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The roles of the phosducin family proteins in the regulation of heterotrimeric G proteins in vertebrate photoreceptors

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The Roles of the Phosducin Family Proteins in the Regulation of Heterotrimeric G Proteins in Vertebrate Photoreceptors

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ABSTRACT

The Roles of the Phosducin Family Proteins in the Regulation of Heterotrimeric G Proteins in Vertebrate Photoreceptors

Hongman Song

Phosducin (Pdc) and phosducin-like protein 1 (PhLP1) are homologous proteins of the phosducin gene family that specifically interact with heterotrimeric G proteins. Both Pdc and PhLP1 are expressed in photoreceptors, sensory neurons of the retina responsible for acquisition of visual information; however their functions in these cells remains enigmatic. Photoreceptors maintain remarkably high and tightly controlled levels of the heterotrimeric G protein, transducin, utilized by these cells for visual signal transduction. Our central hypothesis was that Pdc and PhLP1 are engaged in the maintenance of transducin homeostasis in the photoreceptors.

To test this hypothesis, we have studied phosphorylation of Pdc, which regulates its interaction with transducin in a light-dependent manner. For that, we have determined light-dependence, time-course, and localization of phosphorylated Pdc within the photoreceptor cell under various physiologically relevant conditions of illumination. We found that phosphorylation of Pdc *in vivo* occurs in a site- and compartment-specific manner, and is specifically enriched in the border between the inner and outer segments of rod photoreceptors. These findings are described in Part I of Chapter 2.

An abundance of Pdc phosphorylation at the entrance to the outer segment allowed us to hypothesize that Pdc regulates trafficking of transducin to this compartment. To test our hypothesis directly, we generated transgenic mice expressing Pdc lacking the principal phosphorylation site, serine 54 and serine 71, under the control of a rhodopsin promoter. Using this Pdc phosphorylation mutant, and transgenic mice expressing full-length Pdc as a control, we compared the rates of transducin trafficking to the rod outer segments, and found that phosphorylation of Pdc significantly accelerates trafficking of transducin to the rod outer segments. This ongoing research project is described in Part II of Chapter 2.

To explore the role of PhLP1, we have suppressed its endogenous activity in photoreceptors using transgenic overexpression of a dominant-negative N-terminally truncated splice-isoform of PhLP1. We found that suppressing PhLP1 activity triggered fast and severe photoreceptor degeneration, apparently due to a profound disruption in transducin expression. These findings, described in Chapter 3, strongly support our hypothesis that PhLP1 plays a central role in the post-translational stabilization of transducin subunits, which is essential for visual function and rod viability.

In summary, our findings have demonstrated that phosducin and phosducin-like protein 1 function as specific chaperones in the folding, assembly and trafficking of transducin subunits in photoreceptors.

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List of Abbreviations

CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
COX I	Subunit I of cytochrome c oxidase
ERG	Electroretinogram
$G\alpha\beta\gamma$	Heterotrimeric G protein
$G\beta\gamma$	The $\beta\gamma$ subunits of heterotrimeric G protein
$G\alpha\beta\gamma t$	Transducin
$G\alpha t$	Transducin α subunit
$G\beta\gamma t$	Transducin $\beta\gamma$ subunits
GPCR	G protein-coupled receptor
HSP90	Heat shock protein 90
IS	Inner segment
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
Pdc	Phosducin
Pdc54p	Phospho-specific antibody recognizing phosducin phosphorylated at residue 54
Pdc71p	Phospho-specific antibody recognizing phosducin phosphorylated at residue 71
PDE6	cGMP phosphodiesterase 6
PhLP1	Phosducin-like protein 1
PKA	cAMP-dependent protein kinase
PP2A	Protein phosphatase 2A
RGS-9	Regulator of G protein signaling-9
Rho	Rhodopsin
ROM-1	Retinal outer segment membrane protein 1
TCP1	Tailless complex polypeptide 1
TRiC/CCT	TCP1-ring complex/chaperonin containing TCP1
UPR	Unfolded protein response

Chapter 1

Literature Review

Significance

Heterotrimeric G proteins, consisting of α, β, γ subunits (Gαβγ), are well-known for their function as key mediators for a wide variety of physiological responses to extracellular stimuli including hormones, neurotransmitters, and sensory signals [1-3]. To convey these signals perceived by the cell surface G protein-coupled receptors (GPCRs) to downstream effectors, three subunits of heterotrimeric G proteins must be associated and localized to the cytoplasmic surface of plasma membranes. This process is critical to determine the cellular content of G proteins and, consequently, the efficiency of G protein signal transduction. Although, over the past decade, significant progress has been made in understanding the role of G proteins in signal transduction, there are still a lot of fundamental questions that need to be clarified, such as the molecular mechanisms contributing to G protein assembly and intracellular trafficking, etc [4]. More importantly, dysregulation of G protein signaling is linked to many diseases [5-7]. As a result of such ubiquitous importance, the cell exquisitely regulates these signaling pathways at different levels, in which G proteins are one of the major regulatory targets.

Transducin, a rod photoreceptor-specific G protein (Gαβγt), is one of the best-studied G proteins [8, 9]. It mediates visual signal transduction, known as phototransduction, from the light-activated GPCR, rhodopsin, to the downstream effector, cGMP phosphodiesterase 6 (PDE6) in rod photoreceptors. Several regulatory proteins can modulate transducin-mediated signaling by directly affecting the activity of

each transducin subunit, such as the regulator of G protein signaling (RGS-9), which is a GTPase-activating protein for transducin α subunit (Gαt) [10-12], and the phosducin family proteins (phosducin, Pdc, and phosducin like-protein 1, PhLP1), which have been known to bind transducin βγ subunits (Gβγt) with high affinity [13-20]. Recent reviews about the genetic mechanisms of several retinal degenerative diseases suggest that abnormal expression of phototransduction proteins could lead to cell death and retinal degeneration [21, 22]. More directly, disruption of the transducin β or γ gene led to the development of retinal degeneration, indicating that normal expression of transducin βγ subunits is essential for survival of rod photoreceptors [23, 24]. Therefore, our studies are dedicated to the investigation of how expression and trafficking of transducin βγ subunits are regulated by Pdc and PhLP1 in rods. Understanding the regulatory mechanisms responsible for transducin expression and trafficking in rod photoreceptors will contribute not only to an increased knowledge of the cell biology of G proteins, but also provide new insights into novel pathways involved in retinal degeneration, which will benefit the development of new strategies to cure, or even prevent, such retinal degenerative diseases.

Specialized organization of vertebrate photoreceptors

The vertebrate retina is the neural part of the eye, which contains neurons sensitive to light and capable of transmitting light information to the brain [25]. There are two classes of light-sensing neurons in the vertebrate retina, rod and cone photoreceptors. Approximately 97% of the photoreceptor cells in the mouse retina are rods that are necessary for the extremely light-sensitive monochromatic vision [26]. These cells are organized in highly ordered and layered compartments with respect to their structure (Figure 1) and function. The outer segment (OS) is the most specialized ciliary

compartment of the photoreceptor cell, where light is absorbed and converted into an electrical signal. This segment is composed of membranous disks that contain light-sensitive photopigment and other proteins involved in visual signal transduction. The outer segment is linked to the inner segment (IS) by a connecting cilium, which contains the essential metabolic and biosynthetic machinery of the cell, including mitochondria, endoplasmic reticulum and ribosomes, etc. The photoreceptor cell bodies house the nuclei that make up the outer nuclear layer (ONL) and are connected to the layer of synapses, the thin outer plexiform layer (OPL) that signals to the inner retinal neurons.

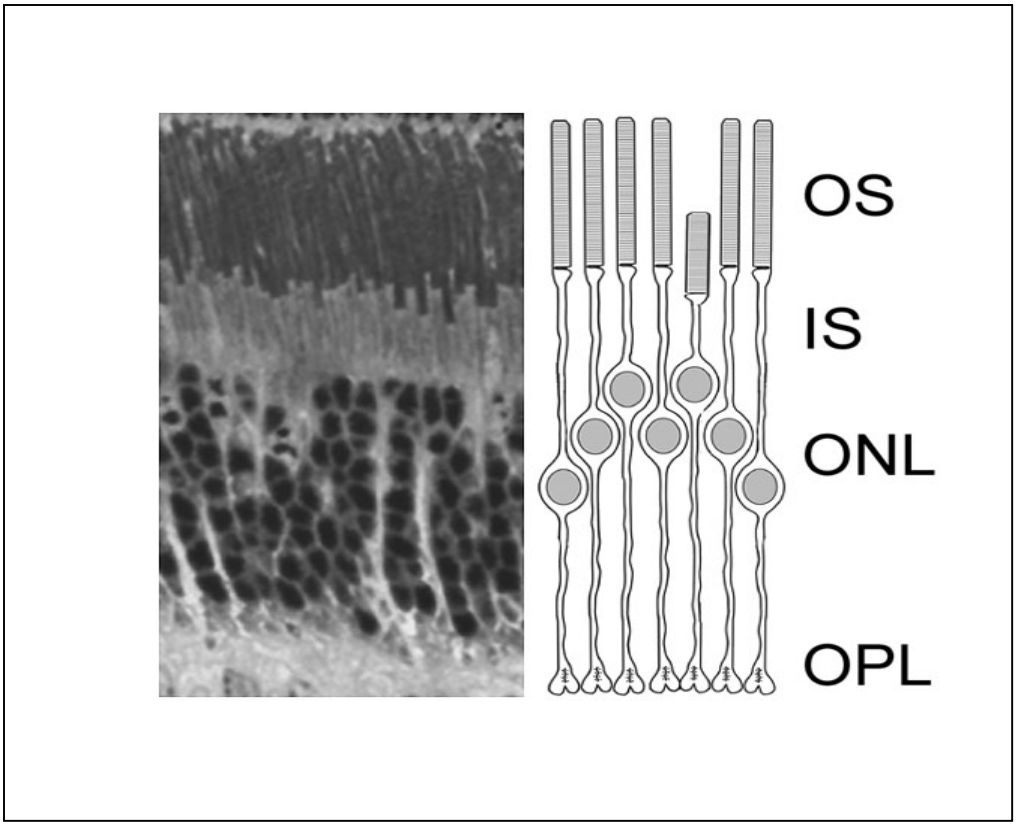

Figure 1. Structure of vertebrate photoreceptors shown by cross-section of the mouse retina (left) and a schematic illustration (right). Photoreceptor layers are OS-outer segments, IS-inner segments, ONL-outer nuclear layer, and OPL-outer plexiform layer.

Transducin as a central mediator of rod signal transduction

Rods use the visual specific G protein, transducin, -mediated signaling cascade for visual signal transduction, which has been a model system for studying G protein signaling for many years [8, 9]. In the rod outer segment, the G protein coupled receptor (GPCR), rhodopsin, is directly activated by absorbing light and initiates the transducin-mediated phototransduction pathway. Transducin is composed of the α subunit (Gαt) and βγ subunits (Gβγt). When the photoactivated rhodopsin catalyzes a nucleotide exchange on transducin, the heterotrimeric transducin dissociates into the Gαt-GTP subunit and Gβγt heterodimer, resulting in the activation of cGMP phosphodiesterase 6 (PDE6) and closure of the cGMP-gated channels. Subsequent deactivation of the signaling cascade is triggered by phosphorylation of the C-terminus of rhodopsin, which promotes arrestin binding and effectively blocks the interaction between rhodopsin and transducin. Hydrolysis of GTP to GDP on Gαt results in transducin re-association into a Gαβγt heterotrimer and signal termination. Thus, to relay the light signal from the activated rhodopsin to PDE6, the heterotrimeric transducin undergoes a continuous activation-inactivation cycle, which allows transducin to function as a central and regulatory mediator.

As a central transducer of rod visual signaling, the transducin heterotrimer is maintained at a high but optimal concentration to ensure that rod photoreceptors continue to be extremely sensitive to light. The sensitivity of rods to light is mainly determined by the rate of transducin activation [27], which has been shown to depend on the transducin concentration in rod outer segments (OS) [28]. The rod inner segment (IS) houses essential biosynthetic machinery, which is responsible for synthesis, folding and assembly of transducin. After formation of heterotrimeric transducin in the IS, it must

be delivered to the OS, where visual signal transduction takes place. Moreover, transducin localization is dynamic, and activation upon light exposure can drive reversible translocation of Gαt and Gβγt out of the rod outer segment [4, 28]. The dynamic distribution of the three transducin subunits within rods will affect transducin content in the outer segment, which will eventually determine the sensitivity of rods to bright light. Accordingly, a mechanistic understanding of folding, assembly and dynamic translocation of transducin subunits will help to identify novel ways that rods regulate transducin signaling.

Cytosolic chaperonin TRiC/CCT (TCP1-ring complex or chaperonin containing tailless complex polypeptide 1 (TCP1) is required for folding transducin β and/or its assembly with transducin γ subunit

Formation of G protein βγ subunits occurs at an early stage during assembly of the heterotrimeric G protein [29]. Under physiological conditions, The Gβγ complex is very stable and always exists as a functional unit. Contrary to the established function of Gαt as the effecter regulatory subunit, Gβγt was thought to turn off the activated Gαt subunit and to enhance membrane binding of the transducin heterotrimer, nevertheless its primary role in rods is very poorly defined. A recent study suggests that the expression level of Gβγt is critical in setting the normal content of the entire heterotrimer in rod photoreceptors [24]. Additionally, new evidence has demonstrated that the key step for Gβγ formation is dependent on the cytosolic chaperonin TRiC/CCT for Gβ folding [30-32]. The crystal structure reveals that Gβ has a seven-bladed β-propeller structure; each blade is formed from a WD40 repeat made up of four β-strands [33-35]. Completely regular β-sheet edges are inherently aggregation prone and difficult to fold to the desired final structure [36-38] . As a result, unfolded protein intermediates with an enrichment in

β-sheet structure, such as that occurring in Gβ, are particularly sensitive to misfolding and aggregation. One strategy that cells use to prevent these problems is to take advantage of the chaperone machinery.

The eukaryotic chaperonin TRiC/CCT is an essential chaperone that assists in the folding of nascent and non-native polypeptides and/or in the assembly of substrates with their respective partner in an ATP-dependent manner in the cytosol [39-41]. It has a unique double ring-like shape, each ring composed of eight different but homologous subunits of ~ 60 KDa, which constitutes a central cavity to provide a protected folding chamber for unfolded polypeptide substrates. TRiC/CCT plays a crucial role in maintaining cellular homeostasis by promoting cellular protein folding and/or protein complex assembly. As estimated from a previous study, approximately 9-15% of newly synthesized cellular proteins fold in a TRiC/CCT-assisted manner [42]. Furthermore, the proteomic and bioinformatic characterization of TRiC/CCT substrates demonstrates that the chaperonin TRiC/CCT binds a variety of polypeptides that participate in many cellular functions and is required for folding proteins with complex topologies that are slow folding and aggregation prone, such as those with a high β-strands tendency [43]. This report clearly identified Gβ as a TRiC/CCT folding substrate. Intriguingly, Gαt is also defined as a TRiC/CCT substrate. Using an *in vitro* transcription/translation system, it has been directly shown that the chaperonin TRiC/CCT binds newly synthesized $G\beta_1$, prevents its aggregation and assists in Gβ folding [30]. As an absolute partner, Gγ does not associate with the chaperonin TRiC/CCT, suggesting that TRiC/CCT is specifically involved in the folding of Gβ. Knockdown of the TRiC/CCT α subunit in mammalian cells by siRNA caused down-regulation of Gβγ [32]. Thus, the cytosolic chaperonin TRiC/CCT is required for Gβ folding and/or subsequent Gβγ assembly. Nonetheless the physiological significance of TRiC/CCT in vertebrate photoreceptors, which produce large amounts of transducin, has never been explored. Therefore, we are conducting a

project using rod photoreceptors as a model system to determine the functional significance of this chaperonin complex.

Light-driven translocation of transducin subunits in rod photoreceptors

In addition, the subcellular localization of transducin within the rod photoreceptors is also properly regulated during the dark/light cycle, in which most transducin is present in the outer segment (OS) of dark-adapted retinas, but bright light evokes transducin translocation from the outer segment (OS) to the other compartments of the rod photoreceptors, a phenomenon known as light-driven transducin translocation [44-47]. Such translocation is bidirectional, in which transducin will return to the OS in the dark after light exposure.

There are several hypotheses about the function of light-driven transducin translocation in rods [48, 49]. Transducin translocation was first proposed as a mechanism of photoreceptor light adaptation and this adaptive hypothesis was further supported experimentally [28, 44-46]. Due to light-driven transducin translocation, less transducin in the outer segment is available for activation by rhodopsin, which lowers rod light sensitivity. Such an adaptive response allows rods to function effectively during light adaptation. However, redistribution of rod transducin requires bright light levels that are saturating for rods and that exceed the range where rods can adapt. Therefore, another more appealing hypothetical function of transducin translocation is related to the mechanisms of photoreceptor survival and degeneration. Fain and Lisman have proposed that continuous light may cause photoreceptor cell death and degeneration by constitutively activating the visual signal transduction pathway [50-52]. This hypothesis was directly proven by Hao et al. [53], in which retinal degeneration, produced in arrestin knockout or rhodopsin kinase knockout mice by continuous illumination, was effectively

prevented by introducing a second mutation, knockout of the Gαt gene that blocked the rod transduction cascade. Therefore, transducin translocation, which reduces visual signaling during prolonged light exposure to bright light illumination, would be expected to provide a protective mechanism that photoreceptors use to defend themselves against light-induced cell damage.

The phosducin family proteins in rod photoreceptors: phosducin and phosducin-like protein 1

Phosducin (Pdc) and phosducin-like protein 1 (PhLP1) belong to the widely expressed phosducin family proteins [54]. Being the only other known binding partners for Gβγt in rod photoreceptors, besides Gαt, makes Pdc and PhLP1 more attractive for researchers to study the cell biology of G protein [13-20].

Phosducin is a 33KDa cytosolic phosphoprotein first identified in retinal extracts [55] and highly expressed in photoreceptor cells [13]. As a soluble, cytosolic protein, Pdc is present in all compartments of the rod cells [56, 57]. The most interesting feature of Pdc is its ability to form a specific complex with the βγ subunits of transducin in its dephosphorylated state [13, 17], and light modulates phosphorylation state of phosducin [58, 59].

On the other hand, PhLP1 was initially identified and isolated in neural cell cultures during a screen for ethanol-inducible genes [60]. This protein shares 41% identical and 65% similar amino acids with Pdc (figure 2). Unlike the restricted expression pattern of Pdc, PhLP1 is a protein with widespread expression in most tissues and cell types [60-62]. As indicated by quantitative Western blot analyses, PhLP1 was expressed in retina but 5~6 fold less than the level of Pdc [63]. More importantly, it has been shown that PhLP1 can bind Gβγ with high affinity, similar to Pdc [19, 20]. This finding was supported

by the analysis of homology between Pdc and PhLP1, in which an 11 amino acid sequence corresponding to helix 1 of Pdc, a major binding site for Gβγt, is highly conserved [17, 60]. Also, the interaction of PhLP1 with Gβγ was not regulated by phosphorylation [63, 64], which occurred constitutively and was not modulated by light.

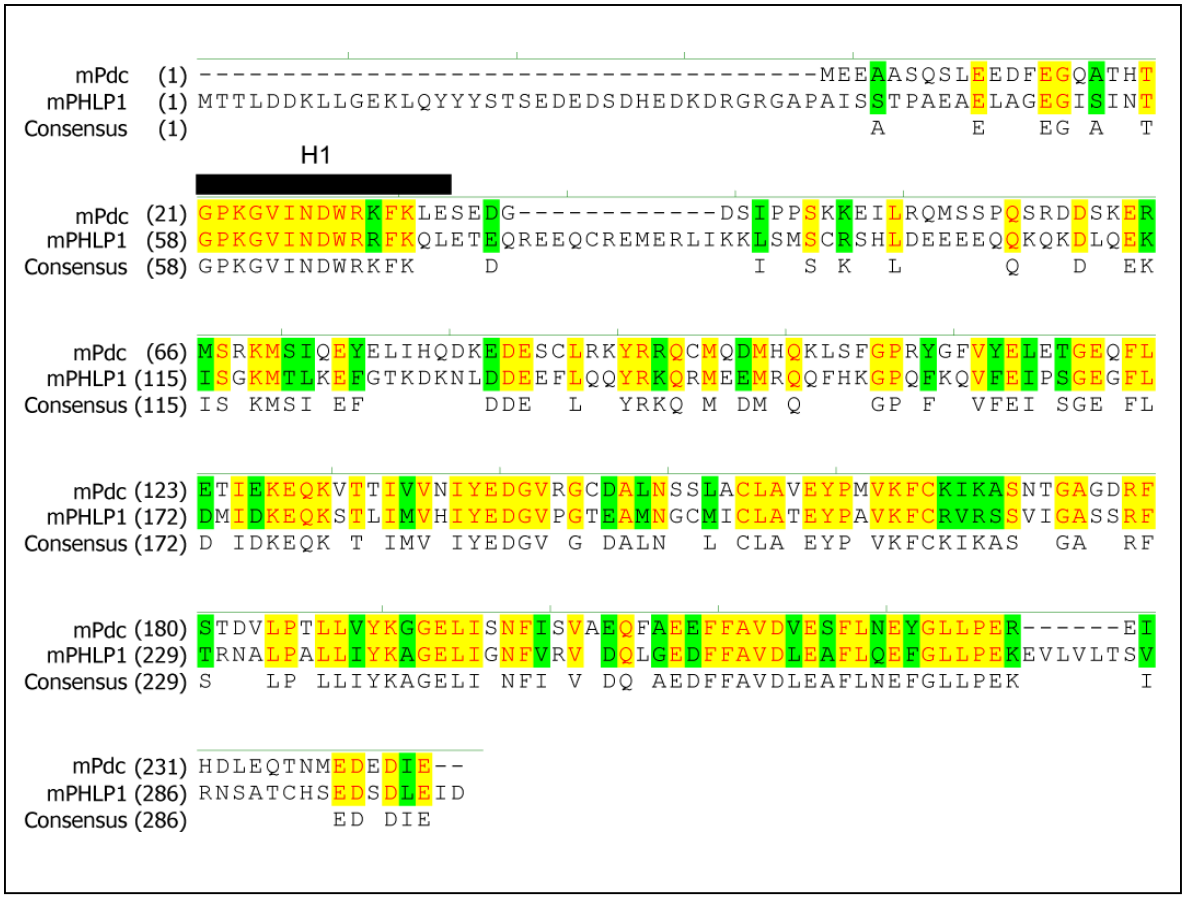

Figure 2. Sequence alignment of mouse phosducin (mPdc) and phosducin-like protein 1 (mPhLP1). The amino acid sequences of mPdc and mPhLP1 were aligned using Invitrogen Vector NTI Advance 10. Homology between sequences is marked by different colors (yellow – identical residue, green – similar residue). Gaps are indicated by dashes. Major binding sites of phosducin to transducin βγ subunits are represented above the sequence (black bar – H1: helix 1 of Pdc) (17).

Together, these observations point to divergent roles of Pdc and PhLP1 in the regulation of transducin in rods, although it cannot be excluded that their regulatory functions on transducin might be complemented with each other, given the similarities of structure and shared Gβγt binding property between Pdc and PhLP1.

