The N termini of the inhibitory γ-subunits of phosphodiesterase-6 (PDE6) from rod and cone photoreceptors differentially regulate transducin-mediated PDE6 activation

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Running title: N-terminus of Pγ modulates transducin activation of PDE6

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ABSTRACT

Phosphodiesterase-6 (PDE6) plays a central role in both rod and cone phototransduction pathways. In the dark, PDE6 activity is suppressed by its inhibitory γ-subunit (Pγ). Rhodopsin-catalyzed activation of the G protein, transducin, relieves this inhibition and enhances PDE6 catalysis. We hypothesized that amino acid sequence differences between rod- and cone-specific Pγs underlie transducin’s ability to more effectively activate cone-specific PDE6 than rod PDE6. To test this, we analyzed rod and cone Pγ sequences from all major vertebrate and cyclostome lineages, and found that rod Pγ loci are far more conserved than cone Pγ sequences, and that most of the sequence differences are located in the N-terminal region. Next, we reconstituted rod PDE6 catalytic dimer (Pαβ) with various rod or cone Pγ variants and analyzed PDE6 activation upon addition of activated transducin α-subunit (Gtα*-GTPγS). This analysis revealed a rod-specific Pγ motif (amino acids 9-18) that reduces the ability of Gtα*-GTPγS to activate the reconstituted PDE6. In cone Pγ, Asn-13 and Gln-14 significantly enhanced Gtα*-GTPγS activation of cone Pγ truncation variants. Moreover, we observed that the first four amino acids of either rod or cone Pγ contribute to Gtα*-GTPγS-mediated activation of PDE6. We conclude that physiological differences between rod and cone photoreceptor light responsiveness can be partially ascribed to ancient, highly conserved amino acid differences in the N-terminal regions of Pγ isoforms, demonstrating for the first time a functional role for this region of Pγ in the differential activation of rod and cone PDE6 by transducin.

Although the speed and sensitivity of the photoresponses of rod and cone photoreceptors are quite different, the underlying molecular components of the visual excitation pathway are believed to be homologous in both classes of photoreceptors (1,2). In rod photoreceptors, the cGMP signaling cascade is initiated when light-activated rhodopsin binds the heterotrimeric G protein, transducin (Gt). This leads to guanine nucleotide exchange of GDP to GTP and dissociation of the activated Gtα*-GTP. Gtα*-GTP then associates with and activates the rod PDE6 holoenzyme (consisting of two catalytic subunits, α and β, and two inhibitory rod-specific γ-subunits, rPγ). Cone photoreceptors express homologous forms of the visual pigment (cone opsins), cone transducin, and cone PDE6 holoenzyme [comprised of two identical α' catalytic subunits and two cone-specific Pγ subunits, cPγ; for review, see (3)].

One approach to identifying the biochemical differences in the rod and cone visual transduction pathways has been to
genetically introduce a cone-specific isoform of a phototransduction gene into rod photoreceptor cells. As recently summarized (2), transgenic incorporation of various cone opsin genes, cone transducin α-subunit, or cone PDE6 α'-subunit into mouse rod photoreceptor cells had very modest or no effect on the light sensitivity of the rod photoresponse. Biochemical and molecular approaches have identified several intrinsic differences between rod and cone opsins (e.g., spontaneous activation, chromophore dissociation and regeneration), but evidence is lacking that differences in photoactivated rod and cone opsins can account for the different light sensitivity and photoresponse kinetics of rods and cones (4). Characterization of the biochemical properties of rod and cone transducin α-subunits have failed to reveal significant functional differences (5-7). Likewise, the PDE6 catalytic dimers in rods and in cones have equivalent hydrolytic activities (8-10).

The photoreceptor PDE6 is the only member of the eleven-member phosphodiesterase superfamily that is known to be regulated by a distinct regulatory protein, the Pγ subunit (3,11). The mechanism of rod PDE6 activation involves the binding of Gtα*-GTP to the PDE6 holoenzyme causing displacement of the C-terminal domain of rPγ from the entrance of the active site to accelerate the hydolysis of cGMP. The lifetime of Gtα*-GTP and activated rod PDE6 is determined by the GTPase activity of the transducin α-subunit which is regulated by a complex of three GTPase accelerating proteins (RGS9-1, Gβ5L, and R9AP (12). Importantly, the rPγ subunit also serves as feedback regulator of this GAP complex in rods (13). The cone cPγ sequence is highly homologous to rPγ, and both Pγ isoforms bind to and inhibit rod PDE6 catalytic dimer with similar affinities (10). However, cone PDE6 holoenzyme is more easily activated by rod Gtα*-GTPγS than rod PDE6 holoenzyme (8).

The rod and cone Pγ genes (gene names PDE6G and PDE6H) appear to have evolved with our earliest vertebrate ancestors, at the same time that PDE6 catalytic subunit genes arose (14,15) with their unique catalytic and regulatory properties (3). It is noteworthy that the sea lamprey (P. marinus), a cyclostome that diverged from other vertebrate lineages about 500 million years ago, has a duplex retina in which rod and cone photoreceptors show similar physiological differences to light as is observed with mammalian photoreceptors (16,17). Interestingly, lamprey rods and cones express the same PDE6 catalytic subunit along with distinct rod- and cone-specific Pγ isoforms (18).

To test the hypothesis that differences in rPγ and cPγ subunits may contribute to the physiological differences in rod and cone light responsiveness, we examined whether amino acid sequence differences between rPγ and cPγ underlie the ability of transducin to more effectively activate cone PDE6 relative to rod PDE6. We first carried out a phylogenetic analysis of available Pγ subunit sequences to identify rod- and cone-specific sequence differences that might underly their different biochemical properties. We then reconstituted purified rod PDE6 catalytic dimer (Pαβ) with different rPγ or cPγ mutants and tested the relative affinity and extent of catalytic activation upon addition of persistently activated transducin α-subunit (Gtα*-GTPγS). Our results demonstrate that the N-terminal amino acids of rPγ and cPγ are responsible for the differential activation of PDE6 by transducin, from which we conclude that the evolution of separate genes for rod and cone Pγ represents one of the regulatory mechanisms distinguishing the rod and cone phototransduction pathways in vertebrate photoreceptors.

**Results**

**Evolutionary analysis reveals that rod Pγ is far more conserved than cone Pγ**

In order to identify differences in the sequences of rod (PDE6G) and cone (PDE6H) Pγ genes, we sampled the largest phylogenetic diversity possible from public databases. The sequences we obtained include representation from PDE6G and PDE6H sequences from all major lineages of craniates including cyclostomes (hagfish and lamprey),
N-terminus of Pγ modulates transducin activation of PDE6

chondrichthyans (sharks and rays), neopterigian fishes (including teleosts), squamates (lizards and snakes), archosaurs (crocodilians and birds) and mammals. At present, PDE6G and PDE6H sequence data are lacking only for sturgeons, paddlefishes and bichirs.

