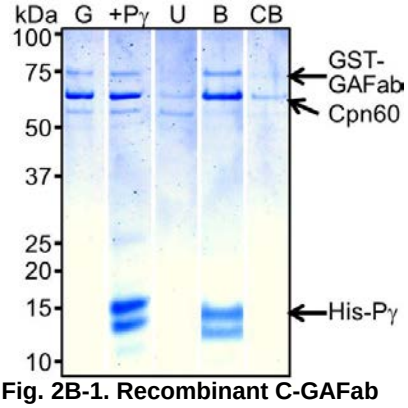

Fig. 2B-1. Recombinant C-GAFab binds Pγ. GST-GAFab1-445 (77 kDa MW) was expressed in *E. coli* Arctic Express cells and affinity purified on glutathione-agarose beads. **G.** GAFab (~75 kDa) co-purifies with bacterial chaperonin Cpn60. **+Pγ.** His-tagged Pγ (app. MW ~15 kDa) added to "G." **U.** Unbound fraction after addition of Ni-NTA beads. **B.** His-Pγ (and binding partners) bound to beads. **CB.** Control beads in absence of His-Pγ.

To increase bacterial expression of C-GAFab to yields similar to that of PDE5 GAFab (Wang et al., 2010) and as needed for experiments in Aim 2B-2, we will optimize: (1) start and stop positions of the construct and fusion tag placement (Muradov et al., 2004); (2) *E. coli* expression strain (e.g., cold temperature-optimized expression with Arctic Express cells, Agilent; or enhanced disulfide bond formation with Rosetta-gami cells, Novagen), and; (3) co-expression of C-GAFab with Pγ. If the yield and purity of C-GAFab is still insufficient for structural determination (Aim 2B-2), we will resort to the baculovirus system that Artemyev's lab has developed for expressing PDE6 C-GAFab domains linked to PDE5 catalytic domains (Muradov et al., 2004) and that we have used for PDE5 expression (Cahill et al., 2012).

Once we have optimized C-GAFab expression, we will also co-

express the GAF domains from the rod α- and β-subunits in the pET-DUET-1 vector ("R-GAFab"). If sufficient quantities of purified R-GAFab are obtained, we will focus subsequent characterization and structural determination experiments on the rod heterodimeric GAFab. Recombinant GAFab proteins will be biochemically characterized for: oligomeric state, cGMP binding affinity/ stoichiometry, and Pγ binding affinity. Expression of both R- and C-GAFab will permit us to test the hypothesis that the GAF domains of rod and cone PDE6 are functionally distinct (see Aim 1C-2).

Evaluation of cGMP- and Pγ-induced conformational changes of the isolated GAFab domain. We recently discovered a large conformational change in PDE6 upon binding of cGMP and Pγ1-45 to Pαβ catalytic dimer (Matte et al., 2012). Because the N-terminal half of Pγ interacts with the GAFab domain, we hypothesize that C-GAFab and R-GAFab should undergo similar changes in shape even when the catalytic domain is absent. We will test this idea by fluorescently labeling both GAFab constructs with 5-iodofluorescein and comparing the hydrodynamic properties of the constructs in the presence and absence of Pγ and/or cGMP using analytical ultracentrifugation with fluorescence detection (Matte et al., 2012). We will conduct this work in consultation with Dr. Tom Laue (*see letter*), a long-time collaborator and leading expert in the field.

These experiments represent the first opportunity to test whether cGMP binding to the PDE6 GAFa domain can by itself cause allosteric changes to PDE6 (similar to PDE2 and PDE5), since the catalytic domain will not be present to degrade the ligand. The reciprocal positive cooperativity of cGMP and Pγ (Cote et al., 1994) can also be analyzed more thoroughly when the catalytic domains are absent, but we also acknowledge the concern expressed by Reviewer 2 that Pγ- or cGMP-induced allosteric effects may differ when the PDE6 catalytic domain is attached to the GAFab domains.

