Membrane Protein Transport in Photoreceptors: The Function of PDE δ

The Proctor Lecture

Wolfgang Baehr

Department of Ophthalmology, John A. Moran Eye Center, University of Utah Health Science Center, University of Utah, Salt Lake City, Utah, United States

Department of Neurobiology and Anatomy, University of Utah Health Science Center, University of Utah, Salt Lake City, Utah, United States

Department of Biology, University of Utah, Salt Lake City, Utah, United States

Correspondence: Wolfgang Baehr, John A. Moran Eye Center, University of Utah Health Science Center, 65 Mario Capecchi Drive, Salt Lake City, UT 84132, USA; wbaehr@hsc.utah.edu.

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This lecture details the elucidation of cGMP phosphodiesterase (PDE δ), discovered 25 years ago by Joe Beavo at the University of Washington. PDE δ , once identified as a fourth PDE6 subunit, is now regarded as a promiscuous prenyl-binding protein and important chaperone of prenylated small G proteins of the Ras superfamily and prenylated proteins of phototransduction. Alfred Wittinghofer's group in Germany showed that PDE δ forms an immunoglobulin-like β -sandwich fold that is closely related in structure to other lipid-binding proteins, for example, Uncoordinated 119 (UNC119) and RhoGDI. His group cocrystallized PDE δ with ARL (Arf-like) 2^{GTP}, and later with farnesylated Rheb (ras homolog expressed in brain). PDE δ specifically accommodates farnesyl and geranylgeranyl moieties in the absence of bound protein. Germline deletion of the *Pde6d* gene encoding PDE δ impeded transport of rhodopsin kinase (GRK1) and PDE6 to outer segments, causing slowly progressing, recessive retinitis pigmentosa. A rare *PDE6D* null allele in human patients, discovered by Tania Attié-Bitach in France, specifically impeded trafficking of farnesylated phosphatidylinositol 3,4,5-trisphosphate (PIP3) 5-phosphatase (INPP5E) to cilia, causing severe syndromic ciliopathy (Joubert syndrome). Binding of cargo to PDE δ is controlled by Arf-like proteins, ARL2 and ARL3, charged with guanosine-5'-triphosphate (GTP). Arf-like proteins 2 and 3 are unprenylated small GTPases that serve as cargo displacement factors. The lifetime of ARL3^{GTP} is controlled by its GTPase-activating protein, retinitis pigmentosa protein 2 (RP2), which accelerates GTPase activity up to 90,000-fold. *RP2* null alleles in human patients are associated with severe X-linked retinitis pigmentosa (XLRP). Germline deletion of *RP2* in mouse, however, causes only a mild form of XLRP. Absence of *RP2* prolongs the activity of ARL3^{GTP} that, in turn, impedes PDE6 δ -cargo interactions and trafficking of prenylated protein to the outer segments. Hyperactive ARL3^{GTP}, acting as a hyperactive cargo displacement factor, is predicted to be key in the pathobiology of *RP2*-XLRP.

Keywords: phototransduction, protein prenylation, protein trafficking

Photoreceptors are polarized neurons, with specific subcellular compartmentalization and unique requirements for protein synthesis and trafficking. Each photoreceptor contains an outer segment (OS) housing the phototransduction machinery, an inner segment (IS) where proteins are biosynthesized, and a synaptic terminal for signal transmission. Outer and inner segment are joined by a connecting cilium (CC), corresponding to the transition zone of primary cilia. Proteins that participate in phototransduction (rhodopsin, transducin, cGMP phosphodiesterase 6 [PDE6], cyclic nucleotide-gated [CNG] channel subunits) and accessory proteins (rhodopsin kinase or GRK1, arrestins, guanylate cyclase [GC], GC-activating proteins or GCAPs, and the GTPase-activating protein [GAP] complex) are synthesized in the IS and must be transported through the CC to the OS. These proteins are either transmembrane (TM) proteins or peripherally associated membrane proteins that are attached to the membrane surface. How TM proteins (e.g., rhodopsin)

and peripherally associated proteins (e.g., transducin and PDE6) traffic through the IS to incorporate eventually in the nascent disc membrane is unknown.

PHOTOTRANSDUCTION

Vision begins in the OSs of rod (dim light) and cone (bright light) photoreceptors upon the absorption of photons by visual pigment molecules; the opsins become activated and start the phototransduction cascade (Fig. 1) (reviewed recently in Refs. 1, 2; see also Yingbin Fu in Webvision [http://webvision.med.utah.edu/, part V; in the public domain]). Activated opsins, acting as guanine nucleotide exchange factors (GEFs), catalyze guanosine diphosphate (GDP/GTP) exchange on the α -subunit of transducin (T α) followed by dissociation from T $\beta\gamma$.^{3,4} T α ^{GTP} activates the cGMP PDE6 by dislocating the inhibitory PDE6 γ subunit bound to the active site of the enzyme.^{5,6} Activity of PDE6 is controlled by diffusion and degrades cGMP with a maximal rate

FIGURE 1. Rod phototransduction cascade. In the dark, rhodopsin and transducin are inactive, and PDE is inhibited with only basal activity. High cGMP levels maintain a limited number of CNG channels in the open state. In light, rhodopsin (R^*) serially activates many copies of $T\alpha$ (gain 1), and $T\alpha$ in turn, activates PDE6, which degrades cytoplasmic cGMP with rapid turnover (gain 2). Polypeptides involved in phototransduction are TM proteins or are peripherally attached to the membrane surface. Number of integral membrane protein TM domains (rhodopsin, guanylate cyclase, NCKX, CNG channel) is shown (yellow). Posttranslational modification (prenyl or acyl) is indicated for membrane association of transducin α , transducin γ , PDE $\alpha\beta$, and GRK1.