Phylogenetic analyses revealed strong support for two clades representing PDE6G and PDE6H for all vertebrate classes; however, owing to strong sequence conservation, we found little support for shallow nodes across the tree (Fig. 1). Overall, we found that PDE6G sequences are highly conserved throughout craniates. In contrast, the PDE6H clade has undergone greater amino acid sequence diversification, especially outside the mammalian class. In particular, PDE6H sequences for neopterigean fishes show radical alterations compared to other PDE6H sequences. In addition, we identified eight N-terminus residues that are highly conserved in PDE6G but divergent or missing in PDE6H. We also uncovered two residues that are differentiated, but highly conserved, within PDE6G and PDE6H (Fig. 1).

According to the consensus logo (Fig. 2), vertebrate PDE6G and PDE6H residues show high sequence similarity except for the N-terminal region of the protein sequence (Fig. 2A). Within this N-terminal region, PDE6G sequences are significantly more highly conserved than PDE6H sequences, as judged by comparison of the consensus logo for all vertebrates (Fig. 2A). This pattern of greater conservation of rod versus cone residues in the N-terminal region is even more evident when the analysis is confined to mammalian sequences (Fig 2B).

Our analyses also reveal four highly conserved rod-cone differences among inhibitory subunit sequences that are virtually invariant since the last common ancestor of PDE6G and PDE6H (Fig. 2A and 2B, indicated with vertical lines). With vertebrate PDE6G as a reference, these include residues V21, Q48, N74 and Y84 (Fig. 2A). The mutually exclusive nature of these residues is even more strongly pronounced when considering mammalian sequences alone (Fig. 2B), perhaps indicating purifying selection during the radiation of mammals. [Note that the insertion after G59 of PDE6G (Fig. 2A) was found in only a single species and is unlikely to have biological significance.] In summary, PDE6H sequences have diversified much more than their rod sisters in the evolutionary history of craniates and much of this sequence diversity is found within the first ~15 N-terminal amino acid residues.

**Gt*-GTPγS activation of PDE6 is more effective with cone Pγ than rod Pγ**

It has been reported that the rod and cone PDE6 holoenzymes are very similar in their catalytic properties and their interactions with their inhibitory Pγ subunits, but differ in how rod and cone PDE6 are activated by transducin (10). We hypothesized that structural differences in rod and cone Pγ isoforms may be responsible for these different interactions between transducin and rod and cone PDE6.

To test this, we first compared full-length recombinant rPγ and cPγ reconstituted with purified rod Pαβ catalytic dimers, and tested the ability of rod Gt*-GTPγS to activate PDE6 and accelerate cGMP hydrolysis. Fig. 3A shows that PDE6 containing cPγ is activated to a much greater extent (72% of the maximum catalytic activity of Pαβ lacking Pγ) than rPγ (16% maximum activation) when tested under identical experimental conditions. In contrast to the 4.5-fold difference in the extent of activation, the concentration dependence of Gt*-GTPγS activation (K1/2) did not significantly differ for the two PDE6 enzyme preparations (Table 1).

To evaluate whether this 4.5-fold difference in maximum transducin activation of PDE6 could be accounted for by a lower intrinsic binding affinity of cPγ for Pαβ, we measured the concentration dependence of rPγ and cPγ to inhibit cGMP hydrolysis of Pαβ. As shown in Fig. 3B, no significant difference between IC50 values of rPγ and cPγ was observed. We conclude that the differences in overall binding affinity of Pγ to Pαβ cannot account for the
greater efficacy of cPγ to promote transducin activation of PDE6.

The N-terminal region of cone Pγ is the locus for the differences in transducin activation of PDE6

To identify the structural basis for the differences in the ability of rPγ and cPγ to permit efficient activation of PDE6 by transducin, we first hypothesized that one or more of the four highly conserved amino acid differences identified in the Pγ sequence alignment (vertical lines in Fig. 2B) were responsible. To test this, we generated site-directed mutants of cPγ to see whether converting the cone residue to its rod counterpart would suppress the extent of transducin activation of reconstituted PDE6. As seen in Fig. 4A, the cPγ triple mutant (K44Q, S70N, F80Y) was as effective as wildtype cPγ in supporting Gtα*-GTPγS activation of PDE6; a similar result was observed for the cPγT17V construct (Fig. 4A). From this result we concluded that the variable N-terminal region of rPγ and cPγ was most likely responsible for the observed differences in the ability of transducin to activate PDE6.

To evaluate the role of the N-terminal region of Pγ, we created two rod-cone Pγ chimeras consisting of the N-terminal region of one Pγ with the remaining C-terminal portion of the other Pγ: rPγ1-18-cPγ15-83 and cPγ1-14-rPγ19-87. We first verified that the two chimeric Pγ constructs inhibited Pαβ with affinities similar to that of the wildtype Pγ proteins (Table 1). As seen in Fig. 4B, the chimera containing the cPγ N-terminal region behaved essentially the same as wildtype cPγ, while the N-terminal region of rPγ enhanced Gtα*-GTPγS activation of PDE6 less than two-fold (30% maximum activation) when compared to wildtype rPγ (16% maximum activation). We conclude that differences in the N-terminal region of rPγ and cPγ, previously thought to lack functional significance, are responsible for regulating the ability of transducin to bind to and efficiently activate catalysis of the PDE6 holoenzyme.

Residues in the N-terminal region of rod Pγ impair the activation efficiency of Gtα*-GTPγS

Because of sequence dissimilarity in the N-terminal region of rPγ and cPγ sequences (Fig. 2), it was difficult to predict potential functional sites responsible for suppressing the ability of transducin to activate Pαβ reconstituted with rPγ. Instead, we generated several N-terminal truncation mutants of rPγ to test whether removing a portion of the N-terminus would enhance Gtα*-GTPγS activation of PDE6. As shown in Fig. 5A, removal of the first four or eight amino acids from the rPγ N-terminus did not alter Gtα*-GTPγS activation efficiency, while removal of the first 18 amino acids resulted in a less than 2-fold increase in transducin-activated PDE catalysis.