2B-2. Determine the atomic-level structure of the dimeric GAF domains of PDE6 in a complex with Pγ and structural rearrangements induced by cGMP binding

The crystal structure of PDE5 GAFab (Wang et al., 2010) has provided important insights into the dimeric structure and the mechanism of allosteric communication in PDE5, and served as a template for structural homology modeling of PDE6 [(Gao et al., 2013); see Aim 3A]. However, as mentioned above, we lack structural information that would explain the molecular mechanism of allosteric regulation of PDE6. To address this, we are collaborating with Dr. Hengming Ke (*see letter*), a highly accomplished crystallographer of mammalian PDE structures who solved the structure of the PDE5 GAFab dimer (Wang et al., 2010).

We will optimize preliminary conditions for crystallization, processing of diffraction data, structural determination, and model refinement as recently described (Wang et al., 2010). We will attempt crystallization of purified, dimeric R-GAFab or C-GAFab proteins under the following conditions: no ligands, Pγ (no cGMP), cGMP (no Pγ), and both cGMP and Pγ present. *In the event that we are unable to obtain crystals that diffract well or obtain structures with a significant amount of disordered segments, we would test additional GAFab constructs of different lengths that may be more suitable for structural determination.*

Expected *o*utcomes*:* Upon completion of Aim 2 we expect: (1) a greater knowledge of the mechanism by which Pγ (complexed with PDE6) joins the Tα*-RGS9-1 complex to regulate the GTPase activity of transducin; (2) a critical evaluation of the hypothesis that cGMP- or phosphorylation-dependent regulation of Pγ interactions with the GAF domain enhance Pγ participation in the GAP complex; (3) the first structural determination of the PDE6 GAFab domain that will advance our understanding of the mechanism of the reciprocal allosteric regulation of PDE6 by cGMP and Pγ binding.

Aim 3. Define the topological relationship of known PDE6-interacting proteins and the mechanism by which they modulate PDE6 activity

A thorough understanding of the molecular etiology of retinal diseases that result from dysfunction of the central phototransduction pathway will require greater knowledge of the structure, function, regulation, and macromolecular organization of the PDE6 signaling complex.

Aim 3A. Characterize the molecular architecture of the PDE6 signaling complex, and define changes in topological relationships during activation and inactivation of PDE6

[Feasibility studies and revised experimental strategies have been changed in Sub-aim 3A.]

Background and Preliminary Data: Previous studies with x-ray crystallography, NMR, and cryo-EM of PDE6 holoenzyme and its interacting partners have attempted to address the absence of atomic-level structural information on PDE6 (Kameni Tcheudji et al., 2001; Ke and Wang, 2007; Wensel, 2008; Goc et al., 2010). Prior cross-linking studies of the PDE6 signaling complex most commonly utilized reactive groups covalently incorporated into the Pγ molecule to form cross-linked products with Pαβ, transducin, and/or RGS9 (Artemyev et al., 1993; Liu et al., 1996; Liu et al., 1999; Muradov et al., 2002; Guo et al., 2006; Guo and Ruoho, 2011).

While important insights have been gained, it is important to complement this approach with other strategies to examine protein-protein interactions that occur in the PDE6 signaling complex. *Chemical cross-linking combined with MS-based sequence determination (Rappsilber, 2011) offers a powerful alternate approach for overcoming past limitations to obtaining structural information for PDE6 and its binding partners at the atomic level.*