of several thousand cGMP molecules hydrolyzed/s/PDE6.^{7,8} Breakdown of cytoplasmic cGMP releases cGMP from the ion channels where its binding sustains the inward-flowing cation current that keeps visual cells depolarized in darkness.^{9,10} Continued extrusion of cations, including Ca^{2+} , by the cation exchanger NCKX causes hyperpolarization of the photoreceptor. The net effect of one photon being absorbed is hydrolysis of millions of cGMP molecules on a millisecond time scale. Photoreceptors return to the dark by (1) inactivating rhodopsin through phosphorylation by rhodopsin kinase (GRK1) and arrestin binding,^{11–13} (2) accelerating hydrolysis of GTP bound to $T\alpha$ by a GAP complex,^{14,15} and (3) resynthesizing cGMP via GC that is activated by GCAPs in low free $[Ca^{2+}]$.^{16–19} Defects in genes encoding any of these proteins lead to retina dystrophies, for example, retinitis pigmentosa,^{20,21} cone-rod dystrophy,²² cone dystrophy, or nonprogressive congenital stationary night blindness.^{23–25}

HIGH BIOSYNTHETIC DEMAND IN PHOTORECEPTORS

Photoreceptors are an excellent model system to study synthesis, post-Golgi trafficking, and ciliary transport of proteins. The IS contains the “machinery” necessary for protein synthesis, modification, sorting, and trafficking (endoplasmic reticulum [ER], ribosomes, Golgi, microtubules, mitochondria) (Fig. 2A). Mechanisms that regulate disc membrane assembly at the proximal OS, concomitant disc shedding at the distal end, and phagocytosis of shed disc membranes by the adjacent retinal pigment epithelium (RPE) are incompletely understood^{26,27} (for review, see Refs. 28, 29).

Pioneering work of Richard Young provided evidence that photoreceptor OSs are renewed approximately every 10 days.^{30–32} In a pulse-chase experiment,³³ newly synthesized proteins were identified by autoradiography and observed to migrate toward the CC and synaptic region (Fig. 2B); proteins destined for the OS then trafficked through the CC to incorporate into nascent discs. Labeled discs reached the distal end of the OS in 10 days and were removed by phagocytosis. Later experiments revealed that new discs assemble at the rod OS base at a rate of 80 discs/d, requiring synthesis of ~1000 rhodopsins/min. Daily renewal of ~10% of the OS membrane requires an extremely high rate of biosynthesis and highly reliable transport and targeting pathways.^{34–36} Disc shedding appears to be regulated by circadian processes, as distal rod OS are shed in the morning and distal cone OS at dusk.^{37,38}

A mouse retina contains approximately 6 million rods and 200,000 cones, while the human retina contains 110 million rods and 6.4 million cones³⁹ (see also <http://webvision.med.utah.edu/>, part XIII, in the public domain). Each mouse ROS contains ~800 discs arranged in a vertical stack,⁴⁰ and each disc houses roughly 30,000 molecules of rhodopsin, 2500 molecules of transducin, and 250 molecules of PDE6. Accordingly, the rhodopsin concentration is quite high, the highest for any G protein-coupled receptor (GPCR), approaching 3 mM per mouse retina and up to 8 mM per disc,⁴⁰ a major reason why rhodopsin was the first GPCR isolated 140 years ago at the beginning of biochemistry.⁴¹ Replacement of 80 discs per rod requires synthesis and transport of 2.4 million rhodopsin molecules/day, quite an astonishing number relative to traffic across the George Washington Bridge in New York

FIGURE 2. Diagram of outer segment renewal. (A) Photoreceptor compartments. (B) Outer segment renewal. (1) Radioactively tagged amino acids are injected into the retina, chased, and labeled proteins accumulate. (2) Autoradiography reveals radioactively tagged proteins moving toward the connecting cilium and incorporated into discs. (3–5) Bands of labeled discs are displaced distally to the OS tip within 10 days. (6) Labeled discs undergo phagocytosis by the RPE. Inspired by Richard Young's drawing³³ and redrawn.

City, which is ~250,000 cars/d (http://en.wikipedia.org/wiki/George_Washington_Bridge, in the public domain), or 10% of the number of rhodopsin molecules traversing the CC in photoreceptors.

INTEGRAL MEMBRANE PROTEIN TRAFFICKING

Rhodopsin, a GPCR with seven TM domains, is synthesized by ER-associated ribosomes, glycosylated cotranslationally, and integrated into the ER membrane (for a review, see Ref. 42). Rhodopsin then traffics, likely as a homodimer,⁴³ to its rod outer segment (ROS) destination following the canonical secretory pathway.⁴⁴ Details of trafficking to the Golgi, post-Golgi sorting of membrane proteins, and incorporation into transport vesicles are currently under intense investigation.^{45,46} Vesicles presumably fuse with the cell membrane, likely by exocytosis, at the periciliary ridge complex, and cargo is assembled for intraflagellar transport (IFT). Cargo (consisting of rhodopsin and other TM proteins⁴⁷) may traffic through the cilium by anterograde IFT powered by kinesin motors to be deposited in nascent disc membranes; however, mechanistic details are unknown. Rhodopsin has been visualized in the CC by immuno-electron microscopy (EM), but involvement of kinesin-II in rod IFT has been questioned.⁴⁸ An alternate model proposes that rhodopsin-laden vesicles traffic through the transition zone without molecular motors and fuse with and enlarge OS nascent discs.⁴⁹

POSTBIOSYNTHESIS OS TRAFFICKING PATHWAYS OF PERIPHERAL MEMBRANE PROTEINS

Phototransduction proteins are membrane associated to enable lateral diffusion on the disc surface. Rhodopsin, GCs, NCKX, and the cGMP-gated channel subunits are TM proteins with 7,

1, 11, or 6 TM domains, respectively, whereas PDE, T, and GRK1 are peripherally associated proteins anchored to the membrane by lipid modifications (Fig. 1). Transducin is acylated at the N-terminal of $T\alpha$ ⁵⁰ and farnesylated at the C-terminal of $T\gamma$.^{51,52} Cyclic GMP-specific phosphodiesterase 6 catalytic subunits⁵³ and GRK1^{54,55} are prenylated at their C-termini with farnesyl and geranylgeranyl chains. $T\beta\gamma$ assembly requires the molecular chaperone CCT (chaperonin containing TCP-1) and phosducin-like protein 1 (PhLP1) as a cochaperone in the folding process.^{56,57} $G\alpha$ subunits carrying the myristoylation consensus sequence⁵⁸ are acylated cotranslationally or by an unknown ER-resident acyltransferase. It is generally assumed that G protein heterotrimer formation is required for targeting to the cell membrane.⁵⁹ Consistent with this model, genetic deletion of $T\gamma$ results in the mistargeting and degradation of $T\alpha$ and $T\beta$.⁶⁰ It is presumed that membrane-associated transducin traffics to the distal IS by vesicular transport using molecular motors, highly coordinated with its GPCR, rhodopsin.

