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The Experiences of a Biochemist in the Evolving World of G protein-dependent Signaling

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Abstract

This review describes how a biochemist and basic researcher (i.e. myself) came to make a career in the area of receptor-coupled signal transduction and the roles cellular signaling activities play both in normal physiology and in disease. Much of what has been the best part of this research life is due to the time I spent with Bob Lefkowitz (1982–1985), during an extraordinary period in the emerging field of G-protein-coupled receptors. Among my laboratory colleagues were some truly outstanding scientists including Marc Caron, the late Jeffrey Stadel, Berta Strulovici, Jeff Benovic, Brian Kobilka, and Henrik Dohlman, as well as many more. I came to Bob's laboratory after being trained as a physical biochemist and enzymologist. Bob and his laboratory exposed me to a research style that made it possible to connect the kinds of fundamental biochemical and mechanistic questions that I loved to think about with a direct relevance to disease. Indeed, I owe Bob a great deal for having imparted a research style and philosophy that has remained with me throughout my career. Below, I describe how this has taken me on an interesting journey through various areas of cellular signaling, which have a direct relevance to the actions of one or another type of G-protein

Keywords

signaling; G-proteins; GPCRs; Ras; Cdc42; GAPs; GEFs

2. The early days of G protein-coupled receptors (GPCRs): Simplicity

I arrived in the Lefkowitz laboratory just after they had achieved the first purification of the β 2-adrenergic receptor (HPLC) [1]. This was a heroic achievement, much of which was driven by Rob Shorr and Marc Caron, and soon moved to even greater heights by Jeff Benovic [2,3]. The purification of the putative β 2-adrenergic receptor, comprised of just a single polypeptide chain, was based solely on the successful detergent-solubilization of a protein that exhibited the proper ligand-binding capability. Thus, the question remained as to whether this protein was capable of the other critical function that such a receptor needed to fulfill, namely, the ability to couple to its G-protein partner and transmit a signal. My goal upon arriving at the Lefkowitz laboratory in the summer of 1982 was to demonstrate that the

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newly purified $\beta 2$ -adrenergic receptor was capable of both hormone binding and signal propagation, by reconstituting the signaling interactions between the purified receptor, the purified Gs protein, and ultimately the purified effector protein, adenylyl cyclase. Eventually, I was able to do this but not without the help and efforts from others in the Lefkowitz laboratory (e.g. Caron, Benovic, Strulovici), as well as with the aid of some superb outside collaborators including Allen Spiegel, who at the time was an investigator at NIH (now the Dean of Albert Einstein Medical School), Lutz Birnbaumer, then at Baylor Medical College (now at the National Institute of Environmental Health), and the late Eva Neer of Harvard Medical School [4,5]. The reconstitution of the $\beta 2$ -adrenergic receptor signaling pathway showed us just how beautifully simplistic was the design of GPCR-signaling pathways (Figure 1). Upon the binding of a hormone (agonist), the $\beta 2$ -adrenergic receptor (e.g. R^* in Figure 1) was able to associate with the heterotrimeric Gs protein, which had recently been purified by the laboratories of the late Al Gilman and Lutz Birnbaumer, stimulating the exchange of GDP for GTP on the alpha subunit of the G-protein ($G\alpha$). This resulted in the dissociation of the GTP-bound $G\alpha$ subunit from its beta ($G\beta$) and gamma ($G\gamma$) subunit partners, enabling its ensuing interaction with its effector protein, adenylyl cyclase (i.e. as depicted by the conversion of E_1 to E_2 in Figure 1).

From this beginning came the discovery of additional layers of regulation. One critically important mode of regulation, as discovered by Jeff Benovic, results in the desensitization of the receptor-coupled signaling pathway as an outcome of receptor phosphorylation by G protein-coupled receptor kinases GRKs [6–8]. Receptor phosphorylation enabled members from a family of proteins called arrestins to directly bind to the receptor, resulting in a steric interference of G-protein coupling [9–11]. This not only shuts down receptor communication with its G-protein partner, but it also leads to receptor endocytosis and ultimately receptor ubiquitylation and degradation.

