Phosphorylation of Phosducin Accelerates Rod Recovery from Transducin Translocation

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Purpose. In rods saturated by light, the G protein transducin undergoes translocation from the outer segment compartment, which results in the uncoupling of transducin from its innate receptor, rhodopsin. We measured the kinetics of recovery from this adaptive cellular response, while also investigating the role of phosducin, a phosphoprotein binding transducin βγ subunits in its de-phosphorylated state, in regulating this process.

Methods. Mice were exposed to a moderate rod-saturating light triggering transducin translocation, and then allowed to recover in the dark while free running. The kinetics of the return of the transducin subunits to the outer segments were compared in transgenic mouse models expressing full-length phosducin, and phosducin lacking phosphorylation sites serine 54 and 71, using Western blot analysis of serial tangential sections of the retina.

Results. In mice expressing normal phosducin, transducin α and βγ subunits returned to the outer segments with a half-time ($t_{1/2}$) of ~24 and 29 minutes, respectively. In the phosducin phosphorylation mutants, the transducin α subunit moved four times slower, with $t_{1/2}$ ~95 minutes, while the movement of transducin βγ was less affected.

Conclusions. We demonstrate that the recovery of rod photoreceptors from the ambient saturating levels of illumination, in terms of the return of the light-dispersed transducin subunits to the rod outer segments, occurs six times faster than reported previously. Our data also support the notion that the accumulation of transducin α subunit in the outer segment is driven by its re-binding to the transducin βγ dimer, because this process is accelerated significantly by phosducin phosphorylation. (*Invest Ophthalmol Vis Sci.* 2012;53:3084–3091) DOI:10.1167/iovs.11-8798

Phosducin (Pdc) is a cytosolic phosphoprotein implicated in the regulation of heterotrimeric G proteins based on its high affinity towards Gβγ subunits.[1–5] Pdc is highly expressed in the rod and cone photoreceptors of the vertebrate retina.[6,7] Knockout of the Pdc gene causes a reduction in the cellular levels of and a partial mislocalization of the visual G protein, transducin, in rod photoreceptors.[8,9] An in depth analysis of phosducin knockout mice also revealed the role of phosducin in regulating synaptic transmission,[10] hypothesized previously.[11] Some evidence suggests that Pdc also may be expressed in a variety of other tissues, however, at much lower levels.[12,13] The affinity of Pdc towards Gβγ is down-regulated by phosphorylation and consequent binding of the 14-3-3 protein.[11,14] In photoreceptors, the phosphorylation status of Pdc depends on the history of illumination. Two phosphorylation sites of Pdc, serine 54 and 73 (71 in the mouse sequence), are regulated by the concerted interplay of the activities of PKA, CaMKII, and PP2A, resulting in Pdc's dynamic phosphorylation in the dark and dephosphorylation in the light.[14–18] The physiological significance of light-dependent Pdc phosphorylation has remained unknown.

We tested the hypothesis that phosphorylation of phosducin regulates the trafficking of transducin subunits to the rod outer segment. For that, we generated transgenic mice expressing normal Pdc or a Pdc phosphorylation mutant in rod photoreceptors, and analyzed the kinetics of the return of the light-dispersed transducin subunits to the rod outer segments in these models. Our study reveals the physiological rate of the trafficking of transducin subunits to the outer segment, and the role phosducin phosphorylation has in accelerating this process.

Methods

Generation of Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG Transgenic Mice

All experiments involving animals were performed according to the procedures approved by the Animal Care and Use Committee of West Virginia University, and in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To prepare the wild-type mouse phosducin (Pdc) construct with a 3′-FLAG tag, total RNA was isolated first from the retina of a 129Sv mouse using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA), and the RNA was reverse transcribed using the mouse Pdc gene-specific RT primer 5′-CCC GAG TTT AAA TAG CC with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene). The polymerase chain reaction, using the Easy-A High-Fidelity PCR Master Mix (Stratagene) and the following primers, was used to amplify the coding sequence of the subsequent Pdc cDNA, and to add a Kozak sequence (GCCACCAUGG) to the 5′ end and a FLAG sequence to the 3′ end of Pdc, as well as two new stop codons: forward primer 5′-GCC ACC ATG GAA GAA GCC GCC AGC CAA AGC and reverse primer 5′-CTA CTA CTT GTC ATC GTC GTC CTT GTA ATC TTC AAT GTC CTC GTC TTC CAT GTT GG. The PCR product originally was cloned into the StrataClone PCR Cloning Vector pSC-A (Stratagene), but ultimately subcloned into

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the pBAM4.2 vector containing a 4.4 kb mouse rhodopsin promoter and a mouse protamine I polyadenylation sequence (MPI).[19] PCR amplification of the Kozak-WT Pdc-FLAG sequence from its pSC-A template was done using the forward primer 5′-GAG AGT CGA CGC CAC CAT GGA AGA AGC CGC C to add a *Sal*I site to the 5′ end, and using the reverse primer 5′-GCC TGG ATC CCT ACT ACT TGT CAT CGT CGT CC to add a BamHI site to the 3′ end. The subsequent PCR product and the pBAM4.2 vector were digested with *Sal*I and BamHI restriction endonucleases, gel purified, and ligated so the Kozak-WT Pdc-FLAG sequence would be inserted after the mouse rhodopsin promoter and before the mouse protamine I polyA sequence, creating pRhop4.4k-Pdc. An additional serine-to-alanine variant transgene of mouse Pdc at positions 54 and 71 was created from the pRhop4.4k-Pdc vector by using a PCR-based strategy and the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the following primers in subsequent order: For S54A conversion – forward primer 5′-CCT CAG ACA AAT GGC CTC TCC TCA GAG CAG and reverse primer 5′-CTG CTC TGA GGA GAG GCC ATT TGT CTG AGG. For S71A conversion – forward primer 5′-GAA AGA ATG AGC AGA AAG ATG GCC ATT CAA GAA TAT GAA CTC ATT C and reverse primer 5′-GAA TGA GTT CAT ATT CTT GAA TGG CCA TCT TTC TGC TCA TTC TTT C (with all underlined bases above indicating those changed from the wild-type sequence). The integrity of both constructs was confirmed by sequence analysis, and the final transgenes (4.4 kb rhodopsin promoter-Kozak-WT Pdc-FLAG-MPI polyA or 4.4 kb rhodopsin promoter-Kozak-Pdc$^{S54A/S71A}$-FLAG-MPI polyA) were removed from pRhop4.4k-Pdc and pRhop4.4k-Pdc$^{S54A/S71A}$, respectively, by digestion with restriction endonucleases *Kpn*I and *Spe*I, gel purified, and submitted to the West Virginia University Transgenic Animal Core Facility, where they were injected into the pronuclei of zygotes from superovulated FVB females. Transgene integration was determined in the offsprings by PCR analyses of tail DNA, and then transgenic expression of Pdc-FLAG or Pdc$^{S54A/S71A}$ -FLAG in the retinas was confirmed by Western blotting. Two independent transgenic lines expressing epitope-tagged Pdc of each kind were established by crossing selected founders with Pdc$^{-/-}$ mice.[8,9]