BIDIRECTIONAL TRANSDUCIN TRAFFICKING BY DIFFUSION

Light activation of rhodopsin triggers GTP/GDP exchange on $T\alpha$ causing $T\alpha^{GTP}$ and $T\beta\gamma$ to dissociate. Under persistent illumination, $T\alpha^{GTP}$ and $T\beta\gamma$ diffuse into the IS with a $t_{1/2}$ of 3 to 5 minutes for $T\alpha$ and a $t_{1/2}$ of approximately 12 minutes for $T\beta\gamma$.^{61–63} Upon arrival in the IS, $T\alpha^{GTP}$ hydrolyzes to $T\alpha^{GDP}$, which then recombines with $T\beta\gamma$, reforming a heterotrimer that associates with IS membranes. Upon resumption of dark adaptation, both $T\alpha$ and $T\beta\gamma$ subunits return to the OSs with a $t_{1/2}$ of ~2 hours. UNC119 (Unc-119 homolog *Caenorhabditis elegans*) plays a key role in the elution of $T\alpha^{GTP}$ from the IS membranes and controls its return to the OS. UNC119 is an acyl-binding protein, which interacts with the acylated N-

FIGURE 3. GRK1 prenylation. (A) Structures of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, the substrates prenyl transferase. (B) Prenylation of GRK1 in the cytoplasm and docking to the ER. The endoprotease RCE1 (Ras-converting enzyme 1) removes the amino acids VLS of the CAAX box,^{72,77} and the S-adenosine L-methionine (SAM)-dependent isoprenylcysteine-O-carboxyl methyltransferase (ICMT) methylates the -COOH of cysteine.⁷³

terminal of T α and serves as a solubilization factor. Upon solubilization of T α ^{GTP} by UNC119, mediated by spontaneous GDP/GTP exchange, the complex diffuses passively, depositing T α onto discs in the OS.^{64,65} The slow return of T α may be explained by the low rate constant of spontaneous GDP/GTP exchange in the absence of its GEF (rhodopsin) and the low abundance of UNC119 compared with transducin. A key role in discharging T α may be played by Arf-like (ARL) 3^{GTP}, which has recently been identified as a cargo displacement factor or GDI (GDP dissociation inhibitor [GDI] displacement factor).⁶⁶

PROTEIN PRENYLATION

Prenylation provides C-terminal hydrophobic anchors that allow soluble proteins to attach to membrane surfaces or to interact with proteins (Fig. 3). C₁₅ farnesol or a C₂₀ geranylgeraniol is attached to the C-terminal cysteine of a protein carrying a 4 amino acid prenylation motif, called a CAAX box.⁶⁷ Protein prenylation is a common posttranslational modification in eukaryotic cells affecting up to 2% of all proteins, as defined by open reading frames (ORFs) followed by a CAAX box and stop codon, expressed in mammalian cells (referred to as the prenylome).⁶⁸ Prenyl side chains are synthesized in all living organisms via the mevalonate pathway⁶⁹ and attached to newly synthesized cytosolic proteins by prenyl transferases.^{70,71} The C-terminal amino acid X determines the nature of the lipid chain as leucine specifies geranylgeranylation and all other residues result in farnesylation with high efficiency. The prenyl chain is attached to the CAAX box cysteine via a stable thioether bond by cytosolic prenyl transferases.⁷¹ Prenylated proteins dock to the ER and are further processed by the ER-associated enzyme, RCE1

protease (ras-converting enzyme 1), which cleaves AAX of the CAAX box,⁷² and an isoprenyl cysteine carboxymethyl transferase (ICMT), which methylates the COOH of cysteine.^{73,74} Each enzyme is required for mouse development, as deletion of either RCE1 or ICMT is embryonically lethal.^{75,76} Deletion of RCE1 in retina prevents transport of rod PDE6 to the ROS, but with no effect on GRK1.⁷⁷ Apart from visual cascade components, known prenylated CAAX proteins include members of the Ras superfamily (Rac, Rho, and Rab GTPases), G protein γ -subunits, and others^{74,78} (Table 1; PRENbase, annotated database of known and predicted prenylated proteins at <http://mendel.imp.ac.at/PrePS/PRENbase/> [in the public domain]).

PDE6 IDENTIFICATION AND KEY PROPERTIES

Cyclic GMP-specific phosphodiesterase 6 belongs to a large PDE superfamily (PDE1–11) whose members regulate cellular concentrations of cAMP and cGMP.⁷⁹ Light activation of PDE6 and identification of cGMP as the internal transmitter of phototransduction were discovered in the early 1970s, following the first formulation of a light-induced enzymatic cascade as a mechanism for phototransduction.⁸⁰ Cyclic GMP-specific phosphodiesterase 6 was first isolated from frog retina⁸¹ and later from bovine retina membranes.⁷ Its purification relies on the observation that phosphodiesterase cosediments with membranes in isotonic buffers but can be eluted from the membrane surface by hypotonic shock (Fig. 4, lanes 4–6), suggesting peripheral-membrane attachment (Fig. 3, showing GRK1 as an example). In this way, PDE6 and transducin may be eluted from unbleached ROS membranes. Separation of PDE6 and transducin (termed 80K protein in Fig.

FIGURE 4. Purification of membrane-associated bovine PDE6. Coomassie-stained SDS-PAGE, generated in 1978.⁷ PDE6 purification steps: (1) retina lysate; (2) 30% sucrose 4K supernatant; (3) ROS membranes before sucrose gradient; (4) ROS membranes after sucrose gradient—rhodopsin, 80K protein (transducin), and PDE6 $\alpha\beta$ are major constituents; (5) hypotonic supernatant; (6) purified PDE6 $\alpha\beta$ after DEAE column chromatography. S, size markers (bacterial RNA polymerase subunits, bovine serum albumin, and lysozyme).