Light-dependent phosphorylation of phosducin

Initially discovered as a major retinal phosphoprotein, phosphorylation of phosducin was thought to be an important regulatory mechanism for Pdc and its function. Extensive studies have been conducted to enhance our understanding on Pdc phosphorylation. Pdc phosphorylation is light-dependent, where it is phosphorylated in the dark and dephosphorylated in response to light [58, 59]. Pdc specifically interacts with the βγ subunits of transducin, and light-modulated phosphorylation of phosducin reduces its affinity towards Gβγt subunits [16, 65, 66]. Interestingly, it was found that Pdc in retinal degeneration 1 (rd1) retina was highly phosphorylated irrespective of light status, indicating that abnormal phosphorylation behaviors of Pdc were related to retinal degeneration [67]. Based on the analysis of Pdc amino acid sequence, it was found that Pdc contained consensus phosphorylation sites at serine 73 (according to the rat sequence: equivalent to serine 71 in the mouse sequence) and serine 54 for cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), respectively, which was further demonstrated *in vitro* and in isolated retina [59, 65-68]. It has also been shown that PP2A is a putative phosducin phosphatase [69]. However, no *in vivo* information about regulation of these sites, crucial for understanding the physiological function of phosducin in rod photoreceptors, was currently available, which prompted us to quantitatively analyze light-dependent phosphorylation of phosducin at serine 54 and serine 71 in the retinas of free-running animals.

Putative roles of phosducin in the regulation of rod transducin

Phosducin was originally proposed to negatively regulate rod visual signaling by sequestering transducin βγ subunits from transducin-mediated phototransduction, which decreases photoreceptor light sensitivity [14, 16, 70]. This hypothesis, however, was directly rejected by evidence from recent studies on the light adaptation property of phosducin knockout (Pdc-/-) rods [71]. The first clues for *in vivo* phosducin function were revealed by the analysis of Pdc-/- mice [56]. No developmental and morphological abnormalities were observed in the rods of Pdc-/- mice. The study of long-term light adaptation in Pdc-/- mice suggested that phosducin facilitated light-driven transducin translocation, which is thought to be involved in light adaptation and neuroprotection of photoreceptor cells by regulating the amount of transducin engaged in phototransduction and, thus, controlling rod responsiveness [28, 48, 49]. On the basis of previous studies, Pdc forms a specific complex with Gβγt subunits and controls its association with Gαt subunit in a light- and phosphorylation-dependent manner. Since the association of Gαt with Gβγt, which forms the transducin heterotrimer, is one of the key events required for their trafficking to the outer segment membranes, we are further investigating the molecular mechanisms by which Pdc regulates transducin trafficking to the outer segment membranes by determining the role of Pdc phosphorylation in this process using transgenic mice expressing Pdc lacking phosphorylation sites, serine 54 and 71.

Another important observation in the analysis of Pdc-/- mice was that deletion of the phosducin gene also resulted in significantly reduced protein levels of transducin β and γ subunits in rod photoreceptors [56, 71]. This phenotype appeared to be generally consistent with the several putative roles of Pdc in photoreceptors, as proposed by other researchers, in which Pdc may participate in the transcriptional [72-76] and posttranslational regulation of transducin expression [77, 78]. All these proposed

mechanisms are based on *in vitro* studies, our previous studies, however, showed that there were no significant differences in the mRNA levels of transducin α, β, and γ subunits between the retinas of wild-type and Pdc-/- mice (Belcastro, M, Song, H and Sokolov, M, unpublished data), suggesting that Pdc most likely participates in posttranslational regulation of transducin expression. The elucidation of such regulatory mechanisms is one of the major challenges of future investigations. It is noteworthy that abnormal expression of Pdc itself was observed in early diabetic rat retina, suggesting that Pdc might be involved in the pathogenesis of diabetic retinopathy [79].

New clues about PhLP1 function in the regulation of the folding and assembly of transducin βγ subunits

As a close homolog of Pdc, previous *in vitro* and overexpression studies proposed PhLP1 as an inhibitor for G protein signaling through $G\beta\gamma$ sequestration [18, 20, 80]. However, this initial hypothesis was later challenged by several *in vivo* data that indicated a positive regulatory role for PhLP1 in $G\beta\gamma$ function [54, 81, 82]. Particularly, disruption of the PhLP1 gene in the soil amoeba *dictyostelium* produced a phenotype similar to that in $G\beta$ null mutants. These previous studies also showed that lacking a functional $G\beta\gamma$ dimer resulted in defects in G protein signaling [82]. Furthermore, in mammalian cells depleted of PhLP1 by RNA interference, the level of $G\beta$ expression was significantly decreased without affecting $G\beta$ mRNA levels, and, as a result, G protein signaling was inhibited [83]. Using pulse-chase experiments, it was demonstrated that this inhibition upon PhLP1 depletion was caused by an inability to form the $G\beta\gamma$ dimer. Conversely, overexpression of PhLP1 increased the rate of

Gβγ assembly. In addition, PhLP1 was shown to associate only with nascent Gβ, but not Gγ [83].

Now, it is well-established that the function of PhLP1 in cell culture is to facilitate the formation of the Gβγ dimer by promoting the folding of Gβ. Clues into such an important function of PhLP1 stem from the identification of PhLP1 *in vivo* binding partners by proteomics in which a high affinity interaction of PhLP1 with the cytosolic chaperonin TRiC/CCT complex was determined [84]. More importantly, PhLP1 binds TRiC/CCT in its native conformation as a regulator rather than a folding substrate. This observation was further confirmed by the structure of the PhLP1-TRiC/CCT complex solved by cryo-electron microscopy [85]. Unlike folding substrates that bind TRiC/CCT within the central folding cavity, PhLP1 sat above the cavity through contacts with only the tips of the apical domains of the TRiC/CCT ring and thus effectively sealed the folding cavity. From this structure, it can be seen that binding of PhLP1 to TRiC/CCT occurs in a manner similar to prefoldin, a TRiC/CCT cochaperone that binds and delivers actin to TRiC/CCT for folding [86]. Indeed, as determined by co-immunoprecipitation, PhLP1 could form a ternery complex with TRiC/CCT and Gβ subunit that did not include Gγ [64]. Considering that the cytosolic chaperonin TRiC/CCT is required for Gβ folding and/or subsequent Gβγ assembly, these observations suggested that PhLP1 might act as a cofactor of the chaperonin TRiC/CCT for the folding and assembly of the Gβγ subunits by stabilizing an interaction between Gβ, Gγ and TRiC/CCT until Gβ reaches its native structure and then associates with Gγ to form the Gβγ dimer. However, such a CCT/PhLP1 function has never been tested in vertebrate photoreceptors, and its physiological significance in these cells remains largely unknown. For this end, we plan to investigate the role of CCT/PhLP1 in the regulation of transducin βγ expression within rod photoreceptors.

Research project objectives

In this research, we want to understand the role of phosducin (Pdc) and phosducin-like protein 1 (PhLP1) in the biosynthesis and trafficking of the rod visual G protein, transducin, in vertebrate photoreceptors. For this purpose, we tested two specific hypotheses.

1) We proposed that Pdc is engaged in the trafficking of transducin to the outer segments (OS) of photoreceptors, according to the following mechanism illustrated in Figure 3. Pdc binds to free Gβγt with high affinity in its dephosphorylated state [13, 16, 65, 66], and by doing so prevents the association of Gαt with Gβγt [17]. Association of Gαt and Gβγt is required for targeting of the transducin heterotrimer to the outer segment membrane [4, 49]. Therefore, in order to associate with Gαt to form functional, membrane-bound heterotrimeric transducin, Gβγt must first dissociate from Pdc. This dissociation is triggered by phosphorylation of Pdc *in vitro* and in isolated retinas [13, 16, 65, 66]. To test this hypothesis on the role of Pdc in transducin trafficking in photoreceptors, in Chapter 2, we first characterized Pdc phosphorylation *in vivo* in Part I and then directly tested our hypothesis using transgenic mice expressing Pdc without principle phosphorylation sites in Part II.

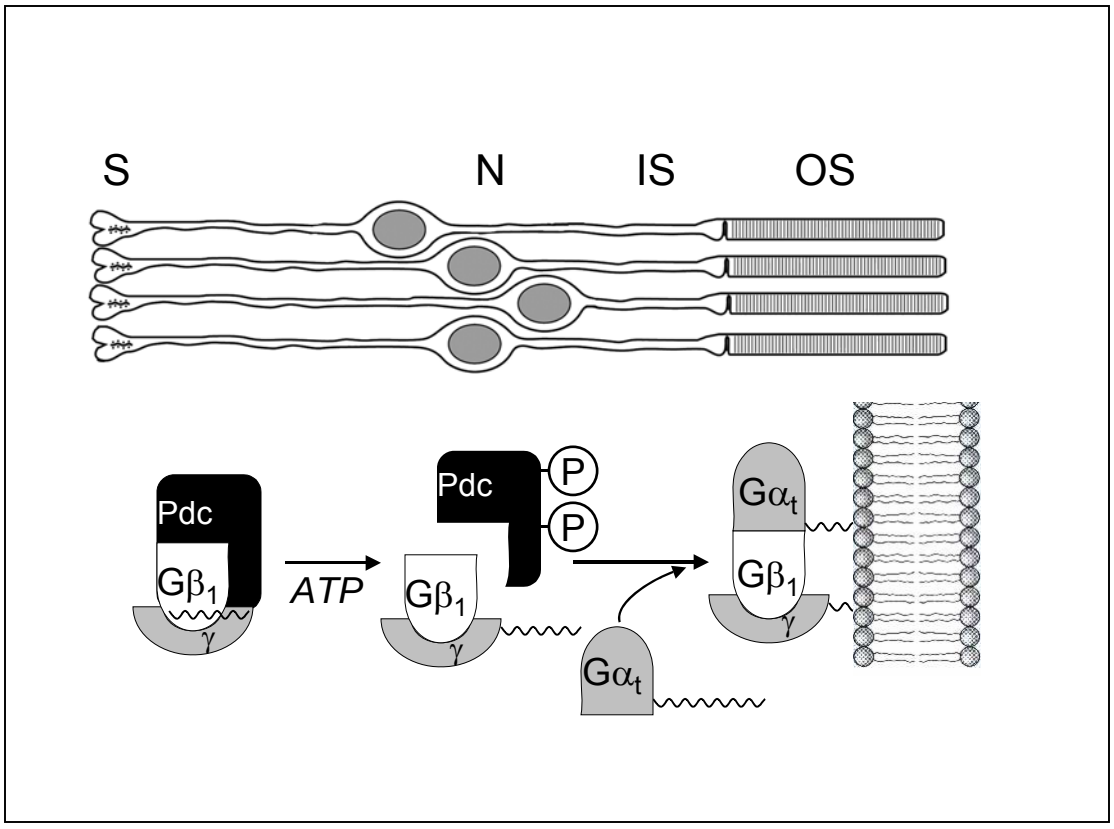

Figure 3. Model of the role of phosducin (Pdc) in transducin ($G\alpha\beta\gamma t$) trafficking to the outer segments of rod photoreceptors (discussed in the text). Photoreceptor layers are OS-outer segments, IS-inner segments, N-nucleus, S-synase

2) We hypothesized that the chaperone CCT/PhLP1 complex plays a central role in the folding and assembly of transducin βγ subunits in photoreceptors. This hypothesis was based on previous studies, which demonstrated that PhLP1, in cell culture and several organisms, acts as a cofactor of the chaperonin CCT complex that is specifically required for the folding and assembly of Gβγ subunits [32, 54, 64, 81, 83]. The proposed model of PhLP1 function in the folding of Gβt is shown in Figure 4. Nascent Gβt binds to the folding chamber within the CCT and then PhLP1 binds the CCT/Gβt complex, forming a ternary complex. PhLP1 binding facilitates the release of folded Gβt from CCT, which is further stabilized by dimerization with Gγt. Thus, in chapter 3, we set out to explore the role of CCT/PhLP1 in rod photoreceptors using transgenic mouse models, in which endogenous CCT/PhLP1 activity is suppressed in photoreceptors.

Our data provide new insights not only into the roles of Pdc and PhLP1 in the regulation of transducin homeostasis in vertebrate photoreceptors, but also into novel pathways involved in retinal degeneration, which will assist in designing new therapies.

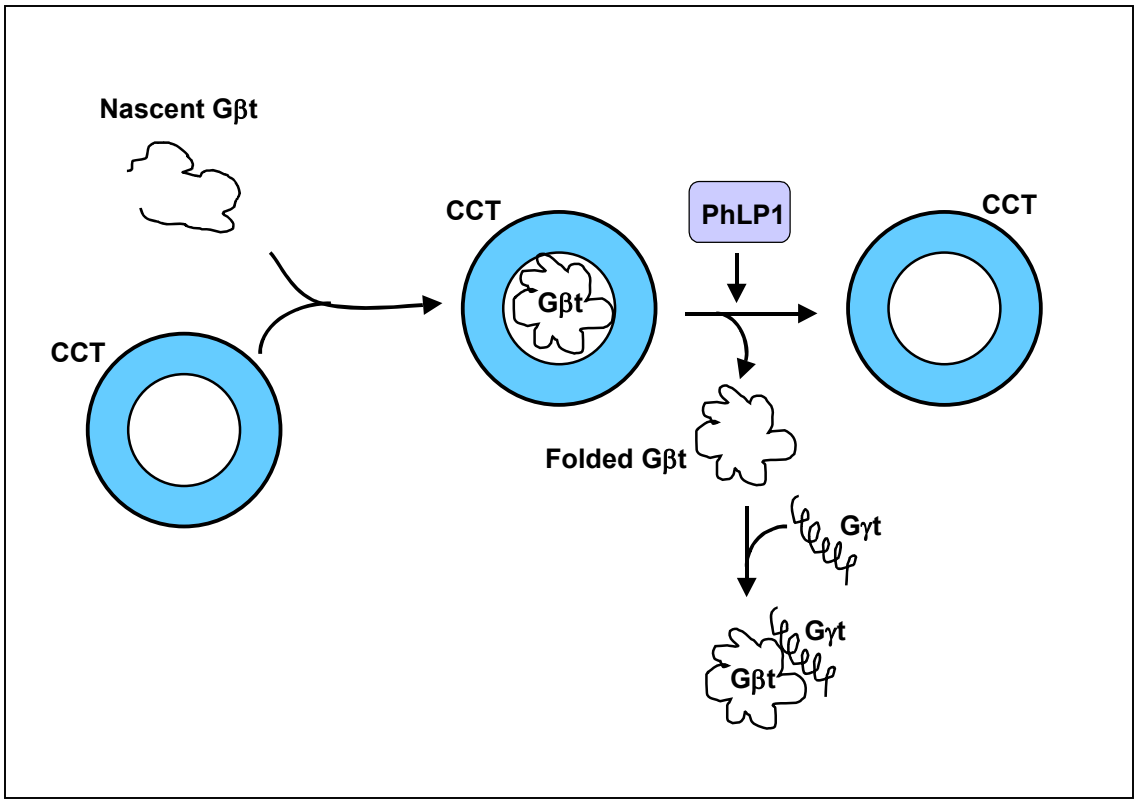

Figure 4. Proposed model of phosducin-like protein 1 (PhLP1) function in the folding and assembly of transducin βγ subunits (Gβγt) (refer to the text). CCT, chaperonin containing TCP1 complex.

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Chapter 2

In vivo Characterization and Physiological Function of Phosducin Phosphorylation in Rod Photoreceptors

Preface

Phosducin (Pdc) is one of the most abundant proteins in vertebrate photoreceptors. It was primarily identified as a major retinal phosphoprotein that undergoes a cycle of light-dependent phosphorylation. More intriguingly, Pdc specifically interacts with free G protein $\beta\gamma$ subunits, and this interaction is modulated by Pdc phosphorylation. Thus, elucidation of the nature of Pdc phosphorylation is critical in identifying and understanding the physiological function of Pdc in regulating G protein-mediated signaling. Previous studies identified serine 54 and 71 as principal light-modulated phosphorylation sites of Pdc, however regulation of these sites has never been studied *in vivo.* Therefore, we generated phospho-specific antibodies against serine 54 and 71 of Pdc, and analyzed the phosphorylation status of these sites under various physiologically relevant conditions of illumination in free-running mice and rats. Our data, described in Part I, allowed us to hypothesize that Pdc is engaged in the process of subcellular trafficking of transducin. In Part II, we have directly tested this hypothesis using transgenic mice expressing Pdc lacking serine 54 and 71 phosphorylation sites. Our results provide important clues on the role of Pdc in the regulation of G protein-mediated signaling in rod photoreceptors.

Part I

Compartment-specific Phosphorylation of Phosducin in Rods Underlies Adaptation to Various Levels of Illumination

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The co-author Marycharmain Belcastro was responsible for the generation and testing phospho-specific antibodies. E.J. Young participated in the experiment shown in Figure 2C.

COMPARTMENT-SPECIFIC PHOSPHORYLATION OF PHOSDUCIN IN RODS UNDERLIES ADAPTATION TO VARIOUS LEVELS OF ILLUMINATION

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Phosducin is a major phosphoprotein of rod photoreceptors that interacts with the Gβγ subunits of heterotrimeric G proteins in its dephosphorylated state. Light promotes dephosphorylation of phosducin, thus it was proposed that phosducin plays a role in the light adaptation of G protein-mediated visual signaling. Different functions, such as regulation of protein levels and subcellular localization of heterotrimeric G proteins, transcriptional regulation, and modulation of synaptic transmission have also been proposed. Although the molecular basis of phosducin interaction with G proteins is well understood, the physiological significance of light-dependent phosphorylation of phosducin remains largely hypothetical. In this study, we quantitatively analyzed light dependence, time course and subcellular localization of two principal light-regulated phosphorylation sites of phosducin, serine 54 and 71. To obtain physiologically relevant data, our experimental model exploited free running mice and rats subjected to controlled illumination. We found that in the dark adapted rods, phosducin phosphorylated at serine 54 is compartmentalized predominantly in the ellipsoid and outer segment compartments. In contrast, phosducin phosphorylated at serine 71 is present in all cellular compartments. The degree of phosducin phosphorylation in the dark appeared to be less than 40%. Dim light within rod operational range triggers massive reversible dephosphorylation of both sites, whereas saturating light dramatically increases phosphorylation of serine 71 in rod outer segment. These results support the role of phosducin in regulating signaling in the rod outer segment compartment and suggest distinct functions for phosphorylation sites 54 and 71.

Phosducin (Pdc) was originally identified in the retina as an abundant 33-kDa cytosolic phosphoprotein phosphorylated in the dark and dephosphorylated in the light (1). Within the retina, Pdc is expressed in both rod and cone photoreceptors (2,3). The most distinguished feature of Pdc is its ability to form a specific complex with the βγ subunits of visual heterotrimeric G protein, transducin (4,5), and other heterotrimeric G proteins (6-8). Affinity of Pdc towards Gβγ is down-regulated by multiple phosphorylation and probably consequent binding of 14-3-3 protein (9,10). Although the identity of Pdc kinase and phosphatase in photoreceptors remains unknown, the analysis of Pdc phosphorylation *in vitro* and *ex vivo* revealed that Pdc possesses multiple cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation sites (10-12) and identified protein phosphatase (PP2A) as the putative Pdc phosphatase (13).

Despite the obvious progress in understanding the molecular basis of Pdc/Gβγ interactions, the role of Pdc and its light-dependent phosphorylation in photoreceptors is poorly understood. Originally it was proposed that, upon activation by light, Pdc scavenges transducin βγ subunits

from phototransduction and by doing so reduces photoreceptor light sensitivity (14-16). This hypothesis, however, is yet to be substantiated by physiological data. The first clues into *in vivo* Pdc functions were obtained from the analysis of Pdc-null mice (17). The analysis of long term light adaptation in this mutant revealed that Pdc significantly increases light-driven transducin translocation, another adaptive mechanism regulating the amount of transducin engaged in phototransduction (for a recent review, see Ref. (18)). It was also noticed that the deletion of Pdc gene reduced the level of transducin β subunit by 30%.

To assess the individual contributions of two known light-regulated phosphorylation sites of Pdc, serine 54 and serine 71 (11,19), to the regulation of transducin translocation, we quantitatively analyzed their status and subcellular localization in free running mice under physiologically relevant levels of illumination. We found that Pdc phosphorylated at serine 54 is present predominantly in the ellipsoid and rod outer segment, whereas phosphorylation of serine 71 occurs throughout the entire rod. We also found that rapid and reversible dephosphorylation of both sites was triggered by a very dim light below the threshold of transducin translocation. Prolong exposure to moderate ambient light initiated light-driven transducin translocation and also caused massive phosphorylation of serine 71 in the rod outer segments. Our results provide evidence for Pdc playing potential roles in both light adaptation and light protection of rod photoreceptors and point to the distinct functions of phosphorylation sites serine 54 and 71.

EXPERIMENTAL PROCEDURES

Antibodies − The phospho-specific antibodies against Pdc were generated by 21st Century Biochemicals (Marlboro, MA) as follows. Peptides corresponding to amino acids 50-59 and 67-77 of mouse Pdc containing phosphoserine at positions 54 and 71 (peptide sequences LRQMpSSPQSR (pS, Ser(P)-54) and SRKMpSIQEYEL (Ser(P)-71)) were synthesized and used to immunize rabbits according to the standard procedure. Phospho-specific antibodies against Pdc, designated as Pdc54p and Pdc71p, were affinity-purified from the immune serum using immobilized specific peptides. To eliminate binding to dephosphorylated Pdc, each antibody was depleted against LRQMSSPQSR and SRKMSIQEYEL peptides. Antibody against full length Pdc was described previously (17). Antibody against rod transducin α subunit was sc-389 from Santa Cruz Biotechnology. Antibody against subunit I of cytochrome c oxidase, COX I, was MS404 from MitoSciences. Monoclonal 4D2 antibody against rhodopsin was a gift from Dr. Robert S. Molday, (University of British Columbia).

Light Conditioning of Animals − All experiments involving animals were performed according to the procedures approved by the West Virginia University Animal Care and Use Committee. Wild type pigmented Long Evans rats and 129SV mice were purchased from Charles River Laboratories. Pdc knockout mice described previously (17) were back-crossed into 129SV background for three generations. Animals were maintained in standard cage rooms with cyclic light (12 h light/12 h dark) until used. Before all experiments animals were dark adapted overnight. Light conditioning of mice was performed in a 23 x 15 x 20-cm white box illuminated from a white diffuser embedded into the lid and connected to the light guide of an adjustable light source ACE I (Schott). Light conditioning of rats was carried out in 40 x 50 x 30-cm white box evenly illuminated from the top by the bench light source. The levels of illumination in the box were measured using a Traceable light meter (Fisher Scientific) (units: photopic lux) and a calibrated photodiode attached to a PDA-750 amplifier (Terahertz Technologies) (units: microamperes, μA). The light collecting surface of the photodiode was covered by a blue glass filter (BG 39, Newport Franklin Inc., Franklin, MA) with a spectral sensitivity closely matching

that of rhodopsin. The luminance of the walls was calculated, assuming the white box to be a cube, according to the formula 1 cd/m^2= 2π/3 lux. At the end of the experiment animals were euthanized by flashing the box with CO_2 followed by cervical dislocation, and their eyes were harvested and either frozen on the dry ice or dissected to obtain the retinas.

The rate of rhodopsin activation in the photoreceptors during light conditioning in the box was determined experimentally. Dark adapted 129SV mice were anesthetized to reduce rhodopsin regeneration (20), and their corneas were protected by applying the methylcellulose ophthalmic lubricant, Murocel (Bausch and Lomb). Mice were exposed to 10^3 lux (240 scotopic cd/m^2) light in the center of the box for various durations of time. After the exposure, mice were sacrificed, their eyes were harvested and frozen on dry ice. The rhodopsin content of the eyes was determined as described below. We found that during the first 15 min of exposure the bleaching of rhodopsin could be approximated as a linear process with a rate of 0.0072% sec^{-1}. Assuming that the rod cell has 7 X 10^7 molecules of rhodopsin (21), this rate corresponds to activation of 5 x 10^4 rhodopsin rod^{-1} sec^{-1}. The rates of rhodopsin activation under all other levels of illumination were calculated from this value.

