Lecture

Timing Is Everything: GTPase Regulation in Phototransduction

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As the molecular mechanisms of vertebrate phototransduction became increasingly clear in the 1980s, a persistent problem was the discrepancy between the slow GTP hydrolysis catalyzed by the phototransduction G protein, transducin, and the much more rapid physiological recovery of photoreceptor cells from light stimuli. Beginning with a report published in 1989, a series of studies revealed that transducin GTPase activity could approach the rate needed to explain physiological recovery kinetics in the presence of one or more factors present in rod outer segment membranes. One by one, these factors were identified, beginning with PDEγ, the inhibitory subunit of the cGMP phosphodiesterase activated by transducin. There followed the discovery of the crucial role played by the regulator of G protein signaling, RGS9, a member of a ubiquitous family of GTPase-accelerating proteins, or GAPs, for heterotrimeric G proteins. Soon after, the G protein β isoform Gβ5 was identified as an obligate partner subunit, followed by the discovery or R9AP, a transmembrane protein that anchors the RGS9 GAP complex to the disk membrane, and is essential for the localization, stability, and activity of this complex in vivo. The physiological importance of all of the members of this complex was made clear first by knockout mouse models, and then by the discovery of a human visual defect, bradyopsia, caused by an inherited deficiency in one of the GAP components. Further insights have been gained by high-resolution crystal structures of subcomplexes, and by extensive mechanistic studies both in vitro and in animal models.

Photoreceptor cells of the retina have a unique ability to translate the information entering the eye in the form of photons to the language of neuronal electrical activity. The molecular mechanisms by which the first steps of this process occur in rod and cone photoreceptors are known as phototransduction. Two major questions historically facing researchers in this area have been (1) how do the cells generate graded responses to different intensities of impinging light ranging from individual photons, in the case of rods, to stimuli 10^7 times brighter, in the case of cones, and (2) how is temporal resolution achieved, ranging from a few hertz in the case of rods to nearly 100 Hz in the case of cones; that is, how do the molecules that provide the answer to question 1 activate and inactivate within these short time windows? By the mid-1980s, the model of phototransduction outlined below (Fig. 1) was sufficiently developed to explain the rapid onset and the high degree of signal amplification in the light response, both originating from the high rate of the interaction between the G protein transducin and photoexcited rhodopsin (metarhodopsin II, referred to here as R*), enabling the activation of multiple transducin molecules in the course of the light response. However, a large gap in the model was recognized with respect to the kinetics of photoresponse recovery. Our topic today is the process of filling in a large portion of that GAP, which was the focus of much of our work during the subsequent 2 decades.

The current model of phototransduction[1–3] (see Arshavsky and Burns[4] for recent updates) begins with photon absorption by the visual pigment molecules tightly packed within the disc membranes located inside the ciliary outer segment organelles of rods and cones (Fig. 1A). Visual pigments are G protein–coupled receptors. Their light excitation results in the activation of a signaling cascade, called the phototransduction cascade, which includes the G protein transducin and the effector enzyme, the type 6 cGMP phosphodiesterase (PDE) (Fig. 1B).

Photoexcited visual pigment, R*, activates transducin by allowing a rapid exchange of GDP bound to transducin's α subunit ($G\alpha_t$) for GTP. At the next step, $G\alpha_t$-GTP stimulates the activity of PDE by binding to one of the 2 small PDE γ subunits (PDEγ) and releases the inhibitory constraint that PDEγ imposes on the catalytic sites located within the PDE α and β subunits. These reactions result in the hydrolysis of cGMP in photoreceptor cytoplasm and the closure of the cGMP-gated channels located in the plasma membrane of the outer segment. Channel closure hyperpolarizes photoreceptors, leading to decreased release of glutamate, the neurotransmitter at their synaptic terminals conveying light signals to the downstream neurons in the retina.

Is the Rate of Transducin GTPase Too Slow to Explain Rapid Photoresponse Recovery?

As illustrated in Figure 1C, both rising and recovery phases of a photoreceptor's response to a flash of light are very rapid. Even a relatively slow single photon response produced by a mammalian rod is completed on the subsecond timescale. This suggests that all activated components of the phototransduction cascade become deactivated within a fraction of 1 second. By the time the model was developed, it had already been