We next constructed several internal deletion mutants of rPγ to search for possible “inhibitory” elements within the N-terminal region that could suppress the ability of transducin to activate rod PDE6. Deletion of amino acid residues 5-8 (i.e., rPγ1-4_9-87) resulted in a modest increase in the maximum extent of Gtα*-GTPγS activation of PDE6 (25% of Pαβ activity; Fig. 5B and Table 1). Further enhancement of Gtα*-GTPγS activation of PDE6 was observed when amino acids 5-18 were deleted (55% of maximum Pαβ activity; Table 1). A positive role for the first four amino acids of the rPγ sequence in promoting transducin activation can be inferred by comparing the rPγ19-87 truncation mutant (25% of maximum Pαβ activity) to the internal deletion mutant (rPγ1-4_9-87; 55% of maximum activity). We further localized the region of rPγ that suppresses transducin activation of rod PDE6 by testing another internal deletion mutant of rPγ lacking only amino acids 9-18; in this instance, Pαβ reconstituted with rPγ1-8_19-87 achieved close to the same level of transducin activation (64%) as we observed for wildtype cPγ (72%; Fig. 5B and Table 1). These results demonstrate that amino acids 9-18 of mammalian rPγ represent a highly conserved sequence (E(I/F)RSATRV*G) that is absent in mammalian cPγ that significantly reduces the ability of transducin to maximally activate
N-terminus of Pγ modulates transducin activation of PDE6

PDE6. These results also indicate that the first eight amino acids of rPγ can enhance the ability of Gtα*-GTPγS to activate PDE6, although this effect is only observed when residues 9-18 have been deleted.

Having identified a rPγ-specific element that decreased the ability of transducin to fully activate PDE6, we sought to determine whether substituting the nearby highly conserved rod-cone Pγ difference (V21 in rPγ, T17 in cPγ) would enhance transducin activation when the rod “inhibitory” region was deleted. Fig. 5C shows that the rPγ construct (Pγ1-8_19-87V21T) achieved a slightly greater maximum extent of activation (80%) compared with wildtype cPγ (72%); a similar enhancing effect of this V21T substitution was also observed when the N-terminal 18 amino acids were deleted (rPγ18-87V21T; Fig. 5D). When tested as a single site substitution, the rPγV21T construct caused a 10% increase in Gtα*-GTPγS activation of PDE6 (Table 1). These results indicate that the valine at position 21 of the rPγ sequence contributes to the decreased ability of Gtα*-GTPγS to bind to and effectively activate PDE6.

Cone Pγ residues N13 and Q14 contribute to efficient Gtα*-GTPγS activation of PDE6.

To determine which N-terminal residues of cPγ modulate the ability of transducin to effectively activate PDE6, several cPγ N-terminal mutants were created. Fig. 6A shows that removal of the first four (cPγ5-83) or eight (cPγ9-83) residues of cPγ have an insignificant effect on the ability of Gtα*-GTPγS to activate PDE6 containing these mutant cPγ constructs. Whereas truncation of the first 14 amino acids from cPγ (cPγ15-83) significantly reduced the maximum extent of transducin activation, inclusion of amino acids N13 and Q14 (cPγ13-83) was sufficient to restore transducin activation to levels observed for wildtype cPγ (Fig. 6B, Table 1).

Since the first four or eight N-terminal amino acids of rPγ were able to enhance the ability of Gtα*-GTPγS to bind to and activate PDE6 when the rPγ inhibitory region was deleted (Fig. 5B), we also investigated whether the N-terminal amino acids of cPγ contributed to transducin activation efficacy. Comparison of cPγ9-83 (62% of Pαβ activity; Fig. 6A and Table 1) with cPγ1-4_9-83 (80% maximal activation; Fig. 6C and Table 1) revealed a significant enhancing effect of the first four amino acids of cPγ. More significantly, the impaired activation efficiency of cPγ15-83 (Fig. 6B) could be completely reversed by inclusion of the first four or eight N-terminal amino acids (Fig. 6C), rising from 45% for cPγ15-83) to 83% of the Pαβ activity for either cPγ1-4_15-83 or cPγ1-8_15-83 (Table 1). We conclude from Fig. 6 that either residues N13 and N14 or the first several N-terminal amino acids of cPγ are sufficient for Gtα*-GTPγS to maximally activate PDE6 containing these cPγ constructs.

Discussion

Using a phylogenetic analysis to identify functionally important differences between the rod and cone isoforms of Pγ in conjunction with biochemical assays of transducin activation of PDE6 reconstituted with different rPγ and cPγ mutants, we have determined that some of the well-established physiological differences in light responsiveness of rod and cone photoreceptors (1,2) can be ascribed to structural differences in the N-terminal region of rod and cone Pγ. This work also identifies for the first time a regulatory role for the N-terminus of Pγ in modulating the ability of transducin to bind to PDE6 holoenzyme and relieve the inhibitory constraint on cGMP hydrolysis imposed by the last ten C-terminal residues of Pγ (19).

Current evidence suggests that the physiological differences in the response to light exhibited by rods and cones had already evolved prior to the last common ancestor of living vertebrates (16,18,20). Our analyses of Pγ sequence evolution provides additional context to this suggestion as we demonstrate that these physiological differences are explained in part by ancient divergences in the N-terminus region.
of Pγ. A recent paper by Lagman and colleagues analyzed Pγ sequences but did not attempt a phylogenetic analysis due to high sequence conservation and proposed lack of phylogenetic signal (15). However, our phylogenetic analyses of Pγ demonstrate two well-supported clades consisting of PDE6G (rod) and PDE6H (cone) sequences. We do observe generally low support for internal nodes by either bootstrapping or approximate likelihood ratio indices but delineation between the major clades is clear. Lagman et al. also proposed a new clade of Pγ called PDE6I (15). In our analyses, PDE6I sequences are found within the PDE6H clade. This finding does not dispute the existence of PDE6I genes, but our phylogenetic results indicate that they are best interpreted as duplicate PDE6H genes. We chose to root our phylogeny in the most evolutionarily parsimonious manner, which places Pγ sequences from the hagfish Eptatretus stoutii at the base of both the rod and cone clades. However, we note that electrophysiological evidence for cyclostome photoreceptor light responsiveness exists only for lamprey (17,21,22), and other rootings for the Pγ phylogenetic tree could change the affinity of the hagfish Pγ sequences.

On the amino acid sequence level, we find that PDE6G sequences are generally far more conserved than PDE6H sequences (Figs. 1 and 2). Because rod phototransduction shows single-photon sensitivity and has been considered a paragon of the efficacy of natural selection (23,24), this strong conservation among PDE6G sequences could be interpreted as evidence for purifying selection. Alternatively, sequence divergence among PDE6H sequences might be an indication of diversifying selection as the kinetics of cone phototransduction—and its role in color vision—might facilitate selection for adaptive, but alternative, regulatory properties. These two scenarios are not mutually exclusive, and functional studies from the cyclostomes will be needed to provide further resolution.