A second key mode of regulation was first discovered by Cassel and Selinger [12], who showed that GTP-hydrolysis by the Gs protein serves as a mechanism for halting the stimulation of adenylyl cyclase. Additional studies showed that $G\alpha$ subunits were capable of an intrinsic GTP hydrolytic reaction that occurs within 30 seconds, although this shut-off mechanism can be greatly accelerated by members of the family of RGS (Regulators of G-protein signaling) proteins [13–15]. Any disruption of these regulatory mechanisms can have dire consequences. One classic example is cholera toxin-mediated pathogenesis, as this toxin catalyzes the modification of an arginine residue on the $G\alpha$ subunit of the Gs protein that is essential for GTP-hydrolysis. Consequently, the cholera toxin-modified Gs- α subunit persists in a GTP-bound state, unable to shut-off, disrupting normal ion channel function in the intestine and resulting in severe diarrhea.

Perhaps even more remarkable was the realization of just how often this signaling architecture is used in biology [16]. A striking example is our ability to see in dim light through the phototransduction pathway operating in retinal rods [17,18]. In this case, the absorption of light by the photoreceptor, rhodopsin, stimulates GDP-GTP exchange on the G-protein transducin, which generates a GTP-bound $G\alpha$ subunit that binds and activates an effector enzyme, the cyclic GMP phosphodiesterase, converting cyclic GMP to GMP. The reduction in the levels of cyclic GMP causes the closing of a sodium channel in retinal rods,

resulting in a hyperpolarization of the rod membranes, which represents the signal that is sent to the optic nerve. Through the years, it became clear that similar types of GPCR-signaling systems were responsible for other sensory response systems, such as those that recognize different odorants and tastants (i.e. our senses of smell and taste), as well as for the regulation of smooth muscle contraction, platelet activation, various neurotransmitter activities and metabolic functions.

3. A starting assumption: Growth factor-dependent signal transduction utilizes similar signaling systems as those used by GPCRs

It was with this as a backdrop that I started to think about the similarities that might exist between GPCR-signaling systems and those stimulated by growth factors which control the normal growth of cells, and when de-regulated, give rise to cancer. Thus, upon starting my independent academic career at Cornell, I decided I would set out to reconstitute epidermal growth factor receptor (EGFR)-coupled signaling, with the idea that the EGFR would activate a G-protein, possibly through an EGFR-catalyzed phosphorylation of the G-protein. I assumed that the activated G-protein would then stimulate the activity of an effector protein, transmitting a signal to help drive cell cycle progression and mitogenesis. What made this idea particularly attractive was the existence of an obvious candidate for the G-protein functioning in EGFR-signaling, namely Ras.

The Ras (for Rat sarcoma) protein was first identified as the causative agent in the rat sarcomas caused by the Harvey and Kirsten retroviruses. Ras was subsequently identified by a number of laboratories, including the Weinberg, Wigler, Barbacid, and Cooper groups, as the first human oncogene product [19–22]. Importantly, the Ras mutations driving tumorigenesis involved substitutions that prevented Ras from hydrolyzing GTP. Therefore, it seemed logical to assume that a normal mitogenic signaling pathway could start with the EGFR prompting the exchange of GDP for GTP on the Ras protein, with GTP-bound Ras then activating an effector protein for a defined period of time, before GTP-hydrolysis shuts down signal propagation. Those oncogenic mutations that prevent Ras from shutting off would give rise to uncontrolled cell growth and thereby represent an initial and important step in tumorigenesis.