Quantification of Proteins by Western Blotting

Each retina was homogenized in 0.2 mL of urea sample buffer (USB) by short ultrasonic pulses, and the resulting extract was cleared by centrifugation. The total protein concentration was determined on a Nanodrop ND-1000 spectrophotometer, and then 5 mM N-ethylmaleimide (Sigma, St. Louis, MO) was added. To prepare SDS-PAGE samples, the protein concentration in each of the compared samples was adjusted to 0.5 mg/mL, and bromophenol blue tracking dye and 5% β-mercaptoethanol were added. Then, 5 μL of each retinal extract from Pdc-FLAG, Pdc$^{S54A/S71A}$-FLAG and wild-type (129Sv) mice were separated side-by-side on a 10% Tris-HCl acrylamide gel. Quantification of the specific bands corresponding to transducin α and β subunits, phosducin and β-tubulin, was performed simultaneously in each sample using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) according to the manufacturer's protocols. The fluorescence values of the transducin α, β and phosducin bands in each sample were normalized first to that of the β-tubulin band used as the loading control. Then, normalized bands in the adjacent wild-type, Pdc-FLAG, and Pdc$^{S54A/S71A}$-FLAG samples were compared. Data from two independent measurements using four mice of each genotype each time were averaged.

Pull Down of Epitope-Tagged Phosducin

Two retinas of each type were homogenized by short ultrasonic pulses delivered from a Microson ultrasonic cell disruptor equipped with a 3 mm probe (Misonix, Farmingdale, NY) in 0.2 mL of buffer containing 10 mM Tris/HCl, pH 7.0, 1.5% Igepal CA-630 (Sigma), and 5 mM N-ethylmaleimide. Cellular insoluble parts were cleared by centrifugation, and each supernatant was incubated with 10 μL of anti-FLAG M2-Agarose beads (Sigma) for 1 hour at room temperature. Beads were washed 4 times for 3 minutes with 1.0 mL of buffer, and bound proteins were eluted with 0.1 mL of USB buffer containing 125 mM Tris/HCl, pH 6.8, 4% SDS, and 6M urea. The samples then were analyzed by Western blotting using antibodies against transducin β and phosducin.

Light Conditioning of Mice and Analysis of Protein Localization in Rod Photoreceptors

Mice were anesthetized with a 2% isoflurane/98% oxygen mixture, and their pupils were dilated with one drop per eye of a mixture of 1.25% phenylephrine hydrochloride and 0.5% tropicamide ophthalmic solution for 10 minutes. Anesthetized mice were exposed to a pre-set 200 cd/m^2 white background light inside the light dome of a UTAS-E4000 Visual Electrodiagnostic Test System (LKC Technologies, Gaithersburg, MD), equivalent to 600 ± 50 photopic lux luminance at the cornea as measured by a traceable light meter (Fisher Scientific, Pittsburgh, PA) for 10 minutes. Following light conditioning, mice were allowed to awaken from gas anesthesia, which usually took a minute, and then they were placed in their original cages to recover in the dark. Mice were sacrificed at different time points of dark recovery, and the protein localization in rod photoreceptors was analyzed using Western blotting of serial tangential sections of the frozen retina, according to the latest modification[20] of a previously described procedure.[21] For indirect immunofluorescence detection of rod transducin, eyes were enucleated first, and fixed for 10 minutes with Oculo Fix (BBC Biochemical, Pittsburgh, PA). The anterior portions of the fixed eyes then were removed, and the eyecups were fixed further with Oculo Fix for 3 hours. Fixed eyecups then were cryoprotected with 30% sucrose in 1X PBS overnight. The eye cups then were transferred to a 1:1 tissue freezing medium (TFM; TBS Triangle Biomedical Sciences, Inc., Durham, NC) −30% sucrose solution for 2 hours, and then embedded in TFM, and frozen. The frozen blocks then were cut radially in 16 μm sections on a Leica CM1850 Cryostat, and the sections stored at −20°C. Before immunostaining, frozen sections were thawed at room temperature for 10 minutes, hydrated in 1× PBS for 5 minutes, and blocked with goat-serum containing 5% Triton-X 100 for 30 minutes. Sections were probed first with primary antibody against transducin α at a 1:1000 dilution overnight, and then probed with Alexa Fluor 488 or 568 conjugated secondary antibody (Invitrogen) for 1 hour. The tissue sections also were counterstained with either propidium iodide (EMD Biosciences/Calbiochem, San Diego, CA) or 100 ng/mL DAPI (Roche, Indianapolis, IN) for rod nuclear staining. Images were acquired on a Zeiss LSM 510 Meta confocal microscope after adjusting the detector gain for all the fluorescent channels, and the amplifier offset for background corrections, and while keeping the amplifier gain of the lasers to a minimum. Identical parameters were used when comparing samples.

Determination of the Levels of Rhodopsin

A protocol modified from that of Song et al.[18] was used as follows. All procedures were performed under dim red light. Each retina was harvested, and homogenized in 0.2 mL of water containing 2.5% n-octyl β-D-glucopyranoside (BP585-1; Fisher Scientific) and 1.25% cetyltrimethylammonium chloride (292,737, Sigma) by short ultrasonic pulses. The extract was cleared by centrifugation on a Centrifuge 5415 D (Eppendorf, Hauppauge, NY) at 16,000 revolutions per minute for one minute. The absorption 400–700 nm spectrum of supernatant then was obtained in a UV-Mini 1240 spectrophotometer (Shimadzu, Columbia, MD), and assigned as a baseline, after which the sample was bleached for 1 minute with bright white light, and the spectrum was obtained again. Rhodopsin concentration was determined from a 500 nm negative peak. The average level of rhodopsin bleaching was calculated by comparing light-conditioned and dark-adapted mice using retinas from at least 4 mice of the same age and genetic background in each group.