In collaboration with Dr. Feixia Chu (*see letter*), an expert in mass spectrometric determinations of protein structure (Chu et al., 2006; Wu et al., 2013), we have carried out an initial structural study of purified PDE6 heterotetramer isolated from rod outer segments using several bifunctional (lysine-lysine, carboxyl-amine, and amine-sulfhydryl) cross-linkers to map the topology of these protein-protein interactions (Fig. 3A-1). After forming covalent cross-links within the PDE6 holoenzyme, we proteolytically digested cross-linked bands isolated from SDS-PAGE, and performed LC-MS/MS analysis and searched the database with Protein Prospector to identify the sequences of the cross-linked peptides. To date, we have identified 20 intersubunit, 26 intrasubunit, and 12 intrapeptide cross-linked pairs (Zeng et al., 2012; Gao et al., 2013). To interpret these data, we created structural homology models using the Integrative Modeling Platform (IMP) in Modeler (Russel et al., 2012) for the PDE6 catalytic dimer, relying on information currently available for the PDE5/6 chimera of the catalytic domain (Barren et al., 2009), the PDE5 GAFab homodimer structure (Wang et al., 2010), the overall molecular organization of the GAF and catalytic domains derived from the PDE2 crystal structure (Pandit et al., 2009), and from electron microscopic density maps for PDE6 (Kameni Tcheudji et al., 2001; Goc et al., 2010). One early outcome has been our exciting observation that compared to PDE2 (Pandit et al., 2009), the *PDE6 catalytic domains are in a more "open" configuration to each other*, a result with implications for the diffusion-controlled catalytic mechanism and the allosteric regulation of PDE6 (Zeng et al., 2012; Gao et al., 2013).

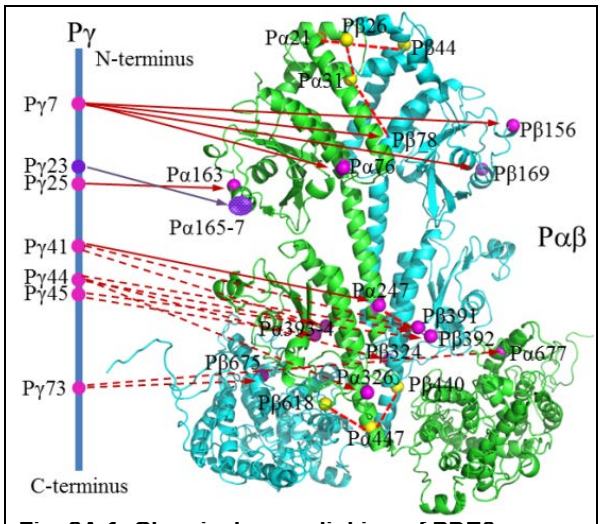

Fig. 3A-1. Chemical cross-linking of PDE6 holoenzyme. Homology model of rod Pαβ is shown next to linear Pγ. *Pink* and *purple* are locations of Pγ cross-links with a catalytic subunit. *Yellow* are locations of α-β cross-links. *Dashed lines* are linkages to either α- or β-subunit.

Most of the intersubunit cross-links we identified (Fig. 3A-1, *pink and purple*) link Pγ residues in the N-terminal half of the molecule with residues in the GAFab domains of the catalytic subunits [Fig. 3A-1; (Zeng et al., 2012; Gao et al., 2013)]. Of particular note: (1) PγK7 interacts at a single site on Pα but forms cross-links with three distinct regions of the Pβ GAFa domain; (2) PγK25 cross-links to the Pα GAFa domain at the so-called "lid" of the noncatalytic cGMP binding site, whereas the corresponding Pβ sequence does not form cross-links with Pγ; this is consistent with asymmetric binding of Pγ to Pα versus Pβ reported in Aim 1B-1 and may represent the structural basis for the two distinct classes of cGMP binding sites and Pγ binding sites documented previously (Mou et al., 1999); (3) each of the three lysines located within Pγ41-45 form cross-links with the GAFb domain of the α- and β-subunits just prior to the linker region to the catalytic domain; this region may be important in allosteric communication between GAFab and the catalytic domain. *These results demonstrate the feasibility of the work proposed in this aim to define the topological relationship of each protein known to interact with Pγ within the PDE6 signaling complex.*

3A-1. Determine the topological relationships of the catalytic and inhibitory subunits of the PDE6 holoenzyme, and evaluate structural changes resulting from cGMP binding to the GAFa domain