4, lane 5) is achieved by size chromatography or by bleaching of ROS membranes during which transducin associates tightly with rhodopsin, as long as GTP is absent.^{82,83} These procedures established the subunit composition of PDE6 as PDE6 $\alpha\beta\gamma$. Two large subunits (α and β) are catalytically active, whereas the small subunit, PDE6 γ , is inhibitory⁵ and minimizes the dark activity of PDE6. The final subunit stoichiometry of rod PDE6 was later adjusted to PDE6 $\alpha\beta\gamma_2$.^{84,85} The catalytic subunits have internal tandem repeats (termed GAF-A and GAF-B), which have been implicated as sites of noncatalytic cGMP binding.^{86,87} Mammalian PDE6 β is predicted to be geranylgeranylated, while PDE6 α is farnesylated. The differential prenylation of PDE6 α and PDE6 β subunits was confirmed *in vivo*.⁵³ Among other phototransduction components, only transducin- γ (T γ)^{52,88} and rhodopsin kinase (GRK1 or G protein-coupled receptor kinase 1)^{54,55,89} are known to be farnesylated.

THE PRENYL-BINDING PROTEIN PDE δ

PDE δ ⁹⁰ (also PDE6 δ , PrBP/ δ , PDE6D) was discovered in 1989 as a protein copurifying with soluble PDE6 $\alpha\beta\gamma_2$ (Figs. 5A, 5B). Surprisingly, purified PDE δ did not affect PDE6 activity. PDE δ was present in several bovine tissue mRNA preparations by Northern blotting, but the strongest PDE δ signal was present in the retina.⁹¹ Addition of PDE δ -GST fusion protein to permeabilized ROS reduced the maximal rate of cGMP hydrolysis in response to light,⁹² demonstrating that PDE δ -glutathione-S-transferase (GST) can modify activity of the phototransduction cascade *in vitro* due to a functional uncoupling of PDE6. However, it was realized later that amounts of PDE δ in OS are insufficient to downregulate the phototransduction cascade.⁹³ A low-resolution cryo-EM structure of PDE6 $\alpha\beta\gamma_2\delta_2$ at 18-Å resolution identified the probable location of isoprenylation, PDE6 γ subunits, and catalytic sites.⁹⁴

Identification of PDE δ as a PDE6 subunit became problematic when it was shown that human PDE δ specifically interacts with Rab13, a small GTPase associated with vesicles in fibroblasts and epithelial cells.⁹⁵ Recombinant PDE δ dissociat-

TABLE 1. Polypeptides (Mouse) Interacting With PDE δ (Column 1, Proteins Involved in Phototransduction, Small G Proteins, and Other Interacting Proteins; Column 2, CAAX Box Sequences; Column 3, Prenyl Side Chains at the C-terminal Cys; Column 4, References)

Target	CAAX	Prenyl	References
PDE α *	CCIQ	f	92
PDE β *	CCIL	gg	92
GRK1*	CLIS	f	102
GRK7*	CLLL	gg	102
cT γ *	CVLS	f	100
T γ *	CVIS	f	100
G γ 13	CTIL	ff	122
DmPDE5/6	CALL	gg	127
Rab13†	CLLG	f	95
Rab28†	CAVQ		128, 129
Rheb†	CSVM	f	99, 66, 130
Rho6 (Rnd1)†	CSIM	f	98
Rap1a†	CLLL	gg	98
Rap1b†	CQLL	gg	98
Rap2b†	CVIL	gg	98
H-Ras†	CVLS	f	98
N-Ras†	CVVM	f	98
K-Ras†	CVIM	f	98
RhoA†	CLVL	gg	98
RhoB†	CKVL	gg	98, 99
Prostacyclin-R	CSLC	f	131
INPP5E	CTVS		101
RPGR-RCC1	-	-	132, 133
ARL2-GTP	-	-	103
ARL3-GTP	-	-	134

f, farnesyl; gg, geranylgeranyl.

* Proteins involved in phototransduction.

† Small G proteins.

ed Rab13 from cellular membranes, similarly to the way it dissociates PDE6 from ROS disc membranes (Fig. 5C). Further, the eyeless nematode *C. elegans* expressed a PDE δ ortholog (CE δ), which solubilized bovine PDE6 from ROS membranes nearly identically to PDE δ ⁹⁶ (Fig. 5C). GST-CE δ pulled down PDE α , PDE6 β , and an unprenylated N-terminal fragment of retinitis pigmentosa GTPase regulator (RPGR) containing the RCC1 domain.⁹⁶ PDE6 $\alpha\beta\gamma_2\delta_2$ structure at 18-Å resolution was determined by cryo-EM (Fig. 5D). Subsequent protein sequence analyses showed that PDE δ was expressed in all species for which protein sequences were available. PDE δ is now viewed as a promiscuous, ubiquitous prenyl-binding protein whose orthologs have been identified throughout the animal kingdom, from unicellular ciliated organisms (*Paramecium*) and nematodes (*C. elegans*) to humans (Fig. 6).

INTERACTION OF PDE δ WITH PRENYLATED PROTEINS

In photoreceptors, PDE δ bound preferentially to carboxymethylated PDE6.⁹⁷ PDE δ dissociated slowly from the catalytic subunits with a half-life of approximately 3.5 hours.⁹⁷ Micromolar concentrations of C-terminal peptides blocked the PDE6/PDE δ interaction only when the peptides were both prenylated and carboxymethylated. Soluble PDE6 from these rod OSs was 5-fold more methylated than the membrane-bound PDE6, suggesting that PDE δ bound preferentially to carboxymethylated PDE6 subunits.