Starting with this working model, my laboratory set out to reconstitute the functional coupling between highly purified preparations of the EGFR and Ras in liposomes, examining whether Ras was tyrosine phosphorylated and/or stimulated to bind radiolabeled GTP. However, this approach failed miserably, as we did not see the EGFR stimulate the phosphorylation of Ras or its GTP-binding activity. We now know that these experiments failed because we were missing a key class of regulatory proteins that normally links the EGFR to the stimulation of GDP-GTP exchange on the Ras proteins. At the time, I chalked up these failures to perhaps having the wrong G-protein for the EGFR, since it was becoming clear through the work of Yoshimi Takai and others that a family of Ras-related G-proteins exists (now often referred to as small G-proteins or small GTPases) [23]. This led us to search for the proper G-protein partner for the EGFR by reconstituting the receptor with membrane preparations from different sources. Through these efforts, we found what

appeared to be a potential signaling partner that was phosphorylated by the EGFR in reconstituted liposomes, with the phosphorylation being eliminated in the presence of micromolar amounts of the GTP-analog, GTP γ S, leading us to believe it was a Ras-related G-protein [24]. Upon the purification and molecular cloning of this phospho-substrate, we were able to identify it as the mammalian/human homolog of a yeast (*Saccharomyces cerevisiae*) gene product that had been discovered at the same time and was classified as a cell-division-cycle protein, named Cdc42 [25,26]. Thus, despite our incorrect starting assumption that the EGFR should be capable of directly binding to and phosphorylating its G-protein target, which led to our wrong conclusion that Ras might not be a G-protein signaling partner of the EGFR, we were nonetheless able to discover a new member of the Ras family that would keep our laboratory and many others consumed for a number of years.

4. Understanding the regulation of Cdc42 and the functional similarities to the regulation of G-proteins by GPCRs

Like the G-proteins activated by GPCRs, Cdc42 and other Ras-related small G-proteins mediate their signaling functions through a tightly regulated GTP-binding/GTP hydrolytic cycle. However, unlike the large G-proteins, the small G-proteins are not directly activated by membrane receptors. Indeed, the phosphorylation of Cdc42 observed in our reconstituted systems containing the EGFR was actually catalyzed by c-Src, which was also present in these preparations, and did not directly result in stimulating GDP-GTP exchange. Rather, three primary regulatory proteins are responsible for setting the timing of the GTP-binding/GTP hydrolytic cycle of Cdc42 and related small G-proteins. These are guanine nucleotide exchange factors (GEFs), GTP-hydrolysis-activating proteins (GAPs), and Rho guanine nucleotide dissociation inhibitors (GDIs) (Figure 2).

4.1. GEFs serve a similar function as GPCRs but through a unique mechanism

As is the case for the G_{α} subunits of large G-proteins, Cdc42 and other Ras-related small G-proteins bind GDP with high affinity, such that in the absence of an activating signal, they remain in a GDP-bound state. Once it became clear that Cdc42 was not directly activated by the EGFR, we were interested in understanding how this exchange reaction was catalyzed. The major breakthrough came when we realized that the product of a potent oncogenic protein named Dbl (for Diffuse B-cell lymphoma) shared sequence similarity with a yeast protein, Cdc24, that was suspected to function upstream of the *S. cerevisiae* Cdc42 protein in a pathway that regulates bud-site assembly [27,28]. This led to our discovery that the oncogenic Dbl protein was a potent activator of Cdc42 [28,29], and the founding member of a family of Rho-GEFs [30,31].

With regard to how members of the Dbl family of GEFs stimulate the GDP-GTP exchange reaction within their cognate G-protein substrates, the initial interaction between portions of a highly conserved region on the GEF, designated the Dbl-homology (DH) domain, and a conformationally sensitive region on the G-protein (designated as Switch 2), leads to a restructuring of the guanine nucleotide-binding pocket. Two specific changes are especially important, the insertion of the methyl side chain of Ala59 (Cdc42 numbering) into the coordination sphere of the Mg^{2+} ion (which plays a key role in the high affinity binding of

GDP), and an ion pairing between Lys16 with Glu62 (Cdc42 numbering). It is the combination of Ala59 blocking the binding of Mg^{2+} , together with a loss of the interaction between the β -phosphate of GDP with Lys16 within the “P-loop” of the G-protein, that results in a significant reduction in the affinity for GDP, paving the way for GDP-GTP exchange. Interestingly, although members of the family of GEFs for the G-protein Ras do not exhibit significant sequence similarity with the Dbl-family, they use a similar mechanism to catalyze GDP-GTP exchange [32,33]. The same is true for a genetically distinct family of GEFs that have evolved for Cdc42 and related small G-proteins, called the DOCK180/CED-5/Myoblast City family [31].