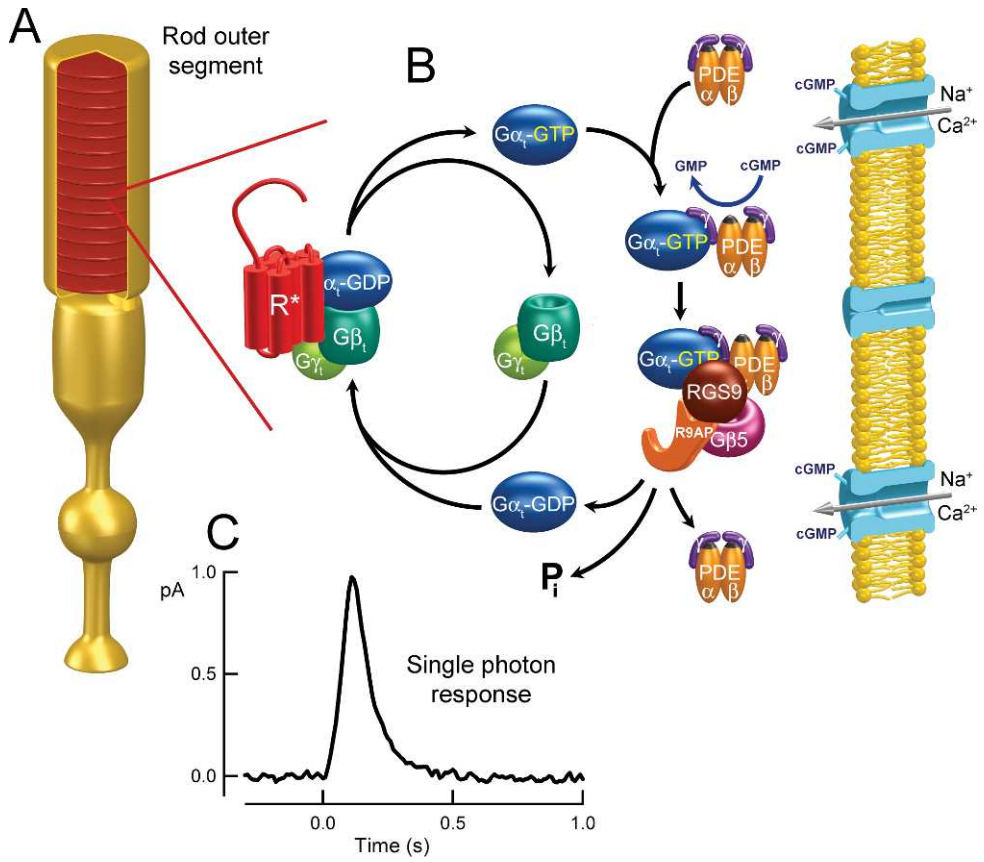

Figure 1. Overview of phototransduction and the physiological light response. (**A**) Schematic representation of a rod photoreceptor cell. Phototransduction takes place in the outer segment organelle filled with several hundreds of flat disc membranes. (**B**) The place of transducin activation/deactivation cycle in phototransduction (note that all protein–protein interactions illustrated here take place on the surface of a photoreceptor disc). Vision begins with rhodopsin photoexcitation to produce its R* conformation, which is capable of activating transducin. Transducin activation consists of the exchange of bound GDP for GTP, followed by the separation of the trimer into $G\alpha_t$-GTP and the $\beta\gamma$ subunits. Next, $G\alpha_t$-GTP stimulates the activity of PDE by binding to PDEγ and relieving the inhibitory action that PDEγ imposes on the PDE catalytic subunits. This results in vigorous cGMP hydrolysis in photoreceptor cytoplasm and closure of the cGMP-gated channels located in the plasma membrane enclosing the outer segment. PDE activation persists until the GTP molecule bound to $G\alpha_t$ is hydrolyzed to GTP, the process facilitated by the RGS9·Gβ5·R9AP complex. The GTP hydrolysis is followed by the return of both transducin and PDE into their inactive states. (**C**) An example of single photon photoresponse recorded from a mouse rod cell. Reprinted with permission from Arshavsky VY, Lamb TD, Pugh EN Jr. G proteins and phototransduction. *Annu Rev Physiol*. 2002;64:153–187. Copyright 2002 Annual Reviews, Inc. Arshavsky VY, Burns ME. Photoreceptor signaling: supporting vision across a wide range of light intensities. *J Biol Chem*. 2012;287:1620–1626. Copyright 2012 American Society for Biochemistry and Molecular Biology. Burns ME, Arshavsky VY. Beyond counting photons: trials and trends in vertebrate visual transduction. *Neuron*. 2005;48:387–401. Copyright 2005 Elsevier.[87]

shown that deactivation of R* is accomplished by the 2-stage mechanism initiated with R* phosphorylation by rhodopsin kinase and completed upon arrestin binding to phosphorylated R*.[5,6] Furthermore, early biochemical studies suggested that rhodopsin phosphorylation may be sufficiently rapid to deactivate R* on the physiological timescale.[7] However, the mechanism responsible for the rapid deactivation of transducin remained unclear. The theory of G protein signaling suggested that transducin acts as a molecular switch. It was expected to activate PDE until the GTP molecule bound to $G\alpha_t$ is hydrolyzed to GDP and P_i. Yet, multiple biochemical measurements showed that the rate at which purified transducin hydrolyzes its bound GTP is nearly 100-fold slower than the photoresponse recovery rate.[8,9] This discrepancy was particularly puzzling because the current dogma in the field was that the rate at which heterotrimeric G proteins hydrolyze their bound GTP is determined by their intrinsic properties and is not regulated by the interacting partners.[10] There were two potential solutions to this puzzle: either transducin GTPase rate is much faster in real photoreceptors than in the test tube, or PDE is deactivated by a GTPase-independent mechanism, whereas GTPase role is restricted to subsequent recycling transducin into its inactive state.