In addition to highly reliable single photon detection, rod photoreceptors differ from cone photoreceptors in having a lower level of continuous “dark noise” in their dark-adapted state (25,26), as well as a longer latency period for the initial, “rising” phase of the response to flash stimuli (27). Recently, it has been reported that rod PDE6 activation by transducin requires the binding of two activated transducins to the rod PDE6 holoenzyme (28); furthermore, computer simulations of this dimeric activation model for rod PDE6 (29) support the idea that this dimeric transducin activation mechanism serves to reduce rod PDE6 spontaneous activation (i.e., reduced dark noise) as well as account for the longer delay in the rising phase of visual excitation compared with the cone photoresponse in vivo. Since cone PDE6 consists of a catalytic homodimer, Lamb et al. postulated that the symmetrical cone catalytic dimer may permit each cone catalytic subunit to be activated by transducin binding in an independent, non-cooperative manner (29). Our results support an alternative hypothesis in which differences between rod and cone Pγ isoforms in the N-terminal region of the protein—and not differences between rod and cone PDE6 catalytic subunits—determine whether transducin can activate each catalytic subunit independently (in the presence of cPγ) or alternatively require the binding of two transducins to the rod PDE6 holoenzyme (containing rPγ) for maximal activation. However, it should be pointed out that our in vitro biochemical data are obtained with protein concentrations several orders of magnitude lower than in the photoreceptor outer segment, and it will be important to evaluate the
importance of the N-terminal region of $\gamma$ under physiological conditions.

Previous work delineating the sites of interaction of the intrinsically disordered rP$\gamma$ subunit (11) with the rod PDE6 catalytic dimer have identified several functionally important regions (Fig. 7): (1) the approximately ten C-terminal amino acid residues of rP$\gamma$ are known to directly interact with the catalytic domain to regulate access of substrate to the active site and directly control catalytic activity (19,30); (2) a region in the N-terminal half of rP$\gamma$ (amino acids 10-30) enhances the affinity of cGMP to noncatalytic binding sites in the GAFa regulatory domain of the catalytic dimer (31), most likely by direct binding of this region of rP$\gamma$ to the cGMP binding pocket (32,33); and (3) multiple binding sites of rP$\gamma$ with P$\alpha\beta$ have been identified along practically the entire length of the rP$\gamma$ sequence, with its central region making a major contribution to the high overall affinity of rP$\gamma$ for P$\alpha\beta$ (31). In contrast to these functionally important regions of rP$\gamma$, the N-terminal region of rP$\gamma$ (specifically its first seventeen residues) was not observed to contribute to the ability of P$\gamma$ to inhibit cGMP hydrolysis or to stimulate cGMP binding (31). Because of the difficulty in isolating biochemical quantities of purified cone PDE6 holoenzyme, comparable characterizations of the interactions of cP$\gamma$ with cone catalytic homodimer are lacking.

Upon rod PDE6 activation by transducin, Gt$\alpha*$-GTP interacts with multiple sites within the C-terminal half of rP$\gamma$ (Fig. 7, black boxes) to displace rP$\gamma$ from the catalytic domain, thereby allowing diffusion of cGMP into the active site (34-36). Studies of the interactions of rP$\gamma$ with activated transducin $\alpha$-subunit have also identified a second region of interaction in the polycationic central region of rP$\gamma$ (37,38) whose function is uncertain. The present work is the first report that the N-terminal region of P$\gamma$ (preceding its polycationic region) plays an important role in Gt$\alpha*$-GTP activation of PDE6. For both rP$\gamma$ and cP$\gamma$, the first four amino acids significantly contribute to enhancing the maximum extent of transducin activation under conditions where neighboring amino acids have been deleted (Δ5-8; Figs. 5 and 6). Of greater significance are the inhibitory (rP$\gamma$ residues 9-18, and V21; Fig. 5) and stimulatory loci (cP$\gamma$ N13 and Q14; Figs. 4 and 6) we discovered that modulate the efficacy with which transducin can bind to P$\gamma$ and activate PDE6 catalysis.

The fact that these sites near the N-terminus of P$\gamma$ overlap with the region of rP$\gamma$ previously shown to enhance cGMP binding affinity to the GAFa domain (Fig. 7) are consistent with the following allosteric mechanism. We hypothesize that rP$\gamma$ residues 9-21 may be allosterically coupled to the central polycationic region of rP$\gamma$ (residues ~22-45) and reduce the affinity of transducin binding to this region of rP$\gamma$ which in turn reduces the ability of transducin to bind to and displace the C-terminal residues of rP$\gamma$ required for disinhibition of PDE6 catalysis. Conversely, residues 13-17 in cP$\gamma$ may serve to allosterically enhance transducin binding to the central region of cP$\gamma$, facilitating full activation of PDE6 catalysis. The lower affinity of cGMP for the GAFa domains of cone PDE6 than for rod PDE6 (8,39) and the well-established reciprocal regulation of rP$\gamma$ and cGMP binding affinity to the PDE6 catalytic dimer [reviewed in (3)] is also likely contribute to differences in the allosteric regulation of rP$\gamma$ and cP$\gamma$ interactions with activated transducin.

In conclusion, this work demonstrates that the N-terminal region of the rod and cone inhibitory P$\gamma$ subunits allosterically regulate the efficiency with which activated transducin is able to displace P$\gamma$ from the active site of PDE6 holoenzyme. This supports the hypothesis that
N-terminus of Pγ modulates transducin activation of PDE6

the activation mechanism of PDE6 differs in rods and cones and can account for at least some of the physiological differences in rod and cone light responsiveness. These results will also contribute to a better understanding of the molecular etiology of disease-causing mutations in the catalytic and inhibitory subunits of photoreceptor PDE6, particularly those that occur in the regulatory domains of these proteins [(40) and references cited therein].

Experimental Procedures

Materials

Bovine retinas were purchased from W.L. Lawson, Inc. Synthetic peptides (rPγ19-87 and cPγ15–83) were purchased from New England Peptide. All synthetic DNAs of rPγ and cPγ mutants were purchased from ThermoFisher-Invitrogen. Site-directed mutants of Pγ were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Filtration membranes and chemicals were from Millipore-Sigma.