While GEFs serve a similar function as GPCRs, the mechanisms by which they stimulate GDP-GTP exchange on their G-protein partners clearly differ. The G-proteins that couple to GPCRs are heterotrimeric complexes consisting of $G\alpha$, $G\beta$ and $G\gamma$ subunits, with the $G\alpha$ subunits being approximately twice the size of the small G-proteins. The $G\alpha$ subunits contain a region highly homologous to Ras, called the Ras-like domain, as well as a second, largely helical domain. The Ras-like and helical domains of the $G\alpha$ subunits come together to form a ‘clamshell’ that encases the bound guanine nucleotide. GPCRs need to cause structural rearrangements within $G\alpha$ subunits that open the clamshell. This was shown in the X-ray crystal structure for the β 2-adrenergic receptor-Gs complex, an extraordinary achievement by Brian Kobilka and his colleagues, which demonstrated that the helical domain moved away from the Ras-like domain in a dramatic fashion [34]. Small G-proteins do not contain a large helical domain, but rather use Mg^{2+} to ensure a high affinity for GDP, such that GEFs need to displace the Mg^{2+} , in order to loosen the binding of GDP and allow GDP-GTP exchange to occur.

4.2. A unique feature of GEFs compared to GPCRs

Following GPCR-stimulated GDP-GTP exchange, the GTP-bound $G\alpha$ subunit dissociates from the $G\beta$ and $G\gamma$ subunits, as well as from the GPCR, enabling it to engage and alter the activity of a specific biological effector protein. The ability of the activated G-protein to dissociate into its component $G\alpha$ subunit and $G\beta\gamma$ subunit complex ensures that the signal propagation initially triggered by the GPCR is unidirectional.

Interestingly, GEFs for Cdc42 and other small G-proteins can follow quite different signaling rules from those used by GPCRs. In particular, we identified a family of GEFs for Cdc42 and the related Rac G-protein whose members were capable of binding directly to a Cdc42/Rac effector protein, the serine/threonine kinase Pak (for p21-activated kinase). We named these proteins Cool (for cloned-out-of-library) [35,36]. The same proteins were independently discovered by Manser, Lim and colleagues and called Pix (for Pak-interactive exchange factor) [37]. The Cool/Pix proteins contain an SH3 (Src-homology 3) domain that is responsible for binding to a proline-rich motif on Pak, as well as the conserved DH and PH (pleckstrin-homology) domains characteristic of the Dbl family of GEFs. Thus, we surprisingly discovered an upstream activator (GEF) of a small G-protein that is capable of binding to a downstream effector protein. Even more surprising, we found that the Cool/Pix proteins are also capable of binding to GTP-bound (activated) Cdc42 or GTP-bound Rac, at a site distinct from where GDP-bound Cdc42 or Rac interacts with Cool/Pix to undergo

GDP-GTP exchange. Moreover, the interaction of GTP-bound Cdc42 or Rac with Cool/Pix enhances the GEF activity, providing for a positive-feedback mechanism of G-protein activation [38]. This was also shown to be the case for other GEFs including the Ras-GEF Sos (for Son-of-sevenless), in which case GTP-bound Ras can interact with Sos and stimulate its GEF activity toward GDP-bound Ras molecules [39].