While the results summarized in Fig. 3A-1 represent the most detailed structural model of the PDE6 holoenzyme to date, there is a paucity of cross-linking data in the catalytic domains (either between α and β or with Pγ). The absence of lysine residues and the net negative charge in the C-terminal half of Pγ have made it difficult to analyze cross-linked fragments in this region using LC-MS/MS. To remedy this, we will construct Pγ mutants containing lysine residues spread out along the C-terminal half of Pγ; these mutants introduce positive charge, amine-reactive cross-linking sites, and serve as substrates for tryptic digestion. One such mutant, Pγ62K/65K/73K/79K, has been prepared and tested. It retains high affinity interactions with Pαβ, forms 110 and ~220 kDa bands on SDS-PAGE upon exposure to the cross-linker SulfoMBS, and generates cross-links between Pγ K73 and Pα K677 [or Pβ K675; (Fig. 3A-1)]; this agrees with the crystal structure of PDE5/PDE6 chimera complexed with the Pγ C-terminal tail (Barren et al., 2009). We will also use (as needed) photoactivatable cross-linkers designed for Aim 1B-2 to obtain additional topological information for Pγ with Pαβ. Once we can trace the interaction surface of Pγ on the catalytic dimer, we will use label-free, quantitative proteomic analysis (Chu et al., 2006) to identify changes in cross-linking patterns to test the hypothesis that binding of cGMP to the GAFa domains results in a large conformation change in the catalytic domains

[analogous to the cGMP-stimulated PDE2 enzyme (Pandit et al., 2009)].

[The original Aim 3A-2 ("Proteomic analysis of the PDE6...") has been deleted; see Introduction.]

3A-2 (previously 3A-3). Molecular organization of transducin-activated PDE6 before and after formation of the GAP complex with RGS9-1

Upon activation by transducin, some interactions between Pγ and Pαβ are disrupted by binding of Tα*-GTPγS to the PDE6 holoenzyme (Artemyev, 1997; Grant et al., 2006; Zhang et al., 2012), but most evidence suggests that Pγ remains associated with Pαβ throughout the activation/inactivation cycle (Wensel and Stryer, 1986). The crystal structure of the GTPase accelerating protein (GAP) complex [consisting of the C-terminal half of Pγ, a chimeric form of Tα*-GTPγS, and the RGS domain from RGS9-1 (Slep et al., 2001)] offers the best available model for how RGS9-1 interacts with Tα and the C-terminal region of Pγ in the GAP complex. However, as mentioned above, free Pγ (i.e., not complexed with Pαβ) that is reconstituted with transducin and RGS9-1 is likely to assume a different conformation in solution compared to its linearly extended conformation when bound to Pαβ (Song et al., 2008). *Thus, structural information of the GAP complex reconstituted with PDE6 holoenzyme is needed to better approximate the physiological context of rod outer segments.*

Purified PDE6 holoenzyme will be incubated with Tα*-GTPγS at concentrations to elicit 80% or greater of the maximal activation rate of PDE6 (Liu et al., 2009); we have recently enhanced the activation efficiency of transducin by reconstituting Tα*GTPγS and PDE6 on large unilamellar vesicles (Melia et al., 2000). The cross-linking reaction and mass spectrometric analyses will be conducted using similar methods to those described above. Preliminary efforts to cross-link the Tα*-GTPγS-PDE6 complex have resulted in a ~250-300 kDa band on SDS-PAGE from which *four cross-linked peptides linking Tα and Pαβ have been isolated* thus far [two to each catalytic subunit; (Gao et al., 2013)]. With additional cross-linking data, we will be able to "dock" two Tα subunits to their sites of interaction on the PDE6 holoenzyme, providing the first structural model of transducin-activated PDE6. Subsequent experiments will determine conformational changes of the PDE6 signaling complex under the following discrete stages of the activation/inactivation mechanism of PDE6 that can be manipulated by varying the nucleotide bound to Tα: activated state (Tα*-GTPγS), transition state (Tα*-GDP-AlF_4^-), and "ready to leave" state (Tα-GDP). Finally, we will prepare the GAP complex inclusive of PDE6, and will add the isolated RGS domain of RGS9-1 (Slep et al., 2001) to the transducin-activated PDE6 complex described above (integrating information obtained through completion of Aims 1A and 2A to optimize the experimental conditions for these structural determinations). *These experiments will provide the first structural models that define the progression of the PDE6 signaling complex from its nonactivated to transducin-activated and subsequently inactivated states.*