Determination of the Levels of Rhodopsin − A protocol modified from Sokolov *et al*. (22) was used as follows. All procedures were carried out under dim red light. Two retinas were harvested from a dark adapted mouse and homogenized in 0.6 ml of water containing 2.5% n-octyl β-D-glucopyranoside (O3757, Sigma) and 1.25% cetyltrimethylammonium chloride (292737, Sigma) by short ultrasonic pulses delivered from a Microson ultrasonic cell disruptor equipped with a 3 mm probe (Misonix, Farmingdale, NY). When required, 5 μM microcystine LR (Sigma) and 50 mM sodium EDTA were added to inhibit protein phosphatase and kinase activities in the extracts. The extract was cleared by centrifugation on a Centrifuge 5415 D (Eppendorf) at 16,000 rpm for 3 minutes. The supernatant was divided into two equal aliquots; one was kept in the dark, and the second one was exposed to bright light for 1 minute to bleach all rhodopsin. The absorption 400-700 nm spectrum of the dark aliquot was then obtained in a UV-Mini 1240 spectrophotometer (Shimadzu) using the bleached aliquot as a baseline. Rhodopsin concentration was determined from a 500 nm peak using a molar extinction coefficient of 40,500 (23). When required, the same protocol was used to determine rhodopsin content of the whole eye.

Determination of the Levels of Pdc − The amounts of Pdc in the retinas were determined by quantitative Western blotting with a standard curve according to the protocol modified from Sokolov *et al*. (17) as follows. The retinas were harvested from dark adapted 129SV mice, and the whole retina extracts containing known amount of rhodopsin were prepared as described above. The extracts were mixed with SDS PAGE sample buffer and analyzed by Western blotting together with the standard curve comprised of various amounts of purified recombinant Pdc added to the Pdc-null retinal extracts containing the same amount of rhodopsin as the analyzed samples. Phospho-Pdc standards were generated by *in vitro* phosphorylation with an excess of either Ca^{2+}/calmodulin-dependent protein kinase II or cAMP-dependent protein kinase , which was previously reported to cause virtually complete phosphorylation of serine 54 and serine 71, respectively (19). Serine 54-phosphorylated Pdc standard was obtained by incubating 100 pmol of recombinant Pdc with 500 units of Ca^{2+}/calmodulin-dependent protein kinase II (P6060S, New England Biolabs) using buffers, reagents and protocol provided by the manufacturer. Serine 71-phosphorylated Pdc standard was obtained by incubating 400 pmol of Pdc with 100 units of bovine PKA catalytic subunit (Sigma) in phosphate-buffered saline buffer, pH 6.5, containing 2.5 mM ATP, 10 mM $MgCl_2$, for 1 h at 30ºC. Blots were probed with the pan- and phospho-specific antibodies against Pdc, and the amounts of total- and phosphorylated Pdc

in the mouse retinas were calculated from the standard curves and presented as molar ratios with rhodopsin.

Pdc phosphorylation assays − The eyes were harvested from dark adapted and light-conditioned 129SV mice of the same age and frozen on dry ice. An eye was homogenized in 0.2 ml of buffer containing 125 mM Tris/HCl, pH 6.8, 4% SDS, 6 M Urea, and 10 mg/ml dithiothreitol by short ultrasonic pulses resulting in complete disintegration of the eye tissue. The extract was cleared by centrifugation. 15 μl aliquots were separated on 18-well 10% Tris-HCl gels (Bio Rad), transferred to polyvinylidene difluoride membrane Immobilon FL (Millipore), and probed with phospho-specific Pdc54p and Pdc71p antibodies. Total Pdc was determined after diluting original extracts 100 times. Fluorescence values of phosphorylated Pdc bands were divided by those of total Pdc bands, and then the amounts of phosphorylated Pdc in the light-adapted samples were normalized to those in the dark-adapted samples on the same gel. The time course data were fit to a first order rate equation, $\ln[A] = -kt + \ln[A_0]$ (exponential rise to maximum, two parameters), where [A] and $[A_0]$ are the amounts of phosphorylated Pdc at times t and 0 min, respectively, and k is the first order rate constant, using Sigma Plot software. Half-life value ($t_{1/2}$) was calculated from the rate constant k by the equation $t_{1/2} = \ln(2)/k$.

Immunoprecipitation − Two mouse retinas were homogenized into 0.4 ml of radioimmune precipitation assay buffer (R0278, Sigma) containing 10 mM sodium EDTA, 5 μM microcystine LR (Sigma), 5 μM okadaic acid (Sigma) and protease inhibitor mixture (#539131, Calbiochem) by several short ultrasonic pulses. Homogenates were cleared by centrifugation and incubated with 10 μl of protein G-Sepharose beads (Pierce) and 20 μg of Pdc71p antibody for 1 h at room temperature with gentle rocking. Beads were separated from supernatant by centrifugation and washed three times with 1.0 ml of RIPA buffer, and then bound Pdc was eluted from the beads with 0.1 ml of 0.5% trifluoroacetic acid and vacuum-dried in a Vacufuge (Eppendorf). The supernatant was mixed with 9 volumes of 10% trichloroacetic acid, 50% acetone, incubated on ice for 30 min. Precipitates were collected by centrifugation, washed 3 times with 1.0 ml of cold acetone, and vacuum-dried. Both bound and unbound fractions were resuspended in 50 μl of SDS PAGE sample buffer containing 6 M urea, 125 mM Tris-HCl, pH 6.8, 4% SDS, bromophenol blue tracking dye, and 10 mg/ml dithiothreitol for the subsequent Western blot analysis.

Serial Tangential Sections of the Retina − Tangential sectioning of the rat retina was carried out as previously described (17) with several optimizations. Eyes were dissected under a stereo microscope in HEPES-Ringer's solution containing 130 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.02 mM EDTA, 10 mM HEPES-NaOH (pH 7.4), osmolarity adjusted to 313 mosM. When necessary all tissue manipulations were conducted in the dark under a Stemi 2000-C stereomicroscope (Zeiss) equipped with OWL Gen 3+ intensifiers (B.E. Meyers and Co. Inc) and an infrared light source. The anterior portion of the eye was cut away and the lens was removed. The eye cup with the retina attached to it was cut into four pieces of the same size. Each piece was transferred to the flattening chamber filled with HEPES-Ringer, where the retina was gently pulled away from the eyecup and mounted photoreceptors up onto a supporting polyvinylidene difluoride membrane, which was positioned on top of a flat glass capillary array (GCA 09/32/25/0/20 LM, BURLE Electro-Optics, Sturbridge, MA). The retina was flattened by first applying a suction force from underneath the filter slowly removing all solution from the flattening chamber and then by clamping the retina on the supporting membrane between two glass slides separated by 0.5 mm spacers. The assembly was frozen on dry ice. To align the retina with the cutting plane of the cryostat blade, optimal cutting temperature compound was allowed to freeze at −20ºC on the specimen holder and then sectioned through to create a flat surface large enough to accommodate the glass slide. The clamps, the cover glass and the

spacers were removed; the base slide with the retina on the supporting membrane attached to it was gently pressed against the optimal cutting temperature surface and secured by addition of water drops to the sides of the glass base. The retina and its supporting membrane were generously trimmed around the perimeter to remove uneven and folded parts and 5-μm-sectioned. When the first sizeable section was cut out, all other parts of the retina not included in this section were trimmed away, and then sectioning was resumed. Each section was collected and thawed in 50 μl of SDS PAGE sample buffer containing 6 M urea, 125 mM Tris-HCl, pH 6.8, 4% SDS, bromophenol blue tracking dye, and 10 mg/ml dithiothreitol. To assess the quality of the sectioning, 0.5 μl aliquots were applied on dry nitrocellulose membrane and the dot blots were probed with anti-rhodopsin antibody. Only the sets containing rhodopsin in the upper 4-6 sections were selected and stored at −80ºC until analyzed.

15 μl aliquots were analyzed by Western blotting using a Criterion Cell and Blotter system and 26-well 10% Tris-HCl gels (Bio Rad). Polyvinylidene difluoride membrane Immobilon FL (Millipore) was used. For the detection of rhodopsin, the original extracts were diluted 100 times. For the detection of Pdc and transducin subunits, the original extracts were diluted 25 times.

The profiles of protein distribution in rods were obtained as previously described (22). In brief, fluorescence of a specific band in each section was plotted as a percentage of combined fluorescence in all sections. To determine the degree of Pdc phosphorylation in different rod compartments, the fluorescence value of the phosphorylated Pdc band in each section was divided by that of total Pdc. The experiments were repeated five times with dark adapted animals and four times with light adapted animals.

Western blotting − Quantification of the specific bands was performed on an Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's protocols and using specific primary antibodies and anti-rabbit, anti-sheep and anti-mouse secondary antibodies conjugated to either Alexa Fluor 680 (Invitrogen) or IRDye 800 (LI-COR Biosciences).

RESULTS

Properties of phospho-specific antibodies against Pdc − Phospho-specific antibodies against two known light-regulated phosphorylation sites of Pdc, serine 54 and 73 (11,19), were generated and affinity-purified. The antibodies were designated as Pdc54p and Pdc71p, according to the mouse Pdc sequence. The ability of the phospho-specific antibodies to discriminate phosphorylated and dephosphorylated Pdc is illustrated in Fig. 1. In this experiment, recombinant rat Pdc was phosphorylated *in vitro* with either Ca^{2+}/calmodulin-dependent protein kinase II (Fig. 1, *upper blot*) or cAMP-dependent protein kinase (Fig 1, *lower blot*) to introduce phosphate in position 54 or 71, respectively. This approach was previously validated using mass spectrometry (19). Non-phosphorylated Pdc was used as a control. As evident from the data in Figure 1, each antibody recognizes only phosphorylated Pdc and was blocked by the phosphopeptide corresponding to its immunization antigen but not by the other phosphopeptide. Consistent with a previous report (19), each Ca^{2+}/calmodulin-dependent protein kinase II or cAMP-dependent protein kinase in vitro phosphorylates Pdc at multiple sites, resulting in the appearance of several bands on an SDS-PAGE. We utilized phospho-specific Pdc54p and Pdc71p antibodies to detect site-specific Pdc phosphorylation by Western blotting.

Phosphorylation of Pdc in the dark adapted rods − It is well documented that in the retinas of dark-adapted animals Pdc undergoes phosphorylation; however the degree of Pdc

phosphorylation is unknown. To obtain this information, which is crucial for the understanding of Pdc function, we determined the amounts of serine 54- and serine 71-phosphorylated Pdc in the retinas of dark adapted 129SV mice using three different experimental approaches.

In the first approach the amounts of phosphorylated Pdc in the retina were determined by quantitative Western blotting with a calibration curve (Fig. 2 A). The retinas were harvested under dim red light, and then the SDS extracts of the whole retina containing known amounts of rhodopsin were prepared as described under "Experimental Procedures". To quantify the total amount of Pdc, three different extracts were analyzed together with the calibration curve comprised of various known amounts of recombinant Pdc (Fig. 2A, left panel). To create an identical environment in the extracts and calibration curve, which may potentially affect protein transfer from gel to membrane and exposure to antibody, Pdc standards were premixed with the whole retinal extract of Pdc knockout mice. We found that on average dark-adapted mouse retina contains 26±0.6 molecules of Pdc per 100 molecules of rhodopsin. This number represents the protein level of Pdc in rods, because Pdc expression in the retina is limited to photoreceptors, and cones comprise only 3% of the total photoreceptor pool in the mouse retina (24). Next, we determined the amounts of Pdc phosphorylated at serine 54 and 71. In these assays Pdc standards were phosphorylated *in vitro* by either Ca^{2+}/calmodulin-dependent protein kinase II at serine 54, or cAMP-dependent protein kinase at serine 71, and specific bands were visualized with phospho-specific Pdc54p and Pdc71p antibodies. On average 1±0.03 molecules of serine 54-phosphorylated Pdc and 9±3 molecules of serine 71-phosphorylated Pdc per 100 molecules of rhodopsin were detected in the extracts, respectively. Thus, 4±0.1% and 35±12% of total Pdc in dark-adapted rods appeared to be phosphorylated at serine 54 and serine 71, respectively.

The second approach was to immuno-precipitate all phosphorylated Pdc in the retinal extracts with an excess of immobilized phospho-specific antibody and then to determine the degree of Pdc phosphorylation by comparing the amounts of Pdc in the bound, phosphorylated, and unbound, dephosphorylated fractions. As illustrated in Fig. 2B, after immunoprecipitating almost all serine 71-phosphorylated Pdc in the extract with Pdc71p antibody (upper blot), we found that it comprised only 44±4% of the total amount of Pdc in the retina (lower blot). Unfortunately, we could not estimate the amounts of serine 54-phosphorylated Pdc by this approach due to the poor performance of Pdc54p antibodies in the immunoprecipitation assay.

The third approach was to determine whether or not additional phosphorylation of Pdc in the dark could be achieved by suppressing the activity of protein phosphatase pharmacologically. We reasoned that this would allow endogenous protein kinase to phosphorylate all available Pdc in the retina, thus provide the estimation of the highest achievable level of Pdc phosphorylation in rods. As shown in Fig. 2C, incubation of freshly obtained dark-adapted mouse retinas with phosphatase inhibitors microcystine LR and okadaic acid resulted in the robust increase of the levels of phosphorylated Pdc, providing yet another estimation that on average only 11±4 and 37±10% of total Pdc are phosphorylated at serine 54 and 71, respectively.

Combined these data demonstrate that even during prolonged overnight dark adaptation, only a small 4-10% fraction of total Pdc in the rods of living animals becomes phosphorylated at serine 54, whereas approximately 40% of total Pdc becomes phosphorylated at serine 71.

Light dependence of Pdc phosphorylation − To get a better understanding of the role of Pdc phosphorylation in rod visual function, we monitored the phosphorylation status of serine 54 and serine 71 under various levels of illumination. Seeking physiologically relevant data, we exposed

non-anesthetized pigmented mice to the controlled steady illumination in a white box. The levels of rhodopsin activation in the retinas under different levels of illumination in the box were determined experimentally, as described in “Experimental Procedures”. After 10 min of light conditioning, mice were sacrificed, and their eyes were promptly frozen on the dry ice. The whole procedure of enucleation and freezing was completed within a minute and was easy to reproduce. The levels of serine 54 and 71 phosphorylation were determined in the whole eye extracts by Western blotting, after homogenizing the entire eye in SDS-containing buffer (Fig. 3). We found that exposure of dark adapted mice to a very dim 0.08 lux background light resulted in a reproducible increase in the level of phosphorylation of each site, and was insufficient to trigger dephosphorylation. Increasing the illuminance level to a modest 0.8 lux triggered dephosphorylation of serine 54 and serine 71 by more than 80%. Further increasing background illumination to 8, 10^3 and 10^4 lux illuminance essentially eliminated phosphorylation of serine 54. In contrast, a dramatic increase in the level of serine 71 phosphorylation was observed during exposure to 10^4 lux light. Different from the dark, phosphorylation of serine 71 in the light was accompanied by a shift of Pdc electrophoretic mobility and the appearance of slower running bands, similar to those observed in *in vitro* phosphorylated Pdc (Fig. 1), suggesting the presence of additional phosphorylation sites. These results demonstrate that phosphorylation sites serine 54 and serine 71 of Pdc are regulated differently by light.

Time course of Pdc phosphorylation − Using a similar experimental approach we determined the time course of Pdc phosphorylation. A group of dark adapted mice was exposed to 8 lux constant light, which saturates Pdc dephosphorylation (Fig. 3), and then placed in the dark again. Mice were sacrificed at different time points in the experiment, and the levels of serine 54 and 71 phosphorylation were determined in the whole eye extracts by Western blotting (Fig. 4A) and compared with the dark-adapted levels (Fig. 4B). We found that the levels of serine 54 and serine 71 phosphorylation were reduced by ~ 50% after 1 minute of light exposure (Fig. 4, compare time points −10 min and −9 min). After 10 min of the exposure, dephosphorylation of ~ 97% of serine 54 and ~90% of serine 71 was completed (Figure 4, time point 0 min). The process was rapidly reversed in the dark and appeared to be biphasic. When approximated as a first order reaction, the initial phase of serine 54 phosphorylation had a rate constant of 0.44±0.04 s^{-1} and a $t_{1/2}$ value of ~57 sec. Respective parameters of serine 71 phosphorylation were found to be 0.72±0.13 s^{-1} and ~94 sec, respectively. The complete recovery of the dark levels of serine 54 and 71 phosphorylation on average occurred after 20 min of dark adaptation. Thus, light-dependent Pdc phosphorylation appeared to be a moderately fast reversible process operating in the minutes time scale.

Compartmentalization of phosphorylated Pdc in rod photoreceptors − In our previous studies we successfully utilized an experimental approach combining serial tangential sectioning of the flat mounted frozen retina with the analysis of specific protein content in individual sections (22). This technique takes advantage of the aligned, layered structure of the retina, with the major cellular compartments of the rod present in parallel layers (Fig 5A). Combined with Western blot detection, this technique allowed us to describe protein localization within rod photoreceptors quantitatively. We have applied this technique for determining compartmentalization of Pdc phosphorylated at serine 54 or serine 71 under different conditions of illumination. Retinas were obtained from either dark-adapted rats or rats that were allowed to run in moderately bright ambient 10^3 lux light for 1 h. The retinas were flat-mounted, frozen, and serially sectioned. Each set of serial sections was then analyzed by Western blotting (Fig. 5, B and D). The rod outer segments were identified using antibodies against rhodopsin confined in this cellular compartment. A mitochondrial marker subunit I of cytochrome c oxidase was used to highlight the ellipsoid, a subcellular compartment of the inner segment, densely packed with mitochondria and adjacent to the outer segment. Total Pdc and phosphorylated Pdc were determined using

pan- and phospho-specific antibodies and expressed as a ratio of phosphorylated Pdc to total Pdc in each section (Fig. 5, C and E). We found that site-specific phosphorylation of Pdc in rod photoreceptors is strongly compartmentalized. As shown in Fig. 5B, in the retinas of dark-adapted animals, Pdc phosphorylated at serine 54 was specifically enriched in the ellipsoid of the inner segment, and from there it appeared to spread into the outer segment, but it was virtually undetectable in the sections corresponding to rod nuclei and synaptic termini. Under these conditions, Pdc phosphorylated at serine 71 was readily detectable in all cellular compartments of rods, with some enrichment also observed in the inner segments. When rat was exposed to a moderate 10^3 lux light for 1 h (Fig. 5D), phosphorylation of residue 54 was efficiently eliminated in all cellular compartments, whereas significant amounts of Pdc phosphorylated at serine 71, apparently exceeding those in the dark adapted retinas, appeared in the rod outer segments. The light-induced phosphorylation of serine 71 in the outer segment was accompanied by the appearance of multiple bands, indicating that a 1-h exposure to 10^3 lux light has the same effect on Pdc phosphorylation as a shorter 10-min exposure to 10^4 lux light (Fig. 3), which was further confirmed experimentally (data not shown). The gradient distribution of serine 71-phosphorylated Pdc within rod outer segment, as in Fig. 5D, was not common for all preparations and, therefore, not discussed.

To determine whether compartment-specific phosphorylation of Pdc coincides with its redistribution within rods, we compared the fractions of Pdc present in the outer segment compartments in dark and light. A similar analysis was previously utilized to study light-driven transducin translocation in rods (22). In brief, the specific fluorescence in each section was expressed as a percent fraction of the combined fluorescence in all sections. Then the fraction of the Pdc confined in sections containing rhodopsin and free from subunit I of cytochrome c oxidase was calculated and normalized to the fraction of rhodopsin present in these sections. For example, in the experiment shown in Fig. 5B, sections 1-5 contain 81% of rhodopsin, 35% of Pdc and are essentially free from subunit I of cytochrome c oxidase. Thus, the fraction of Pdc in the outer segment, calculated as 35/85 x 100, is equal to 43%. Our analysis of five dark-adapted and four light-conditioned rats revealed that similar fractions of Pdc, 44±9 and 39±8% (S.E., *t* test $p > 0.7$), respectively, are present in the outer segments of rods under these conditions, which suggests that, contrary to the early report (25) and in good agreement with the most recent reports (17,26) no significant translocation of Pdc from the outer segment has occurred as a result of prolong light conditioning.

Using the same analysis, we monitored light-driven translocation of rod transducin. During prolonged dark adaptation transducin is accumulated in the membranes of the rod outer segment. For example, in the experiment shown in Fig. 5B, 83% of the total transducin α subunit was found to be present in the rod outer segment. Exposure of free running rats to a moderate ambient 10^3 lux light for 1 h resulted in reproducible transducin translocation from the rod outer segment, and in average only 53 ± 1% (S.E., n=4) of transducin α subunit remained in this cellular compartment. This data indicates that phosphorylation of Pdc at serine 71 in the outer segments coincides with translocation of transducin from this cellular compartment; thus dephosphorylation of serine 71 is unlikely to be a mechanism triggering transducin translocation.

DISCUSSION

To complement previous studies conducted in isolated retina preparations (1,11,19) and to obtain clues crucial to our understanding of the role of Pdc in rod dark and light adaptation, we have quantitatively analyzed phosphorylation of two known light-regulated phosphorylation sites of Pdc, serine 54 and serine 71, in free running animals exposed to physiologically relevant

levels of illumination. Our data demonstrates that in rods phosphorylation of Pdc at serine 54 and 71 has distinct spatial and temporal distribution, which is governed by the intensity of background illumination as follows.

Phosphorylation of Pdc in the dark − Pdc phosphorylated at serine 71 is distributed throughout the entire rod, whereas phosphorylation of serine 54 appears predominantly in the ellipsoid and the outer segment. The most important observation was that, even during prolong dark adaptation, the percent fraction of phosphorylated Pdc in any cellular compartment of rod is unlikely to exceed 40%, with the exception of the ellipsoid, where the degree of Pdc phosphorylation may be higher. This suggests that, contrary to the commonly accepted notion, significant amounts of dephosphorylated Pdc are available for interaction with the $\beta\gamma$ subunits of transducin or other heterotrimeric G proteins in the dark-adapted rods. It also refutes the idea that light-evoked dephosphorylation of either serine 54 or 71 is setting up an activation threshold for rod transducin translocation, which most recently was demonstrated to be determined primarily by the capacity of the GTPase-activating complex (27,28).

Dephosphorylation of Pdc in operating rods − Exposure of animals to dim and moderate light triggers massive and reversible dephosphorylation of Pdc, reducing the levels of serine 54 and 71 phosphorylation nearly 10-fold, and operating in the minutes time scale. The light sensitivity threshold of Pdc dephosphorylation was estimated to be near 0.8 lux (27 rhodopsin rod^{-1} sec^{-1}) of steady light, which is within rod operational range. Indeed, even 3-fold higher 2.5 lux background light was reported to be still comfortable for rod-meditated vision in living pigmented mice (21). The low light sensitivity threshold of Pdc dephosphorylation suggests its potential role in the regulation of rod visual signaling.

Hyperphosphorylation of Pdc in the outer segments of saturated rods − A surprising observation of this study was that phosphorylation of Pdc at serine 71 is significantly increased rather than decreased during prolong exposures to saturating light. In our experiments massive Pdc phosphorylation was evident when free running animals were exposed to a moderate ambient 10^3 lux steady light estimated to activate $5{\cdot}10^4$ rhodopsin rod^{-1} sec^{-1}, which would completely saturate rod responses in electrophysiological experiments (29). Most remarkably, virtually all serine 71-phosphorylated Pdc was present in the rod outer segment, while serine 54 remained completely dephosphorylated. An obvious hallmark of light-induced Pdc phosphorylation, a multiple banding pattern on SDS PAGE, which is absent in dark-adapted rods, suggests the presence of additional phosphorylation sites or other covalent modifications on Pdc.