These findings implied that GEFs like Cool/Pix could serve to assemble a complete signaling complex, where the GEF initially activates a G-protein and then the GTP-bound activated G-protein engages and regulates an effector protein (like Pak). Indeed, such a mechanism has significant implications for EGFR-signaling. As shown in Figure 3, following the EGFR-dependent activation of Cdc42 (as well as Ras [40]), the GTP-bound Cdc42, through its ability to bind to Cool-1/ β -Pix, helps to stabilize a complex between Cool-1/ β -Pix and the E3 ubiquitin ligase, c-Cbl, which uses a poly-proline-rich motif to bind to the SH3 domain of the Cool/Pix protein (i.e. similar to how Pak binds). When c-Cbl is part of this complex, it is not free to engage and catalyze the ubiquitylation of the EGFR, thus significantly extending the EGFR signaling lifetime. Therefore, we were finally able to establish a signaling connection between the EGFR and Cdc42, although through a very different mechanism than we had assumed when we discovered the mammalian/human Cdc42 protein, many years earlier.

4.3. GAPs share functional roles with RGS proteins

GTP-hydrolysis-activating proteins (GAPs) have a critical role in regulating the lifetime of the activated state of small G-proteins like Cdc42. There is a large family of GAPs for Cdc42 and related G-proteins whose members share three conserved regions that are responsible for their catalytic function [41]. X-ray crystallographic studies of a Cdc42-GDP-AlF₃-GAP complex, thought to mimic the transition-state for the GTP hydrolytic reaction, shed light on how the GAP catalyzes GTP-hydrolysis [42]. Specifically, the GAP performs two functions: 1) it introduces an essential arginine residue (often referred to as the 'arginine finger') that stabilizes the bound GTP in a transition-state for hydrolysis, and 2) it stabilizes the conformationally sensitive Switch 2 domain on Cdc42 so that an essential glutamine residue (Gln61) is properly positioned to help introduce the catalytic water. These two functional roles give rise to a significant (several-fold) stimulation of GTP-hydrolysis by Cdc42. A similar mechanism is used by GAPs that stimulate GTP-hydrolysis by Ras, although in these cases, a 10–100-fold stimulation of the reaction can occur because of the exceedingly slow intrinsic GTP hydrolytic activity of the Ras proteins.

Somewhat analogous functional roles are played by the RGS (Regulator of G-protein signaling) proteins that stimulate GTP-hydrolysis by the heterotrimeric G-proteins. However, unlike the GAPs for Cdc42 and Ras, the RGS proteins do not need to introduce an essential arginine residue into the catalytic site, since the large helical domain of G α subunits provides the 'arginine finger' [13–15]. The RGS proteins stabilize Switch 2 in G α subunits, ensuring the proper orientation of the essential glutamine residue.

4.4. GDIs-Regulatory proteins specific for Ras-related small G-proteins

The major distinction between small G-proteins like Ras and Cdc42, and the large G-proteins which couple to GPCRs, is that large G-proteins contain two additional subunits, $G\beta$ and $G\gamma$, which remain tightly associated under non-denaturing conditions (i.e. the $G\beta\gamma$ complex). An important functional role of $G\beta\gamma$ complexes is to enhance the affinity of the $G\alpha$ subunit for a GPCR, as well as contribute to the specificity of the GPCR-G-protein interaction. However, upon GPCR-stimulated GDP-GTP exchange, the dissociation of the $G\alpha$ -GTP species from the $G\beta\gamma$ complex provides for the possibility that signal propagation can be mediated not only by the GTP-bound $G\alpha$ subunits but also by $G\beta\gamma$ complexes [43–45]. Moreover, it has been suggested that $G\beta\gamma$ complexes play critical roles in G-protein activation by working together with GPCRs to help change the juxtaposition of the large helical domain relative to the Ras-like domain on $G\alpha$ subunits, thereby facilitating GDP release and GDP-GTP exchange [46–49].