Sequence alignments and phylogenetic analyses

PDE6G and PDE6H sequences from a diversity of vertebrate species were obtained from Uniprot or from the literature (14,18). Additional sequences were obtained from the gene models of 56 additional craniate species with whole genome sequences using a custom phylogenomics pipeline. This pipeline uses BLAST (e value = 0.0001) to search individual datasets using PDE6G and PDE6H query sequences (CNRG_HUMAN, P18545.1; CNCG_BOVIN, P22571.1) (41). Redundant sequences are then removed using cdhit [-c = 1.0; (42)] and the resulting dataset aligned using the progressive algorithm implemented in PASTA with default parameters (43). Following the initial PASTA alignment, we removed four sequences that were either shorter than 50 amino acid residues long or had internal deletions greater than 30 amino acids long. Sequences were then realigned using PASTA under default parameters; we confirmed that additional iterations of progressive alignment did not produce improvements. The resulting Pγ amino acid sequences from different vertebrate species are listed in Supporting Information, Table S1.

The phylogeny of PDE6G and PDE6H was then estimated using IQ-tree under the best fit model (44); see Supporting Information, Fig. S1, for the resulting tree with species labels. Node support was ascertained using both bootstrap and approximate likelihood ratio scores (45). The final tree with tip label and approximate likelihood ratio scores is available as a .tre file in the Github repository associated with this paper. All scripts and command lines used in sequence analyses are located at https://github.com/plachetzki/PDE6_GAMMA.

Construction and purification of Pγ mutants

Codon-optimized, synthetic DNA fragments coding for various bovine rPγ and cPγ constructs used in this study (Supporting Information, Fig. S1) were inserted into the NdeI and BamHI sites of the pET11a vector, followed by transformation into the E. coli BL21(DE3) strain. The sequence of all Pγ mutants was confirmed by DNA sequencing (Functional Biosciences).

Following expression of recombinant Pγ mutants in E. coli BL21(DE3), the bacterial extract was purified by HiTrap SP FF column from GE. The Pγ mutants were further purified by C18 reverse-phase high pressure liquid chromatography following standard procedures (46). The purity of these proteins was determined to be >90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined by the bicinchoninic acid protein assay using bovine γ-globulin as a standard.

PDE6 and Pαβ purification and activity assays

Bovine rod PDE6 holoenzyme was purified from bovine retinas as described (47). Pαβ catalytic dimers were prepared by limited trypsin proteolysis and re-purified by Mono Q anion exchange chromatography prior to use (47). PDE6 catalytic activity was measured in 20 mM HEPES, 10 mM MgCl₂, and 0.5 mg/ml
bovine serum albumin using a colorimetric assay (48). The PDE6 concentration was estimated based on the rate of cGMP hydrolysis of trypsin-activated PDE6 and the knowledge of the $k_{cat}$ of the enzyme (5600 mol cGMP hydrolyzed per mol Pαβ per s (39). The inhibition potency (IC$_{50}$) of all $\gamma$ constructs reported in Table 1 was determined using 0.2 nM Pαβ and 2 mM cGMP as substrates (39,49); in all cases, the IC$_{50}$ values did not differ significantly from those observed for wildtype r$\gamma$ and c$\gamma$ (Fig. 3B). Note that the difficulty in obtaining biochemical quantities of purified bovine cone photoreceptor PDE6 holoenzyme and bovine cone transducin precluded carrying out complementary experiments with these cone isoforms.

**Purification of Gt$_{a^*}$-GTP$_{\gamma}$S and measurements of transducin activation of PDE6**

Transducin $\alpha$-subunits were extracted from the PDE6-depleted ROS membranes by addition of 50 $\mu$M GTP$_{\gamma}$S. The extracted Gt$_{a^*}$-GTP$_{\gamma}$S was purified on a Blue Sepharose column as described (50,51), followed by gel filtration chromatography to remove residual PDE6. The concentration of Gt$_{a^*}$-GTP$_{\gamma}$S was determined by a colorimetric protein assay. Purified Gt$_{a^*}$-GTP$_{\gamma}$S (in 50% glycerol) was stored at -20 °C.

To measure transducin activation of PDE6, 1 nM Pαβ was pre-incubated with 10 nM of a $\gamma$ mutant for 10 min prior to addition of the indicated concentrations of Gt$_{a^*}$-GTP$_{\gamma}$S for 1 hr at room temperature. The PDE6 activity was then assayed at a final concentration of 0.2 nM using 2 mM cGMP as substrate. Note that these assay conditions were chosen to maximize differences between Pαβ reconstituted with r$\gamma$ and c$\gamma$; however, even with a 10-fold higher concentration of purified rod PDE6 holoenzyme (2 nM) and a 1000-fold excess of Gt$_{a^*}$-GTP$_{\gamma}$S, we failed to observe greater than 50% maximal activation by Gt$_{a^*}$-GTP$_{\gamma}$S [similar to literature values; see (8,10,31)].

**Data analysis**

All experiments were repeated at least three times. The transducin concentration dependence of PDE6 activation (Fig. 3A, Figs. 4-6) was analyzed by nonlinear regression (Sigmaplot v.12.5, SPSS, Inc.) using a 3-parameter hyperbolic equation: $y = y_0 + a*x/(b+x)$ where $a$ is the maximum percent activation, $b$ is the K$_{1/2}$ and $y_0$ is the basal PDE6 activity in the absence of Gt$_{a^*}$-GTP$_{\gamma}$S. $\gamma$ inhibition potency (Fig. 3B) was determined using a 3-parameter logistic equation: $y = a/(1 + (x/x_0)^b)$ where $a$ is the amplitude, $b$ is the slope, and $x_0$ is the IC$_{50}$ value.

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**Conflicts of interest:** The authors declare they have no conflicts of interest with the contents of this article.

**References**

1. Korenbrot, J. I. (2012) Speed, sensitivity, and stability of the light response in rod and cone photoreceptors: Facts and models. *Prog. Retin. Eye Res* **31**, 442-466
2. Ingram, N. T., Sampath, A. P., and Fain, G. L. (2016) Why are rods more sensitive than cones? *J Physiol* **594**, 5415-5426
3. Cote, R. H. (2006) Photoreceptor phosphodiesterase (PDE6): a G-protein-activated PDE regulating visual excitation in rod and cone photoreceptor cells. in *Cyclic Nucleotide Phosphodiesterases in Health and Disease* (Beavo, J. A., Francis, S. H., and Houslay, M. D. eds.), CRC Press, Boca Raton, FL. pp 165-193
4. Kefalov, V. J. (2012) Rod and cone visual pigments and phototransduction through pharmacological, genetic, and physiological approaches. *Journal of Biological Chemistry* **287**, 1635-1641
N-terminus of γ modulates transducin activation of PDE6

5. Deng, W. T., Sakurai, K., Liu, J., Dinculescu, A., Li, J., Pang, J., Min, S. H., Chiodo, V. A., Boye, S. L., Chang, B., Kefalov, V. J., and Hauswirth, W. W. (2009) Functional interchangeability of rod and cone transducin alpha-subunits. *PNAS* **106**, 17681-17686