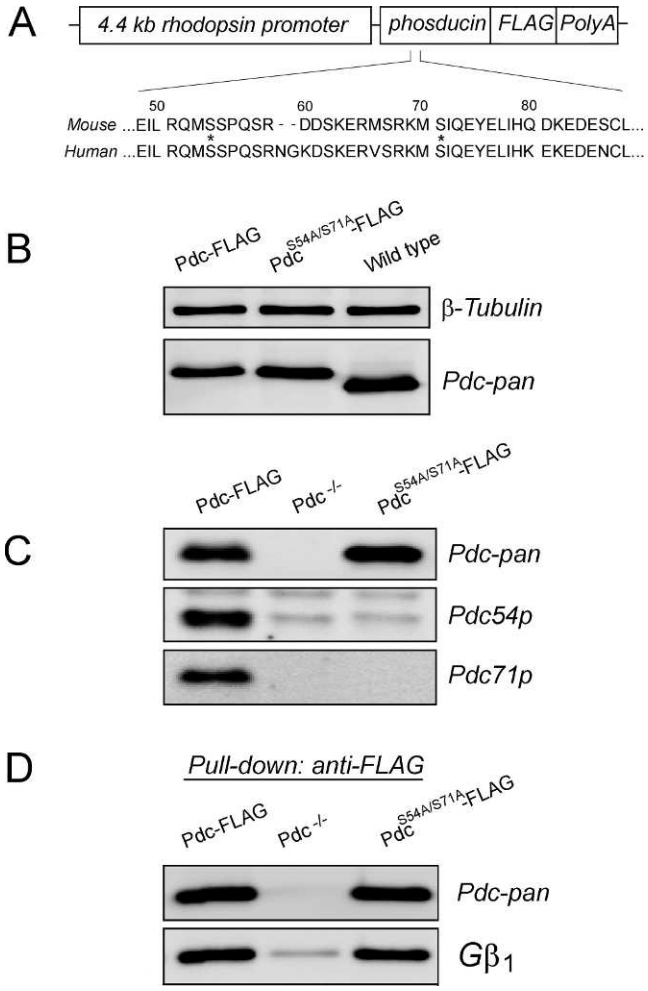

Figure 1. (**A**) Transgenic construct used for the generation of Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG mutant mice. Partial amino acid sequence alignment of human and mouse phosducin showing the positions of serine 54 and 71 (*), which were substituted with alanine in the Pdc$^{S54A/S71A}$-FLAG mutant. (**B**) Representative Western blot shows the levels of epitope-tagged or endogenous phosducin in whole retina extracts of Pdc-FLAG, Pdc$^{S54A/S71A}$-FLAG, and wild-type (129Sv) mice. (**C**) Freshly dissected retinas from dark-adapted mice of the indicated genotypes were incubated with 10 nM okadaic acid in the dark for 20 minutes, and the phosphorylation of phosducin at serines 54 and 71 was assayed by Western blotting, using pan-specific (Pdc-pan) and phospho-specific antibodies against Pdc phosphorylated at serine 54 (Pdc54p) and serine 71 (Pdc71p). (**D**) Phosducin was pulled-down by its FLAG tag from retinal extracts, and $G\beta_1$ protein in the pull-downs was detected by Western blotting using an anti-$G\beta_1$ antibody. Pdc-pan antibody blots show equal levels of the FLAG-tagged Pdc in the extracts.

Antibodies

We used primary antibodies against transducin α ($G_t\alpha_1$) (sc-389; Santa Cruz Biotechnology, Santa Cruz, CA), transducin β (PA1-725; Applied BioReagents; Golden, CO), Gβ (sc-378; Santa Cruz Biotechnolgy), $G\gamma_1$ (sc-373; Santa Cruz Biotechnology), β-tubulin (T0198; Sigma Aldrich, St. Louis, MO), visual arrestin (V4389; Sigma), and cytochrome c oxidase subunit I (COX I; MS404, MitoSciences, Eugene, OR). Antibody against rod outer segment membrane protein (rom1) was provided generously by Andrew Goldberg, Oakland University. Pan-specific (Pdc-pan) and phospho-specific (Pdc54p and Pdc71p) antibodies against phosducin were generated in the lab.[18]

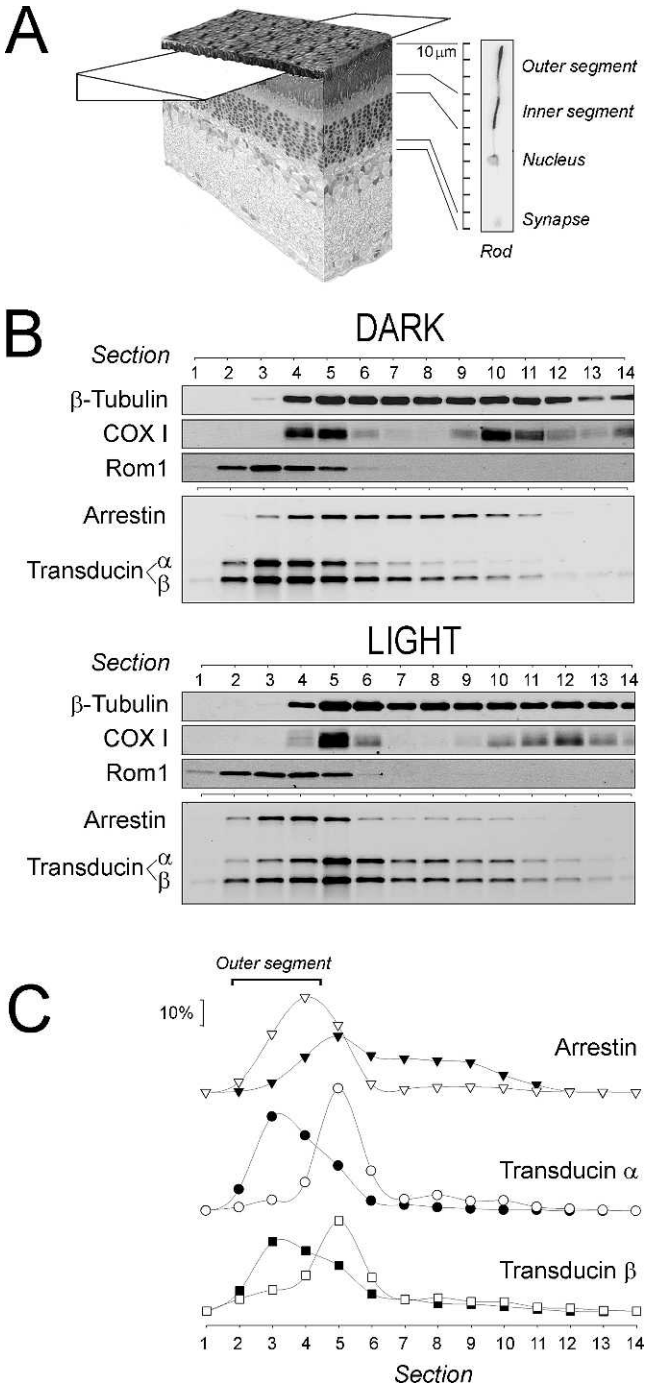

Figure 2. (**A**) Schematic illustration of how a flat-mounted fragment of retina was sectioned. *Inset*: cellular compartments of an individual mouse rod expressing GFP (unpublished image obtained in this laboratory) and their distribution in serial sections of the retina. (**B**) Representative blots showing the distribution of protein markers of different rod cellular compartments (β-tubulin, COX I, and rom1), and arrestin, and transducin α and β subunits throughout a set of serial tangential sections (1–14) of the retinas of a mouse dark-adapted for 840 minutes (DARK) following 10 minutes of light conditioning with 600 lux (LIGHT). (**C**) Quantification of arrestin (*triangles*), and transducin α (*circles*) and β (*squares*) subunits distribution in blots shown in (**B**). The fluorescence value of a specific band in each section on the corresponding blot was plotted as a percentage of the total fluorescence value of all bands in all sections on the blots, dark-adapted mouse (*closed symbols*), light-conditioned mouse (*open symbols*).

Table 1. Fractions of Protein Found in the Rod Outer Segment at Different Times of Dark Recovery after Light Conditioning

		Time of Dark Recovery (Percent of Total Present in the Outer Segment)			
Mouse Model	**Protein**	**0 Mins.**	**60 Mins.**	**120 Mins.**	**840 Mins.**
Pdc-FLAG	Transducin α	12 ± 3%	64 ± 3%	79 ± 4%	74 ± 8%
	Transducin β	33 ± 2%	59 ± 1%	71 ± 6%	67 ± 4%
	Arrestin	64 ± 7%	28 ± 3%	16 ± 4%	6 ± 1%
Pdc $^{S54A/S71A}$-FLAG	Transducin α	23 ± 6%	42 ± 5%	53 ± 4%	75 ± 2%
	Transducin β	39 ± 5%	62 ± 10%	49 ± 5%	62 ± 1%
	Arrestin	59 ± 1%	27 ± 3%	11 ± 2%	9 ± 1%

SEM, $n = 4$.