Model − A putative mechanism of Pdc phosphorylation under three different levels of background illumination, which is consistent with our data, is presented in Fig. 6. This model is based on the assumption that the same protein phosphatase catalyzes dephosphorylation of Pdc and rhodopsin (13,30). In dark-adapted rods (Fig. 6, *upper panel*), the near 1:1 molar ratio of phosphorylated and dephosphorylated Pdc is due to the comparable rates of Pdc phosphorylation by protein kinase and dephosphorylation by protein phosphatase. Under these conditions phosphorylation of rhodopsin is negligible. In the operating rods (Fig. 6, *middle panel*), closure of cyclic nucleotide gated channels causes reduction in intracellular Ca^{2+} (31,32). As a result, suppression of Ca^{2+}-dependent Pdc kinase, protein kinase (10,33), shifts the equilibrium toward Pdc dephosphorylation. Under these conditions, phosphorylated Pdc is a primary substrate for protein phosphatase because the amounts of phosphorylated rhodopsin in the cell are still modest. In the saturated rods (Fig. 6, *lower panel*), significant amounts of phosphorylated rhodopsin competitively inhibit the reaction of Pdc dephosphorylation by protein phosphatase, shifting the equilibrium toward Pdc phosphorylation. Translocation of protein

phosphatase activity between cytoplasm and membrane originally described by Brown *et al*. (13) may occur under these conditions.

In summary, our studies provide the first quantitative account of compartment-specific phosphorylation of two principal light-regulated phosphorylation sites of Pdc, serine 54 and serine 71, in rod photoreceptors of living animals exposed to different levels of illumination. Our data support Pdc playing plural roles in a photoreceptor dark/light adaptation and in light protection. Analysis of Pdc phosphorylation mutants lacking serine 54 and serine 71 phosphorylation sites is required to get a better understanding of the physiological role of light-dependent Pdc phosphorylation.

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FOOTNOTES

We thank Norman A. Michaud, Massachusetts Eye and Ear Infirmary, for providing microphotographs of rat retina cross-sections and Dr. Visvanathan Ramamurthy, West Virginia University Eye Institute, for critically reading this manuscript. This work was supported by a grant from the National Institutes of Health (NCRR 2P20 RR15574-06 COBRE in Sensory Neuroscience, Subproject #5, to M.S.)

The abbreviations used are: Pdc, phosducin; G$\beta\gamma$, the $\beta\gamma$ subunits complex of heterotrimeric G protein; Pdc54p and Pdc71p, phospho-specific antibodies recognizing phosducin phosphorylated at residue 54 and 71, respectively; PP2A, protein phosphatase 2A.

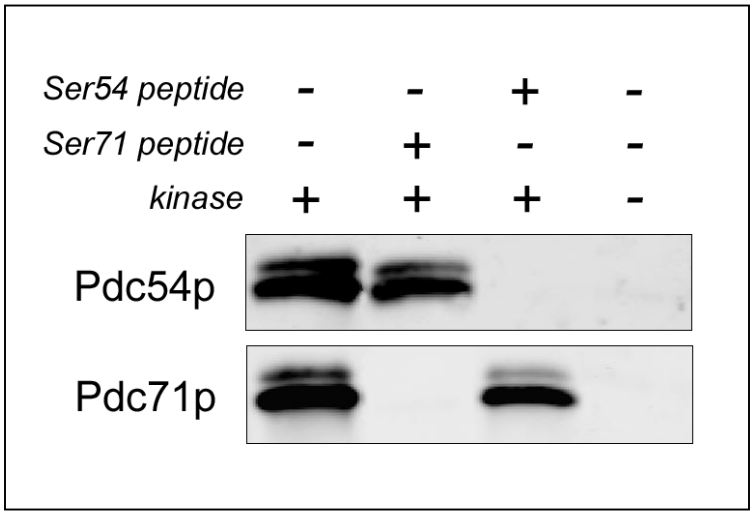


Figure 1 Characterization of phospho-specific antibodies against Pdc. Aliquots containing 2.0 pmol of Ca^{2+}/calmodulin-dependent protein kinase II-phosphorylated and non-phosphorylated purified recombinant Pdc (upper blot) and 0.75 pmol of cAMP-dependent protein kinase -phosphorylated and non-phosphorylated Pdc (lower blot) were analyzed by Western blotting using Pdc54p and Pdc71p antibodies. When stated, a 1 mM concentration of the corresponding phosphopeptide described under "Experimental Procedures" was present during incubation of the blots with primary antibodies.

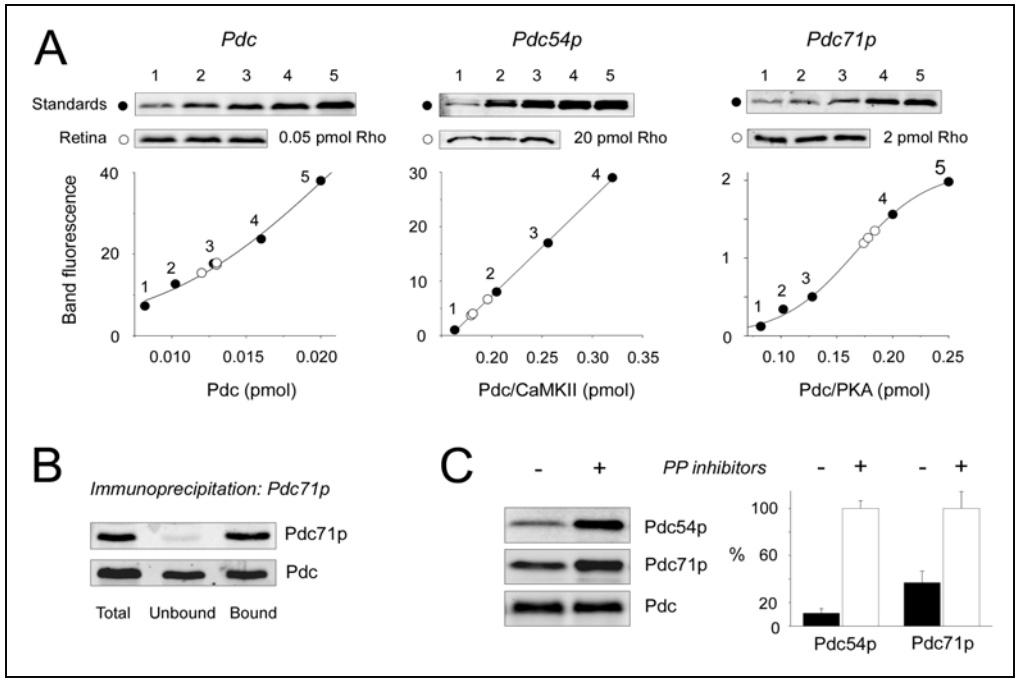


Figure 2 Quantification of the levels of serine 54- and serine 71-phosphorylated Pdc in the dark adapted retina. Retinas were harvested from dark-adapted mice under dim red light. *A*, SDS-extracts of the whole retina (*open circles*) containing the indicated amounts of rhodopsin (Rho) were analyzed by Western blotting together with a calibration curve comprised of various known amounts of purified recombinant Pdc premixed with the retinal extracts of Pdc knockout mice containing the same amount of rhodopsin (*closed circles*). When indicated, Pdc standards were phosphorylated at serine 54 and 71, with Ca^{2+}/calmodulin-dependent protein kinase II (*CaMKII*) and cAMP-dependent protein kinase (*PKA*), respectively, as described under "Experimental Procedures". Blots were probed with Pdc, Pdc54p, and Pdc71p antibodies. The amounts of Pdc in the retinal extracts were calculated from the calibration curve, which was obtained by plotting the fluorescence values of the specific bands (1-5) against the amounts of Pdc in that standard. The amounts of total- , serine 54-, and serine 71-phosphorylated Pdc were determined to be 26±0.6 (100%), 1±0.03 (4%), and 9±3 (35%) molecules per 100 molecules of rhodopsin, respectively (S.E., n=6). *B*, Each retina was extracted in radioimmune precipitation assay buffer, and then serine 71- phosphorylated Pdc was immunoprecipitated with an excess of immobilized Pdc71p antibody. The amounts of Pdc in the unbound and bound fractions were analyzed by Western blotting using Pdc71p (*upper blot*) and Pdc antibody (*lower blo*t). Comparison of fluorescence values of the Pdc bands in the unbound and bound fractions revealed that 44 ± 4% (S.E., n=5) of total Pdc in the retina was phosphorylated at serine 71 under these conditions. *C*, Retinas were incubated in HEPES-Ringer's solution containing 10mM glucose, with (+) or without (−) protein phosphatase (PP) inhibitors, including 10 μM microcystine LR and 10 μM okadaic acid for 20 min in the dark. The amounts of serine 54- and serine 71-phosphorylated Pdc was determined by Western blotting. Comparison of fluorescence values of the phospho-Pdc bands in the PP (+) and (−) retinas, shown as a bar graph, indicated that 11 ± 4% and 37 ± 10% (S.E., n=3) of total Pdc is phosphorylated at serine 54 and 71, respectively.

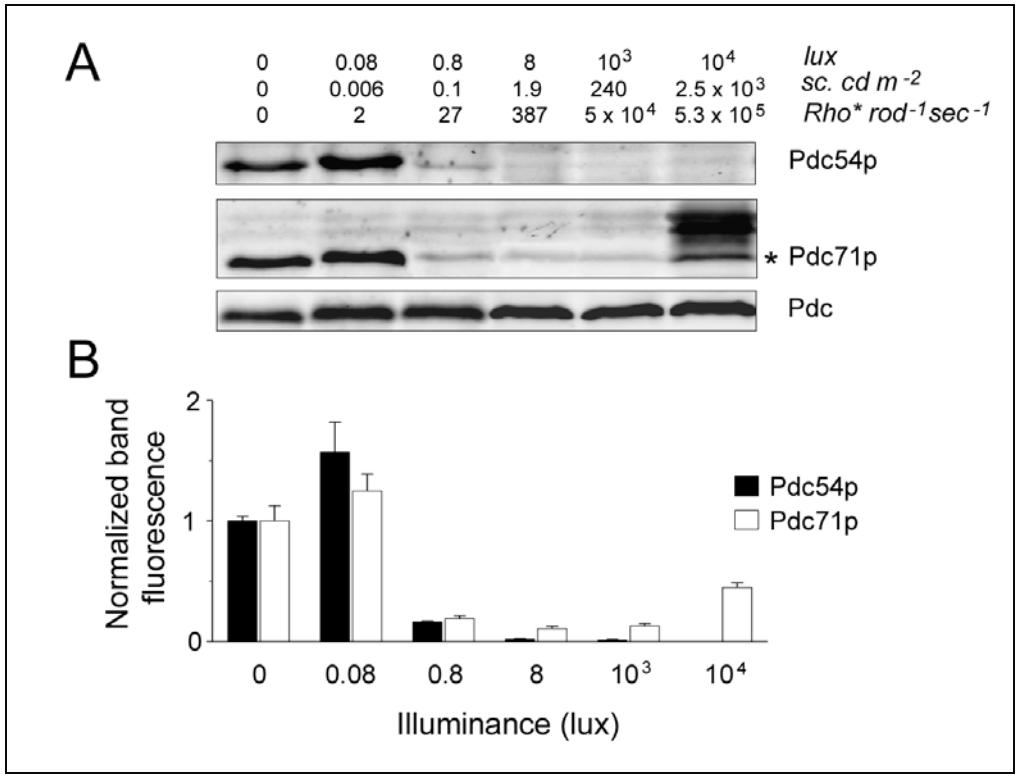


Figure 3 Light dependence of Pdc phosphorylation. Dark adapted mice were exposed to the designated levels of constant illumination for 10 min and sacrificed, and their eyes were harvested and frozen. Each eye was homogenized in SDS-containing buffer. *A*, 15-μl aliquots of the whole eye extracts were analyzed alongside each other by Western blotting with Pdc54p and Pdc71p antibodies. For the detection of total Pdc, original extracts were diluted 100 times. *B*, Fluorescence values of Pdc54p *(black)* and Pdc71p *(white)* bands were divided by fluorescence of corresponding total Pdc band, and then the amount of phosphorylated Pdc in the light was normalized to the dark-adapted value, designated as 0 lux (S.E., n=6). Note that the cluster of Pdc71p bands of reduced electrophoretic mobility that appears in the 10^4 lux light-conditioned samples was excluded from the analysis, and only the 33-kDa band (*) was analyzed.

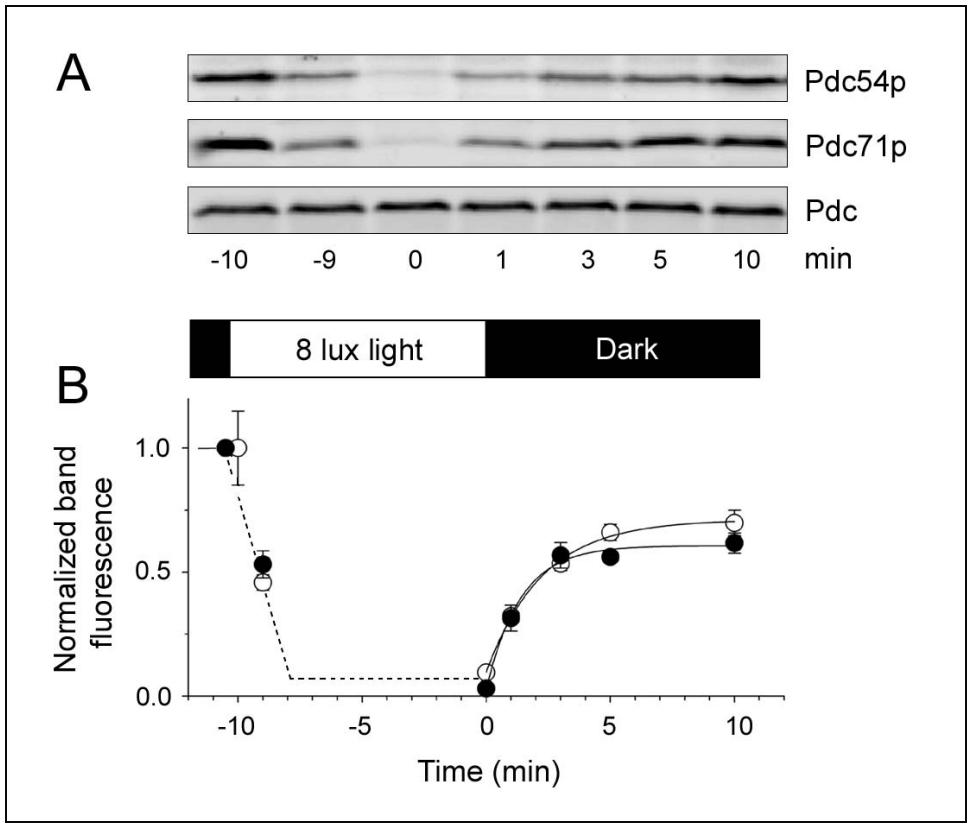
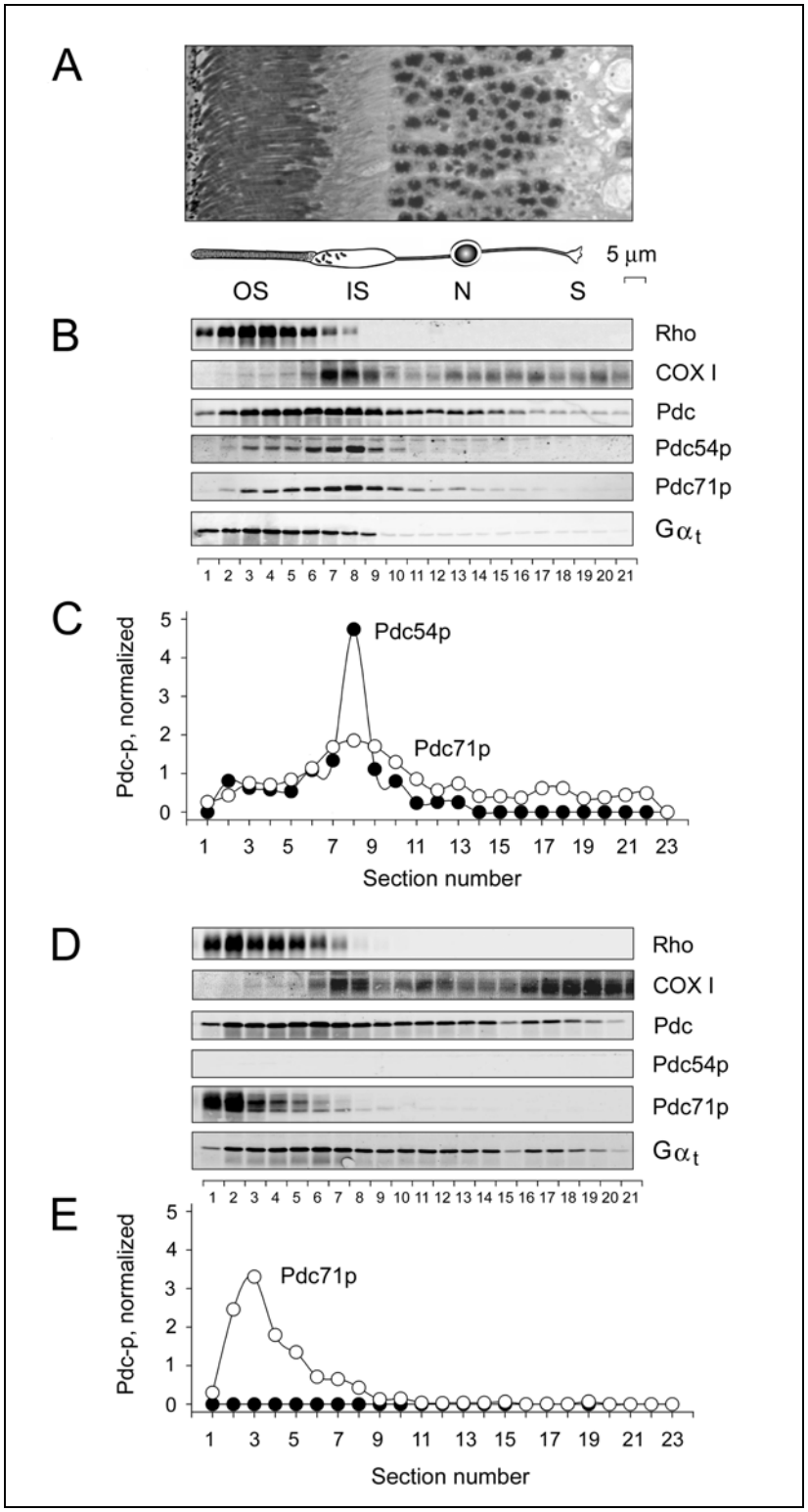


Figure 4 Time course of Pdc phosphorylation. Dark-adapted mice were exposed to 8 lux light from −10 min to 0 min and then placed in the dark again. Mice were sacrificed at different time points of the experiments, and their eyes were harvested and frozen. Each eye was homogenized in SDS-containing buffer. *A*, Western blots showing the levels of Pdc phosphorylated at serine 54 (Pdc54p), serine 71 (Pdc71p), and total Pdc in the extracts at different time points of the experiment. *B*, Fluorescence values of Pdc54p *(closed circles)* and Pdc71p *(open circles)* bands were divided by fluorescence of corresponding total Pdc band, and then the amounts of phosphorylated Pdc in each sample were normalized to the dark-adapted value, designated as −10 min (S.E., n=6). The initial rate of Pdc dephosphorylation is shown as a straight dotted line. The lines represent fits of time points 0, 1, 3, 5 and 10 min to the pseudo-first order rate equation for phosphorylation.


A
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Gαt
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<u>Figure 5</u> Analysis of the distribution of phosphorylated Pdc in the retina by Western blotting of serial sections. Retinas were obtained from either dark-adapted rat (*B*) or rat exposed to 1000 lux light for 1 h (*D*), flat-mounted, frozen, and serially 5-μm-sectioned (1-21). Each section was dissolved in SDS sample buffer and analyzed by Western blotting with antibodies against proteins indicated on the right of each blot. *A*, cross-section of rat retina stained with toluidine blue and a schematic drawing of a rod photoreceptor, shown to juxtapose rod morphology and the appearance of protein bands in serial sections. The following abbreviations are used to denote rod subcellular compartments: OS, outer segment; IS, inner segment; N, nucleus; S, synapse. *B* and *D,* blots showing distribution of the outer segment marker rhodopsin (Rho), inner segment marker (COX I), total phosducin (Pdc), phosducin phosphorylated at serine 54 (Pdc54p) and serine 71 (Pdc71p), and rod transducin α subunit ($G\alpha_t$) in serial tangential sections of the retina. *C* and *E,* The fluorescence values of each phosphorylated Pdc band was divided by the fluorescence value of the corresponding total Pdc band, and the resulting normalized Pdc54p *(closed circles)* and Pdc71p *(open circles)* amounts were plotted against the section number to show the relative abundance of phosphorylated Pdc in different cellular compartments. Data from blots *B* and *D* are shown on *C* and *E*, respectively.

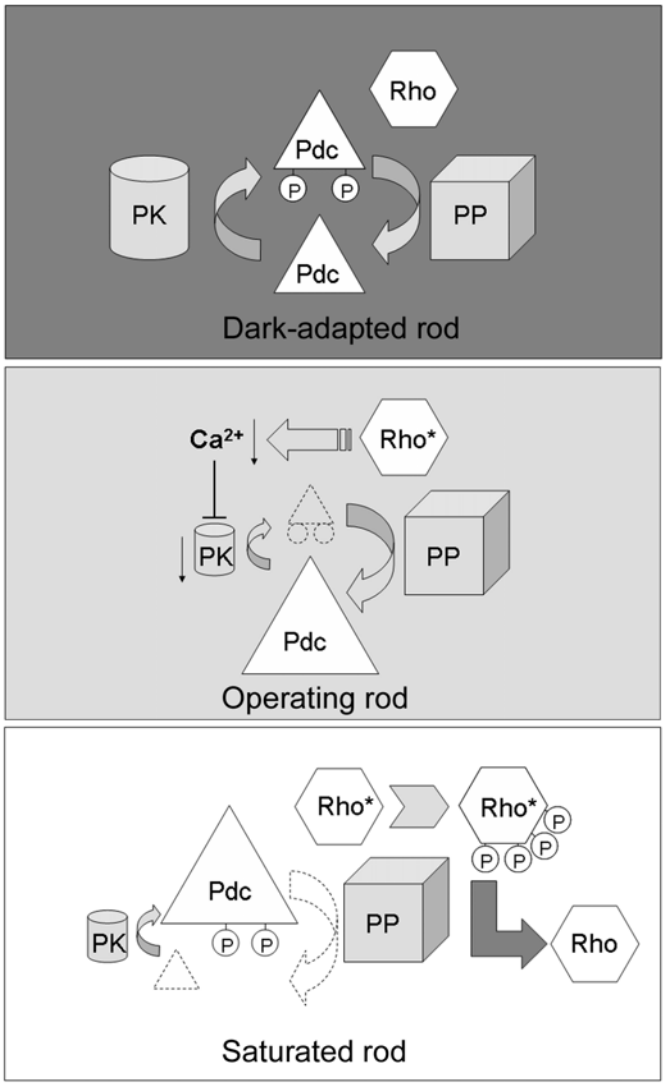


Figure 6 Phosphorylation cycle of Pdc under different conditions of illumination. *Dark-adapted rod*, comparable levels of dephosphorylated and phosphorylated Pdc are due to the equal rates of its phosphorylation by protein kinase (PK) and dephosphorylation by protein phosphatase (PP). No appreciable phosphorylated rhodopsin (Rho) is present. *Operating rod*, activation of rhodopsin (Rho*) and phototransduction cascade by dim background light suppresses activity of protein kinase via negative Ca^{2+} feedback, resulting in Pdc dephosphorylation. The amount of phosphorylated rhodopsin is insignificant. *Saturated rod*, large amounts of phosphorylated rhodopsin exceeding those of phosphorylated Pdc competitively inhibit dephosphorylation of Pdc by protein phosphatase. As a result, protein kinase activity prevails, and Pdc becomes phosphorylated.