PDE δ apparently interacts nonselectively with a large number of prenylated G proteins of the Ras superfamily including Rac, Rap, Rho, Rheb, RhoA, RhoB, and Rho6^{98,99} (Table 1). The physiological significance of interaction with

FIGURE 5. Membrane-bound and soluble PDE6. (A) Cartoon contrasting membrane-bound (*top*) versus soluble (*bottom*) PDE6, with PDEδ associated with the soluble form. (B) SDS-PAGE of membrane and soluble PDE6. Membrane-bound PDE consists of PDE6α (100 kDa), PDE6β (100 kDa), and PDE6γ (10 kDa). Soluble PDE has an apparent fourth subunit, PDEδ (~17 kDa). (C) Elution of PDE6 from ROS membranes as a function of human PDEδ or *C. elegans*, Ceδ. (D) Structure of PDE6αβγδ2 by cryo-EM at 18Å resolution. The research in (B) was originally published in *The Journal of Biological Chemistry*. Gillespie PG, Prusti RK, Apel ED, Beavo JA. A soluble form of bovine rod photoreceptor phosphodiesterase has a novel 15-kDa subunit. *J Biol Chem.* 1989;264:12187-12193. Copyright The American Society for Biochemistry and Molecular Biology. (C) Adapted from Li N, Baehr W. Expression and characterization of human PDEδ and its *Caenorhabditis elegans* ortholog CEδ. *FEBS Lett.* 1998;440:454-457. Copyright 1998 Federation of European Biochemical Societies. (D) Reprinted with permission from Goc A, Chami M, Lodowski DT, et al. Structural characterization of the rod cGMP phosphodiesterase 6. *J Mol Biol.* 2010;401:363-373. Copyright 2010 Elsevier Ltd.

PDEδ likely consists of trafficking membrane-bound G proteins from one membrane (place of biosynthesis or posttranslational modification) to a destination membrane (transport vesicle or plasma membrane). Proteins shown to mistraffic in the absence of PDEδ are photoreceptor PDE6 and GRK1,¹⁰⁰ Gγ13 in olfactory epithelia (Zhang H, Baehr W, unpublished observation, 2012), and INPP5E (phosphatidylinositol 3,4,5-trisphosphate [PIP3] 5-phosphatase) in human patients with a *PDE6D* null allele¹⁰¹ (see below).

Yeast two-hybrid screens indicate that prenylation alone may be insufficient for binding to PDEδ. Several prenylated proteins do not interact with PDEδ, suggesting that specificity is mediated in part by protein-protein interactions. Examples of noninteracting prenylated GTPases include Rala, Ralb, and Rac1.^{98,99}

FLUORESCENCE RESONANCE ENERGY TRANSFER WITH FLUORESCENT PRENYL SIDE CHAINS

The question arose as to whether prenyl side chains form stable complexes with PDEδ in the absence of polypeptide chains. To solve this question, fluorescence resonance energy transfer (FRET) between PDEδ and a fluorescently labeled prenyl ligand was used to determine interaction and binding constants.¹⁰² Fluorescence resonance energy transfer is a distance-dependent interaction between the excited states of two dye molecules. Excitation from a donor molecule (PDEδ) is transferred to an acceptor molecule (dansyl-Cys-farnesyl) without emission of a photon. We synthesized an interactant molecule in which a dansyl (a fluorescent green dye) was linked covalently to cysteine, the C-terminal amino acid of prenylated proteins, and a farnesyl chain. This molecule was used as a probe to measure the strength of interaction with its target PDEδ using FRET. PDEδ, when excited at 290 nm, strongly emits at 315 nm based on excitation of its aromatic tryptophan residues. Dansyl-Cys-farnesyl, when excited at 290 nm, shows low-fluorescence emission at 500 nm. When dansyl-

Cys-farnesyl and PDEδ are combined and allowed to interact, an increase of fluorescence at 500 nm is observed; the FRET signal can be used to quantify binding of farnesyl to PDEδ in a titration experiment (Fig. 7). As interaction between prenyl chains and PDEδ occurs in the range of 1 to 20 μM (Fig. 7C), this result is interpreted as evidence that prenyl side chains are sufficient for interaction with PDEδ. Interaction of PDEδ with domains of the prenylated protein may strengthen, weaken, or even abolish interaction.

FIGURE 6. Phylogram of 21 PDEδ orthologs. PDEδ orthologs are highly conserved among vertebrates, invertebrates, and even unicellular organisms (*Paramecium*). A common ancestor of PDEδ orthologs is the acyl-binding protein Uncoordinated (UNC) 119, the unc-119 homolog of *C. elegans*.^{64,105,135}

FIGURE 7. PDE δ interacts with prenyl side chains in the absence of polypeptide. (A) Structure of the fluorescent ligand, dansyl-Cys-farnesyl. (B) Fluorescence emission spectra of PDE δ (black), dansyl-Cys-farnesyl (green), and PDE δ in the presence of dansyl-Cys-farnesyl. Fluorescence resonance energy transfer (FRET) is indicated by a yellow double arrow. (C) Binding curves. Δ , relative fluorescence as a function of dansyl-Cys-farnesyl or dansyl-Cys-geranylgeranyl.

STRUCTURES OF PDE δ /ARL2 AND PDE δ /RHEB AND GDF FUNCTION OF ARL3-GTP

Cocrystal structures of PDE δ /ARL2^{GTP} (Fig. 8A) and PDE δ /Rheb in complex with ARL3^{GTP} were published by the Wittinghofer group in Germany. These structures and subsequent work were significant breakthroughs in our understanding of the PDE δ /cargo trafficking pathway.^{99,103} PDE δ structure consists of an immunoglobulin-like β -sandwich fold composed of two β -sheets forming a hydrophobic pocket. The N-terminal region forms an α -helix ($\alpha 1$); one sheet is formed by four β -strands ($\beta 1$, $\beta 2$, $\beta 4$, and $\beta 7$), and the other is formed by five β -strands ($\beta 3$, $\beta 5$, $\beta 6$, $\beta 8$, and $\beta 9$).⁹⁹ A loop connecting $\beta 7$ and $\beta 8$ is flexible and disordered (not revealing structure). The β -sandwich structure can accommodate farnesyl (C15) and geranylgeranyl (C20) lipids,¹⁰² but not fatty acids such as myristoyl.⁶⁴ The β -sandwich fold of PDE δ (Fig. 8C) is closely related to those of Rho GTPase guanine nucleotide dissociation inhibitor (RhoGDI)¹⁰⁴ (Fig. 8B) and UNC119⁶⁴ (Fig. 8D)