6. Gopalakrishna, K. N., Boyd, K. K., and Artemyev, N. O. (2012) Comparative analysis of cone and rod transducins using chimeric Ga subunits. *Biochemistry* **51**, 1617-1624

7. Mao, W., Miyagishima, K. J., Yao, Y., Soreghan, B., Sampath, A. P., and Chen, J. (2013) Functional comparison of rod and cone Gα on the regulation of light sensitivity. *Journal of Biological Chemistry* **288**, 5257-5267

8. Gillespie, P. G., and Beavo, J. A. (1988) Characterization of a bovine cone photoreceptor phosphodiesterase purified by cyclic GMP-Sepharose chromatography. *J. Biol. Chem* **263**, 8133-8141

9. Mou, H., and Cote, R. H. (2001) The catalytic and GAF domains of the rod cGMP phosphodiesterase (PDE6) heterodimer are regulated by distinct regions of its inhibitory γ subunit. *JBC* **276**, 27527-27534

10. Muradov, H., Boyd, K. K., and Artemyev, N. O. (2010) Rod phosphodiesterase-6 PDE6A and PDE6B subunits are enzymatically equivalent. *J. Biol. Chem* **285**, 39828-39834

11. Guo, L. W., and Ruoho, A. E. (2008) The retinal cGMP phosphodiesterase gamma-subunit - a chameleon. *Curr. Protein Pept. Sci* **9**, 611-625

12. Arshavsky, V. Y., and Wensel, T. G. (2013) Timing is everything: GTPase regulation in phototransduction. *Invest Ophthalmol. Vis. Sci* **54**, 7725-7733

13. Skiba, N. P., Hopp, J. A., and Arshavsky, V. Y. (2000) The effector enzyme regulates the duration of G protein signaling in vertebrate photoreceptors by increasing the affinity between transducin and RGS protein. *Journal of Biological Chemistry* **275**, 32716-32720

14. Lamb, T. D., Patel, H., Chuah, A., Natoli, R. C., Davies, W. I., Hart, N. S., Collin, S. P., and Hunt, D. M. (2016) Evolution of Vertebrate Phototransduction: Cascade Activation. *Mol Biol Evol* **33**, 2064-2087

15. Lagman, D., Franzen, I. E., Eggert, J., Larhammar, D., and Abalo, X. M. (2016) Evolution and expression of the phosphodiesterase 6 genes unveils vertebrate novelty to control photosensitivity. *BMC Evol. Biol* **16**, 124

16. Lamb, T. D. (2013) Evolution of phototransduction, vertebrate photoreceptors and retina. *Prog. Retin. Eye Res* **36**, 52-119

17. Morshedian, A., and Fain, G. L. (2017) Light adaptation and the evolution of vertebrate photoreceptors. *J Physiol* **595**, 4947-4960

18. Muradov, H., Boyd, K. K., Kerov, V., and Artemyev, N. O. (2007) PDE6 in lamprey Petromyzon marinus: implications for the evolution of the visual effector in vertebrates. *Biochemistry* **46**, 9992-10000

19. Granovsky, A. E., Natochin, M., and Artemyev, N. O. (1997) The γ subunit of rod cGMP-phosphodiesterase blocks the enzyme catalytic site. *J. Biol. Chem* **272**, 11686-11689

20. Morshedian, A., and Fain, G. L. (2017) The evolution of rod photoreceptors. *Philos Trans R Soc Lond B Biol Sci* **372**

21. Asteriti, S., Grillner, S., and Cangiano, L. (2015) A Cambrian origin for vertebrate rods. *Elife* **4**

22. Morshedian, A., and Fain, G. L. (2015) Single-photon sensitivity of lamprey rods with cone-like outer segments. *Curr Biol* **25**, 484-487

23. Hecht, S., Shlaer, S., and Pirenne, M. H. (1942) Energy, Quanta, and Vision. *J Gen Physiol* **25**, 819-840

24. Pugh, E. N., Jr. (2018) The discovery of the ability of rod photoreceptors to signal single photons. *J Gen Physiol*
N-terminus of $\gamma$ modulates transducin activation of PDE6

25. Rieke, F., and Baylor, D. A. (1996) Molecular origin of continuous dark noise in rod photoreceptors. *Biophys. J* 71, 2553-2572
26. Rieke, F., and Baylor, D. A. (2000) Origin and functional impact of dark noise in retinal cones. *Neuron* 26, 181-186
27. Rotov, A. Y., Astakhova, L. A., Firsov, M. L., and Govardovskii, V. I. (2017) Origins of the phototransduction delay as inferred from stochastic and deterministic simulation of the amplification cascade. *Mol Vis* 23, 416-430
28. Qureshi, B. M., Behrmann, E., Schoneberg, J., Loerke, J., Burger, J., Mielke, T., Giesebercht, J., Noe, F., Lamb, T. D., Hofmann, K. P., Spahn, C. M. T., and Heck, M. (2018) It takes two transducins to activate the cGMP-phosphodiesterase 6 in retinal rods. *Open Biol* 8
29. Lamb, T. D., Heck, M., and Kraft, T. W. (2018) Implications of dimeric activation of PDE6 for rod phototransduction. *Open Biol* 8
30. Zhang, X.-J., Skiba, N. P., and Cote, R. H. (2010) Structural Requirements of the Photoreceptor Phosphodiesterase gamma-Subunit for Inhibition of Rod PDE6 Holoenzyme and for Its Activation by Transducin. *Journal of Biological Chemistry* 285, 4455-4463
31. Zhang, X. J., Gao, X. Z., Yao, W., and Cote, R. H. (2012) Functional mapping of interacting regions of the photoreceptor phosphodiesterase (PDE6) $g$-subunit with PDE6 catalytic dimer, transducin, and Regulator of G-protein Signaling9-1 (RGS9-1). *J. Biol. Chem* 287, 26312-26320
32. Muradov, K. G., Granovsky, A. E., Schey, K. L., and Artemyev, N. O. (2002) Direct interaction of the inhibitory $g$-subunit of rod cGMP phosphodiesterase (PDE6) with the PDE6 GAFa domains. *Biochemistry* 41, 3884-3890
33. Zeng-Elmore, X., Gao, X. Z., Pellarin, R., Schneidman-Duhovny, D., Zhang, X. J., Kozacka, K. A., Tang, Y., Sali, A., Chalkley, R. J., Cote, R. H., and Chu, F. (2014) Molecular architecture of photoreceptor phosphodiesterase elucidated by chemical cross-linking and integrative modeling. *J. Mol. Biol* 426, 3713-3728
34. Skiba, N. P., Artemyev, N. O., and Hamm, H. E. (1995) The carboxyl terminus of the gamma-subunit of rod cGMP phosphodiesterase contains distinct sites of interaction with the enzyme catalytic subunits and the $a$-subunit of transducin. *J. Biol. Chem* 270, 13210-13215
35. Liu, Y., Arshavsky, V. Y., and Ruoho, A. E. (1996) Interaction sites of the COOH-terminal region of the gamma subunit of cGMP phosphodiesterase with the GTP-bound subunit of transducin. *J. Biol. Chem* 271, 26900-26907
36. Guo, L. W., Hajipour, A. R., and Ruoho, A. E. (2010) Complementary interactions of the rod PDE6 inhibitory subunit with the catalytic subunits and transducin. *J. Biol. Chem* 285, 15209-15219
37. Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P., and Hamm, H. E. (1992) Sites of interaction between rod G-protein $a$-subunit and cGMP-phosphodiesterase gamma-subunit. Implications for phosphodiesterase activation mechanism. *J. Biol. Chem* 267, 25067-25072
38. Artemyev, N. O. (1997) Binding of transducin to light-activated rhodopsin prevents transducin interaction with the rod cGMP phosphodiesterase gamma-subunit. *Biochemistry* 36, 4188-4193
39. Mou, H., Grazio, H. J., Cook, T. A., Beavo, J. A., and Cote, R. H. (1999) cGMP binding to noncatalytic sites on mammalian rod photoreceptor phosphodiesterase is regulated by binding of its $g$ and $d$ subunits. *JBC* 274, 18813-18820
40. Gopalakrishna, K. N., Boyd, K., and Artemyev, N. O. (2017) Mechanisms of mutant PDE6 proteins underlying retinal diseases. *Cell Signal* 37, 74-80
41. Altschul, S. F., and Koonin, E. V. (1998) Iterated profile searches with PSI-BLAST—a tool for discovery in protein databases. *Trends Biochem Sci* 23, 444-447
42. Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150-3152
N-terminus of Pγ modulates transducin activation of PDE6