The Dawn

The first attempt to solve this problem was made in 1987 by Arshavsky and colleagues[11] who proposed that the rate at which transducin hydrolyses bound GTP is actually very fast and so is the rate of PDE deactivation. However, the rate of the P_i release from the active site on $G\alpha_t$ may be slow and rate limiting for the overall transducin activation–deactivation cycle. Accordingly, any value of transducin GTPase activity measured under conditions when transducin is activated multiple times (as in all experiments conducted until then) would reflect the rate of phosphate release rather than the true GTP hydrolysis rate. To test this hypothesis, they measured the formation of P_i in a course of a single synchronized turnover of transducin GTPase reaction. A complex between purified transducin and R* was preformed in a preparation of bleached photoreceptor discs, a fraction of transducin was then rapidly

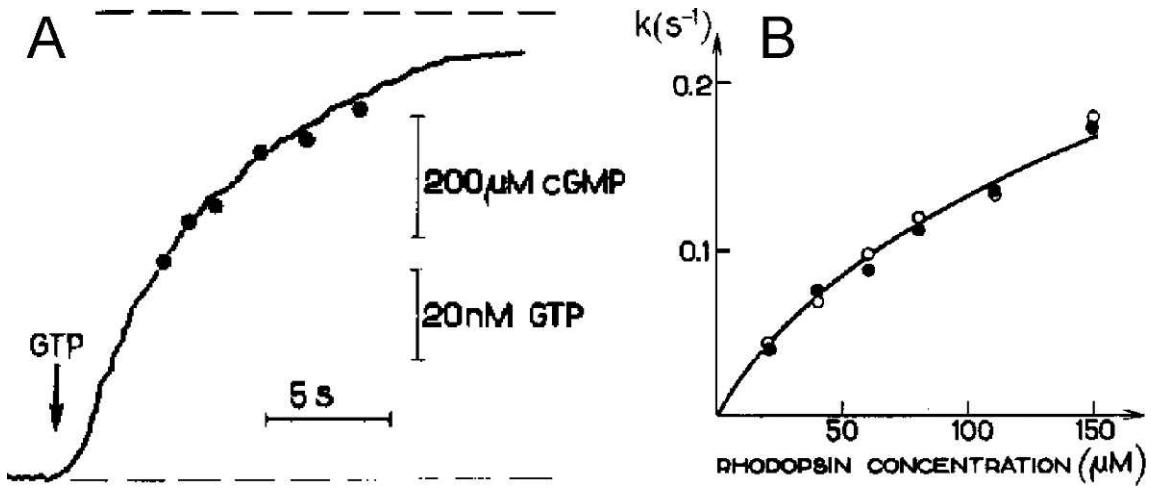

FIGURE 2. Acceleration of transducin GTPase by a component of rod outer segments. Simultaneous measurements of transducin GTPase and cGMP hydrolysis by PDE were performed in suspensions of bleached photoreceptor membranes. Both reactions were initiated by an addition of GTP taken in the amount significantly smaller than that of transducin, thereby allowing only a single synchronized cycle of transducin and PDE activation/deactivation. (**A**) The time courses of 2 reactions followed a similar exponential trajectory. *Solid line* represents continuously monitored cGMP hydrolysis; *solid circles* represent the measurements of P_i produced in the course of the GTPase reaction. Rhodopsin concentration in this experiment was 100 μM. (**B**) Summary of the data obtained at multiple photoreceptor membrane concentrations. The exponential time constants of transducin GTPase (*solid circles*) and PDE deactivation (*open circles*) increased with the increase in membrane concentration, following the same trend. Reprinted with permission from Arshavsky VY, Antoch MP, Lukjanov KA, Philippov PP. Transducin GTPase provides for rapid quenching of the cGMP cascade in rod outer segments. *FEBS Lett*. 1989;250:353–356. Copyright 1989 Federation of European Biochemical Societies.

activated by an addition of radiolabeled GTP in the amount less than the amount of transducin, and the rate of transducin GTPase was determined from the exponential time course of the $^{32}P_i$ formation. These experiments showed that this rate (1 turnover per ~15 seconds at the body temperature) was essentially indistinguishable from the rate measured by traditional "multiple turnover" methodology, which rejected the hypothesis.

However, a major breakthrough was made when these authors applied the same "single turnover" methodology to measure transducin GTPase in suspensions of homogenized rod outer segments[12] (Fig. 2A) and found that an increase in the photoreceptor membrane concentration is accompanied by a significant increase in transducin GTPase rate (Fig. 2B). When projected to cellular conditions of membrane concentration and body temperature, this rate was predicted to exceed 1 turnover per second, thereby falling into the physiological range. A particular advantage of using the single turnover methodology was that it also allowed the authors to compare the kinetics of transducin-bound GTP hydrolysis with the time course of PDE activation and deactivation measured in the same experiment. These measurements revealed a very strong correlation between the rates of transducin GTPase and PDE deactivation (Fig. 2), therefore demonstrating that transducin GTPase indeed serves as the primary PDE deactivation mechanism and that both processes are highly regulated in photoreceptors. This critical observation has launched an over decade-long journey to identify the molecular nature of the GTPase-activating component of the photoreceptor membranes.

The Age of Discovery

The Rate of Transducin GTPase Is Regulated by PDE

The next insight into the mechanism of transducin GTPase regulation came with the demonstration that the rate of this reaction measured in suspensions of frog rod outer segments can be inhibited by cGMP.[13] While the physiological significance of this phenomenon remains poorly understood even today,[14,15] this result stimulated the authors to propose that the effect is mediated by the noncatalytic cGMP-binding sites of PDE and that PDE is directly involved in the GTPase activation. Indeed, an experiment reported in the next paper[16] showed that the addition of PDEγ (an immediate $G\alpha_t$-GTP partner) to photoreceptor membranes, depleted of endogenous PDE but retaining endogenous transducin, resulted in a several-fold increase in the transducin GTPase rate. The same degree of GTPase activation was achieved by the addition of the PDE holo-enzyme and, as predicted, the effect was partially reversed by cGMP.