FIGURE 8. Ribbon representation of PDE δ ARL2^{GTP} complex. (A) Ribbon diagram (PDB 1KSG) based on the crystal structure solved by the Wittinghofer group.⁹⁹ The PDE δ β -sandwich structure (yellow), α -helical structures (red), and loops (green) are indicated; ARL2 β -strands (gray), helical structure (blue) with GTP position (black), and loops (green) are shown for comparison. (B) RhoGDI,¹⁰⁴ PDE δ ,⁶⁶ and UNC119⁶⁴ structures with lipid chains inserted; figures were drawn with PyMOL (www.pymol.org, in the public domain).

despite low sequence similarity (10% overall identity with RhoGDI and 24% overall amino acid sequence identity with UNC119). Major structural differences among these three proteins consist of the loop lengths and structures connecting β -sheet and the N-terminal regions.⁹⁹ RhoGDI extracts prenylated Rac, Rho, and Cdc42 GTPases from membranes and is active only on Rho family GTPases.¹⁰⁴ UNC119A and UNC119B are acyl-binding protein with specificity for a subset of myristoylated N-termini of G-protein α -subunits in *C. elegans* olfactory neurons (ODR-3, GPA-13),⁶⁴ mouse retina photoreceptors (T α),⁶⁴ and kidney epithelial-derived cell lines (NPHP3 in IMCD3 cells).¹⁰⁵ A common feature of these lipid-binding proteins is that they solubilize lipidated proteins, thereby facilitating traffic from one membrane to a target membrane.

In contrast, ARL proteins function as key molecular switches by exchanging GDP with GTP catalyzed by a GEF. Arf-like proteins play important roles in a variety of membrane- and cytoskeleton-associated cellular processes, which are critical for cell homeostasis (reviewed in Refs. 106–108). GTP binding at the P-loop near the ARL2 N-terminus changes the ARL2 conformation at the adjacent switch 1 and switch 2 regions. Nucleotide binding to the P-loops of ARL2 and ARL3 is very strong with nM range affinity.¹⁰⁹ Binding of ARL2, which is not lipidated, to PDE δ is enabled by β -sheet interactions. The interface between ARL2^{GTP} and PDE δ is formed mainly by the $\beta 2$ strand of ARL2 representing switch regions and $\beta 7$ from PDE δ . PDE δ interacts specifically with ARL2 and ARL3 in the

FIGURE 9. Structure of ARL3^{GTP} complexed with RP2.¹¹⁶ Ribbon diagram of the ARL3₁₇₋₁₇₇ (Q71L)-GppNHp-RP2 complex (PDB code: 3BH6): β -strands (red), α -helices (blue for ARL3 and green for RP2), and loops (purple) are shown with GppNHp bound to ARL3 (representing GTP, black). Figure was drawn with PyMOL (www.pymol.org, in the public domain).

GTP-bound form, and does not interact with other ARL proteins.

The 1.7-Å structure of PDE δ in complex with farnesylated Rheb (Ras-like homolog expressed in brain, Fig. 8C) shows the farnesyl group of Rheb buried deeply in the hydrophobic pocket of PDE δ .⁶⁶ The hydrophobic binding pocket of the Rheb/PDE δ complex exists in an “open” conformation to accommodate prenyl side chains. Interaction of Rheb with PDE δ is regulated allosterically by ARL2 and ARL3 with GTP bound. Rheb, PDE δ , and ARL3 form a fast-dissociating ternary complex in which ARL3^{GTP} acts as GDI displacement factor (GDF). When bound to ARL3-GTP, the pocket constricts and evicts its cargo. In this way, ARL3^{GTP} displaces small prenylated G proteins (Rheb) from their lipid-binding proteins, and prevents further interaction by stabilizing the “closed” form of PDE δ . ARL3^{GTP} also promotes cargo release from UNC119,^{105,110} but by an entirely different mechanism in which the β -sandwich opens, rather than constricts, to release cargo.¹¹⁰ ARL2^{GTP} is ineffective with UNC119, thus revealing functional difference from its closely related ARL3^{GTP}.

THE ARL3-RP2 COMPLEX

Retinitis pigmentosa protein 2 (RP2) localizes to the cytoplasmic face of the plasma membrane of cells throughout the retina, including photoreceptors and synaptic terminals as well as inner retina cells.^{111,112} The RP2 N-terminus is myristoylated at G2 and palmitoylated at C3, and acylation is required for plasma membrane targeting.^{111,113} G2A-RP2 and Δ 6S-RP2 mutants lack acyl side chains and are cytosolic.¹¹³ Biochemical analysis showed that RP2 is a GAP for ARL3-GTP.¹¹⁴ Retinitis pigmentosa protein 2 accelerates GTPase activity of ARL3-GTP more than 90,000-fold under saturating conditions and 1400-fold with catalytic amounts of RP2.¹¹⁴ Retinitis pigmentosa protein 2 catalyzes GTPase activity at ARL3 but has no effect on ARL2^{GTP}, and conversely the ARL2-GAP, Elmod 2, has little effect on ARL3^{GTP}.¹¹⁵

A truncated version of ARL3 (residues 17–177) with guanosine 5'-[β , γ -imido]triphosphate (GMPPNP) bound was cocrystallized with RP2, again in elegant experiments by the

FIGURE 10. *Pde6d*^{-/-} phenotype. (A–D) Confocal immunolocalizations of rod PDE6, T γ , GRK1, and cone PDE6 in WT (left) and knockout (right) retina cryosections. (A) Although most PDE6 (MOE antibody) trafficked to the ROS, aberrant distribution of some PDE6 was observed in *Pde6d*^{-/-} rod inner segments (arrow). (B) Distributions of rod T γ in WT and *Pde6d*^{-/-} retinas, respectively. T γ mislocalizes, in part, to the inner segment. (C) GRK1 is nearly undetectable in *Pde6d*^{-/-} rod and cone OS. (D) Cone PDE is undetectable in COS. Nuclei are counterstained with propidium iodide (red). Scale bars: 10 μ m. (E, F) Paired-flash ERGs of WT (left) and *Pde6d*^{-/-} (right). Intensity of the first and second flashes is 1.4 log cd/s/m². The interval between two flashes increases from 600 ms to 5 seconds. Figure adapted with permission from Zhang H, et al. Deletion of PrPb/ δ impedes transport of GRK1 and PDE6 catalytic subunits to photoreceptor outer segments. *Proc Natl Acad Sci U S A*. 2007;104:8857–8862. Copyright 2007 The National Academy of Sciences of the USA.