While the family of regulatory proteins known as GDP-dissociation inhibitors (GDIs) is unique to the small G-proteins, GDIs do not play roles analogous to those performed by $G\beta\gamma$ complexes in GPCR-dependent signaling functions. The GDIs were named for their ability to stabilize the GDP-bound state of Cdc42 and related small G-proteins (e.g. Rac1 and RhoA) [50]. The GDI for Cdc42 also stabilizes the GTP-bound state by blocking GTP hydrolysis [51]. However, perhaps the most significant function of the GDIs is its ability to promote the transition of G-proteins like Cdc42 from a membrane-associated state to a soluble state. Unlike what was previously assumed, the GDI does not actively extract Cdc42 from membranes. Instead, the membrane association of Cdc42 is a dynamic process, such that the G-protein associates and dissociates from membranes with measurable rates [52]. The GDI binds to Cdc42 while it is still associated with membranes, such that the GDI-Cdc42 complex dissociates from membranes with a rate that is essentially identical to the rate of dissociation of Cdc42 from membranes in the absence of the GDI. Once the GDI-Cdc42 complex dissociates from the membrane, the GDI sequesters the isoprenoid geranylgeranyl moiety attached to the C-terminal tail of Cdc42, which is required for membrane-association, as shown in the X-ray crystal structure that we determined for the complex [53]. A key role played by the GDI is to ensure that Cdc42 does not bind promiscuously to any membrane in its vicinity, but rather binds only to membrane sites that contain its signaling partners.

5. The idea of a ‘fast-cycling’ G protein

Because point mutations that cause Ras to be oncogenic are due to defects in its ability to hydrolyze GTP (e.g. G12V or Q61L mutations), we initially assumed the same would be true for Cdc42. However, we found that the ectopic expression of GTP-hydrolysis-defective mutants of Cdc42 typically gave rise to detrimental effects on cell growth. This caused us to consider that in order for Cdc42 to properly signal so as to promote (and not inhibit) cell growth, it was necessary that the G-protein cycles between its GDP- and GTP-bound states. For example, the necessity of cycling between GDP- and GTP-bound states might occur if Cdc42 were playing a role in some type of intracellular trafficking activity, such that it needed to engage a specific cargo protein (or vesicle-cargo) at one membrane location and

then subsequently discharge it at another membrane site. Indeed, Cdc42 has been shown to bind to the γ -coatomer subunit of the COPI complex and to have roles in intracellular trafficking events [54]. Given these considerations, we set out to use an alternative strategy for the activation of ectopically expressed G-proteins. Rather than introducing mutations that block GTP-hydrolysis, we generated mutants capable of enhanced intrinsic GDP-GTP exchange rates while maintaining normal GTP hydrolytic activity. We called these 'fast-cycling' mutants because of their ability to rapidly cycle between the GDP- and GTP-bound states.

The first of these fast-cycling mutants was generated by substituting a phenylalanine residue at position 28 in Cdc42 that is highly conserved among small G-proteins and plays a role in stabilizing the guanine base of GDP [55,56]. The substitution of this phenylalanine residue to a leucine yielded a Cdc42 protein (Cdc42(F28L)) that exhibited reduced affinity for GDP and was capable of constitutive GDP-GTP exchange. When the Cdc42(F28L) mutant was expressed in NIH 3T3 cells, it induced cellular transformation. We took advantage of the fast-cycling Cdc42(F28L) mutant to determine the role of Cdc42 in intracellular trafficking [54] and in extending the signaling lifetime of EGFRs [40], and to demonstrate how these capabilities contributed to the transformed state.

Interestingly, we found that introducing an asparagine residue in place of a conserved aspartic acid residue at position 118, which is involved in stabilizing the guanine ring moiety of GDP through an interaction with the N7 ring nitrogen (a similar interaction occurs with G α subunits), yielded a Cdc42 mutant (Cdc42(D118N)) capable of constitutive GDP-GTP exchange, but at a rate slower than that for the Cdc42(F28L) mutant [57]. However, the Cdc42(D118N) mutant was more potently transforming, compared to Cdc42(F28L), suggesting that the actual rate that Cdc42 cycles between its GDP- and GTP-bound states might be critical for optimal activation of signaling pathways necessary for transformation.