This result was particularly striking not just because it appeared to explain the mechanism of transducin GTPase activation, but also because it provided the first direct evidence that the GTPase activity of a heterotrimeric G protein can be regulated by its interacting partner. Just a couple months later, it was reported that yet another G protein effector, the β_1 isoform of phospholipase C, exerts a similar effect on its upstream G protein, G_q.[17] Together, these observations refuted a longstanding dogma that only small GTPases, such as Ras, have GTPase-activating proteins (GAPs) and raised the hypothesis that heterotrimeric G protein effectors serve as GAPs regulating their own lifetime in the activated state.[18]

Discovery of Membrane-Bound GAP Activity Distinct From PDEγ

This simple concept was soon challenged by Angleson and Wensel[19] who argued that PDEγ cannot activate transducin GTPase without another membrane-bound protein present in the photoreceptor membranes. They added PDEγ to transducin and dilute (4 μM R*) bovine rod outer segment membranes and found no effect on GTPase kinetics, although a modest enhancement by holo-PDE was observed. Moreover, they were able to reproduce with these membranes the acceleration of GTPase kinetics with increasing membrane concentration even when the PDE inactivation assays showed that virtually all $G\alpha_t$-GTP was bound to PDEγ.

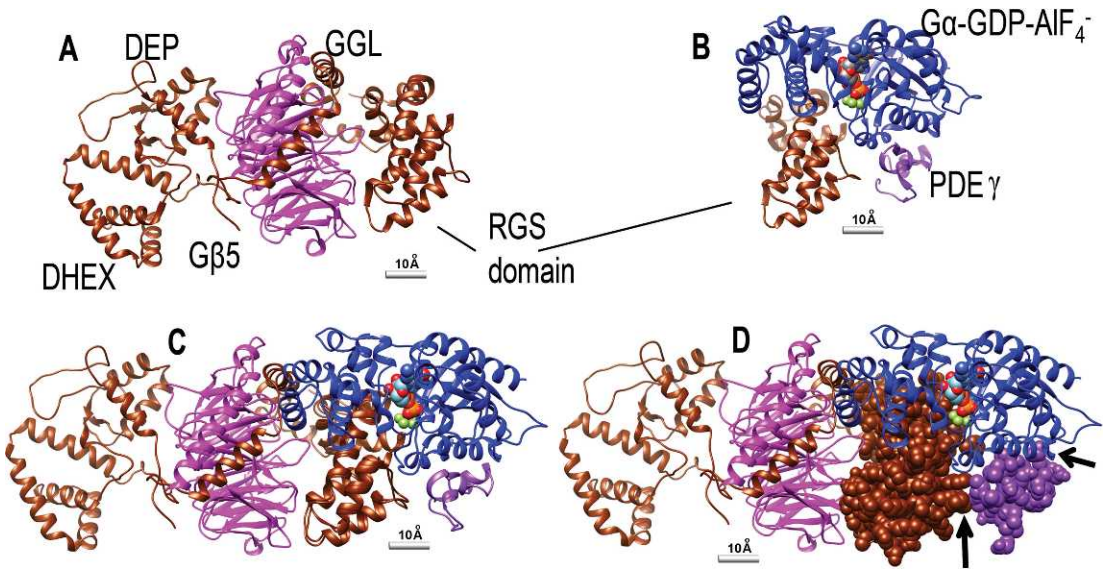

Figure 3. Structures of GAP complexes. (**A**) Complex of RGS9 residues 1–422 with Gβ5 (PDB file 2PBI[39]). The N-terminal DEP domain, DEP helical extension (DHEX), GGL, and RGS domains of RGS9 are labeled. (**B**) The tertiary complex of the RGS domain of RGS9, a $G\alpha_{t/i1}$ chimeric protein in transition state for GTP hydrolysis (mimicked by bound GDP-AlF_4^-) and the C-terminal fragment of PDEγ (residues 46–87) (PDB file 1FQJ[36]). (**C**) Alignment of the RGS domains of the 2 structures in (**A**, **B**) suggests how the components may be arranged under physiological conditions. (**D**) Same as in (**C**) with space-filling representation of the RGS domain and PDEγ showing the interactions of PDEγ with both RGS9 and Gα (*arrows*) that allow it to overcome the inhibitory constraint imposed by Gβ5, and enhance the interactions between RGS9 and Gα to achieve highly specific acceleration of transducin GTP hydrolysis.

Further work confirmed this conclusion and demonstrated that PDEγ acts by enhancing the activity of the membrane-bound protein factor.[20–22] These results led to a continued search for the elusive GAP by several groups, and established one criterion for the authentic transducin GAP: its activity toward transducin should be enhanced by PDEγ.