Wittinghofer group¹¹⁶ (Fig. 9). Retinitis pigmentosa protein 2 structure shows an N-terminal, right-handed β -helix consisting of three stacked β -sheets.¹¹⁴ The β -helix interacts with ARL3, providing the GTPase active site. Arf-like protein 3 forms an interface with RP2 through the P-loop and the switch regions. Many RP2 mutations identified in patients, including the catalytically important arginine finger R118, are located in this area.^{114,116} Apart from ARL3, other known RP2-interacting partners are polycystin 2,¹¹⁷ N-ethyl-maleimide sensitive factor (NSF), a protein promoting vesicle-membrane fusion,¹¹² transducin β (T β),^{118,119} and UNC119.^{64,105,109,110}

PDE6D DELETION IMPEDES TARGETING OF PRENYLATED PROTEINS TO OUTER SEGMENTS

To explore the function of PDE δ in vivo, we generated a *Pde6d*^{-/-} mouse by loxP/Cre-mediated recombination.¹⁰⁰ Deletion of exons 2, 3, and 4 destroyed the β -sandwich structure preventing binding of prenyl side chains. The *Pde6d*^{-/-} mouse was viable, developed normally, and was fertile, but exhibited significantly reduced body size early in life.¹⁰⁰ Phenotypically, the *Pde6d*^{-/-} mouse exhibited transport deficiency of several prenylated membrane-associated proteins, for example, GRK1, PDE6, and T β , to rod and cone OSs (Figs. 10A–D), which correlated with anomalous physiology. In

TABLE 2. Intrinsic GTPase Activity and GAP Acceleration of $T\alpha^{GTP}$ Versus $ARL3^{GTP}$

	Intrinsic GTPase	GAP Acceleration	Fold Activation
$T\alpha^{GTP}$	1–2/min, 0.016/s	6000/min, 100/s	6,000
$ARL3^{GTP}$	0.007/min, 0.000012/s	65/min, 1.2/s	90,000

Pde6d^{−/−} rod single-cell recordings, sensitivity to single photons was increased.¹⁰⁰ Double-flash electroretinograms (ERGs) indicated a >20-minute delay in recovery to the dark state in *Pde6d*^{−/−} rods (Fig. 10F), which is likely due to severely reduced levels of GRK1 in ROS.¹⁰⁰ Surprisingly, rod PDE6 trafficked nearly normally (Fig. 10A), whereas geranylgeranylated cone PDE6 α' was undetectable by immunofluorescence in *Pde6d*^{−/−} COS (Fig. 10D).¹⁰⁰ Under photopic (bright light) ERG conditions, the *Pde6d*^{−/−} cone response was diminished, which is consistent with reduced PDE6 α' levels in cone outer segments (COS).¹⁰⁰ Taken together, PDE δ deletion in photoreceptors results in defective transport of a subset of prenylated proteins (PDE6 subunits and GRK1) to the OS. Transport defects vary, suggesting presence of additional, unidentified prenyl-binding proteins or alternative trafficking pathways. Visual pigments and other TM proteins trafficked normally.¹⁰⁰ The *Pde6d* knockout mouse phenotype resembled a slowly progressing recessive rod–cone dystrophy (retinitis pigmentosa).

PDE6D NULL ALLELES IN HUMAN PATIENTS ARE ASSOCIATED WITH JOUBERT SYNDROME

In contrast to the *Pde6d*^{−/−} mouse, a homozygous *PDE6D* null mutation in human patients caused a severe syndromic ciliopathy (recessive Joubert syndrome).¹⁰¹ An in-frame deletion of *PDE6D* exon 3 removes β -strands β 3 through β 6, destroys the β -sandwich structure of PDE δ , and impairs targeting of farnesylated INPP5E protein to primary cilia.⁸⁵ Patients with *PDE6D* deletion present with postaxial polydactyly, renal hypoplasia, microphthalmia, coloboma, and extinguished ERG, among other dysfunctions. No additional *PDE6D* mutation was found in more than 900 patients with Meckel syndrome, Senior-Løken syndrome, Leber congenital amaurosis (LCA), and other ciliopathies, indicating that *PDE6D* mutations represent a rare cause of ciliopathy. INPP5E is a farnesylated phosphatidyl inositol 3,4,5-trisphosphate (PIP3) 5-phosphatase that removes a phosphate at position 5 of the PIP3 inositol ring and controls activity of phosphatidylinositol 3-kinase (PI3K). That PI3K is an important enzyme in photoreceptors cannot be overemphasized, as the PI3K signaling pathway is essential for rod and cone survival in the mouse retina.^{120,121} These results are consistent with PDE δ functioning as a GDI-like solubilization factor (GDF), serving to extract a subset of prenylated proteins from the ER surface where the C-terminal CAAX box is modified.^{100,122}

THE RP2 KNOCKOUT MOUSE PHENOTYPE: A MILD FORM OF XLRP

As PDE δ , $ARL3^{GTP}$, and RP2 are closely linked in function, we generated an RP2 knockout mouse to gain insight into the function of $ARL3^{GTP}$. Absence of RP2, the GAP of $ARL3^{GTP}$, is predicted to affect strongly the lifetime of active $ARL3^{GTP}$ and increase its levels.¹²³ Intrinsic GTPase activity of $ARL3^{GTP}$ is very low, approximately 100-fold lower than that of $T\alpha^{GTP}$ (Table 2). Abnormal abundance of $ARL3^{GTP}$ is predicted to

force PDE δ to assume a predominantly “closed” conformation, thereby impeding interaction with cargo. The consequence should be vastly reduced effectiveness of PDE6 and GRK1 trafficking.