We also applied the fast-cycling concept to other G-proteins, in particular the Ran GTPase, where its ability to both bind and hydrolyze GTP is a critical aspect of its function in nucleocytoplasmic transport. By taking advantage of structural information for Ran, we predicted that a lysine residue at position 152 would be important in binding to the ribose moiety of GDP, and thereby changed it to alanine [58]. We found that the Ran(K152A) mutant was capable of rapidly cycling between the GDP- and GTP-bound states, and when expressed in fibroblasts, was capable of inducing transformed phenotypes.

6. Circling back to GPCR-G protein interactions: Development of new G-protein mutants

Given the success of the fast-cycling mutants for providing both mechanistic and biological information for small G-proteins like Cdc42, we were interested in seeing if the generation of similar types of mutants in G α subunits might be equally beneficial. As described above, the X-ray crystal structures of different G α subunits showed that the helical and Ras-like domains envelop the bound GDP, essentially forming a clamshell. The helical and Ras-like domains are connected by linkers, which help the clamshell to open and close. Indeed, the X-ray crystal structure obtained by Kobilka and colleagues for the β 2-adrenergic receptor-

Gs complex suggested that the receptor opens the clamshell by altering the orientation of the helical domain, relative to the Ras-like domain, enabling GDP release to occur [59]. However, before this structure was published, we had set out to test the idea that perturbing the linker regions would accelerate the intrinsic rate of GDP-GTP exchange on $G\alpha$ subunits. We examined this for the transducin- α subunit (αT), in which linker 1 consists of residues 54–58 and linker 2 includes residues 173–179. We altered the inherent flexibility of the linker regions with the idea being to generate an open state for the clamshell, so as to facilitate intrinsic GDP-GTP exchange. Molecular dynamic studies had suggested that the helical domain of αT moves toward the Ras-like domain, via a hinge-bending motion centered on residues Gly56 and Gly179 [60], and so we changed each of these residues to proline [61]. We found that both the $\alpha T(G56P)$ and $\alpha T(G173P)$ mutants could undergo GDP-GTP exchange in the absence of light-activated rhodopsin and the $G\beta\gamma$ complex. Thus, these represent $G\alpha$ subunit fast-cycling mutants. We then showed that while rhodopsin provides an additional stimulation to the GDP-GTP exchange activity of the $\alpha T(G56P)$ mutant, this stimulatory effect was much less sensitive to the $G\beta\gamma$ complex ($\beta 1\gamma 1$) compared to the wild-type αT subunit [62]. Additional biochemical and structural studies showed that the G56P substitution results in a concerted series of changes that are transmitted to the conformationally sensitive Switch regions of αT , as well as to the $\alpha 4/\beta 6$ loop and the $\beta 6$ strand. The $\alpha 4/\beta 6$ loop on $G\alpha$ subunits has been suggested to be a critical site for GPCR-mediated activation. The Switch domains are contact sites for $G\beta\gamma$ complexes. Thus, taken together, these findings suggested that the $\alpha T(G56P)$ mutant adopts a conformational state that is normally induced within $G\alpha$ subunits by the combined actions of the GPCR and the $G\beta\gamma$ complex during G-protein activation.

We discovered another interesting $G\alpha$ fast-cycling mutant when examining the consequences of changing a conserved serine residue within the guanine nucleotide-binding P-loop of αT . Both small and large heterotrimeric G-proteins have either a conserved serine or threonine residue within their P-loops. In the case of small G-proteins like Cdc42, Rac or Ras, the mutation of this residue results in the stabilization of the nucleotide-free state, thus yielding small G-proteins with an enhanced affinity for their GEFs, such that they act as dominant-negative inhibitors. Mutations of the corresponding residue in $G\alpha$ subunits have been suggested to give rise to enhanced affinities for $G\beta\gamma$ complexes and GPCRs [63–65]. However, when we changed the corresponding serine residue within the αT background (i.e. as S43N substitution), the resultant S43N mutant was capable of undergoing intrinsic GDP-GTP exchange at a significantly increased rate, compared to the wild-type protein [66]. Light-activated rhodopsin caused only a modest increase in the rate of GDP-GTP exchange within the S43N mutant. Moreover, the S43N mutant, when in a $GTP\gamma S$ -bound state, was capable of binding to rhodopsin and stimulating the PDE. Our findings were consistent with a model in which the S43N mutant stabilizes a ternary complex between the GPCR, the $G\alpha$ subunit, and $G\beta\gamma$ that exists following GDP-GTP exchange. We are very interested in comparing the structures for the GDP- versus $GTP\gamma S$ -bound forms of this αT mutant, as well as determining a high resolution structure for this mutant bound to light-activated rhodopsin, as this would shed important light on an interesting intermediate state along the G-protein activation pathway.