Transducin GTPase Is Activated by an RGS Protein

Following the discovery of a new family of proteins known as RGS proteins, which act as negative Regulators of G protein Signaling,[23–26] it was hypothesized that one mechanism by which such negative regulators could work might be to function as GAPs for G protein α subunits.[24] This possibility, which was soon confirmed experimentally,[27] led several groups to look for RGS proteins that might act as the GAP for transducin.[28–31] Multiple (eventually upwards of 11) RGS-encoding mRNAs were found to be expressed in the retina. Of these, only RGS9, and specifically its retina-specific splice variant, RGS9-1,[32,33] was found to be enriched in photoreceptor outer segments,[34] especially in cones.[35] Of all RGS core domains tried up to that time, only the one from RGS9 had GAP activity toward transducin that was enhanced by PDEγ.[34] In contrast, PDEγ inhibited the GAP activity of other tested RGS domains.[28,31] These results pointed very strongly to RGS9-1 as the major PDE-enhanced GAP for transducin. The results also suggested that a high-affinity ternary complex is formed by PDEγ, the core RGS domain of RGS9, and $G\alpha_t$ in the transition state of GTP hydrolysis and, indeed, within a few years, a crystal structure of this complex was solved.[36] Figures 3A and 3B show two RGS9 complex structures solved by crystallography, and Figures 3C and 3D show how their components might fit together.

RGS9 Exists as a Constitutive Complex With a Nonconventional G Protein β Subunit

The story, however, proved not to be so simple because the attempts to purify RGS9 from detergent-solubilized rod outer segments membranes consistently revealed the presence of the second protein band of ~44 kDa.[37] This band represented the long splice isoform of the type 5 G protein β subunit (Gβ5), a distant member of the G protein β subunit family.[38] Subsequent experiments revealed that RGS9 and Gβ5 form a tight complex[37] via a highly specific coiled–coil interaction between Gβ5 and the G protein γ-subunit–like (GGL) domain of RGS9.[39] The 3-dimensional structure of this complex, solved a decade later[39] (Fig. 3B), revealed its remarkable similarity to the interactions of G protein γ subunits with conventional Gβ subunits. Importantly, a similar tight association with Gβ5 isoforms was documented for a group of GGL domain–containing RGS proteins, known as the R7 RGS subfamily.[40–42] Experiments with recombinant proteins,[43,44] as well as the analysis of protein expression in the Gβ5 knockout mouse,[45] indicated that proper folding and stability of all R7 proteins, including RGS9, are crucially dependent on their binding to Gβ5. Therefore, the complex formation between RGS9 and Gβ5 is constitutive, resembling that in conventional G protein βγ-subunit complexes.

RGS9·Gβ5 Is Tethered on the Membrane Surface by an Anchor Protein

The inventory of the GAP complex was not yet complete. The discovery of the partnership of Gβ5 made possible the expression of RGS9 and its purification from insect cells as a fully soluble complex with Gβ5.[43,46] In some ways this result was not surprising, because neither subunit is predicted to have transmembrane or lipidation motifs. However, RGS9 in rod outer segments behaved like a transmembrane protein, requiring as much detergent to solubilize it as rhodopsin, with its 7 transmembrane helices.[35] The solution to this puzzle came from immunoprecipitation experiments, in which RGS9-specific antibodies were used to pull down associated proteins from detergent extracts of rod outer segments.[47] The results were remarkably simple and clear: there were only 4 major bands visible upon staining an SDS-PAGE gel of the pellet, all

present at roughly equal stoichiometric ratios: RGS9, $G\alpha_t$-GDP (whose role in this complex remains unknown), Gβ5, and a novel protein whose migration was consistent with a molecular mass of approximately 25 kDa. This protein was fragmented into peptides and their sequences were determined by Edman degradation, which allowed the isolation of bovine cDNA clones, and identification of intronless genes encoding a 25-kDa protein in the mouse and human genome databases. This protein, which has homology to the syntaxin family SNARE proteins, and a single predicted transmembrane helix, was named R9AP (also known as RGS9-1 binding protein 1) for RGS9 Anchor Protein.[47] Further studies confirmed that R9AP has a transmembrane helix, revealed that its cytoplasmic domain binds tightly to the RGS9·Gβ5 complex, and showed that full-length recombinant R9AP reconstituted into lipid vesicles could anchor RGS9·Gβ5 to the membranes. When co-expressed in insect cells, R9AP caused translocation of EGFP-tagged RGS9·Gβ5 from the cytoplasm to the plasma membrane. Antibodies raised against R9AP stained the photoreceptor outer segments, with the brightest signal in the cones, in addition to weak staining of the outer plexiform layer. Experiments with recombinant fragments of RGS9·Gβ5 and R9AP revealed that the interacting sequences involve primarily the N-terminal 214 amino acids of RGS9, which include a DEP domain and the N-terminal putative trihelical domain of R9AP.[47–50] These same studies revealed a dramatic enhancement of the GAP activity of RGS9-1 upon binding of the complex to R9AP, only a small part of which is attributable to a simple increase in local concentration upon membrane binding.[51]

Knockout Mice Reveal the Physiological Role of the GAP and Its Components

Although studies of biochemical properties and localization of the RGS9·Gβ5·R9AP complex strongly suggested that these proteins control the kinetics of photoresponse recovery, the most conclusive evidence came from mice with engineered deletion of each of these proteins.[45,52–54] The photoresponses of rods from each of these lines, as measured by single-cell suction electrode recording, are virtually identical, but strikingly different from those of wild-type mice. The responses to subsaturating flashes have fairly normal rising phases and peak amplitudes, but dramatic, more than 14-fold slowing of their recovery phases. Recovery from saturating flashes was even slower, more than 33-fold slower than from wild-type rods. These mice also revealed the dependence of the subunits of the GAP complex on the others for stability and subcellular localization. RGS9 knockouts had no detectable Gβ5 in their photoreceptors, and the Gβ5 knockouts had no detectable RGS9, but both had normal levels of R9AP. In contrast, R9AP knockouts had severe depletion of both RGS9 and Gβ5 in their photoreceptors. Additional studies, discussed below, revealed in further depth not only the function of each subunit of the GAP complex, but also their constituent domains.