To verify this prediction, we used a gene-trapped ES cell line to generate an *Rp2b*-null mouse¹²⁴ (Fig. 11). The gene trap cassette was located in the first intron of the mouse *Rp2b* gene, upstream of most truncation mutations associated with XLRP in the human gene. Scotopic a-wave and photopic b-wave amplitudes were reduced in the *Rp2b* knockout animals as early as 1 month of age and more severely at 6 months.¹²⁴ Cone pigments, rhodopsin, transducin, and GC1 target normally to *Rp2b*-null OSs. Trafficking defects were observed with a subset of prenylated proteins in *Rp2b*^{−/−} rods and cones. As shown in Figure 11B, rod PDE6 mislocalized in part to the ISs and the synaptic terminals in the *Rp2b*^{−/−} mouse, similarly to what was reported in *Pde6d*^{−/−} rods.¹⁰⁰ Defects in OS transport of cone PDE6 and GRK1 were much more severe; PDE6 and GRK1 polypeptides were nearly absent in *Rp2b*^{−/−} COS (Figs. 11C, 11D).

FIGURE 11. Mouse *Rp2b* gene knockout. (A) The gene trap located in intron 1 of mouse *Rp2b* truncates RP2 after lysine 32 (K31). (B–D) Cryosections of WT (left) and *Rp2b* knockout (right) retinas were labeled with anti-PDE6 (B), anti-cone PDE6 α (C), and anti-GRK1 (D) antibodies. Scale bars: 10 μ m. Mislocalized PDE6 (right arrow [B]) appears in synaptic terminals.

FIGURE 12. GRK1 trafficking from the ER to its destination membrane. **(A)** Model of PDE δ /ARL3/RP2-dependent trafficking of prenylated proteins in WT photoreceptors. After biosynthesis and prenylation in the cytosol, GRK1 and other prenylated proteins dock to the ER membrane. Following CAAX processing, PDE δ extracts GRK1 from the ER membrane to form a soluble, diffusible complex. PDE δ -GRK1 then combine with ARL3^{GTP} to form a ternary complex, which docks to the destination membrane. The docking station is likely RPGR, as PDE δ is known to interact with RPGR's N-terminal region.¹³⁶ ARL3^{GTP} functions as a GDF by altering the PDE δ "open" conformation to a "closed" one, thereby evicting cargo (GRK1) to bind to membranes. ARL3^{GTP} dissociates from PDE δ and interacts with RP2, accelerating GTP hydrolysis. PDE δ is released to begin another cycle of GRK1 transport. GDP/GTP exchange catalyzed by a guanine nucleotide exchange factor (GEF) regenerates ARL3^{GTP}. **(B)** Perturbed trafficking in *Rp2b* knockout photoreceptors. Absence of RP2 shuts down hydrolysis of GTP bound to ARL3. The ARL3^{GTP}/PDE δ complex extracts GRK1 in minor amounts, and subsequent steps are performed inefficiently.

PROPOSED PDE δ -CARGO TRAFFICKING PATHWAY IN PHOTORECEPTORS

A PDE δ /ARL3^{GTP}/RP2-dependent pathway is modeled in Figure 12. In wild-type (WT) photoreceptors, PDE δ extracts prenylated protein from the ER, forming a soluble complex in which the GRK1 prenyl side chain inserts into the hydrophobic pocket of PDE δ . The soluble GRK1-PDE δ complex then forms a transient ternary complex with ARL3^{GTP} and docks to the destination membrane (Fig. 12A). The docking station has not been determined, but we propose that PDE δ docks to RPGR, as PDE δ is known to interact with the RPGR N-terminal region (RCC1 domain). ARL3^{GTP} forces PDE δ to assume a "closed" pocket conformation to release its GRK1 cargo. Membrane association of ARL3^{GTP}/PDE δ -cargo is essential for RP2 to execute its GAP activity. Retinitis pigmentosa protein 2 catalyzes hydrolysis of GTP bound to ARL3 to GDP by accelerating ARL3's intrinsic GTPase activity, up to 90,000-fold. Failing this (Fig. 12B), ARL3^{GTP} accumulates and PDE δ assumes predominantly a "closed" pocket conformation incompetent to extract prenylated proteins from the ER membrane.

The best-characterized GEF is light-activated rhodopsin that catalyzes GDP/GTP exchange on transducin. The GAP of transducin, a membrane-bound complex of RGS9/G β 5L and R9AP, accelerates the intrinsic GTPase activity ($\sim 1/\text{min}$)⁸³ several thousand-fold to $\sim 6000/\text{min}$ ¹⁰⁹ (Table 2). Absence of photoreceptor GAP activity causes bradyopsia (slow vision) in patients with null mutations in *RGS9* or *R9AP* genes.¹²⁵ Prominent symptoms of these patients are photophobia and difficulty in seeing fast-moving objects (e.g., a baseball thrown toward a bradyopsia patient). Intrinsic GTPase activity of ARL3 is much lower than that of the T α GAP, with a k_{cat} of 0.007/min

($1.2 \times 10^{-5} \text{ s}^{-1}$).¹¹⁴ This low activity accelerates over 6000-fold with catalytic RP2 and 90,000-fold at saturating conditions of RP2 (Table 2). Taken together, mutant photoreceptors lacking RP2 protein are unable to accelerate hydrolysis of GTP bound to ARL3. This defect is predicted to result in a persistently high concentration of hyperactive ARL3^{GTP} locking PDE δ in a "closed" conformation, which impedes its interaction with prenylated cargo, specifically GRK1 and PDE6. In human RP2-XLRP, the resulting photoreceptor degeneration is severe, whereas in mouse, the RP2-XLRP is relatively mild and slowly progressing, affecting both rods and cones. The reason for this distinction is unknown; however, a possible explanation may be provided by the ubiquitous presence of nonspecific GAP activities. In particular, RP2 is known to be a GAP for tubulin-GTP in the presence of tubulin folding cofactor D (TBCD).¹²⁶ In mouse photoreceptors, additional ARL3 GAP activities may be present, thereby reducing the level of ARL3^{GTP} and disease severity.

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