43. Mirarab, S., Nguyen, N., Guo, S., Wang, L. S., Kim, J., and Warnow, T. (2015) PASTA: Ultra-Large Multiple Sequence Alignment for Nucleotide and Amino-Acid Sequences. J Comput Biol 22, 377-386

44. Nguyen, L. T., Schmidt, H. A., von Haeseler, A., and Minh, B. Q. (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32, 268-274

45. Anisimova, M., and Gascuel, O. (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55, 539-552

46. Artemyev, N. O., Arshavsky, V. Y., and Cote, R. H. (1998) Photoreceptor phosphodiesterase: Interaction of inhibitory γ subunit and cyclic GMP with specific binding sites on catalytic subunits. Methods 14, 93-104

47. Pentia, D. C., Hosier, S., Collupy, R. A., Valeriani, B. A., and Cote, R. H. (2005) Purification of PDE6 isozymes from mammalian retina. Methods Mol. Biol 307, 125-140

48. Cote, R. H. (2000) Kinetics and regulation of cGMP binding to noncatalytic binding sites on photoreceptor phosphodiesterase. Methods in Enzymology 315, 646-672

49. Hurley, J. B., and Stryer, L. (1982) Purification and characterization of the gamma regulatory subunit of the cyclic GMP phosphodiesterase from retinal rod outer segments. J. Biol. Chem 257, 11094-11099

50. Kleuss, C., Pallast, M., Brendel, S., Rosenthal, W., and Schultz, G. (1987) Resolution of transducin subunits by chromatography on blue sepharose. J. Chromatogr 407, 281-289

51. Wensel, T. G., He, F., and Malinski, J. A. (2005) Purification, reconstitution on lipid vesicles, and assays of PDE6 and its activator G protein, transducin. Methods Mol. Biol 307, 289-314

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The abbreviations used are: phosphodiesterase-6 (PDE6); PDE6 inhibitory γ-subunit (Pγ); rod PDE6 catalytic dimer (Pαβ); rod Pγ (rPγ); cone Pγ (cPγ); heterotrimeric G protein, transducin (Gt); persistently activated transducin α-subunit (Gtα*-GTPγS); GTP-bound, activated transducin α-subunit (Gtα*-GTP); regulatory domain of PDE6 defined by its occurrence in cGMP-regulated PDEs, adenylyl cyclases and the E. coli protein Fh1A (GAF domain).
Table 1. Effectiveness of Pγ constructs in promoting Gtα*-GTPγS activation of PDE6.

| Pγ construct | K1/2 (nM) | % max. activation | IC50 (nM) |
|--------------|-----------|-------------------|-----------|
| rPγ1-87 (WT) | 34 ± 12   | 16 ± 2.3 (19)     | 0.61 ± 0.14 (12) |
| rPγ5-87     | 35 ± 6.5  | 15 ± 3.8 (3)      | 0.81      |
| rPγ9-87     | 32 ± 4.1  | 15 ± 2.6 (3)      | 0.51      |
| rPγ19-87    | 37 ± 9.9  | 28 ± 3.6 (9)      | 0.74 ± 0.16 (5) |
| rPγ19-87V21T| 37 ± 14   | 55 ± 5.5 (5)      | 0.86 ± 0.13 (3) |
| rPγ1-4_9-87 (Δ5-8) | 30 ± 2.1 | 25 ± 4.1 (3) | 0.58 |
| rPγ1-4_19-87 (Δ5-18) | 58 ± 9.0 | 55 ± 5.9 (4) | 0.40 (2) |
| rPγ1-8_19-87 (Δ9-18) | 78 ± 16  | 64 ± 2.6 (8) | 0.64 (2) |
| rPγ1-8_19-87V21T | 66 ± 12  | 81 ± 11 (3) | 0.56 |
| rPγV21T     | 49 ± 1.4  | 27 ± 6.3 (3)      | 0.42      |
| rPγ1-18_cPγ15-83 | 66 ± 18  | 30 ± 1.3 (4)      | 0.46      |
| cPγ1-83 (WT) | 26 ± 10   | 72 ± 10 (20)      | 0.66 ± 0.15 (13) |
| cPγ5-83     | 35 ± 15   | 66 ± 11 (3)       | 0.68      |
| cPγ9-83     | 40 ± 18   | 62 ± 12 (3)       | 0.62      |
| cPγ13-83    | 33 ± 17   | 72 ± 14 (5)       | 0.83      |
| cPγ15-83    | 44 ± 18   | 45 ± 8.3 (11)     | 0.79 ± 0.18 (5) |
| cPγ1-4_9-83 (Δ5-8) | 40 ± 8.6 | 80 ± 1.2 (3) | 0.69 |
| cPγ1-4_15-83 (Δ5-14) | 25 ± 6.5 | 83 ± 11 (3) | 0.70 (2) |
| cPγ1-8_15-83 (Δ9-14) | 52 ± 4.6 | 83 ± 11 (3) | 0.67 |
| cPγT17V     | 32 ± 6.4  | 74 ± 7.8 (3)      | 0.69      |
| cPγK44QS70NF80Y | 25 ± 2.0 | 72 ± 5.4 (4) | 0.59 ± 0.04 (3) |
| cPγ1-14_rPγ19-87 | 40 ± 20 | 67 ± 8.5 (4) | 0.54 |