PDEγ Is an Affinity Adapter

Establishing the RGS9·Gβ5·R9AP complex as an immediate GAP for transducin raised the mechanistic question of how this activity is further potentiated by PDEγ, the historically first protein implicated in transducin GTPase activation. A study by Skiba and colleagues[55] demonstrated that PDEγ acts as an "affinity adapter," enhancing the mutual affinity between RGS9·Gβ5 and $G\alpha_t$-GTP by more than 20-fold. This effect is observed with both free PDEγ and PDEγ as a part of the PDE holo-enzyme, although the latter potentiates the GAP activity of RGS9·Gβ5·R9AP ~2 times less efficiently than free PDEγ.[16,20]

The role of PDEγ as an affinity adapter could be conceptualized by assuming that it provides an orderly sequence of protein–protein interactions during the propagation of a photoresponse.[56–58] The low affinity of RGS9·Gβ5 for free $G\alpha_t$-GTP may be beneficial to prevent transducin from hydrolyzing the bound GTP before it has a chance to activate its effector enzyme, PDE. On the other hand, the high affinity of RGS9·Gβ5 for $G\alpha_t$-GTP associated with PDE allows the completion of GTP hydrolysis on the rapid timescale of photoresponse recovery. Physiological evidence that this function of PDEγ is critical for timely photoresponse recovery was obtained by using a transgenic mouse in which PDEγ was replaced by its W70A mutant.[57] Preceding studies have revealed that mutations in this tryptophan residue drastically reduce the affinity between PDEγ and $G\alpha_t$-GTP and completely abolish the ability of PDEγ to potentiate transducin GTPase.[59,60] Consistently, rod photoresponses in W70A mice were characterized by reduced light-sensitivity and slow recovery, with the latter highlighting the critical role of PDEγ in timely phototransduction cascade deactivation. It is worth noting that W70A rods still recovered from light excitation approximately twice as fast as RGS9 knockout rods, in agreement with the biochemical evidence that the RGS9·Gβ5·R9AP complex maintains a certain level of the GAP activity even without co-operating with PDEγ.

Recognition of the mutual enhancement of the interactions among $G\alpha_t$, PDEγ, and RGS9 led to the crystallization and structure determination of complexes formed by the catalytic domain of RGS9 with $G\alpha_t$ and of a ternary complex that also contained the C-terminal transducin-binding domain of PDEγ.[36] This structure, shown in Figure 3B, helped to explain that the ability of GTP-bound transducin to activate PDE is based on sequestering the PDEγ residues responsible for inhibition of PDE catalytic subunits.[59,61,62] It also revealed the structural basis for the key role of tryptophan-70 of PDEγ in binding to transducin.

To conclude the discussion of affinity adapters, we should add that this biochemical mechanism is not limited to phototransduction. A study by Martemyanov and colleagues[58] pinpointed a high degree of homology between the functionally significant region of PDEγ and the unique C-terminal sequence of RGS9-2, the brain-specific splice isoform of RGS9 regulating dopamine and opioid signaling in the basal ganglia. The authors further demonstrated that this C-terminus enhances the affinity between RGS9-2 and its cognate G protein α-subunit partner, $G\alpha_o$, just as PDEγ does for RGS9-1 and transducin in rods and cones. A functional advantage of having an affinity adapter as a structural component of an RGS protein itself, as opposed to its effector, may arise from the fact that downstream effectors in G_o pathways are usually activated by G_o βγ-subunits,[63] whereas $G\alpha_o$-GTP interacts with RGS proteins to regulate the duration of signaling events. Another hypothesis, provoked by the functional and structural similarities between PDEγ and RGS9-2, is that the PDEγ gene evolved as a duplication of part of the RGS9 gene encoding the RGS9-2 C-terminus.[58,64]

The Complexity of Transducin GTPase Regulation by Gβ5, Noncatalytic Domains of RGS9 and R9AP

Once all molecular partners in the GAP complex were known, and methods developed for expressing and purifying the recombinant proteins, further insights were obtained by observing the effects of mutations and truncations, and from x-ray crystallography. These studies revealed, for example, that RGS9 interactions with Gβ5 are mediated primarily by the GGL

domain of RGS9, as had been determined for other members of the R7 family of RGS proteins.[42] They showed that these 2 proteins are dependent on one another for proper folding and stability and that interactions of RGS9 with Gβ5 are critical for conveying the dependency of the complex' GAP activity on interactions with PDEγ.[43,46,65,66] In later years, the structure of the RGS9·Gβ5 complex was solved[39] (Figs. 3A, 3C, 3D) revealing an interesting molecular arrangement in which the N- and C-terminal portions of RGS9 are separated by the GGL-Gβ5 module whose structure resembles those of the G protein βγ-subunit complexes.