Aim 3B. Determine functional significance of GARP2-PDE6 interactions and of reversible phosphorylation of members of the PDE6 signaling complex

Background and Preliminary Data: Recent work with transgenic mice lacking calcium regulation of guanylate cyclase and the cGMP-gated channel (Chen et al., 2010b) point to regulation of spontaneously activated or transducin-activated PDE6 as the most likely candidate for controlling calcium-independent aspects of light adaptation (Fain, 2011). We will examine two candidates for exerting regulatory control over PDE6 during conditions of light adaptation, namely the PDE6 binding protein GARP2 and reversible phosphorylation of members of the PDE6 signaling complex (specifically, Pγ and RGS9-1).

GARP2 is a rod photoreceptor-specific protein that is expressed as a splice variant of the rod cGMP-gated channel β-subunit (Colville and Molday, 1996; Korschen et al., 1999). Transgenic mice over-expressing GARP2 (with normal levels of the channel β-subunit) exhibit an increase in phototransduction gain and delayed photoresponse recovery (McKeown et al., 2012), while reduction/ablation of GARP2 in mice leads to severe disruptions in the photoresponse and defects in disk morphogenesis (Zhang et al., 2009). The underlying mechanisms of action of GARP2 are unknown. GARP2 is a high-affinity binding partner of PDE6, causing suppression of PDE6 basal activity *in vitro* (Pentia et al., 2006). We have established that GARP2 also decreases cGMP binding to the GAF domains of PDE6 (Yao and Cote, 2013). Both effects of GARP2 on PDE6 function result from specific interactions with the Pγ subunit, not Pαβ (Yao and Cote, 2013a), further supporting the idea that Pγ serves as the scaffold for organizing and regulating the PDE6 signaling complex. Recently, we have discovered an important new

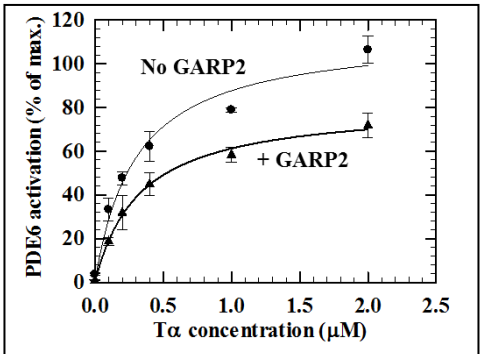

Fig. 3B-1. GARP2 suppresses the extent of PDE6 activation by transducin. Purified PDE6 holoenzyme (5 nM) and a 2000-fold excess of GARP2 were mixed with large unilamellar vesicles, the indicated concentration of Tα*-GTPγS was added, and PDE activity was measured.

activity of GARP2, namely to reduce the extent of transducin activation of PDE6 [Fig. 3B-1; (Yao and Cote, 2013)]. *We hypothesize that interactions of GARP2 with PDE6 represent a novel mechanism for regulating phototransduction by modulating PDE6 basal activity and/or the extent and lifetime of PDE6 activation.*

Pγ can be phosphorylated at several sites [(Guo and Ruoho, 2008) and references cited in Aim 2A]. Evaluation of light-induced changes in Pγ phosphorylation *in vivo* using phospho-specific antibodies suggests that T22 and T35 are partially phosphorylated under dark-adapted conditions, and that T22—but not T35—phosphorylation is increased by light exposure (Janisch et al., 2009). RGS9-1 is also a substrate for phosphorylation at multiple sites (Balasubramanian et al., 2001; Sokal et al., 2003). *We hypothesize that Pγ and/or RGS9-1 are regulated by reversible phosphorylation that alters their interaction within the PDE6 signaling complex during light adaptation.*