Pαβ (1 nM) was pre-incubated with 10 nM of one of the rPγ or cPγ constructs to reconstitute the PDE6 holoenzyme. Various concentrations of Gtα*-GTPγS were then added and incubated for 1 h at room temperature. The PDE activity was measured using 2 mM cGMP as substrate and expressed as the percent activity referenced to fully activated Pαβ. The K1/2 and maximum percent activation were calculated by fitting the data to a 3-parameter hyperbola. The inhibition potency (IC50) of all Pγ constructs was determined using 0.2 nM Puβ and 2 mM cGMP as substrates. The values in the Table represent the mean ± S.D. for n individual experiments.
Figure 1. Phylogenetic analysis of PDE6G (rod) and PDE6H (cone) sequences. Branch colors on the gene tree (left) correspond to the species tree (inset). The tree is rooted between putative rod and cone Pγ subunits. Multiple sequence alignment is shown plotted to the right. In the N-terminal region, several residues (“rod conserved;” grey boxes) are >90% conserved in the PDE6G alignment, but divergent or missing in the PDE6H alignment. Two “rod/cone differentiated” sequences (black boxes) are >80% conserved in either PDE6G and PDE6H alignments, but differentiated between the two genes.
Figure 2. Consensus logo of PDE6G and PDE6H subunits. (A) The vertebrate consensus logo for PDE6G and PDE6H was generated based on the multiple sequence alignment of 101 rod and 103 cone sequences shown in Fig. 1. (B) The mammalian consensus logo is based on a multiple sequence alignment of a subset of the entire set of sequences and consisting of 50 mammalian PDE6G sequences and 51 mammalian PDE6H sequences.
Figure 3. PDE6 reconstituted with cPγ is more effectively activated by Gtα*-GTPγS than PDE6 containing rPγ. (A) Gtα*-GTPγS activation assay. 1 nM Pαβ was pre-incubated with a 10-fold molar excess of wildtype ("WT") rPγ or cPγ to reconstitute the PDE6 holoenzyme. Gtα*-GTPγS was then added at the indicated concentrations and incubated for 1 h prior to measuring PDE activity with 2 mM cGMP as substrate. PDE activity is reported relative to the activity of Pαβ in the absence of Pγ. The data are the mean (± S.D.) for 19 or 20 separate determinations of PDE6 reconstituted with wildtype rPγ or cPγ, respectively. The data were fit by non-linear regression analysis using a 3-parameter hyperbolic equation and reported in Table 1; basal activities for PDE6 reconstituted with rPγ and cPγ were 2 ± 0.8% and 8 ± 3, respectively. (B) Pγ inhibition assay. Pαβ (0.2 nM) was incubated with the indicated concentrations of wildtype rPγ or cPγ for 10 min, and then the catalytic activity measured using 2 mM cGMP as substrate. The data are the mean (± S.D.) of 8 experiments with rPγ and cPγ. A 3-parameter logistic equation was used estimate the IC50: rPγ, IC50 = 0.61 ± 0.14 nM, cPγ, IC50 = 0.58 nM.
Figure 4. Four highly conserved sites that differ between rPγ and cPγ do not alter transducin activation efficacy, whereas the N-terminal region plays a primary role in regulating transducin activation. (A) A rPγ amino acid was substituted for the cPγ residue at three sites (K44Q, S70N, F80Y) or at one site (T17V), and the site-directed mutants were then reconstituted with Pαβ prior to the addition of varying amounts of Gtα*GTPγS. (B) Chimeric Pγ mutants consisting of the N-terminal region of one Pγ fused to the remaining C-terminal sequence of the other Pγ were generated and assayed for the ability of PDE6 to be activated by increasing concentrations of Gtα*GTPγS. Experiments were performed and analyzed as described in the legend to Fig. 3A, with values for K1/2 and percent of maximum activation provided in Table 1.
Figure 5. Residues in the N-terminal region of rPγ affect the Gtα*-GTPγS activation efficiency. (A) Three N-terminal truncated rPγ mutants (rPγ5-87, rPγ9-87 and rPγ19-87) were reconstituted with Pαβ to test Gtα*-GTPγS activation efficiency. (B) Three rPγ mutants with internal deletions were tested to evaluate the role of the N-terminus in promoting PDE6 activation by Gtα*-GTPγS. (C, D) Gtα*-GTPγS activation assays of PDE6 in which rPγ mutants were tested with and without the site-directed mutant V21T. Gtα*-GTPγS activation assays were performed and analyzed as described in Fig. 3A and the K_{1/2} and percent of maximum activation are summarized in Table 1.
Figure 6. Identification of residues in the N-terminal region of cPγ that enhance Gtα*-GTPγS activation efficiency. (A, B) Four N-terminal truncated cPγ mutants (cPγ5-83, cPγ9-83, cPγ13-83, and cPγ15-83) were reconstituted with Pαβ to test Gtα*-GTPγS activation efficiency of PDE6. (C) Three internal deletion mutants of cPγ (cPγ1-4_9-83, cPγ1-4_15-83 and cPγ1-8_15-83) were also tested under the same experimental conditions. The Gtα*-GTPγS activation assay was carried out and analyzed as described in the legend to Figure 3A, and the results summarized in Table 1.
Figure 7. Functionally important sites of the rPγ and cPγ inhibitory subunits. Blue-shaded residues represent highly conserved rod-cone Pγ differences. Solid green boxes represent regions that enhance Gtα* activation. The solid red boxes represent the regions that impair Gtα* activation. The red letters identify two amino acids in the bovine Pγ sequences that differ from the mammalian consensus sequences: M17V (rPγ) and T11A for cPγ. Also shown are previously identified regions that enhance rPγ inhibition potency (white boxes), enhance noncatalytic cGMP binding to the GAFa domain (gray box), or are critical for activation of PDE6 by activated transducin (black boxes); ref. (31).
The N termini of the inhibitory γ-subunits of phosphodiesterase-6 (PDE6) from rod and cone photoreceptors differentially regulate transducin-mediated PDE6 activation

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