Results

Generation of Transgenic Mouse Models Expressing Normal Phosducin or a Phosducin Phosphorylation Mutant in Rods

DNA constructs encoding epitope-tagged full-length mouse phosducin (Pdc-FLAG) and phosducin carrying serine-to-alanine substitutions at residues 54 and 71 (Pdc $^{S54A/S71A}$-FLAG, Fig. 1A) were expressed in transgenic mice under the control of a 4.4 kb rhodopsin promoter[19] targeting expression of the constructs to rod photoreceptors. Founders expressing similar protein levels of epitope-tagged and endogenous Pdc were backcrossed with Pdc-null mice for several generations. As a result, we established several transgenic lines (Tg-Pdc-FLAG$^{+/-}$;Pdc$^{-/-}$ and Tg-Pdc$^{S54A/S71A}$-FLAG$^{+/-}$;Pdc$^{-/-}$ hereafter designated as Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG, respectively) where the endogenous photoreceptors' Pdc was substituted with transgenic Pdc-FLAG or Pdc$^{S54A/S71A}$-FLAG (Fig. 1B). Pdc-FLAG mice expressed their epitope-tagged Pdc at 46 ± 2% (SEM, $n = 4$) of the normal Pdc level. Such levels of Pdc expression were deemed normal, since Pdc$^{+/-}$ mice expressing only half the normal Pdc level essentially have a wild-type phenotype (data not shown). The expression level of Pdc$^{S54A/S71A}$-FLAG was 141 ± 5% (SEM, $n = 4$) compared to that of wild-type Pdc. Western blot analysis using antibodies specific to Pdc phosphorylated at serine 54 and serine 71[18] revealed robust phosphorylation of both sites in the retinas of Pdc-FLAG mice incubated with the protein phosphatase inhibitor, okadaic acid, in the dark (Fig. 1C). This phosphorylation was completely absent in the retinas of Pdc$^{S54A/S71A}$-FLAG mice (Fig. 1C). This result confirmed further that the mutations introduced in Pdc$^{S54A/S71A}$-FLAG eliminated both principal phosphorylation sites of Pdc that are regulated in a light-dependent manner in vivo.[18,22] All other tested properties of Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG were similar. Like the endogenous Pdc, epitope-tagged Pdc was present in all cellular compartments of the rod cell (data not shown). In light-adapted retinas, when Pdc is dephosphorylated, Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG co-precipitated with significant amounts of $G\beta_1$, indicating that the C-terminal epitope-tagging does not affect the high affinity of Pdc towards G-protein β subunits (Fig. 1D). Additional evidence of the functional activity of transgenic Pdc was the rescue of the $G\beta_1$ expression in rods, reduced as a result of the Pdc gene knockout.[8,9] As such 94 ± 2% and 97 ± 3% (SEM, $n = 4$) of the wild-type $G\beta_1$ levels were found in the rods of the Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG transgenic lines, respectively. The level of transducin α subunit, slightly reduced in Pdc-null rods,[9] also was restored completely in Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG lines. Thus, both types of epitope-tagged phosducin, Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG, were similarly efficient in the restoration of transducin levels in vivo, indicating that phosphorylation of Pdc at serines 54 and 71 does not have a significant role in the regulation of this Pdc function.

Comparative Analysis of Transducin Translocation in Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG Mice

We used Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG transgenic mice to assess the effect of Pdc phosphorylation on the light-driven translocation of rod transducin. To initiate translocation of transducin from the rod outer segment, all mice were subjected to a standard 10 minutes of light-conditioning with 600 lux of white light under gas anesthesia, after which animals were allowed to wake up and recover in the dark while free running. Mice were euthanized at 0, 60, 120, and 840 minutes of dark recovery, and their retinas were harvested and frozen. The protein distribution in rods was analyzed quantitatively by Western blotting serial sections of the retina (Fig. 2A). This technique, originally described by Sokolov et al.,[21] was modified recently by us for the sectioning of mouse retina.[20] In these experiments, we monitored simultaneously localization of arrestin and transducin $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits. Two protein markers, rom1 and COX I, were used to identify the rod outer/inner segment boundaries, and β-tubulin was used as a loading control for all compartments except rod outer segment (Fig. 2B). To quantify the data, the fluorescence of a specific band in each section was plotted as a percentage of the combined fluorescence in all sections (Fig. 2C), and then the fraction present in the outer segment was calculated.[21]

We found that when Pdc-FLAG mice were dark-adapted overnight, on average 74% of $G_t\alpha_1$, and 67% of $G\beta_1\gamma_1$ localized to the rod outer segments (Table 1, dark recovery 840 minutes), and a nearly identical distribution of these proteins was observed in Pdc$^{S54A/S71A}$-FLAG mice, indicating that Pdc phosphorylation has no significant effect on the equilibrium distribution of transducin during prolonged dark-adaptation.

Light triggered translocation of $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits from the rod outer segments in both types of mice (Table 1, dark recovery 0 minutes). Immediately after the light conditioning, the fraction of $G_t\alpha_1$ in the outer segment of Pdc-FLAG mice was reduced to 12% (6-fold reduction), whereas 23% of $G_t\alpha_1$ (3-fold reduction) was retained in the outer segment of Pdc$^{S54A/S71A}$-FLAG mice. The corresponding fractions of the $G\beta_1\gamma_1$ subunits complex were 33% (2-fold reduction) and 39% (1.6-fold reduction) in Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG mice, respectively. We applied the same light conditioning protocol to $G_t\alpha_1^{-/-}$ mice. In these mutants, characterized by the complete absence of $G_t\alpha_1$ expression,[23] the free $G\beta_1\gamma_1$ subunits become unable to accumulate in the outer segment under any condition of illumination.[24,25] We found that 30 ± 4% (SEM, $n = 4$) of $G\beta_1\gamma_1$ dimer was present in the outer segments of $G_t\alpha_1^{-/-}$ mice under this condition. Therefore, these data suggest the light conditioning used here evoked the maximum possible translocation of transducin in mouse rods, apparently approaching the equilibrium distributions of fully dissociated $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits in the context of these cells.