3B-1. Define the mechanism of action of GARP2 on the PDE6 signaling complex upon transducin activation, and determine the role of GARP2 in the recovery of PDE6 to its nonactivated state

Because GARP2 only detectibly binds to Pγ, not Pαβ or Tα*-GTPγS (Yao and Cote, 2013), we will first test the idea that GARP2 competes with Tα for binding to one or more shared binding sites on the Pγ "scaffold." Using a reconstituted system of PDE6 holoenzyme, Tα*-GTPγS, and large unilamellar vesicles [to enhance PDE6 activation by transducin; see Fig. 3B-1 and (Melia et al., 2000)], we will identify the region of GARP2 (using truncated fragments of the GARP2 sequence) responsible for suppressing transducin activation of PDE6. To localize the region on Pγ that we hypothesize to be the common locus for GARP2 and Tα*GTPγS binding, we will test the ability of GARP2 to reduce transducin activation when Pαβ is reconstituted with Pγ truncation mutants that we have already constructed (Zhang et al., 2012). As an alternative to direct competition of GARP2 and Tα for a common site(s) on Pγ, we will test the hypothesis that the GARP2 effect on PDE6 activation is mediated through GARP2-induced reduction in cGMP binding to PDE6 (Yao and Cote, 2013) that weakens Tα interactions with PDE6 holoenzyme (Yao and Cote, 2013). Finally, to determine the role of GARP2 in regulating the rate of PDE6 inactivation (McKeown et al., 2012), we will test the ability of exogenous GARP2 added to ROS membranes to alter the Pγ-induced acceleration of transducin GTPase activity (Zhang et al., 2012). *These studies will lead to greater knowledge of the mechanism of GARP2 action on various functions of the PDE6 signaling complex, including transducin activation efficiency and the rate of turnoff of the activated state. The outcome of this work will be a better understanding of the potential role of GARP2 in light adaptation.*

3B-2. Identify light-dependent, post-translational modifications of members of the PDE6 signaling complex in ex vivo rat retinas using MS-based proteomics

In conjunction with the biochemical experiments in Aim 2 to evaluate the effects of Pγ phospho-mimetic mutants T22E and T35E on PDE6 regulation, we need to quantify the physiological occurrence of reversible, light-dependent changes in Pγ and RGS9 phosphorylation. Dark-adapted and light-exposed rat retinas (and, initially, bovine ROS to optimize the method and minimize animal use) will be used to compare the extent and location of phosphorylation sites of Pγ and RGS9-1.; the light intensity threshold will be determined (Strissel et al., 2006) if dark/light changes are observed. We will use the general method of Hu et al. (Hu et al., 2001) to isolate proteins for immunoprecipitation and processing for MS analysis. We will analyze the site(s) and extent of phosphorylation with workflows currently used in Dr. Chu's group (Walther et al., 2007; Casati et al., 2008). If light-induced changes are detected, chemical cross-linking experiments to determine how phosphorylation alters protein-protein interactions. Should we detect light-induced changes in Pγ or RGS9-1 phosphorylation at specific sites on either protein, we would create phospho-mimetic mutations of the protein and analyze their effect on transducin activation of PDE6, and RGS9-catalyzed, Pγ-potentiated transducin GTPase activity.

Expected Outcomes: Upon completion of Aim3 we expect the following outcomes: (1) a comprehensive structural model of the interaction interface of PDE6 holoenzyme with Tα and RGS9-1 during the light activation and inactivation processes; (2) greater understanding of the functional significance of GARP2 binding to the PDE6 signaling complex; (3) increase in our knowledge of light-dependent, reversible phosphorylation of Pγ and/or RGS9-1.

TIMETABLE		Year 1	Year 2	Year 3	Year 4	Year 5
Aim 1	1A		X	X		
	1B	X	X			
	1C				X	X
Aim 2	2A			X	X	X
	2B	X	X	X	X	X
Aim 3	3A	X	X	X		
	3B			X	X	X