Part Ⅱ

Phosducin Regulates Trafficking of Transducin in Rod Photoreceptors

Hongman Song, Marycharmain Belcastro, Antonina Jivotovskaya, Maxim Sokolov

The co-author Marycharmain Belcastro was responsible for the generation of transgenic mice and participated in the experiments shown in Figure 2 and Figure 3. Antonina Jivotovskaya participated in the experiment shown in Figure 1B.

Introduction

Vertebrate rod photoreceptors are highly specialized, polarized sensory neurons, which consist of morphologically and functionally distinct cellular compartments [1]. The most pronounced specification of a rod photoreceptor cell is the outer segment (OS) where light absorption and transduction occurs. The outer segment is linked with an inner segment (IS) via a narrow “connecting cilium”. To achieve a high efficiency of photo capture in dim light, the OS contains high concentrations of visual signaling proteins, including transducin, that are synthesized in the inner segment [3-5]. Transducin, a rod-specific heterotrimeric G protein, composed of α, β and γ subunits (Gαβγt), is a central transducer for visual signal transduction. To relay the extracellular signal from the light-activated photon receptor rhodopsin (Rho), an OS membrane protein, transducin must be delivered to and associated with the disk membrane. Thus, localization of transducin to the OS membrane, which is controlled by illumination conditions, is crucial for the light sensitivity of rod signaling [6-9]. In the dark-adapted photoreceptors, transducin is concentrated in the OS, while bright light evokes massive translocation of transducin from the OS to the IS and the synaptic region. In order to recover its function in night vision, transducin must return to the OS during dark adaptation after light exposure. Thus, transducin is capable of trafficking reversibly between the OS and intracellular locations upon activation, which is thought to be essential for rod visual signal transduction and viability [10-12].

Major advances have been made in understanding the mechanisms underlying light-dependent transducin translocation from the outer segment [13]. There are two possible modes of protein transport, diffusion and active transport, available for transducin trafficking in rod photoreceptors. Recent studies provide prevailing evidence

to support the diffusion model for light-driven transducin translocation. According to the diffusion model, the light-induced dissociation of transducin subunits simply allows them to diffuse from the OS to other compartments of rods, when the rate of transducin activation by photoexcited rhodopsin exceeds the capacity of the GTPase-activating complex to inactivate transducin [14, 15]. The transducin heterotrimer is known to have a high affinity for membranes because of the combined action of two lipid modifications: the N-acyl of Gαt and S-farnesyl of Gβγt [16-19]. When separated from each other, both transducin subunits – Gαt and Gβγt – become more soluble, due to only one lipid modification on each subunit, and readily translocate out of the OS through diffusion [11, 13]. Although strong evidence favors the diffusion model for transducin translocation in the light, there is much less known about the molecular mechanisms responsible for the return of transducin to the OS in the dark, which appears to be governed by a totally different mechanism [10].

Phosducin (Pdc) is an abundant phosphoprotein in vertebrate photoreceptors, which specifically forms a soluble complex with free Gβγt subunits [20, 21]. The binding of Pdc to free Gβγt interferes with the association of Gβγt and Gαt and the membrane as well [21, 22]. Consistent with the notion that transducin subunits need to be separated to transport out of the OS, Pdc has been shown to facilitate light-driven transducin translocation [23]. The interaction of Pdc with Gβγt is regulated by its light-dependent phosphorylation, where Pdc is phosphorylated in the dark and dephosphorylated in response to light [24-30]. Phosphorylation of Pdc at principal sites, serine 54 and 71, significantly decreases its binding affinity towards Gβγt [27, 29, 30]. More importantly, Pdc phosphorylation in the dark could result in the release of Gβγt, and allows for the re-association of Gαt and Gβγt to form heterotrimeric Gαβγt on the membrane. Formation of the transducin heterotrimer is a prerequisite for proper transport of transducin to the OS membrane [12, 31]. Therefore, we hypothesized that phosphorylation of Pdc

regulates subcellular trafficking of transducin to the outer segment membrane. To test this hypothesis *in vivo*, we generated transgenic mice expressing Pdc lacking phosphorylation sites, serine 54 and 71, and compared the *in vivo* rates of transducin trafficking to the rod OS in transgenic mice expressing full-length Pdc and Pdc without phosphorylation sites.

Experimental Procedures

Generation of transgenic phosducin mice

All experiments involving the use of mice were performed according to the protocols approved by the West Virginia University Animal Care and Use Committee. Full-length mouse phosducin (Pdc) cDNA was obtained by RT-PCR with total RNA, which was isolated from 129SV mouse retinas, using a Gene Specific RT Primer (5' – CCC GAG TTT AAA TAG CC – 3') and the AccuScript^{TM} High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene). Pdc cDNA was then engineered by PCR to be flanked by Sal I-Kozak-start codon at the 5' end and FLAG epitope tag-two stop codons-BamHI at the 3' end. This full-length, FLAG-tagged Pdc PCR product was subsequently cloned into the pBAM 4.2 vector [32] between the 4.4 kb mouse rhodopsin (Rho) promoter and the mouse protamine I polyadenylation (MPI) signal (Figure 1A) [33], generating the pRho4.4k-Pdc plasmid. The transgenic construct (4.4 kb rhodopsin promoter-kozak-full-length Pdc-FLAG-MPI polyA) within the pRho4.4k-Pdc plasmid was verified by sequence analysis. In addition, the two serine-to-alanine mutations, eliminating the phosphorylation sites serine 54 and serine 71 on Pdc, were also created from this pRho4.4k-Pdc plasmid using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), named pRho4.4k-Pdc$^{S54/71A}$ plasmid. The transgenic DNA fragments of FLAG-tagged full-length Pdc (Pdc-FL) and phosphorylation double mutant Pdc (serine-to-alanine mutations at serine 54

and serine 71, $Pdc^{S54/71A}$) were released from the pRho4.4k-Pdc and pRho4.4k-$Pdc^{S54/71A}$ plasmids, respectively, by restriction endonucleases *KpnI* and *SpeI* and gel purified. To generate transgenic mice, both purified FLAG-tagged Pdc-FL and $Pdc^{S54/71A}$ DNA fragments, each containing the 4.4 kb rhodopsin promoter and MPI polyadenylation sequences, were separately microinjected into the pronuclei of zygotes from superovulated FVB females in the Transgenic Animal Core Facility at WVU Health Sciences Center. Transgenic founders were identified by PCR using mouse tail DNA with a pair of primers designed to amplify a 255 bp fragment near the 3' end covering the FLAG sequence. To establish the transgenic lines, founders were first crossed with 129SV mice to overcome the $Pdeb^{rd1}$ mutation intrinsic to the FVB strain that causes retinal degeneration [34]. The founder lines with the highest expression of transgenic Pdc in the retinas, which were confirmed by Western blot analysis, were then chosen for breeding with phosducin knockout mice (Pdc-/-) [23] to produce transgenic mice on the hemizygous (Pdc+/-) background. Transgenic mice on the Pdc+/- background were further backcrossed with Pdc-/- mice to express the transgene on the Pdc-/- background, and the appropriate offspring were subsequently determined by the absence of a Pdc PCR product using primers designed previously [23] to amplify a 1kb sequence specifically from the wild–type Pdc allele. Totally, two independent lines for each type of transgenic mice on the Pdc knockout background, Pdc-FL and $Pdc^{S54/71A}$, have been established and maintained for further analysis.

Quantification of transgene expression using Western blotting

Mice were sacrificed by flashing the box with CO_2 followed by cervical dislocation, and their eyes were harvested and quickly frozen on the dry ice. An eye was homogenized in 200 µl of SDS PAGE sample buffer containing 125 mM Tris/HCl, pH 6.8, 4% SDS, 6 M urea, bromphenol blue tracking dye, and 10 mg/ml dithiothreitol by

brief sonication. The extracts were cleared by centrifugation and diluted 20 times. 10 µl aliquots of 20 times diluted original extracts were separated on 10% Tris-HCl gels (Bio-Rad), transferred onto polyvinylidene difluoride membrane Immobilon FL (Millipore), and probed with primary antibody against full-length Pdc [23] and then anti-sheep secondary antibody conjugated to Alexa Fluor 680 (Invitrogen). Fluorescence of the specific bands was quantified on an Odyssey Infrared Imaging System (LI-COR Bioscience) according to the manufacturer's protocols.

Light conditioning of mice

Transgenic mice were maintained in standard cage rooms with cyclic light (12 h light/12 h dark) until used. The light conditioning of mice was performed inside the ganzfeld of a UTAS-E4000 Visual Electrodiagnostic Test System (LKC Technologies). Mice were anaesthetized using an oxygen/isoflurane mixture. The pupils were dilated with a mixture of 1.25% phenylephrine hydrochloride and 0.5% tropicamide ophthalmic solutions for 20 min. To achieve maximal transducin translocation, mice were exposed to 2000 lux background light pre-set in the ERG system for 10 min and then placed in the dark to recover. Mice regained consciousness within a few minutes, which allowed our studies of transducin trafficking in non-anesthetized mice. After 2 h and 12 h (dark adaptation) of recovery, mice were sacrificed with CO_2 followed by cervical dislocation, separately. Their eyes were harvested and analyzed.

Western blot analysis of serial tangential sections of the retina

Serial tangential cryosectioning of the mouse retina was performed following the improved procedure described previously [35] with several optimizations. Eyes were manipulated under a stereo microscope in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 1X (DMEM/F12, 1:1) (GIBCO® 11039, Invitrogen). When necessary, all tissue manipulations were conducted in the dark under a Stemi 2000-C

stereomicroscope (Zeiss) equipped with OWL Gen 3+ intensifiers (B.E. Meyers and Co. Inc.) The anterior half of the eye was cut away, and the lens was removed. The eyecup with the retina attached to it was cut into two pieces. To avoid disturbing the retina morphology, each piece was transferred with a plastic sucker to the flattening chamber containing DMEM/F12 medium. The retina of each piece was gently pulled away from the eye cup, flat-mounted onto a supporting polyvinylidene difluoride (PVDF) membrane with the photoreceptors facing down, and positioned on top of a flat glass capillary array (GCA 09/32/25/0/20 LM, BURLE Electro-Optics, Sturbridge, MA) in the flattening chamber. The retina was flattened by applying gentle suction from underneath the glass capillary array, slowly removing all solution from the flattening chamber. Then the retina on the supporting membrane was flat-mounted, glued, and clamped between two glass slides separated by 0.5 mm spacers to assemble a “sandwich,” and frozen on dry ice. The frozen retina was subsequently sectioned in a Leica CM1850 Cryostat as follows. First, optimal cutting temperature compound was frozen at –20^{o}C on the specimen holder, and sectioned to create a flat surface large enough to accommodate the glass slide. The cover glass and the spacers of the “sandwich” were then removed. The base slide with frozen retina on the membrane was gently placed and fixed on the surface of the freezing compound by the addition of water drops to the sides of the glass base. The retina with the supporting membrane was trimmed to remove any uneven and folded parts, and 10 µm sectioned. When the first section was cut out, all other parts of the retina not included in this section were trimmed away, and then sectioning was resumed. Each section was collected and dissolved in 40 µl of SDS PAGE sample buffer (6 M urea, 125 mM Tris-HCl, pH 6.8, 4% SDS, bromophenol blue, 0.5 µl of benzonase, and 0.5 µl of β-mercaptoethanol). To assess the quality of the sectioning, 0.5 µl aliquots were applied on dry nitrocellulose membrane, and the dot blots were probed with monoclonal

anti-rhodopsin antibody (4D1, Sigma, R5403). Only the sets containing rhodopsin in the upper 3-5 sections were selected, and stored at −80ºC for further analysis.

Western blotting was carried out using the Criterion Cell and Blotter system (Bio Rad). To detect rod transducin α subunit (Gαt), β subunit (Gβt) and visual arrestin, 2 µl aliquots from the original extracts of each section prepared above were separated on a 26-well 10-20% Tris-HCl gel (Bio Rad) and transferred onto PVDF membrane Immobilon FL (Millipore). For the detection of β–tubulin, subunit I of cytochrome c oxidase (COX I) and ROM-1, 10 µl aliquots were used. The tested proteins were probed with specific primary antibodies and anti-rabbit and anti-mouse secondary antibodies conjugated to either Alexa Fluor 680 (Invitrogen) or IRDye 800 (LI-COR Biosciences). Antibodies against Gαt (K-20, sc-389) and Gβt (T-20, sc-378) were obtained from Santa Sruz Biotechnology. Antibodies against visual arrestin (V4389) and β–tubulin (T0198) were obtained from Sigma. Antibody against COX I was MS404 from MitoSciences. A rabbit polyclonal antibody against ROM-1 was a gift from Visvanathan Ramamurthy (West Virginia University). Quantification of the specific bands was then performed on an Odyssey Infrared Imaging System (LI-COR Biosciences) following the manufacturer's protocols.

Results

Generation and characterization of transgenic phosducin phosphorylation mutants

To directly determine the role of phosducin phosphorylation in regulating trafficking of transducin in rod photoreceptors, we generated transgenic mice expressing FLAG-tagged phosducin lacking phosphorylation sites by introducing serine-to-alanine mutations at positions 54 and 71 (Figure 1A, $Pdc^{S54/71A}$) on phosducin (see "Experimental Procedures"). Transgenic mice expressing FLAG-tagged full-length

phosducin (Figure 1A, Pdc-FL) were also created to serve as a control. FLAG-tagged transgenes were driven to express in rod photoreceptors under the control of rod opsin (rhodopsin, Rho) promoter. Both types of transgenic lines were established on the phosducin knockout (Pdc-/-) background by breeding transgenic founders with Pdc-/- mice [23]. Based on the Western blot analysis of the whole eye extracts with anti-full length phosducin (Pdc) antibody, the expression levels of FLAG-tagged Pdc-FL and $Pdc^{S54/71A}$ were 93 ± 7% and 180 ± 14% of the wild-type Pdc level, respectively (Figure 1B). Control experiments demonstrated that the levels of rhodopsin and phosphodiesterase alpha (PDEα) subunit were essentially unchanged in transgenic mice (data not shown), compared with those in wild type mice. Therefore, we concluded that transgenic Pdc-FL and $Pdc^{S54/71A}$ were expressed at high levels in the retinas.

To confirm the elimination of serine 54 and 71 phosphorylation sites, we first enriched phosphorylated Pdc in dark-adapted transgenic rods by suppressing the activity of protein phosphatase pharmacologically, and then detected Pdc phosphorylation using phospho-specific antibodies against serine 54 and 71 (Pdc54p and Pdc71p). This approach was validated in our previous studies [2]. As shown in Figure 1C, even after incubation of freshly obtained dark-adapted mouse retinas with the phosphatase inhibitor okadaic acid, no phosphorylation of Pdc at serine 54 and 71 was detected in transgenic $Pdc^{S54/71A}$ retinas. As a control, the treatment of okadaic acid resulted in strong Pdc phosphorylation at both sites in dark-adapted Pdc-FL retinas.

Finally, we found that overexpression of Pdc-FL and $Pdc^{S54/71A}$ restored the levels of G$\beta\gamma$t subunits to 73 ± 5% and 77 ± 4% (S.E., n=5) of the wild-type Pdc level, respectively, otherwise reduced in the Pdc-null photoreceptors [36], while exerting no detectable detrimental effect on these cells. Therefore, these transgenic models were used to study trafficking of signaling proteins in rod photoreceptors.

Figure 1A

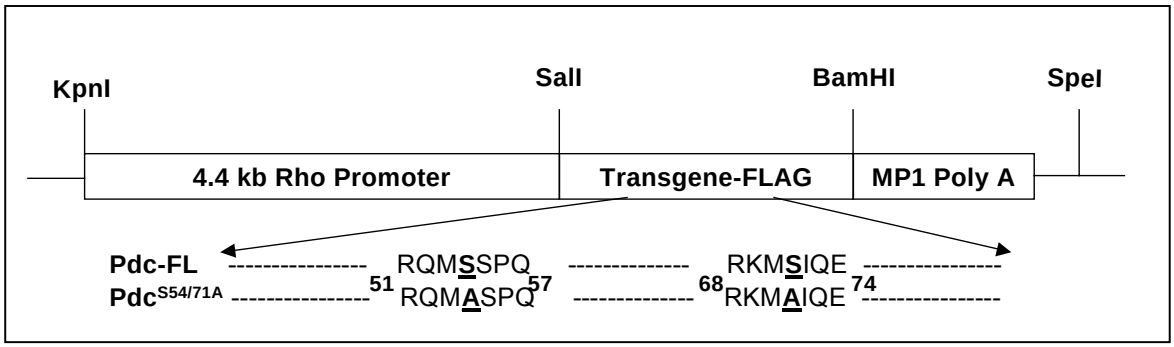


Figure 1B

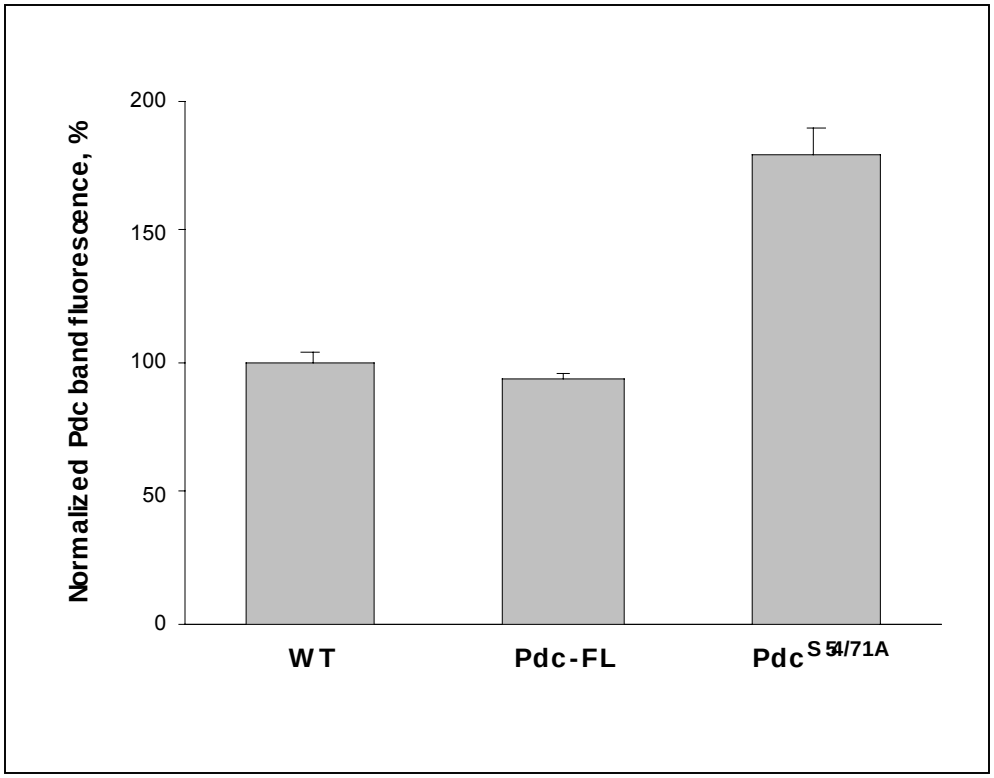


Figure 1C

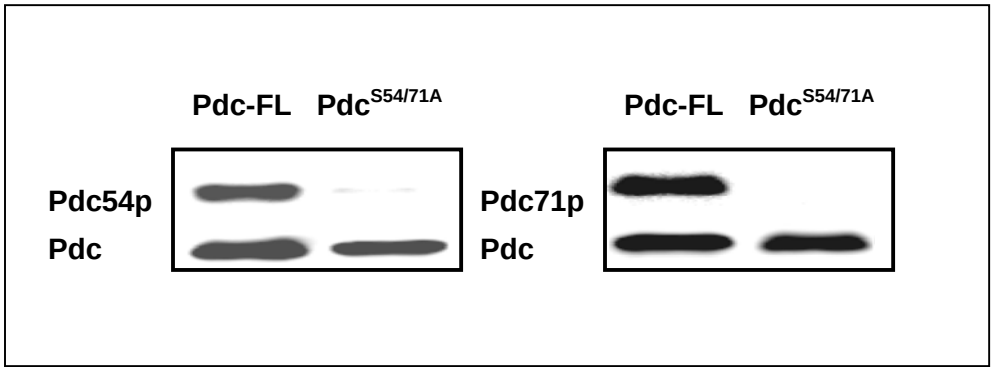


Figure 1. Characterization of transgenic mice expressing FLAG-tagged full-length Pdc (Pdc-FL) or Pdc without two phosphorylation sites (Pdc$^{S54/71A}$). A. Transgenic constructs used for the generation of transgenic Pdc-FL and Pdc$^{S54/71A}$ mice on the phosducin knockout background. Rho, rhodopsion; MPI Poly A, mouse protamine I polyadenylation signal. B. Quantification of the relative protein levels of FLAG-tagged Pdc-FL and Pdc$^{S54/71A}$ in transgenic mice. Whole eyes from transgenic Pdc-FL, Pdc$^{S54/71A}$ and wild-type (WT) mice of the same age were homogenized and analyzed using Western blotting with anti-full length Pdc antibody (Pdc). Fluorescence values of Pdc bands in Pdc-FL and Pdc$^{S54/71A}$ samples were normalized to the wild-type Pdc value (S.E., n=5). C. Transgenic mice expressing Pdc$^{S54/71A}$ lack *in vivo* Pdc phosphorylation at serine 54 and serine 71. Dark-adapted retinas were incubated in DMEM/F12 medium containing 12 nM okadaic acid (protein phosphatase inhibitor) for 20 min. Phosphorylation of Pdc at serine 54 and 71 was analyzed by Western blotting using phospho-specfic (Pdc54p and Pdc71p) [2] and anti-full length Pdc (Pdc) antibodies.

Quantitative study of transducin trafficking in rod photoreceptors by Western blot analysis of serial sections of the retina

In order to study transducin trafficking in rod photoreceptors, we utilized a unique experimental approach combining serial tangential cryosectioning of the flat mounted frozen retina with the analysis of the specific protein content in each section by Western blotting. This technique allows us to describe protein localization within rod photoreceptors quantitatively and has been successfully applied in our previous studies [2, 10, 35]. Using this technique, we measured and compared the subcellular localization of transducin α and β subunits in rod outer segments of transgenic Pdc-FL and $Pdc^{S54/71A}$ mice, which were subjected to controlled lighting conditions, as described in the details under "Experimental Procedures." In brief, both Pdc-FL and $Pdc^{S54/71A}$ mice were anesthetized and their pupils were dilated. First, these mice were exposed to 2000 Lux light for 10 min that saturated transducin translocation from the outer segment. Then they were allowed to recover in the dark for either 2 hr or 12 hr, since the return of Gαt and Gβt to the OS would need several hours to be completed [10, 37]. Finally, after light conditioning, mice were sacrificed at different time points during the experiment and retinas were flat-mounted, frozen, and serially sectioned. Each set of serial sections was then analyzed by Western blotting (Figure 2B and 2C, Figure 3B and 3C). The profile of the expression pattern of a particular protein in serial sections represents the subcellular distribution of that protein in rods. A representative experiment for each genotype at different time points in the dark after light exposure was illustrated in Figure 2 (Pdc-FL) and Figure 3 ($Pdc^{S54/71A}$).