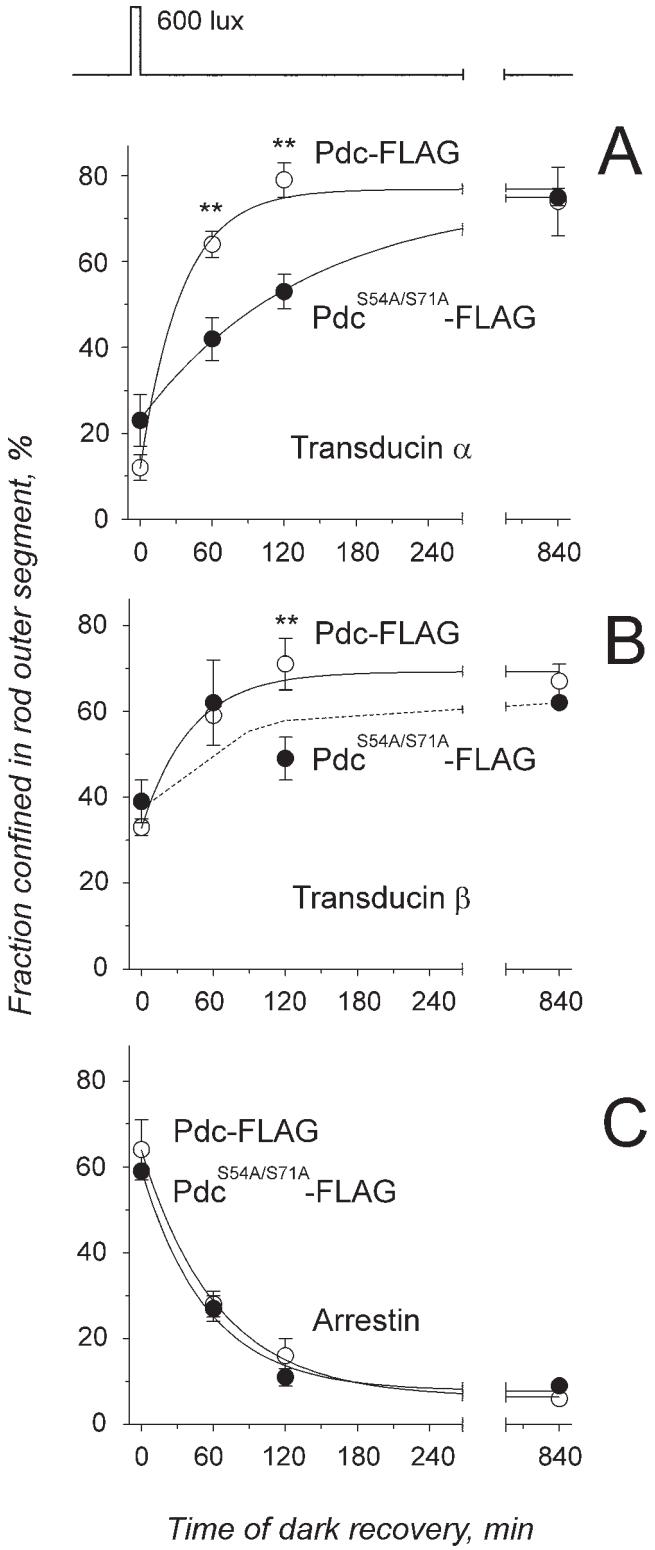

Figure 3. (**A**) Data for the transducin α subunits from Table 1 for Pdc-FLAG mice (*white circles*) and Pdc$^{S54A/S71A}$-FLAG mice (*black circles*) were fitted with exponential rise to maximum function $y = y_o + a(1 - e^{-kx})$. The half-time ($t_{1/2} = \ln[2]/k$) of transducin α return to the rod outer segment was 24 and 95 minutes in Pdc-FLAG mice and in Pdc$^{S54A/S71A}$-FLAG mice, respectively. (**B**) Similar analysis as in (**A**) revealed $t_{1/2} = 29$ minutes for the transducin β movement in Pdc-FLAG mice. Movement of this subunit in the Pdc$^{S54A/S71A}$-FLAG strain did not obey exponential kinetics (*dashed line*) and its $t_{1/2}$ could not be determined. (**C**) Fitting of the arrestin data from Table 1 was performed using an exponential decay function $y = y_o + ae^{-kx}$. Arrestin moves from the outer segment with $t_{1/2} = 44$ and 38 minutes in Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG mice, respectively. For all data a *t*-test was applied: no asterisk indicates a P value > 0.1; (**) indicates a P value < 0.05.

When this process was reversed by allowing mice to recover in the dark, Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG mice showed profound differences in the rates of $G_t\alpha_1$ subunit return to the outer segment (Table 1, dark recovery 60 and 120 minutes). When fitted with an exponential rise to maximum, this process had a half-time ($t_{1/2}$) of 24 minutes in Pdc-FLAG and 95 minutes in Pdc$^{S54A/S71A}$-FLAG mice (Fig. 3A), thus demonstrating that $G_t\alpha_1$ returned to the outer segment four times faster if Pdc could undergo phosphorylation. In all these experiments, the accumulation of $G\beta_1\gamma_1$ dimer in the rod outer segment has similar kinetics in both types of mice during the initial 60 minutes of dark recovery (Fig. 3B). At 120 minutes of dark recovery, the movement of $G\beta_1\gamma_1$ in Pdc$^{S54A/S71A}$-FLAG mice became noticeably irregular and deviated significantly from the exponential kinetics observed in Pdc-FLAG mice (Fig. 3B). These data demonstrate that phosphorylation of Pdc primarily controls the rate of $G_t\alpha_1$ return to the outer segment, while also affecting some later stages of the $G\beta_1\gamma_1$ accumulation. Importantly, subcellular movement of rod arrestin, which also is controlled by illumination, though in the opposite direction of transducin,[26] appeared to have identical kinetics in Pdc-FLAG and Pdc$^{S54A/S71A}$-FLAG mice (Table 1 and Fig. 3C), indicating that this effect of Pdc phosphorylation was specific for the trafficking of transducin.

To rule out the possibility that the slower rate of transducin movement observed in Pdc$^{S54A/S71A}$-FLAG mice was caused by the approximately 3-fold transgene overexpression in this versus Pdc-FLAG animals (46% and 142% of the endogenous level, respectively), we analyzed mice overexpressing wild-type phosducin. These mice were generated by back-crossing Pdc-FLAG mice into a 129Sv background (Tg-Pdc-FLAG$^{+/-}$;Pdc$^{+/+}$ designated as Pdc-FLAG x WT). Although the combined level of phosducin in their photoreceptors exceeded that in Pdc$^{S54A/S71A}$-FLAG mice by 2.5-fold (Fig. 4A), following standard light conditioning and 60 minutes dark recovery, their transducin α subunits still returned to the rod outer segments appreciably faster than in Pdc$^{S54A/S71A}$-FLAG mice (Fig. 4B). We concluded, therefore, that the ability of Pdc to undergo phosphorylation, rather than its intracellular level, is responsible primarily for the acceleration of the trafficking of transducin to the rod outer segments.