A hallmark property of RGS9·Gβ5 is that the enhancement of its GAP activity by PDEγ is much more prominent than that of the isolated RGS9 homology domain, in which it never exceeds 2- to 3-fold.[43,46,65,67,68] Two complementary studies from our laboratories revealed that this difference is explained by opposing contributions from Gβ5 and multiple individual noncatalytic domains of RGS9.[43,46] Interestingly, the molecular module consisting of the core 7-propeller structure of Gβ5 associated with the GGL domain of RGS9 strongly reduced the affinity of the RGS9 catalytic domain for transducin, without affecting the degree of its potentiation by PDEγ. However, all other structural elements of RGS9 (the N-terminal DEP/R7H domain and the short C-terminal extending beyond the RGS homology domain), along with the N-terminus unique for the long splice isoform of Gβ5, reverse this inhibition specifically for $G\alpha_t$-GTP·PDEγ, but not for free $G\alpha_t$-GTP. This complex pattern of intramolecular interactions ultimately enables RGS9·Gβ5 to bind the transducin–effector complex with >20-fold higher affinity than free activated transducin.

The primary role of Gβ5 and noncatalytic domains of RGS9 in setting the substrate recognition specificity of RGS9·Gβ5 was further emphasized in a follow-up study.[65] The authors first engineered an RGS domain mutant whose GAP activity was inhibited instead of potentiated by PDEγ (this was accomplished by introducing the double L353E/R360P mutation, using the strategies introduced in Sowa et al.[69]) and then investigated the substrate specificity of full-length recombinant RGS9·Gβ5 bearing the same mutations. Remarkably, these mutations did not reverse the ability of RGS9·Gβ5 to favor $G\alpha_t$-GTP·PDEγ over free $G\alpha_t$-GTP as a substrate, although they reduced the degree of this effect from 23- to 6-fold.

Yet, even a larger overall role in potentiating the catalytic activity of RGS9·Gβ5 is played by R9AP,[49,51,70] which enhances this activity as much as ~70-fold[49] (!). In fact, deactivation of transducin on the physiologically rapid timescale would not be achievable without this R9AP contribution. Curiously, we now understand in hindsight how this R9AP property doomed our early efforts to purify the transducin GAP, using ion-exchange chromatography: the separation of R9AP from RGS9·Gβ5 on ion-exchange columns impeded our ability to detect the GAP activity in chromatography fractions. In contrast, the preservation of the entire GAP complex upon gel-filtration allowed reliably following its activity upon purification.[37]

R9AP enhances the GAP activity of RGS9·Gβ5 only when it is attached to the membrane.[51] This effect is described by a complex kinetic mechanism combining a significant allosteric activation of RGS9·Gβ5 with a modest increase in its affinity for $G\alpha_t$-GTP. It remains unknown whether both effects are consequential to placing RGS9·Gβ5 at the membrane surface, or either may result from direct protein–protein interactions.

R9AP Also Regulates Intracellular Stability and Localization of the GAP Complex

Another important function of R9AP is to set the cellular content of the entire GAP complex. As already noted, photoreceptors of R9AP knockout mice retained no detectable RGS9 and a very small fraction of Gβ5, despite mRNA levels for both proteins remaining normal.[54] Furthermore, photoresponses recorded from rods of these mice were indistinguishable from those of RGS9 knockouts,[54] consistent with the lack of functional GAP complex in their outer segments. Conversely, R9AP overexpression in photoreceptors (but not overexpression of RGS9[71] or Gβ5[45]) caused a several-fold increase in the expression level of the entire RGS9·Gβ5·R9AP complex.[71] It was originally proposed that intracellular stabilization of RGS9·Gβ5 by R9AP is achieved via protein–protein interactions; however, transgenic replacement of R9AP with a mutant lacking the transmembrane domain failed to stabilize RGS9·Gβ5.[72] The same study revealed that the role of R9AP is limited to recruiting RGS9·Gβ5 to cellular membranes: endowing RGS9 with its own means of membrane attachment stabilized RGS9·Gβ5, regardless of its site of intracellular localization in rods.

Mechanistically, the intracellular instability of soluble RGS9·Gβ5 is explained by the presence of 6 destabilizing KFERQ-like motifs within the N-terminus of RGS9.[73] These motifs facilitate binding of the heat shock protein Hsc70, which targets proteins for lysosomal degradation in a process known as chaperone-mediated autophagy. In this context, the stabilization of RGS9·Gβ5 by membrane association could be explained by either masking these destabilizing motifs or Hsc70 failing to physically extract RGS9·Gβ5 from the membrane.

Lastly, the RGS9·Gβ5 association with R9AP is critical for targeting the entire GAP complex to photoreceptor outer segments.[50] Curiously, the molecule of RGS9·Gβ5 possesses intracellular targeting information that specifies its exclusion from the outer segment.[72] This targeting information is completely "neutralized" by association with R9AP to allow outer segment targeting. A study conducted with frog rods noted that the pattern of intracellular distribution of R9AP coincided with those for untargeted transmembrane proteins and suggested that R9AP may not have specific targeting information and instead is delivered to the outer segment (along with associated RGS9·Gβ5) by incorporating into the rhodopsin carrier vesicles comprising most post-Golgi transport vesicles in frog rods.[74] However, this simple concept is unlikely to apply to mammalian photoreceptors characterized by smaller outer segment volumes and correspondingly smaller fraction of intracellular trafficking flow bound for this destination.[75] Therefore, a detailed mechanism responsible for the intracellular targeting and trafficking of RGS9·Gβ5·R9AP remains an area of active investigation.