As shown in Figure 2B and Figure 3B, the rod outer segments were identified by the presence of retinal outer segment membrane protein 1 (ROM-1), a photoreceptor-specific disk protein confined in this subcellular compartment. A mitochondrial marker

subunit I of cytochrome c oxidase (COX I) was used to highlight the beginning of the inner segment, which is located at the ellipsoid part of the inner segment adjacent to the outer segment and also in rod synapse. In addition, β-tubulin was utilized as a loading control. To monitor transducin trafficking within rods, we calculated and compared the fractions of transducin subunits present in the outer segments in Pdc-FL and $Pdc^{S54/71A}$ retinas at different time points in the experiment using an approach originally developed to study light-driven transducin translocation in rods [10]. In brief, we first measured the fluorescence values of the ROM-1, Gαt and Gβt bands in each section and expressed them as a percent fraction of the combined fluorescence of each protein in all sections (Figure 2B and 2C, Figure 3B and 3C). Then, we plotted the resulting percentage against the section number to show the subcellular localization of individual proteins (Gαt and Gβt) in rods (Figure 2D and Figure 3D). We derived the content of Gαt and Gβt in the rod outer segments as follows. The fractions of Gαt and Gβt confined in sections containing ROM-1 and free from COX I were calculated and normalized to the fraction of ROM-1 present in these sections. For example, in a typical experiment shown in Figure 2 (Pdc-FL) 0 hr, sections 1-2 contain 65% of ROM-I, 4% of Gαt and 20% of Gβt and are essentially free from COX I. So, the outer segment fraction of Gαt was 4%/65% = 6.2%; and the fraction of Gβγt in the outer segment was 20%/65% = 31%. Using the same analysis, we quantified transducin subunit contents in the outer segments of Pdc-FL and $Pdc^{S54/71A}$ mice at different time points in the dark and compared the rate of transducin trafficking back to the outer segments in Pdc-FL and $Pdc^{S54/71A}$.

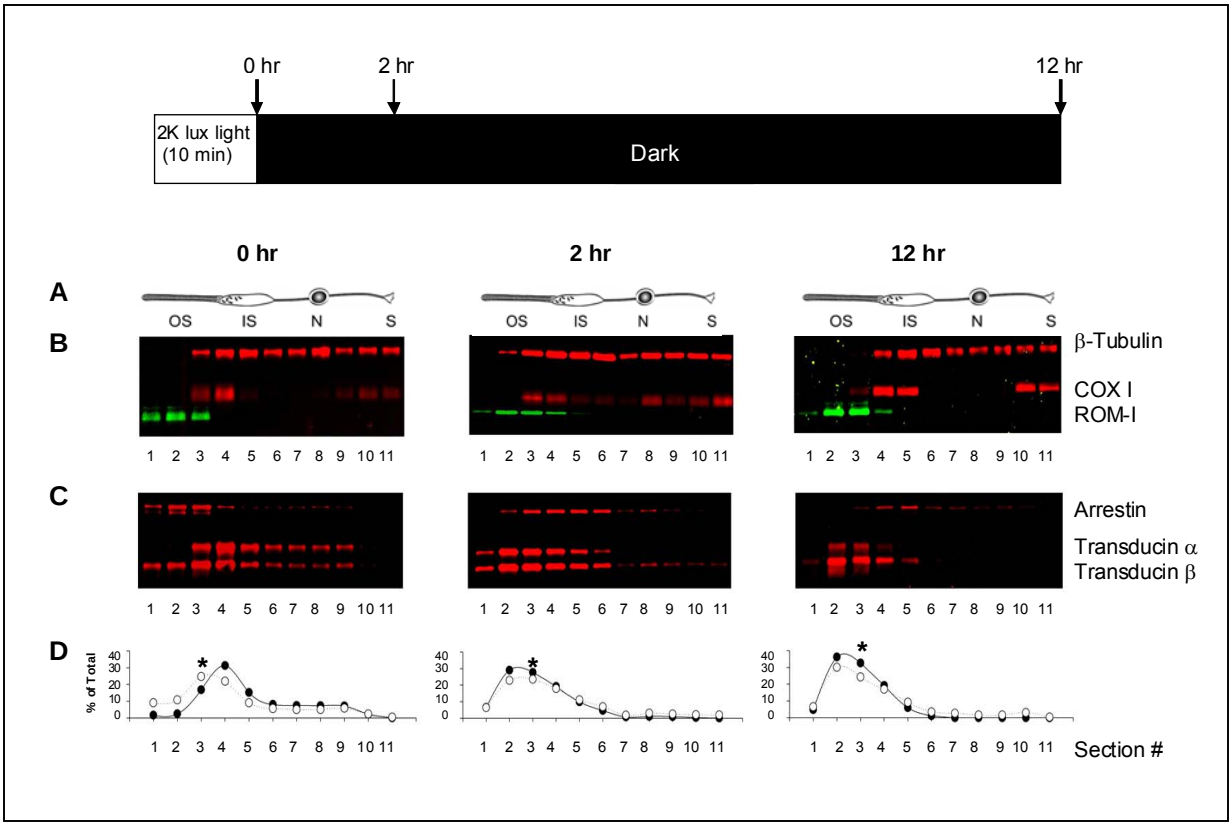


Figure 2. Analysis of the distribution of transducin α and β subunits and arrestin in Pdc-FL rods by Western blotting of serial sections. Anesthetized and dilated Pdc-FL mice were exposed to 2000 lux light for 10 min and then placed in the dark to recover for 0, 2 and 12 hr. Mice were sacrificed at different time points (0 hr, 2 hr and 12 hr) of the experiments. Their retinas were flat-mounted, frozen and serially 10-μm-sectioned (1-11). Each set of serial sections was analyzed by Western blotting with antibodies against specific proteins indicated on the right of the blots. A. A schematic illustration of the corresponding compartments of a rod photoreceptor. OS, outer segment; IS, inner segment; N, nucleus; S, synapse. B and C**.** Western blots showing the distribution of the outer segment marker, retinal outer segment membrane protein 1 (ROM-I), inner segment marker, subunit I of cytochrome c oxidase (COX I), and β-tubulin serving as a loading control (B), and transducin α, β subunits and arrestin (C) in 10-μm serial sections of the retina. D. The fluorescence values of transducin α and β bands in each section of each set shown in C. were quantified using a LICOR Odyssey system and plotted as a percent fraction of the combined fluorescence of the corresponding transducin subunit in all sections against the section number to show the relative abundance of transducin α (*closed circles*) and β (*open circles*) subunits in different subcelluar compartments within rods. The *asterisk* on each graph indicates the border between the outer segment (OS) and the inner segment (IS). The data for each time point are taken from one of three similar experiments with transgenic Pdc-FL mice.

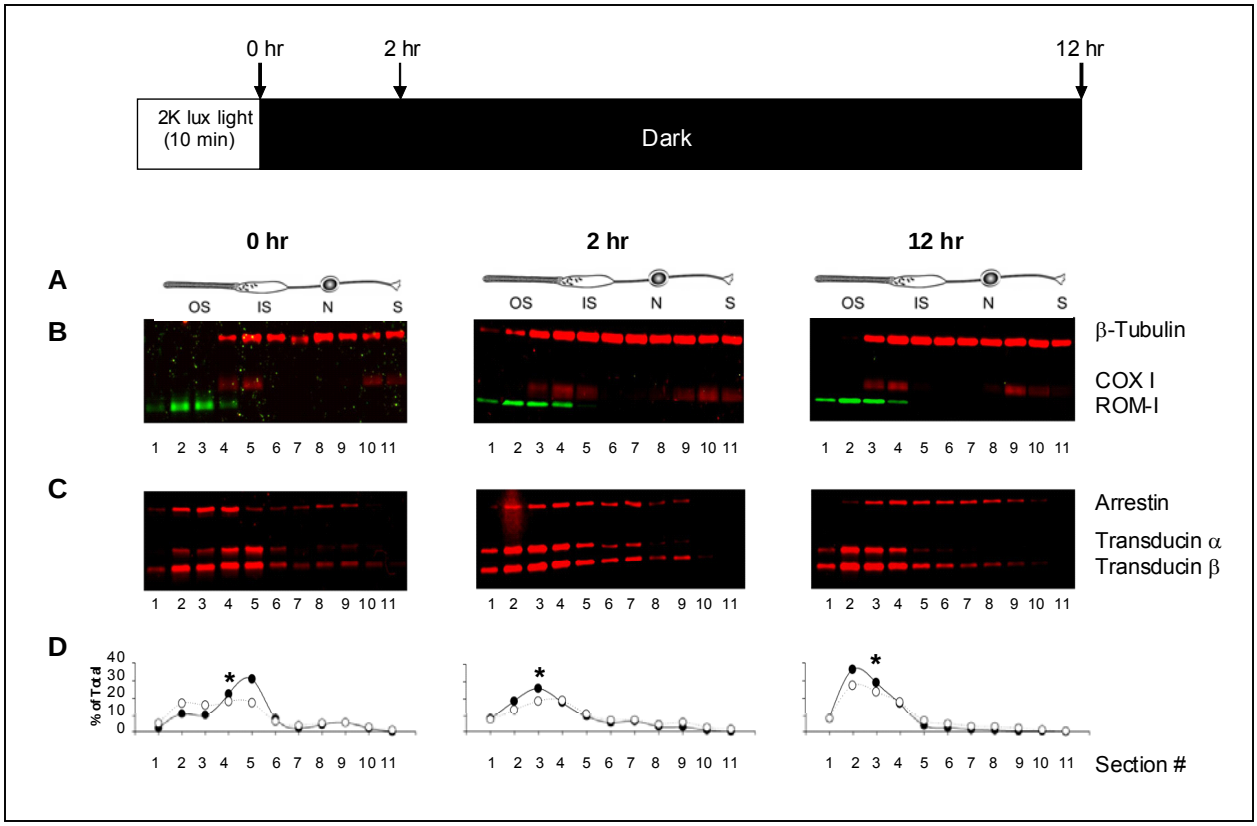


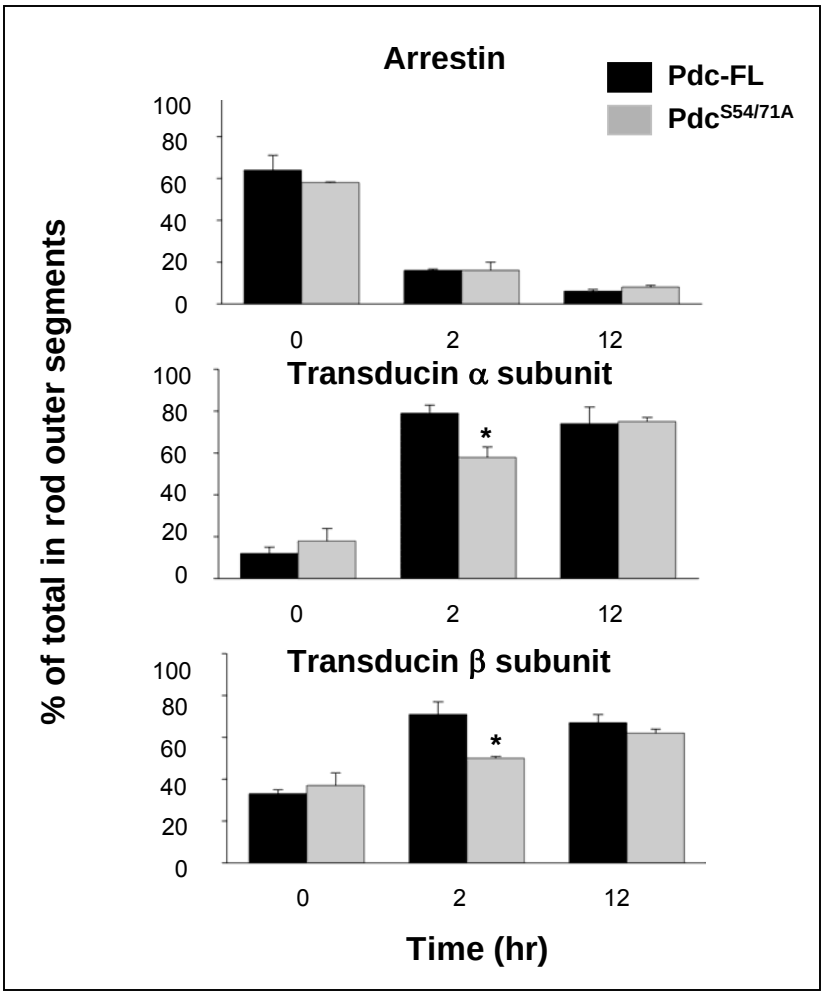
Figure 3. The distribution of transducin α and β subunits and arrestion in Pdc$^{S54/71A}$ rods at different time points in the dark after light exposure. Pdc$^{S54/71A}$ mice were light-conditioned and their retinas were serially 10-μm-sectioned and quantitatively analyzed by Western blotting at 0, 2 and 12 hr in the dark during the experiments, as described in the Figure 2 legend. A. Drawing illustrating the corresponding compartments of a rod photoreceptor. OS, outer segment; IS, inner segment; N, nucleus; S, synapse. B and C. Blots showing distribution of the outer segment marker (ROM-I), inner segment marker (COX I), β-tubulin serving as a loading control (B), and transducin α, β subunits and arrestin (C) in 10-μm serial sections of the retina. D. The fluorescence values of transducin α and β bands in each section of each set shown in C. were plotted as a percent fraction of the combined fluorescence of the corresponding transducin subunit in all sections against the section number to show the relative abundance of transducin α (*closed circles*) and β (*open circles*) subunits in different subcelluar compartments within rods. The *asterisk* on each graph indicates the border between the outer segment (OS) and the inner segment (IS). The data for each time point are taken from one of three similar experiments with transgenic Pdc$^{S54/71A}$ mice.

Trafficking of transducin in transgenic mice expressing Pdc lacking phosphorylation sites

We studied the effects of Pdc phosphorylation on transducin trafficking to the outer segments. The fractions of Gαt and Gβt present in the outer segments were determined and compared in Pdc-FL and $Pdc^{S54/71A}$ mice at 0 hr, 2 hr, and 12 hr in the dark after light exposure. The results are summarized in Table I and Figure 4. In the case of the transducin β subunit, no significant difference was observed in the fractions of Gβt in the outer segments between Pdc-FL and $Pdc^{S54/71A}$ mice at 0 hr and 12 hr in the dark. After 2 hr in the dark, the outer segment content of Gβt in Pdc-FL rods was ~ 71%, which already reached the highest possible value in the dark (12 hr in the dark: ~ 67%), indicating that Gβt completed its return to the outer segments at or even before 2 hr recovery in the dark. In contrast, the corresponding fraction of Gβt in $Pdc^{S54/71A}$ after 2 hr in the dark was ~ 50%, which was ~ 81% of the maximal value in the dark (12hr). These comparative analyses clearly showed significant retardation of transducin β trafficking to the OS in transgenic mice expressing Pdc lacking phosphorylation sites. A similar effect of Pdc phosphorylation mutants on Gαt trafficking to the OS was also detected.

Table I Fractions of transducin α and β subunits and arrestin present in rod outer segments of Pdc-FL and $Pdc^{S54/71A}$ mice at different time points in the dark after light exposure. Pdc-FL and $Pdc^{S54/71A}$ mice were subjected to controlled lighting conditions as described in Figures 2 and 3. The fractions of transducin α and β and arrestin in rod outer segments were quantified as described under "'Results". The data were obtained by averaging the results from three experiments with mice of each type at 0 hr, 2 hr and 12 hr in the dark after light exposure (mean ± S.E., n=3).

	Arrestin			Transducin α subunit			Transducin β subunit		
	0 hr	2 hr	12 hr	0 hr	2 hr	12hr	0 hr	2 hr	12 hr
	%	%	%	%	%	%	%	%	%
Pdc-FL	64 ± 7	16 ± 1	6 ± 1	12 ± 3	79 ± 4	74 ± 8	33 ± 2	71 ± 6	67 ± 4
$Pdc^{S54/71A}$	58 ± 1	16 ± 4	8 ± 1	18 ± 6	58 ± 5	75 ± 2	37 ± 6	50 ± 1	62 ± 2



<u>Figure 4.</u> Comparison of transducin α and β subunits and arrestin fractions present in the outer segments of Pdc-FL and Pdc$^{S54/71A}$ rods at 0, 2 and 12 hrs in the dark after light exposure. The data summarized in Table I was plotted as a percent fraction of the indicated proteins in the outer segments against the corresponding time point. (* p<0.05)

Several control experiments were performed. To confirm that the lighting condition, 10 min of 2000 lux exposure which we used for light adaptation, caused the maximal degree of transducin β translocation from the outer segments in Pdc-FL mice, control experiments were conducted using transducin α knockout (TdKO) mice [38]. The rationale was that Gβγt, in the absence of Gαt, behaved like a soluble protein and was distributed throughout the entire rod cells irrespective of light status, representing the final equilibrium state that Gβγt of wild-type mice would possibly reach in the light [39]. It was found that the outer segment fraction of Gβt in TdKO mice was 30 ± 4 % (S.E., n =4), similar to that in light-adapted (0 hr) Pdc-FL mice (33 ± 2 %). We also monitored arrestin trafficking in Pdc-FL and $Pdc^{S54/71A}$ rods. It has been known that arrestin undergoes bidirectional translocation between the OS and the rest of the compartments of rods in a light-dependent manner, just like transducin, but it moves in a direction opposite to that of transducin [7, 8, 40]. Consistent with previous observations, we were able to detect robust light-driven arrestin translocation in both Pdc-FL and $Pdc^{S54/71A}$ rods (Figure 2C and Figure 3C). Also, there was no significant difference in the outer segment contents of arrestin between Pdc-FL and $Pdc^{S54/71A}$ retinas at 0 hr, 2 hr, and 12 hr in the dark (Table I and Figure 4). These results indicated that only trafficking of transducin subunits, not arrestin, to the OS were specifically affected by Pdc without phosphorylation sites.

Taken together, these data suggest that phosducin increases the rate of trafficking of transducin to the outer segment through its light-dependent phosphorylation. The same effects of Pdc phosphorylation mutants on both Gαt and Gβγt subunits are consistent with the concept that transducin returns to the OS as an intact heterotrimer.

Discussion

In the present study, we established the physiological role of phosducin phosphorylation in regulating transducin trafficking within rod photoreceptors. We generated transgenic mice expressing Pdc lacking phosphorylation sites, and quantitatively analyzed the effects of the Pdc phosphorylation mutants on trafficking of transducin in rods. The central finding of this study was that light-modulated phosphorylation of phosducin accelerated trafficking of transducin to the OS in the rod cell.

To maintain normal rod function, transducin undergoes light-driven translocation from the OS followed by trafficking to the OS upon switching from light to dark. Although it has become increasingly clear that the basic mechanism underlying transducin translocation in the light is diffusion, the mechanisms for transducin trafficking to the OS remain elusive. Furthermore, to completely understand the process of transducin return to the OS, another important but fundamental question is to determine which mechanisms trigger transducin trafficking to the OS. Previous studies on trafficking of transducin and other G proteins suggest that the heterotrimer formation is the triggering event for transport of G proteins to the membrane [12, 31]. Phosducin essentially prevents re-association of $G\alpha t$ and $G\beta\gamma t$ with each other and with the membrane by binding free $G\beta\gamma t$ with high affinity in its dephosphorylated form. Phosphorylation of phosducin in the dark has a potential to reverse its interaction with free $G\beta\gamma t$, allowing the release of $G\beta\gamma t$ and formation of heterotrimeric transducin (Figure 5). By doing so, Pdc phosphorylation would promote transducin trafficking to the OS membrane. Thus, Pdc without phosphorylation sites would bind $G\beta\gamma t$ tightly, slowing down trafficking of transducin to the OS membrane.

This hypothesis is supported by previous research results. First, the light-modulated phosphorylation state of Pdc at serines 54 and 71 controls its interaction with Gβγt *in vitro* and in isolated retinas. Actually, Pdc did indeed bind more Gβγt in the light than in the dark [23, 30]. Second, our recent quantitave studies of Pdc phosphorylation *in vivo* show that site-specific phosphorylation of Pdc is highly compartmentalized, with a high concentration of phosphorylated Pdc localized in the ellipsoid region, a subcelluar compartment of the inner segment near the cilium that connects the IS and the OS [2]. Third, Gαt and Gβγt return to the OS at identical rates [10], indicating that transducin subunits may traffic to the OS as a heterotrimer.

We propose a putative model for the role of Pdc phosphorylation in regulating trafficking of transducin to the OS membrane (Figure 5), which is consistent with our observation. In the inner segment, Pdc interacts with free Gβγt in its dephosphorylated state to keep Gβγt and Gαt apart. Once Pdc is phosphorylated during dark adaptation, Pdc may dissociate from the complex with Gβγt in the appropriate place, most likely the ellipsoid region. In this manner, free Gβγt is available to form the transducin heterotrimer with Gαt in the place adjacent to the OS where heterotrimeric transducin is ready for trafficking to the OS membrane. Thus, Pdc may function as a chaperone for transducin trafficking to the OS membrane.

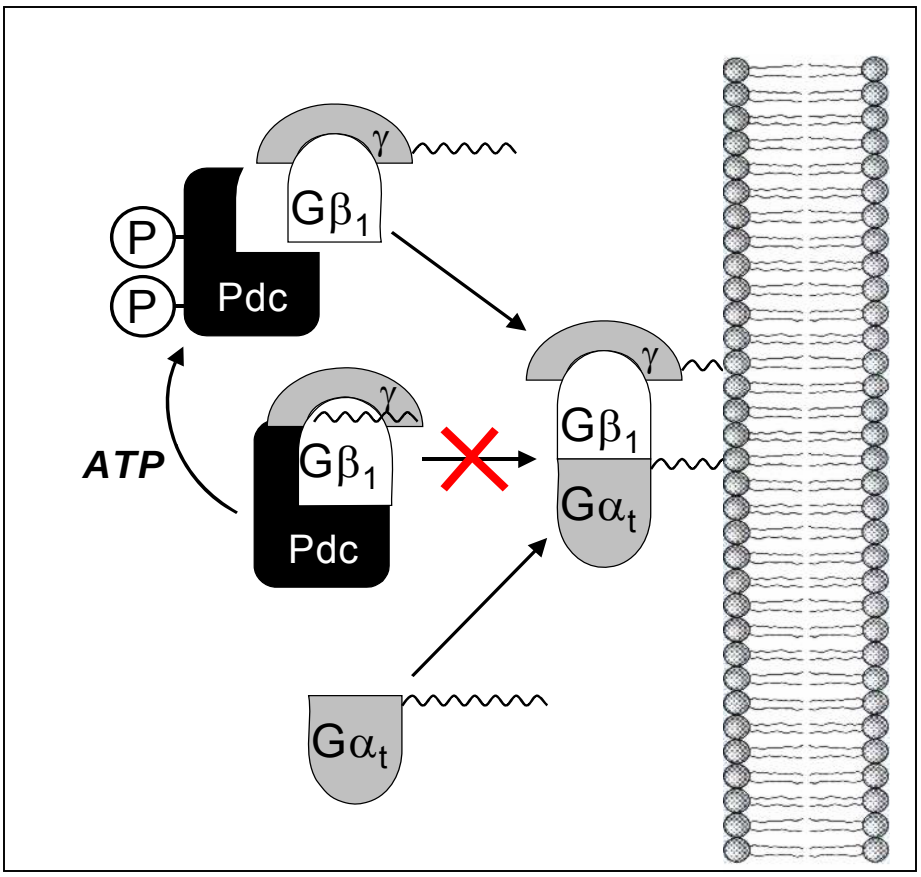


Figure 5. Proposed model of the role of Pdc phosphorylation in regulating trafficking of transducin to the OS membrane. Pdc dissociation from the complex with Gβγt subunits, which is triggered by Pdc phosphorylation, is a prerequisite for the association of Gβγt with Gαt and with the membrane as well. Thus, Pdc phosphorylation could potentially regulate the association of transducin with the membrane, and by doing so participate in transducin trafficking to the outer segments.