Accessing the Effect of Rhodopsin Bleaching on the Rate of Transducin Return to the Outer Segment

We compared the rates of recovery from transducin translocation in rods of wild-type mice conditioned with dim and bright light. For that, we used our standard experimental setup to initiate transducin translocation by exposing one group of mice for 10 minutes to 140 lux light, whereas the second group of mice was conditioned with significantly brighter 3300 lux light. We determined that these light intensities caused bleaching of 37 ± 4% and 98 ± 1% of total rhodopsin in the retina, respectively (SEM, $n = 4$). The subcellular localization of transducin in both groups then was compared at certain time points of dark recovery (Fig. 5). Immediately after light conditioning, we observed the very characteristic pattern of transducin distribution in both groups that was reported previously by us and others.[21,27–30] This subcellular distribution is consistent with transducin undergoing translocation

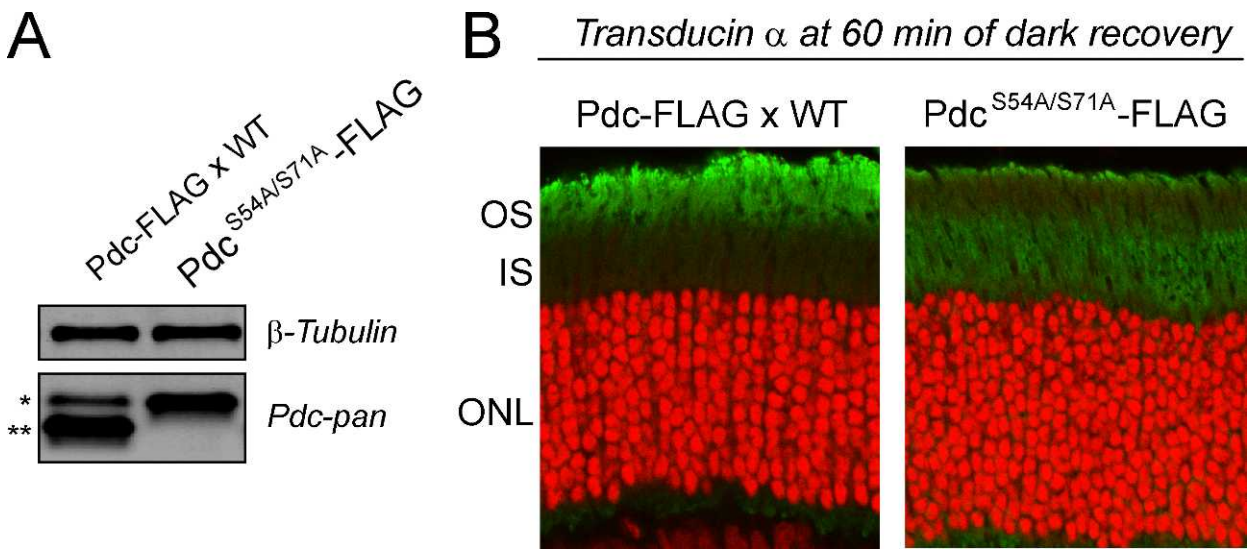

Figure 4. (**A**) Representative Western blot showing the levels of epitope-tagged (*) and endogenous (**) phosducin in the whole retina extracts of Pdc-FLAG mice back-crossed on the wild-type background (Pdc-FLAG × WT) and $Pdc^{S54A/S71A}$-FLAG mice. The combined level of phosducin (* and **) in Pdc-FLAG × WT retinas was 248 ± 17% (SEM, $n = 4$) of that of the mutant phosducin (*) expressed in $Pdc^{S54A/S71A}$-FLAG retinas. (**B**) Subcellular localization of transducin α subunit (*green*), as determined by immunofluorescence microscopy, in Pdc-FLAG × WT and $Pdc^{S54A/S71A}$-FLAG mice dark-adapted for 60 minutes following 10 minutes of light conditioning with 600 lux light. Retinal layers are abbreviated as OS (outer segment), IS (inner segment), and ONL (outer nuclear layer).

from the rod outer segments, its predominant location in dark adapted rods (Fig. 5, 12 hours of dark recovery), all the way toward the synapse, which then makes transducin unambiguously detectable in the outer nuclear and outer plexiform layers of the retina (Fig. 5, 0 minutes of dark recovery). Transducin immunostaining in these layers became reduced appreciably after 30 minutes of dark recovery; however, only in mice conditioned with 140 but not 3300 lux light (Fig. 5, compare 30 minutes of dark recovery, upper and lower panel). Therefore, to our knowledge these results provide the first evidence that rods conditioned with moderate saturating light, resulting in the bleaching of a smaller fraction of the rod's

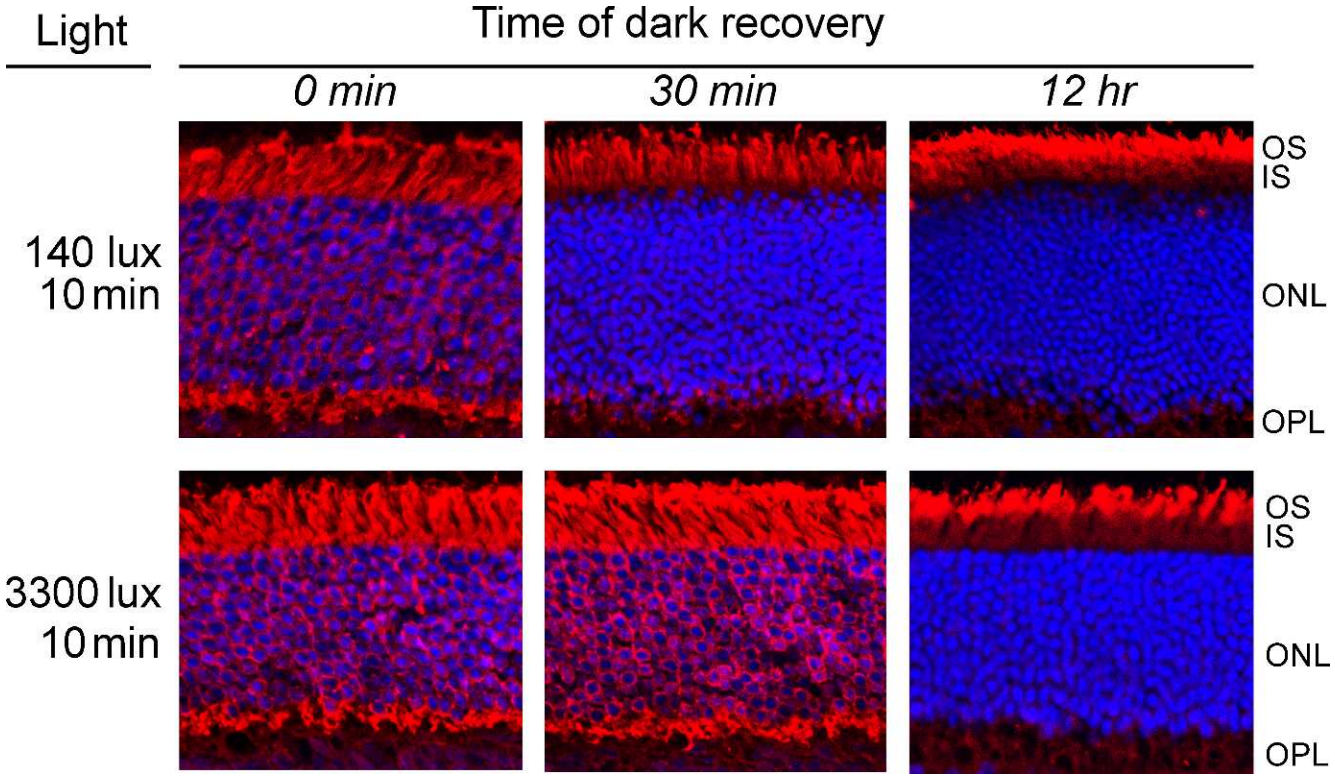

Figure 5. Subcellular localization of transducin α subunit (*red*) in cross-sections of retina from wild-type mice dark-adapted for 0 minutes, 30 minutes, and 12 hours, following 10 minutes of light conditioning with 140 lux and 3300 lux light. Photoreceptor nuclei are shown in *blue*. OPL, outer plexiform layer.

pigment, can recover from transducin translocation faster than rods exposed to excessive illumination.