It is important to stress that engagement of R9AP in regulating the activity, intracellular stability, and subcellular localization of the GAP complex in rods represents yet another general principle in cellular signaling. R9AP and its more ubiquitously expressed homolog R7BP[76,77] regulate the same three functions of all 4 members of the R7 RGS protein family in the retina and throughout the central nervous system.[64,78,79]

Overexpression of RGS9·Gβ5·R9AP Demonstrates That Transducin GTPase Is the Rate-Limiting Reaction in Rod Photoresponse Deactivation

A study by Krispel and colleagues[71] revealed yet another fundamental role for the GAP complex in regulating the visual function. It has been long known that the timely recovery of the photoresponse requires efficient deactivation of both R* and transducin, with the slower, rate-limiting of these two steps playing a critical role in defining the overall rate of response recovery[80] (reviewed in Burns and Pugh[81]). However, it took more than a decade to make an experimental distinction between these two possibilities because the answer

required a molecular manipulation that would accelerate the rate-limiting step, thereby speeding up response deactivation. The opposite manipulations of slowing down any of the recovery steps were easier to achieve, but not very informative because they did not allow to understand whether the rate-limiting reaction became even slower or the nonlimiting one took its place.

Krispel and colleagues[71] achieved overexpression of RGS9·Gβ5·R9AP in rods by overexpressing the R9AP gene and found that this manipulation results in significant acceleration of photoresponse recovery. In contrast, overexpression of rhodopsin kinase, the enzyme responsible for R* deactivation, did not cause a notable effect on the rod response recovery rate, reinforcing the authors' conclusion that this rate is set by transducin GTPase controlled by the GAP complex.

Interestingly, cones express more RGS9 than rods,[35,82] which is likely to contribute to their faster recovery. Therefore, the expression level of RGS9·Gβ5·R9AP serves as a key determinant of the temporal characteristics of individual types of photoreceptor cells. Notably, a recent study of salamander cones suggested that the rate-limiting step for the recovery of cone photoresponses is the deactivation of cone R*.[83] A plausible interpretation of this result is that these cells express so much RGS9·Gβ5·R9AP that transducin GTPase rate surpasses the rate of R* deactivation and the latter becomes rate limiting under these conditions.

Visual Function Without RGS9·Gβ5·R9AP: The Studies of Mice and Men

As already described above, single-cell recordings from RGS9 knockout mice displayed slow rates of photoresponse recovery in rods[52] and the same effect was documented for cones.[84] These results provoked a great interest to understanding how such a defect would affect the overall visual function, both in animals and in humans. The first breakthrough in this direction was achieved by Nishiguchi and colleagues[85] who characterized a group of patients, one of whom lacked the R9AP gene while several others had a point mutation in RGS9 completely abolishing its GAP activity. These patients reported difficulties in adjusting to changes in luminance (such as moving between a poorly lit space and the sunlight and vice versa). They also suffer from photophobia and typically present this problem as the chief complaint when they seek help from an ophthalmologist. Another fascinating feature of their disorder is the inability to see rapidly moving objects, particularly those of low contrast. The condition of these patients was termed "bradyopsia" (slow vision).

The next insight into the slow vision phenotype was obtained in a recent study, which characterized mice lacking the RGS9·Gβ5·R9AP complex by a combination of two in vivo techniques: electroretinographic (ERG) analysis and optomotor behavior assays.[86] Electroretinographic recordings were used to monitor electrical responses of the retina to flickering light stimuli, whereas behavioral assays addressed the ability of animals to detect moving gratings. Somewhat surprisingly, neither slowdown of photoresponse recovery nor its acceleration (achieved by R9AP overexpression in wild-type mice) altered these functions in dim light, at which visual inputs are conveyed by rods acting as single-photon counters. However, under conditions of moderate or bright illumination, when both rods and cones process multiple photon inputs, mice lacking the GAP complex displayed a profound loss in sensitivity to high temporal frequency stimuli, entirely consistent with the phenotype of human patients analyzed at comparable lighting conditions. Furthermore, the sensitivity loss documented by ERG and behavioral assays was comparable, suggesting that control of behavioral temporal contrast sensitivity occurs primarily in the retina.

These important results suggest that rod photoresponse recovery under single photon counting conditions resets the resting state of these cells, but does not determine the temporal characteristics of the downstream responses. On the contrary, timely photoresponse recovery in both rods and cones is critical for maintaining high temporal resolution of vision under conditions when these cells simultaneously process multiple photon inputs and essentially detect light intensity levels as opposed to single photon events.

The New Horizons

Despite a great deal of mechanistic details learned about the molecular components of the GAP complex in photoreceptors, more work is needed to fully understand its functioning. Future studies should incorporate the missing members of the complex into its complete 3-dimensional structure, including the PDE holo-enzyme and R9AP. One of the most pressing functional questions relates to the role of the transducin α subunit molecule tightly and stoichiometrically bound to the GAP complex isolated from the photoreceptor membranes. More needs to be learned about the psychophysical aspects of slow vision, particularly in regard to the applicability of the mouse behavioral studies to properties of human vision.

But perhaps the most exciting aspect of these studies, still awaiting to be fully comprehended, is the degree to which the lessons learned upon investigating the photoreceptor GAP complex are applicable to understanding the functional organization of other signaling pathways in other issues and cell types. We tried to emphasize the general principles revealed so far, as we told our story, from the very demonstration that heterotrimeric G proteins have their own GAPs to revealing the versatility of the interactions between R7 RGS proteins and their membrane anchors. We anticipate further examples to emerge as we learn more about the temporal regulation of cellular signaling in the retina and beyond.

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