7. Structural considerations and the future

Obtaining structural information for GPCRs, and in particular, for active GPCR-G-protein complexes, has garnered tremendous interest, both as a means to better understand the underlying mechanisms by which this important family of receptors directs a wide range of biological outcomes, and as a critical step in the design of more selective and effective drug treatments. X-ray crystal structures for GPCRs had been notoriously difficult to obtain due to their intrinsic low stability and high flexibility. However, we have seen remarkable success during the past five years with the determination of a number of high-resolution GPCR structures. Indeed, what was long regarded as a ‘Holy Grail’ in the field of structural biology was achieved by Brian Kobilka’s laboratory at Stanford University, by solving the crystal structure for an active GPCR-G-protein complex, namely the β_2 -adrenergic receptor (β_2 AR) and its signaling partner G_s [59]. This structure supports the idea [67] that the opening of the nucleotide-binding pocket in a G-protein α subunit, through the movement of the helical domain, is required for G-protein activation. However, it also offers some surprises, as the GPCR contacts the G-protein solely through its α subunit, without an obvious role for its β and γ subunits. Moreover, little is known regarding how GPCR-G-protein specificity is achieved. This highlights the importance of achieving high-resolution structures for other GPCR-G-protein complexes, to see if they use similar mechanisms for G-protein activation. The rhodopsin-transducin complex offers exciting possibilities for achieving such a structure, as it is possible to generate large quantities of the receptor and the G-protein. Slow and steady progress has been made in obtaining structures for activated rhodopsin, as well as activated rhodopsin bound to a C-terminal peptide from the αT subunit [68–70], and recently, for a rhodopsin-arrestin complex [71]. In addition, a number of interesting αT mutants have been identified, including the $\alpha T(S43N)$ mutant that allows stable complex formation between this GTP-bound $G\alpha$ mutant and rhodopsin, offering intriguing possibilities for identifying an important and previously uncharacterized state along the G-protein activation pathway. Thus, I now find myself coming full circle from my initial introduction to GPCR-dependent signaling, perhaps an outcome of the never-ending influence of Bob Lefkowitz on my research career. My colleagues and I are hoping to take advantage of the biochemical benefits offered by the rhodopsin-transducin-coupled phototransduction system, joining the fray with many other laboratories to obtain high resolution structures that hopefully will shed further light on how these beautifully designed signaling systems operate.

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Highlights

- GPCRs use a beautifully conserved signaling architecture.
- Searching for partners for the EGF receptor identified the human Cdc42 protein.
- Interesting analogies exist between the regulation of large and small G-proteins.
- Structural approaches will continue to shed new light on the mechanisms of GPCRs.

Figure 1.
A depiction of GPCR-G-protein-dependent signaling.

Figure 2.
Depiction of the GTP-binding/GTP-hydrolytic cycle of Cdc42. Shown are the three major types of regulatory proteins, namely, a GEF, GAP and GDI.

Figure 3.

Model depicting the Cdc42-mediated regulation of EGF receptor interactions with c-Cbl. EGF receptor activation stimulates GDP-GTP exchange on both Ras and Cdc42, resulting in the formation of Cdc42•GTP-Cool-Cbl complexes and signaling through the Ras-ERK pathway. When the Cdc42-Cool-Cbl complex disassembles, Cbl can then bind to the EGF receptor and is phosphorylated, thus enabling Cbl to function as an ubiquitin E3 ligase that catalyzes receptor ubiquitination and degradation.