Another important observation is that, in transgenic mice expressing Pdc without phosphorylation sites, the rate of transducin trafficking to the OS is significantly affected but not abolished. This finding is consistent with the notion that phosphorylation of Pdc at serine 54 and/or 71 increases the rate of transducin trafficking to the OS, rather than serving as an early triggering event for the return of transducin in the dark. If phosphorylation of Pdc at serine 54 sets up a threshold for transducin trafficking to the OS, as proposed by Willardson and Howlett [41], we would expect that transducin return to the OS should be completely blocked in $Pdc^{S54/71A}$ mutants. This result also suggests that other principal phosphorylation sites may exist in Pdc, which may be key determinants for the interaction of Pdc and $G\beta\gamma t$. Alternatively, transducin may transport to the OS through other phosducin-independent pathways. Identification of such mechanisms triggering transducin movement to the OS is a challenge for future studies.

In summary, our studies provide the first *in vivo* evidence on the physiological role of Pdc phosphorylation in regulating trafficking of transducin in rod photoreceptors. In previous studies, we found that serine 54 and 71 of phosducin showed different phosphorylation responses under various background illuminations, implying distinct functions for these two phosphorylation sites [2]. Moreover, our data support that there may be other phosphorylation sites present on Pdc, in addition to serine 54 and 71 [42]. Further investigation of transgenic mice expressing Pdc lacking serine 54 and/or 71 will be helpful in gaining new insights about the function of Pdc and its phosphorylation state.

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Chapter 3

Phosducin-Like Protein 1 Is Required for Transducin Expression and the Viability of Photoreceptors

Hongman Song, Marycharmain Belcastro, Maxim Sokolov

This chapter is the initial part of the project “To establish the function of phosducin-like protein 1 (PhLP1) and CCT chaperones in photoreceptors”. In this part, I will present our ongoing effort on the generation and characterization of a transgenic mouse model, in which CCT/PhLP1 activity is suppressed in rod photoreceptors.

The co-author Marycharmain Belcastro participated in the experiments shown in Figure 4.

Introduction

In retinal photoreceptors, the rod-specific heterotrimeric G protein, transducin, functions as a key mediator of visual signal transduction responsible for the acquisition of visual information in dim light. High, invariable amounts of transducin, composed of the α and $\beta\gamma$ subunits ($G\alpha\beta\gamma t$), are required for the extremely sensitive response to light produced by rods [1-3]. To maintain physiologically optimal levels of transducin, photoreceptors tightly control synthesis, folding and assembly of transducin subunits. However, the mechanisms underlying this regulation remain poorly understood [4]. Moreover, previous studies have demonstrated that the levels of $G\beta\gamma t$ set the normal content of transducin heterotrimer and are crucial for the viability of photoreceptors [5-8]. It has become increasingly clear that the critical step of $G\beta\gamma t$ assembly depends on chaperone-assisted folding of $G\beta t$ [4]. Therefore, it is essentially important to understand the function of the chaperone proteins involved in the folding of nascent $G\beta t$ and assembly of $G\beta\gamma t$.

Phosducin-like protein 1 (PhLP1) is a ubiquitously expressed member of the phosducin gene family and binds $G\beta\gamma$ subunits with high affinity [9-12]. Recent studies have advanced our understanding towards the physiological function of PhLP1, which have completely reversed the originally proposed role of PhLP1 as a negative-regulator of G protein signaling through $G\beta\gamma$ sequestration [13]. Specifically, targeted disruption of the PhLP1 gene in *Dictyostelium Discoideum* significantly impaired G protein signaling due to the inability of functional $G\beta\gamma$ subunits to form on the membrane [14, 15]. The idea about a new function of PhLP1 has originated from the observation that PhLP1 binds to the chaperonin containing TCP1 (CCT) complex [16]. The chaperonin complex CCT is an essential eukaryotic chaperone that is required to assist the correct folding of

Gβ, a typical protein with seven-blade β–propeller structure, and the assembly of Gβγ subunits [17, 18]. It is now well-established that, in cell culture, PhLP1 acts as a co-chaperone to facilitate CCT-mediated folding of Gβ by first forming a ternary complex with CCT/Gβ and then being released as a PhLP1-folded Gβ complex, which is ready to form a dimer with Gγ [19-21]. Nevertheless, the functional significance of PhLP1 in vertebrate photoreceptors, which require large amounts of the visual G protein, transducin, for their function and viability, has never been explored.

In our previous work, we found that CCT, and in particular PhLP1, are expressed at high levels in photoreceptors, predominantly in the inner segments [22], where photoreceptor proteins are synthesized, folded and assembled. This localization profile suggests the specific demand for CCT/PhLP1 chaperone activity in photoreceptor biosynthetic pathways, most likely in the folding and assembly of Gβγt subunits. The present studies were done to understand the physiological significance of PhLP1 in retinal photoreceptors. To explore the role of PhLP1 in rod photoreceptors, we have suppressed PhLP1 activity in these cells. For this purpose, we have taken advantage of a short and rare splice variant of PhLP1 (PhLPs) that lacks the N-terminal 83 amino acids of PhLP1 because of alternative mRNA splicing (Figure 1) [9, 10, 23]. It has been shown that overexpression of PhLPs strongly inhibits endogenous PhLP1 function, and thus significantly decreases Gβγ expression in transiently transfected cell culture [19, 20, 23]. The potential mechanism of such a dominant-negative effect of PhLPs on PhLP1 activity is illustrated in Figure 2 [13]. According to this model, nascent Gβ binds to the folding chamber within the CCT and then PhLP1 binds the CCT/Gβ complex. PhLP1 binding facilitates the release of folded Gβ from CCT. PhLPs would compete with endogenous PhLP1 for binding of the CCT/Gβ complex to form a very stable ternary complex that would not release Gβ, suppressing the folding and assembly of Gβγ and

also the CCT activity. However, the function of native PhLPs is unclear and its expression levels are vanishingly low in most tissues except for the adrenal gland [10, 23, 24]. Normally, this splice variant is not expressed in the retina.

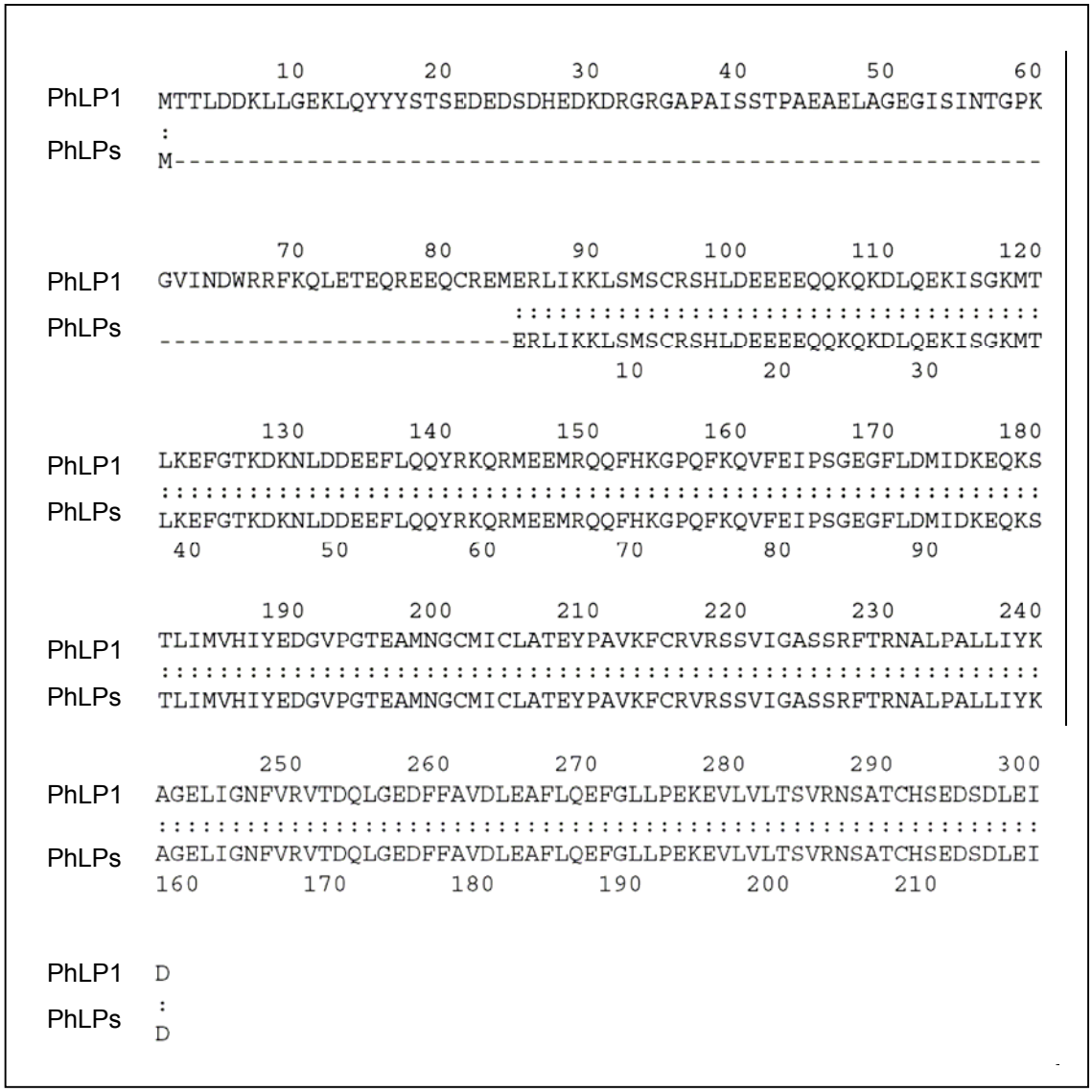


Figure1. Sequence alignment of mouse PhLP1 (NCBI accession number NP-080452) and its N-terminally truncated splice variant, PhLPs. The amino acid sequence of mouse PhLP1 and PhLPs were aligned using the Genestream Align server.

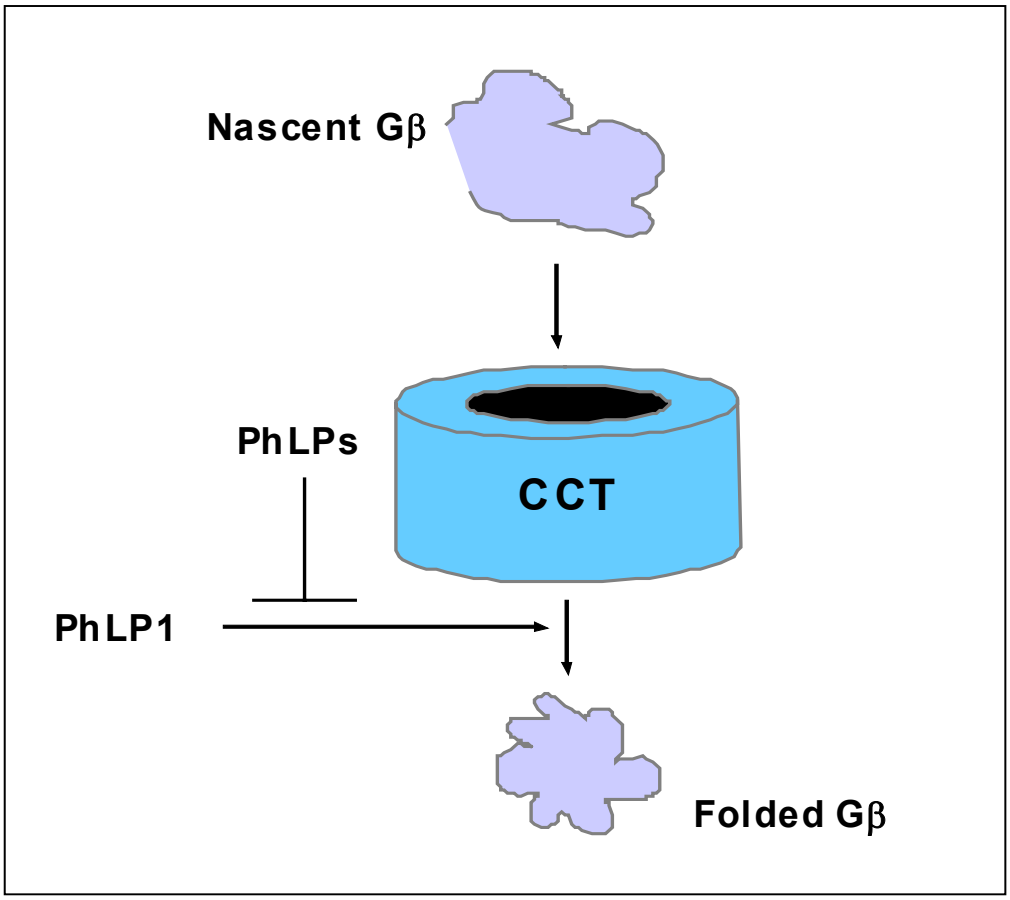


Figure 2. Proposed model of the dominant-negative effect of PhLPs on PhLP1 function. Nascent Gβ binds CCT within the folding chamber and PhLP1 associates with the Gβ–CCT complex to form a meta stable ternary complex, facilitating the release of folded Gβ from CCT. If PhLPs were expressed, it would compete with PhLP1 for binding to the Gβ-CCT complex to form a very stable ternary complex, preventing Gβ release and Gβγ assembly.

Thus, we have generated transgenic mice overexpressing PhLPs in rod photoreceptors to suppress endogenous CCT/PhLP1 activity in these cells and have utilized this transgenic mouse model to test our hypothesis that the CCT/PhLP1 chaperone complex plays a central role in the biosynthesis of transducin, one of the most abundant visual signaling proteins in retinal photoreceptors.

Experimental Procedures

Preparation of PhLP1 Δ1-83 transgene construct for creation of PhLPs transgenic mice

All experiments involving the use of mice were performed according to the protocols approved by the West Virginia University Animal Care and Use Committee. The mouse phosducin-like protein 1 Δ1-83 variant (PhLP1 Δ1-83 or PhLPs) with a 3' FLAG tag was constructed as follows. Wild-type mouse phosducin-like protein 1 (PhLP1) cDNA was obtained by RT-PCR with total RNA, which was isolated from 129SV mouse retinas, using the mouse PhLP1 gene-specific RT primer (5' – ACT AAA TGA GAC TAC AA – 3') and AccuScript^{TM} High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene). To create the PhLP1 Δ1-83 DNA construct, PhLP1 cDNA was amplified starting from the 84th codon and also engineered to be flanked by *Sal I*-Kozak-start codon at the 5' end and FLAG epitope tag-two stop codons-*BamHI* at the 3' end using PCR. The final FLAG-tagged PhLP1 Δ1-83 (PhLPs-FLAG) PCR product was subsequently cloned into the pBAM 4.2 vector [25] between the 4.4 kb mouse rhodopsin promoter and the mouse protamine I polyadenylation (MPI) signal [26] (Figure 3A), generating the pRho4.4k-PhLPs plasmid. The transgenic construct (4.4 kb rhodopsin promoter-kozak-PhLP1 Δ1-83-FLAG-MPI polyA) inserted into plasmid pRho4.4k-PhLPs was verified by sequence analysis.

Generation and genotyping of PhLPs transgenic mice

The purified plasmid pRho4.4k-PhLPs was digested with the restriction endonucleases *KpnI* and *SpeI* to release the PhLP1 Δ1-83 transgene. The digested plasmid DNA was gel purified with the Wizard® SV Gel and PCR Clean-Up system (Promega). To generate transgenic mice, the purified FLAG-tagged PhLP1 Δ1-83 DNA fragment, containing the 4.4 kb rhodopsin promoter, PhLP1 Δ1-83 - FLAG and MPI polyadenylation sequences, was microinjected into the pronuclei of zygotes from superovulated FVB females in the Transgenic Animal Core Facility at WVU Health Sciences Center. Transgenic founders were identified by PCR using mouse tail DNA with a pair of primers designed to amplify a 207 bp fragment near the 3' end covering the FLAG sequence. To establish the transgenic lines, founders were crossed with 129SV mice to overcome the $Pdeb^{rd1}$ mutation intrinsic to the FVB strain that cause retinal degeneration [27]. Protein levels of PhLPs-FLAG expression in different transgenic lines were confirmed by Western blot analysis. Totally, two transgenic mouse lines with various levels of transgene expression, designated as PhLPs-Low and PhLPs-High, have been established and maintained for further analysis.

Quantification of Protein levels using Western blotting

To quantify and compare the levels of proteins of interest, transgene-positive and their transgene-negative littermates were analyzed. Mice were sacrificed by flashing the box with CO_2 followed by cervical dislocation, and their retinas were harvested and quickly frozen on the dry ice. Two whole retinas were homogenized in 100 µl of SDS PAGE sample buffer containing 125 mM Tris/HCl, pH 6.8, 4% SDS, 6 M urea, and 10 mg/ml dithiothreitol by brief sonication. The extracts were cleared by centrifugation. Protein concentration in the extracts was determined and adjusted to 9 mg/ml. 10 µl aliquots of the extracts were separated on 10~20 % Tris-HCl gels (Bio-Rad), transferred onto polyvinylidene difluoride membrane Immobilon FL (Millipore). The tested proteins

were probed with specific primary antibodies (Table 1) and anti-rabbit, anti-mouse and anti-sheep secondary antibodies conjugated to either Alexa Fluor 680 (Invitrogen) or IRDye 800 (LI-COR Biosciences). Visualization and quantification of the specific bands was then performed on an Odyssey Infrared Imaging System (LI-COR Biosciences) following the manufacturer's protocols.

Table 1. Primary antibodies used for detection of specific proteins by Western blotting

Protein name	antibody Source
FLAG™ Conjugated Proteins	600-401-383 Rockland
Transducin alpha subunit (Gαt)	sc-389 Santa Cruz
Transducin beta subunit (Gβt)	sc-378 Santa Cruz
Transducin gamma subunit (Gγt)	sc-373 Santa Cruz
G protein alpha O (Gαo)	sc-387 Santa Cruz
Phosducin (Pdc)	sokolov et al. 2004
Rhodopsin	1D4 Sigma
Phosphodiesterase 6 alpha/beta subunit (PDE6 α/β)	Visvanathan Ramamurthy
Arrestin	Sc-34547 Santa Cruz
Recoverin	James B. Hurley
cAMP-dependent protein kinase regulatory II alpha subunit (PKA RIIα)	612242 BD Biosciences
T-complex polypeptide 1 subunit beta (TCP-1 β)	sc-47717 Santa Cruz
Phosducin-like protein 1 (PhLP1)	Barry M. Willardson
Heat shock protein 90 (HSP90)	sc-7947 Santa Cruz

Electroretinographic (ERG) Measurements

Electroretinograms were recorded in the UTAS-E4000 Visual Electrodiagnostic Test System using EMWIN 8.1.1 software (LKC Technology). In brief, dark-adapted mice were anesthetized with the continuous flow of a 2% isoflurane/50% oxygen mixture using an isoflurane vaporizer. Their pupils were dilated with a mixture of 1% tropicamide and 2.5% phenylephrine hydrochloride for 10 min. A reference needle electrode (LKC Technologies) was inserted underneath the skin on the head, and then a homemade silver wire electrode was placed on the cornea. A drop of the ophthalmic methycellulose was used to cover the cornea and protect the eyes from drying during the experiment.

Histological procedure

Whole eyes were enucleated, cleaned of outside tissue, and fixed in 10% formalin overnight. Fixed eye tissues were then dehydrated and paraffin-embedded. Paraffin-embedded eye tissues were sectioned to obtain 10-μm-thick sections for hematoxylin and eosin (H&E) staining, which were examined using light microscopy.

Results

Overexpression of PhLPs transgene triggers photoreceptor degeneration

To explore the function of PhLP1 in photoreceptors, we employed PhLPs, an effective dominant negative inhibitor for PhLP1, as a tool to suppress endogenous CCT/PhLP1 in photoreceptors. The transgenic construct and strategy used for generation of PhLPs mice is illustrated in Figure 3A and in the Experimental Procedure. FLAG-tagged PhLPs was directed to overexpress in rod photoreceptors under the control of a rhodopsin (Rho) promoter. Several transgenic founders carrying the PhLPs-FLAG transgene were identified by PCR using mouse tail DNA. At P08, protein levels of PhLPs-FLAG expression in different transgenic lines were determined by Western

blotting using anti-FLAG antibody. The blot analysis indicated that the levels of PhLPs-FLAG in the retinas varied between different transgenic lines (Figure 3B). Based on the expression levels of PhLPs-FLAG, two transgenic lines, designated as PhLPs-Low and PhLPs-High, have been established.

To determine the effects of overexpression of PhLPs-FLAG on rod function, electroretinogram (ERG) was used to record the massed field potentials evoked in the dark-adapted retina by light. A typical ERG response consists of the initial negative a-wave, which reflects the light-dependent current suppression in rod photoreceptors, and the following b-wave, which primarily reflects the response of the secondary retinal cells functioning downstream from photoreceptors. We found that the amplitude of the a-wave in 25-day-old PhLPs-Low mice was approximately two-fold smaller than that in PhLPs transgene negative littermates (Figure 3C, compare PhLPs-Low with Control). Moreover, no a-wave was detected in 25-day-old PhLPs-High mice (Figure 3C). These results indicated that transgenic expression of PhLPs in rods correlated with the loss of rod visual function in a dose-dependent manner.

Figure 3A

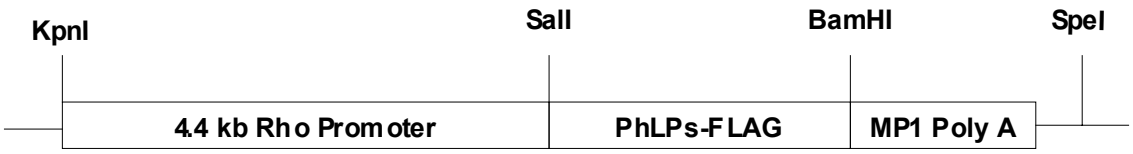


Figure 3B

Figure 3C

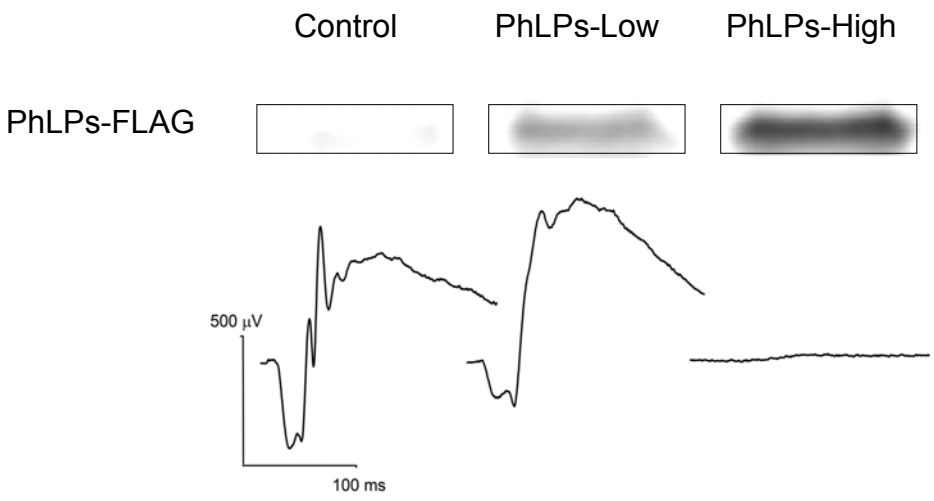


Figure 3 Loss of rod visual function triggered by overexpression of PhLPs in photoreceptor cells. 3A. Transgenic constructs used for the generation of PhLPs-FLAG transgenic mice. Rho, rhodopsion; MPI Poly A, mouse protamine I polyadenylation signal. 3B. Expression levels of FLAG-tagged PhLPs protein in PhLPs-Low and PhLPs-High mice. Retinas from control littlemates, PhLPs-Low, and PhLPs-High mice at P08 were homogenized. Retinal homogenates from each sample containing 90 μg of total retinal protein were subjected to SDS-PAGE and analyzed using Western blotting with anti-FLAG antibody. 3C. Representative ERG responses from dark-adapted control, PhLPs-Low and PhLPs-High mice at P25 evoked by 0.25 cd s^{-1}/m^2 light flashes.