Discussion

As a potential mechanism of long-term light adaptation, bidirectional movement of the visual G protein, transducin, from the light-sensing outer segment compartment of rod photoreceptors has been the focus of numerous studies in the field.[31,32] Our current understanding of the mechanism whereby transducin departs from the outer segment is based on the premise that the sustained dissociation of heterotrimeric transducin reduces its membrane-attachment significantly, and then separated $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits re-distribute through the rod cytoplasm by diffusion.[27–29] However, the molecular mechanism of the reverse event, resulting in the accumulation of transducin in the outer segment, remains controversial. Two distinct models based either on diffusion or active transport often are debated (for review see the study of Artemeyev[33]), and the following observations made in our studies are pertinent to this discussion.

First, the rate of transducin accumulation in the rod outer segments reported in our study is six times faster ($t_{1/2}$ ~30minutes) than that measured previously in rats and mice ($t_{1/2}$ ~200 minutes).[21,30] A possible explanation for this discrepancy is that in our experiments we used a much milder light to evoke transducin translocation. Because rhodopsin decay intermediates produce "equivalent light" that activates transducin,[34] transducin translocation from the outer segment could be sustained during dark recovery if these products are built up in significant amounts. Therefore, it is plausible, under our standard light conditioning (10 minutes of 600 lux light), on average resulting in only 80 ± 2% total rhodopsin bleaching (SEM, $n = 4$), that rods accumulate less rhodopsin decay intermediates than in the aforementioned studies. Indeed, this hypothesis is supported by our data demonstrating that movement of transducin towards the rod outer segments is delayed when a bigger fraction of rod pigment has been bleached during light conditioning (Fig. 5). Importantly, our measurements demonstrate that the transducin subunits dispersed from the outer segment by light could move back to this compartment significantly faster than known previously.

Our central observation, that phosphorylation of Pdc accelerates the trafficking of $G_t\alpha_1$ to the outer segment, perhaps could be explained best in terms of a mechanism of phosphorylation-dependent regulation of heterotrimeric G proteins by Pdc, something originally proposed by Gaudet et al.[35] Based on structural[2] and biochemical[36] evidence, it was proposed that serine residues 54 and 71 of Pdc are located within a loosely assembled N-terminal domain, which makes these sites accessible to a protein kinase even in the Pdc-$G\beta_1\gamma_1$ complex. When such a phosphorylation event occurs, it triggers dissociation of the Pdc-$G\beta_1\gamma_1$ complex, which makes $G\beta_1\gamma_1$ competent to re-associate with $G_t\alpha_1$.[35] Thus, phosphorylation of Pdc within the Pdc-$G\beta_1\gamma_1$ complex could stimulate the re-association of $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits, which is a prerequisite for outer segment targeting.[24,25] Consistently with this mechanism, phosphorylation of both sites of Pdc in the dark occurs in the rod outer segments.[18]

It also is important to note that in our phosducin phosphorylation mutant mice, $Pdc^{S54A/S71A}$-FLAG, transducin $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits return to the outer segment at different rates, something not seen in control mice, where the return of both subunits occurs at similar rates. These differences noted in the rates of transducin subunit return would not be possible if transducin returns to the outer segment as a heterotrimer, but rather suggest that $G_t\alpha_1$ and $G\beta_1\gamma_1$ move to this compartment as individual entities. Intriguingly, our data also suggest that the accumulation of $G_t\alpha_1$ subunits in the outer segment is based predominantly on their re-binding to $G\beta_1\gamma_1$ subunits, either already present or moved to this compartment by another mechanism. Indeed, a nearly 3-fold molar excess of $G\beta_1\gamma_1$ compared to $G_t\alpha_1$ is found in the outer segment after light conditioning (Table 1), because more $G_t\alpha_1$ than $G\beta_1\gamma_1$ undergoes translocation from this compartment. Such a mechanism also would explain why the kinetics of $G_t\alpha_1$ return to the outer segment during dark conditioning shows notably more dependency on Pdc phosphorylation than that of $G\beta_1\gamma_1$ (Figs. 3A and 3B). Two additional lines of evidence further support this hypothesis. First, $G_t\alpha_1$ was found to be competent fully to undergo normal reversible translocation even when $G\beta_1\gamma_1$ doesn't move from the outer segments.[37] Second, rod visual signaling surprisingly appeared to be unperturbed by genetic elimination of the $G_t\alpha_1$ lipid modification,[38] indicating that $G\beta_1\gamma_1$ is fully capable to recruit $G_t\alpha_1$ and form a functional heterotrimer coupled to rhodopsin, even if $G_t\alpha_1$ lacks its membrane anchor. Combined, these data thus appear to corroborate the notion that the light-displaced $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits return to the rod outer segment as individual entities, rather than as a heterotrimer.

Most recently, two groups reported new and exciting data on the mechanism of transducin translocation.[39,40] It was demonstrated that a photoreceptor protein, UNC119, interacts specifically with the $G_t\alpha_1$ lipid moiety, leading to $G_t\alpha_1$ subunit dissociation from $G\beta_1\gamma_1$ and partitioning from the membrane. This mode of action, which resembles closely that of Pdc, makes it tempting to speculate that UNC119 and Pdc act in concert to keep transducin subunits soluble during their return to the rod outer segment. However, it remains to be elucidated how $G_t\alpha_1$ and $G\beta_1\gamma_1$ subunits are released from their trafficking chaperones, which would be required for their assembly on the outer segment membranes.

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References

1. Lee RH, Lieberman BS, Lolley RN. A novel complex from bovine visual cells of a 33,000-dalton phosphoprotein with beta- and gamma-transducin: purification and subunit structure. *Biochemistry*. 1987;26:3983–3990.
2. Gaudet R, Bohm A, Sigler PB. Crystal structure at 2.4 angstroms resolution of the complex of transducin betagamma and its regulator, phosducin. *Cell*. 1996;87:577–588.
3. Hawes BE, Touhara K, Kurose H, Lefkowitz RJ, Inglese J. Determination of the G beta gamma-binding domain of phosducin. A regulatable modulator of G beta gamma signaling. *J Biol Chem*. 1994;269:29825–29830.
4. Xu J, Wu D, Slepak VZ, Simon MI. The N terminus of phosducin is involved in binding of beta gamma subunits of G protein. *Proc Natl Acad Sci U S A*. 1995;92:2086–2090.
5. Muller S, Straub A, Schröder S, Bauer PH, Lohse MJ. Interactions of phosducin with defined G protein beta gamma-subunits. *J Biol Chem*. 1996;271:11781–11786.
6. Lee RH, Whelan J, Lolley RN, McGinnis JF. The photoreceptor-specific 33 kDa phosphoprotein of mammalian retina: generation of monospecific antibodies and localization by immunocytochemistry. *Exp Eye Res*. 1988;46:829–840.
7. von Schantz M, Szél A, van Veen T, Farber DB. Expression of soluble phototransduction-associated proteins in ground

squirrel retina. *Invest Ophthalmol Vis Sci*. 1994;35:3922–3930.