Next, we have established the time course of photoreceptor degeneration in two PhLPs transgenic lines in order to define the onset of degeneration. We monitored retinal morphology in PhLPs-Low and PhLPs-High mice at different ages (P08, P12 and P25) (Figure 4). Specifically, as a sign of photoreceptor degeneration, photoreceptor loss was judged by examining the thickness of the outer nuclear layer (ONL). Compared with control littermates, there was no detectable change in the number of photoreceptor nuclei present in the outer nuclear layer at least up to P12 in PhLPs-Low mice, while approximately half of photoreceptors were lost by P25. On the other hand, no obvious difference in the thickness of ONL was observed between PhLPs-High and control littermates by P08. But at P12, more than half of PhLPs-High photoreceptors were gone with approximately three to four nuclei left per row (compared with ~ 10-12 in control littermates of this age). Moreover, almost all of PhLPs-High photoreceptors were lost at P25. Therefore, the analysis of retinal morphology in all PhLPs transgenic lines displayed a relatively fast but progressive pattern of photoreceptor degeneration. Corresponding to the various levels of PhLPs-FLAG expression, the onset of photoreceptor degeneration occurred earlier in PhLPs-High mice (between P08~P12) than in PhLPs-Low mice (between P12~P25). Taken together, our data revealed that there was a correlation between the levels of PhLPs-FLAG expression and the rate of photoreceptor degeneration. Thus, these results demonstrated that photoreceptor degeneration was triggered by overexpression of PhLPs in rod photoreceptors.

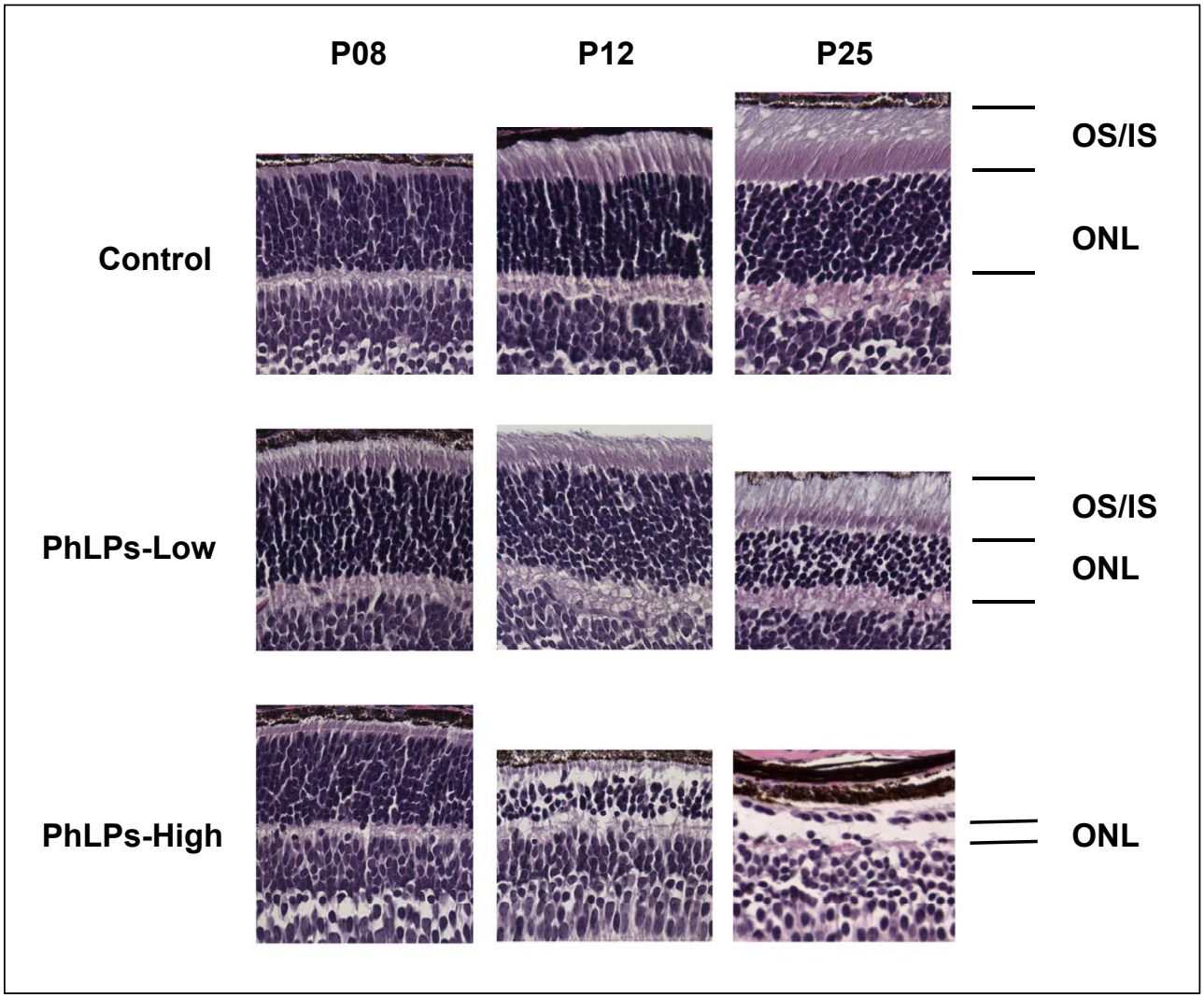


Figure 4 Progressive photoreceptor degeneration in PhLPs transgenic mice. Retinal morphology of PhLPs-Low and PhLPs-High mice at P08, P12, and P25 compared to that of control littermates. OS/IS, outer segment/inner segment; ONL, outer nuclear layer.

Identification of Proteins affected by PhLPs overexpression

We measured and compared the levels of several proteins in retinal extracts from control littermates and PhLPs-High mice at P08, prior to the onset of massive photoreceptor degeneration, using Western blot analysis (Table 2 and Figure 5). A striking effect of the PhLPs-High transgene was a significant reduction of the three transducin subunits, particularly the Gγt subunits (Table 2A). It was found that PhLPs-High retinas contained ~ 40% Gαt, ~ 50% Gβt and ~ 25% Gγt of control amounts. As expected, the content of another heterotrimeric G protein, Gαo, which is expressed in bipolar retinal neurons, was not changed between PhLPs-High mice and control littermates, indicating that the effects of PhLPs-FLAG overexpression was specific to the photoreceptors and did not affect secondary retinal neurons downstream from photoreceptors. These results demonstrated that inhibition of CCT/PhLP1 activity strongly impaired transducin biosynthesis in photoreceptors.

As shown in Table 2B and Figure 5, the expression level of phosducin (Pdc), a close homolog of PhLP1, was reduced by ~ 60%, suggesting that Pdc may be involved in the same biosynthetic pathways responsible for the folding and/or assembly of transducin subunits. Rhodopsin (Rho), an outer segment membrane protein, was also significantly downregulated (~60% reduction). The reduction of Rho is unlikely a consequence of photoreceptor degeneration because cGMP phosphodiesterase 6 (PDE6), another outer segment protein, remained at a similar level as in control littermates, and the morphology of PhLPs-High photoreceptors still looked normal at this age (Figure 4, PhLPs-High, P08). Thus, this phenomenon could be explained by inhibition of CCT function, leading to the downregulation of Pdc and rhodopsin, We also observed a slight decrease in the levels of arrestin and recoverin, which may be an indirect effect caused by suppressing CCT/PhLP1 activity. Finally, several chaperone proteins were analyzed

in PhLPs-High and control retinas (Figure 5 and Table 2C). The expression levels of the TCP-1β subunit of CCT, PhLP1 and heat shock protein 90 (HSP90) were not significantly affected by overexpression of PhLPs-FLAG in rod photoreceptors.

Table 2. Expression levels of indicated proteins in the retinas of 8-day old PhLPs-High mice represented as percentages of the amounts of corresponding proteins in control retinas. Retinas from control littermates and PhLPs-High mice at P08 were homogenized. Retinal extracts from each sample containing 90 μg of total retinal protein were subjected to SDS-PAGE and analyzed using Western blotting with specific antibodies against indicated proteins. Fluorescence values of specific protein bands in PhLPs-High retinas were normalized to the fluorescence of corresponding protein bands in control retinas (mean ± S.E., n=3). 2A, Heterotrimeric G proteins (transducin: Gαt, Gβt and Gγt, and Gαo). 2B, Visual signaling proteins (Pdc, Rho, PDE6 α/β, Arrstin, Recoverin, and PKA RIIα). 2C. Chaperone proteins (TCP-1β subunit of CCT, PhLP1, and HSP90).

2A

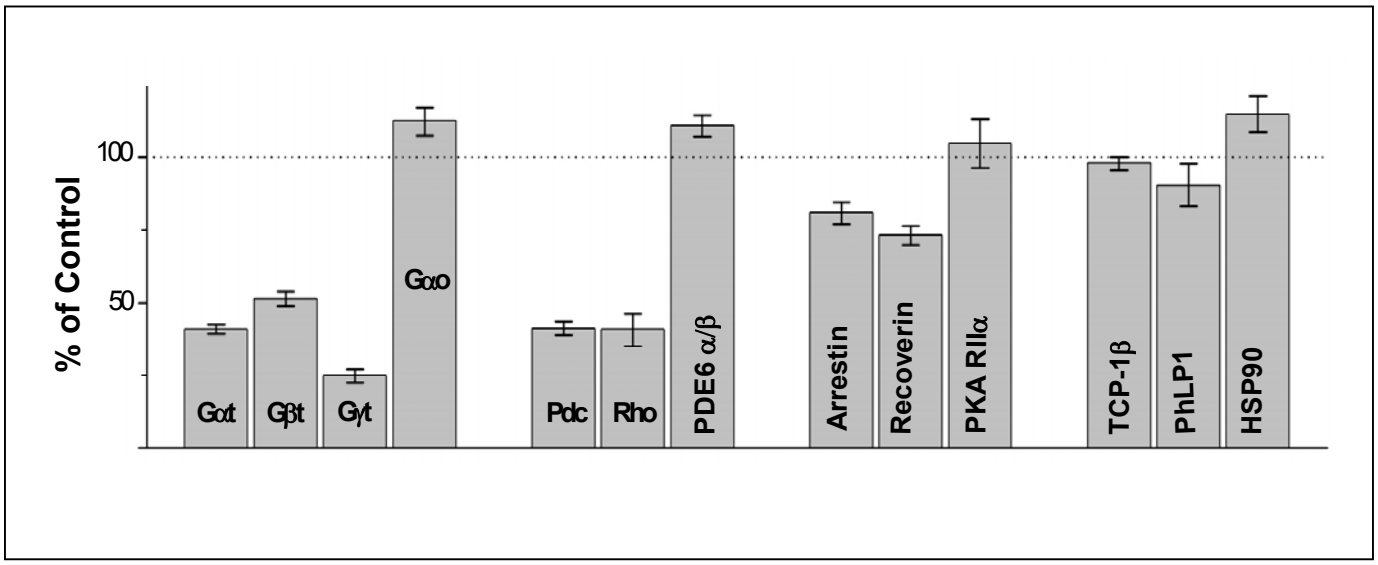
	Gαt (%)	Gβt (%)	Gγt (%)	Gαo (%)
PhLPs-High	40.9 ± 1.5	51.2 ± 2.4	25 ± 2.3	112.2 ± 4.9

2B

	Pdc (%)	Rho (%)	PDE6 α/β (%)	Arrestin (%)	Recoverin (%)	PKA RIIα (%)
PhLPs-High	41.2 ± 2.4	40.7 ± 5.6	110.7 ± 3.6	80.7 ± 3.7	73.2 ± 3.3	104.6 ± 8.3

2C

	TCP-1β (%)	PhLP (%)	HSP90 (%)
PhLPs-High	97.8 ± 2.2	90.2 ± 7.3	114.6 ± 6.3



<u>Figure 5</u> Significant reduction of transducin α, β, and γ protein levels caused by transgenic expression of PhLPs in photoreceptors. The levels of indicated proteins in 8-d old PhLPs-High retinas were plotted as a percent fraction of the levels of corresponding proteins in control retinas. The data were taken from the results summarized in Table 2.

Discussion

This study is the first investigation to address the physiological significance of the CCT/PhLP1 chaperone complex in retinal photoreceptors. The phenotypic analysis of PhLPs mice, in which the function of endogenous CCT/PhLP1 in photoreceptors was suppressed, revealed fast and severe photoreceptor degeneration. Another major finding of this work is that CCT/PhLP1, as an essential chaperone, plays an important role in the folding and assembly of transducin subunits and is required for maintaining transducin homeostasis. Therefore, our results demonstrate for the first time the critical role of healthy CCT/PhLP1 activity in visual signaling function and photoreceptor viability.

It was expected that Gβt was downregulated in PhLPs transgenic retinas, based on the dominant-negative inhibition of PhLPs on Gβ expression in cell culture. The levels of Gβt and Gγt expression depend on each other [5], because free Gβ and Gγ are not stable and susceptible to be degraded. This also is the reason why Gβγt always exists as a complex under physiological conditions [28]. Therefore, the Gγt level was decreased in PhLPs mice. Although the Gβt and Gγt reduction seems very straightforward, the explanation for the downregulation of Gαt is relatively complicated. It has been shown that Gα protein levels rely on the expression of Gβγ [5, 29, 30]. Gβγt can somehow protect Gαt from ubiquitin-dependent proteolysis [31]. Without Gβγt binding, free Gαt would be degraded by proteolysis, resulting in Gαt reduction. Another possible reason could be directly caused by suppression of CCT function, because Gαt is also defined as a folding substrate of CCT [32, 33]. Together, our data support our hypothesis that normal CCT/PhLP1 function is required to maintain the normal content of heterotrimeric transducin in photoreceptors.

The big puzzle of the present study is what mechanism(s) trigger photoreceptor degeneration in PhLPs mice. Elucidation of such mechanisms is an important challenge for future investigations. PhLPs can potentially compete with PhLP1 for binding CCT-Gβt, and prevent the release of Gβt (Figure 2). By doing so, it would interfere with the folding and assembly of Gβγt, causing Gβt to be retained inside the CCT complex and imposing stress on the photoreceptor cells that would eventually trigger cell death. This hypothesis, however, needs more direct and convincing evidence to be proven. There is another possible mechanism. Since the chaperonin CCT is an essential chaperone that assists in the folding of many soluble protein substrates, suppressing CCT function could cause accumulation of a large number of unfolded and misfolded proteins in the cells, which would impose stress on the ER and initiate the unfolded protein responses (UPR). ER stress and UPR could trigger apoptosis in cells [34]. In future studies, we will test this hypothesis by monitoring ER stress - and UPR - induced cell death in PhLPs transgenic retinas. Finally, we will also identify and study new protein candidates that are significantly affected by inhibition of CCT/PhLP1 function before the onset of photoreceptor degeneration using quantitative and comparative proteomic approaches.

In conclusion, PhLPs transgenic mice provide a valuable animal model to study the function of the CCT/PhLP1 chaperone complex in photoreceptors. The initial analysis of PhLPs mice emphasizes the functional importance of CCT/PhLP1 in transducin biosynthesis and photoreceptor survival. We anticipate that the ongoing studies with PhLPs mice will provide new insights into novel pathways leading to photoreceptor degeneration.

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Summary and future directions

Our studies of phosducin (Pdc) phosphorylation *in vivo*, described in Part I of chapter 2, yielded two major findings. First, phosphorylation sites serine 54 and serine 71 of Pdc are regulated differently by light, suggesting distinct functions for these two sites. We will investigate the function of individual sites using transgenic mice expressing Pdc without either phosphorylation site. Moreover, contrary to the accepted notion that light triggers dephosphorylation of Pdc, we found that serine 71-phosphorylated Pdc is significantly increased, rather than decreased, and exclusively localized in the outer segment during prolonged exposure to saturating light. In the future, we will also utilize the Pdc S71A phosphorylation mutant to further address the functional significance of this hyperphosphorylation of Pdc in the light.

The second significant observation is that phosphorylation of Pdc at serine 54 and 71 occurs in a compartment-specific manner, in which phosphorylated Pdc is specifically enriched in the inner segment adjacent to the entrance to the outer segment. This finding indicates that Pdc phosphorylation is engaged in transducin trafficking to the outer segments in photoreceptors. Therefore, in Part II of chapter 2, we have directly tested this hypothesis using transgenic mice expressing Pdc without two principal phosphorylation sites.

Based on the results in Part II of chapter 2, we have concluded that Pdc phosphorylation accelerates the trafficking of transducin to the outer segments. Considering the specific localization of phosphorylated Pdc, our data support the notion that Pdc acts as a chaperone for transducin trafficking in photoreceptors that may keep $G\beta\gamma t$ and $G\alpha t$ apart in the inner segments until a proper place and time during dark adaptation to allow the $G\alpha\beta\gamma t$ heterotrimer to traffick to the outer segments efficiently. To further enhance our conclusion, we plan to add more time points to obtain and compare

the time course of transducin return to the outer segments during dark adaptation in Pdc-FL and $Pdc^{S54/71A}$ mice. Furthermore, to correlate transducin trafficking in photoreceptors with the physiological function of rods, we will measure and compare the recovery of amplitude of the saturated ERG a-waves in Pdc-FL and $Pdc^{S54/71A}$ mice. We expect that $Pdc^{S54/71A}$ expression will slow down the recovery of the ERG a-waves, compared with that in Pdc-FL mice.

In chapter 3, we provided the first *in vivo* evidence to address the functional significance of the CCT/PhLP1 chaperone complex in retinal photoreceptors. From the analysis of PhLPs mouse models, in which the chaperone CCT/PhLP1 activity was suppressed in rod photoreceptors, we demonstrated that normal CCT/PhLP1 function is absolutely required for visual function and rod viability. Also, our data support our hypothesis that the CCT/PhLP1 chaperone complex plays a central role in the folding and assembly of transducin subunits. The big question raised from this study is what mechanisms trigger photoreceptor degeneration. Thus, the next step will be to identify proteins other than transducin that are significantly affected by inhibition of CCT/PhLP1 activity in photoreceptors. We will utilize our PhLPs mouse models to compare the expression levels of a large number of photoreceptor proteins in control littermates versus two PhLPs transgenic lines prior to the onset of photoreceptor degeneration by mass spectrometry-based quantitative proteomic analyses. In addition, as mentioned in chapter 3, we will monitor ER stress- and the unfolded protein response (UPR)-induced cell death in PhLPs transgenic retinas by examining UPR-specific markers, such as levels of the transcription factor CHOP (C/EBP homologous protein) or phosphorylation of eIF2α (the α sununit of eukaryotic translation initiation factor), etc.

Pdc is a close homolog of PhLP1. Moreover, the analysis of phosducin knockout mice indicated that Pdc is involved in posttranslational regulation of transducin expression. Given the similarities of structure and property between Pdc and PhLP1, we

propose that their regulatory roles on transducin homeostasis could be overlapped and complemented with each other. Our future studies will focus on establishing the functional connection between these two proteins, which will help determine the co-chaperone role of Pdc in the folding and/or assembly of Gβγt subunits.

In conclusion, my research provides strong evidence to support that Pdc and PhLP1 function as specific chaperones in the folding, assembly and trafficking of transducin subunits in rod photoreceptors. Further investigations on Pdc phosphorylation mutants and PhLPs mice are expected to more deeply decipher the physiological function of Pdc and CCT/PhLP1 in photoreceptors.

Appendix

List of Publications and Presentations

1. Boesze-Battaglia K, **Song H**, Sokolov M, Pankoski-Walker L, Gretzula C, Gallagher B, Rachel RA, Jenkins NA, Copeland NG, Morris F, Yeagle P, Williams D, Lillo C, Damek-Poprawa M (2007) "The tetraspanin protein, periferin-2 complexes with melanoregulin, a putative membrane fusion regulator" *Biochemistry* 46: 1256 –1272 (PMID: 17260955)
2. Lobanova ES, Finkelstein S, **Song H**, Tsang SH, Chen CH, Sokolov M, Skiba NP, Arshavsky VY (2007) "Translocation of transducin in rods is triggered by saturation of the GTPase activating complex" *Journal of Neuroscience* 27: 1151 – 1160 (PMID: 17267570)
3. Song JH, **Song H**, Wensel TG, Sokolov M, Martemyanov KA (2007) "Localization and differential interaction of R7 RGS proteins with their membrane anchors R7BP and R9AP in neurons of vertebrate retina" *Molecular and Cellular Neuroscience* 35: 311 – 319 (PMID: 17442586)
4. **Song H**, Belcastro M, Young EJ, Sokolov M (2007) "Compartment-specific phosphorylation of phosducin in rods underlies adaptation to various levels of illumination" *Journal of Biological Chemistry* 282: 23613 – 23621 (PMID: 17569665)
5. Krispel CM, Sokolov M, Chen YM, **Song H**, Herrmann R, Arshavsky VY, and Burns ME (2007) "Phosducin regulates the expression of transducin betagamma subunits in rod photoreceptors and does not contribute to phototransduction adaptation" *Journal of General Physiology* 130: 303 –12 (PMID: 17724163)
6. Cao Y, **Song H**, Okawa H, Sampath AP, Sokolov M, and Martemyanov KA (2008) "Targeting of RGS7/Gβ5 to the Dendritic Tips of ON-Bipolar Cells Is Independent of Its Association with Membrane Anchor R7BP" *Journal of Neuroscience* 28: 10443-10449 (PMID: 18842904)
7. **Song H**, Sokolov M (2009) "Analysis of Protein Expression and Compartmentalization in the Retinal neurons Using Serial Tangential Sectioning of the Retina" *Journal of Proteome Research* 8: 346-351 (PMID: 19049346)
8. **Song H**, Belcastro M, Sokolov M "Light controls compartment-specific phosphorylation of phosducin in rod photoreceptors" the Association for Research in Vision and Ophthalmology Annual Meeting 2007
9. **Song H**, Belcastro M, Jivotovskaya A, Sokolov M "Regulation of transducin homeostasis by phosducin in rod photoreceptors" the Association for Research in Vision and Ophthalmology Annual Meeting 2008
10. **Song H**, Belcastro M, Jivotovskaya A, Sokolov M "Phosducin regulates trafficking of transducin in rod photoreceptors" the Association for Research in Vision and Ophthalmology Annual Meeting 2009
11. Sokolov M, Belcastro M, **Song H** "Phosducin-like protein 1 is required for transducin expression and viability of retinal photoreceptors" the Association for Research in Vision and Ophthalmology Annual Meeting 2009