8. Sokolov M, Strissel KJ, Leskov IB, Michaud NA, Govardovskii VI, Arshavsky VY. Phosducin facilitates light-driven transducin translocation in rod photoreceptors. Evidence from the phosducin knockout mouse. *J Biol Chem*. 2004;279:19149–19156.
9. Krispel CM, Sokolov M, Chen YM, et al. Phosducin regulates the expression of transducin betagamma subunits in rod photoreceptors and does not contribute to phototransduction adaptation. *J Gen Physiol*. 2007;130:303–312.
10. Herrmann R, Lobanova ES, Hammond T, et al. Phosducin regulates transmission at the photoreceptor-to-ON-bipolar cell synapse. *J Neurosci*. 2010;30:3239–3253.
11. Nakano K, Chen J, Tarr GE, Yoshida T, Flynn JM, Bitensky MW. Rethinking the role of phosducin: light-regulated binding of phosducin to 14-3-3 in rod inner segments. *Proc Natl Acad Sci U S A*. 2001;98:4693–4698.
12. Danner S, Lohse MJ. Phosducin is a ubiquitous G-protein regulator. *Proc Natl Acad Sci U S A*. 1996;93:10145–10150.
13. Beetz N, Harrison MD, Brede M, et al. Phosducin influences sympathetic activity and prevents stress-induced hypertension in humans and mice. *J Clin Invest*. 2009;119:3597–3612.
14. Thulin CD, Savage JR, McLaughlin JN, et al. Modulation of the G protein regulator phosducin by Ca2+/calmodulin-dependent protein kinase II phosphorylation and 14-3-3 protein binding. *J Biol Chem*. 2001;276:23805–23815.
15. Lee RH, Brown BM, Lolley RN. Light-induced dephosphorylation of a 33K protein in rod outer segments of rat retina. *Biochemistry*. 1984;23:1972–1977.
16. Lee RH, Brown BM, Lolley RN. Protein kinase A phosphorylates retinal phosducin on serine 73 in situ. *J Biol Chem*. 1990;265:15860–15866.
17. Brown BM, Carlson BL, Zhu X, Lolley RN, Craft CM. Light-driven translocation of the protein phosphatase 2A complex regulates light/dark dephosphorylation of phosducin and rhodopsin. *Biochemistry*. 2002;41:13526–13538.
18. Song H, Belcastro M, Young EJ, Sokolov M. Compartment-specific phosphorylation of phosducin in rods underlies adaptation to various levels of illumination. *J Biol Chem*. 2007;282:23613–23621.
19. Lem J, Applebury ML, Falk JD, Flannery JG, Simon MI. Tissue-specific and developmental regulation of rod opsin chimeric genes in transgenic mice. *Neuron*. 1991;6:201–210.
20. Song H, Sokolov M. Analysis of protein expression and compartmentalization in retinal neurons using serial tangential sectioning of the retina. *J Proteome Res*. 2009;8:346–351.
21. Sokolov M, Lyubarsky AL, Strissel KJ, et al. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron*. 2002;34:95–106.
22. Lee BY, Thulin CD, Willardson BM. Site-specific phosphorylation of phosducin in intact retina. Dynamics of phosphorylation and effects on G protein beta gamma dimer binding. *J Biol Chem*. 2004;279:54008–54017.
23. Calvert PD, Krasnoperova NV, Lyubarsky AL, et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha -subunit. *Proc Natl Acad Sci U S A*. 2000;97:13913–13918.
24. Zhang H, Huang W, Zhang H, et al. Light-dependent redistribution of visual arrestins and transducin subunits in mice with defective phototransduction. *Mol Vis*. 2003;9:231–237.
25. Lobanova ES, Finkelstein S, Herrmann R, et al. Transducin gamma-subunit sets expression levels of alpha- and beta-subunits and is crucial for rod viability. *J Neurosci*. 2008;28:3510–3520.
26. Strissel KJ, Sokolov M, Trieu LH, Arshavsky VY. Arrestin translocation is induced at a critical threshold of visual signaling and is superstoichiometric to bleached rhodopsin. *J Neurosci*. 2006;26:1146–1153.
27. Kerov V, Chen D, Moussaif M, Chen YJ, Chen CK, Artemyev NO. Transducin activation state controls its light-dependent translocation in rod photoreceptors. *J Biol Chem*. 2005;280:41069–41076.
28. Lobanova E, Finkelstein S, Song H, et al. Transducin translocation in rods is triggered by saturation of the GTPase-activating complex. *J Neurosci*. 2007;27:1151–1160.
29. Rosenzweig DH, Nair KS, Wei J, et al. Subunit dissociation and diffusion determine the subcellular localization of rod and cone transducins. *J Neurosci*. 2007;27:5484–5494.
30. Elias RV, Sezate SS, Cao W, McGinnis JF. Temporal kinetics of the light/dark translocation and compartmentation of arrestin and alpha-transducin in mouse photoreceptor cells. *Mol Vis*. 2004;10:672–681.
31. Calvert PD, Strissel KJ, Schiesser WE, Pugh EN Jr, Arshavsky VY. Light-driven translocation of signaling proteins in vertebrate photoreceptors. *Trends Cell Biol*. 2006;16:560–568.
32. Slepak VZ, Hurley JB. Mechanism of light-induced translocation of arrestin and transducin in photoreceptors: interaction-restricted diffusion. *IUBMB Life*. 2008;60:2–9.
33. Artemyev NO. Light-dependent compartmentalization of transducin in rod photoreceptors. *Mol Neurobiol*. 2008;37:44–51.
34. Fain GL, Matthews HR, Cornwall MC. Dark adaptation in vertebrate photoreceptors. *Trends Neurosci*. 1996;19:502–507.
35. Gaudet R, Savage JR, McLaughlin JN, Willardson BM, Sigler PB. A molecular mechanism for the phosphorylation-dependent regulation of heterotrimeric G proteins by phosducin. *Mol Cell*. 1999;3:649–660.
36. Wilkins JF, Bitensky MW, Willardson BM. Regulation of the kinetics of phosducin phosphorylation in retinal rods. *J Biol Chem*. 1996;271:19232–19237.
37. Kassai H, Aiba A, Nakao K, et al. Farnesylation of retinal transducin underlies its translocation during light adaptation. *Neuron*. 2005;47:529–539.
38. Kerov V, Rubin WW, Natochin M, Melling NA, Burns ME, Artemyev NO. N-terminal fatty acylation of transducin profoundly influences its localization and the kinetics of photoresponse in rods. *J Neurosci*. 2007;27:10270–10277.
39. Zhang H, Constantine R, Vorobiev S, et al. UNC119 is required for G protein trafficking in sensory neurons. *Nat Neurosci*. 2011;14:874–880.
40. Gopalakrishna KN, Doddapuneni K, Boyd KK, Masuho I, Martemyanov KA, Artemyev NO. Interaction of transducin with uncoordinated 119 protein (UNC119): implications for the model of transducin trafficking in rod photoreceptors. *J Biol Chem*. 2011;286:28954–28962.