MECHANISMS FOR THE STRUCTURAL ACTIVATION OF THE SMALL G PROTEIN CDC42 AND ITS CONSTITUTIVELY ACTIVATED MUTANT, CDC42 (Q61L)

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MECHANISMS FOR THE STRUCTURAL ACTIVATION OF THE SMALL G PROTEIN CDC42 AND ITS CONSTITUTIVELY ACTIVATED ISOFORM, CDC42 (Q61L)

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The x-ray crystal structures for the GTP-bound forms of the small G protein Cdc42 and its constitutively activated isoform, Cdc42 (Q61L), were previously unkown. The mechanism for the activation event has been largely assumed based on x-ray crystal structures for effector-bound-Cdc42 complexes as well as mechanisms for activation for other small G proteins such as Ras.

We used x-ray crystallography, tryptophan fluorescence, and ^{31}P-NMR to study the specific activation event for Cdc42. The x-ray crystal structure revealed a signaling-active GTP-analog-bound Cdc42 conformation that was virtually identical to the conformation for signaling-inactive GDP-bound Cdc42 (1ANO). It was further revealed through the use of tryptophan fluorescence, as well as ^{31}P-NMR, that binding of an effector protein played an important part in inducing a conformational change that resulted in the fully activated structure usually associated with activated G proteins.

Conversely, we used x-ray crystallography to reveal that the constitutively activated isoform of Cdc42, Cdc42 (Q61L), did not require an effector protein to adopt a fully activated structure. ^{31}P-NMR results confirmed these findings and also revealed that the GTP-analog, GMP-PCP, behaved similarly to the physiologically relevant

nucleotide, GTP, during the activation event.

Overall these findings imply that a spectrum of activation mechanisms exist for small G proteins where, on the one end, an effector protein is required to induce the proper activated conformation (i.e. Cdc42), and on the other end, GTP binding is sufficient to drive the G protein to the fully activated structure (i.e. Ras).

BIOGRAPHICAL SKETCH

The author was born in Neptune, New Jersey. At the age of thirteen, after spending most of his childhood in upstate New York, he finally settled in Ithaca, NY. After graduation, he attended Corning Community College and then Tompkins Cortland Community College. Two years later, the author was able to transfer to Cornell University and graduate with a degree in Biology. Fortuitously, the author was hired as a technician in the lab of Professor Rick Cerione at Cornell University where he was first exposed to research. Two years later, Professor Cerione accepted him into his lab as a graduate student through the employee degree program.

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I would like to thank Dr. Rick Cerione for everything he has done for me. If not for him, I would not be writing this dissertation today. He accepted me into his lab as an employee degree participant but allowed me to focus on my graduate work without asking me to perform the duties associated with the employee degree program. He was extremely patient, understanding and helpful throughout all of my time in his lab and I was very fortunate to have him as a boss.

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G protein	GTP-binding protein
AC	Adenylyl Cyclase
GPCR	G protein Coupled Receptor
RGS	Regulators of G protein Signaling
Ras	Rat Sarcoma
RTK	Receptor Tyrosine Kinase
SOS	Son of Sevenless
GEF	Guanine nucleotide Exchange Factor
SH2	Src Homology Domain
Raf	Rapidly growing Fibrosarcomas
GAP	GTPase Activating Protein
GMP-PCP	β,γ-Methyleneguanosine 5´-triphosphate
GTP	Guanosine tri-phosphate

Chapter I

Introduction

G Proteins: Historical perspective

In order to fully appreciate G proteins and their overall contribution to our understanding of biology, it is important to start from the very beginning. Arguably, the beginning of G protein research can be traced back to the discovery of cyclic AMP as a second messenger by Earl Sutherland, for which he received the Nobel Prize in 1971.

In the early 1950's a lot of attention was being paid to glycogen metabolism by biochemists. At that time, scientists had already determined that glycogenolysis was the ultimate outcome of the hormones epinephrine and glucagon in the liver. Based on the work of Carl Cori and others, three enzymes involved in the pathway were elucidated. Sutherland and Cori came to the conclusion that the enzyme named phosphorylase was the rate-limiting enzyme in the reaction and that the hormones glucagon and epinephrine were working by increasing the enzyme's activity (1). An important discovery was made when they found that if they separated membrane fractions from the cytosol, they lost the ability to stimulate phosphorylase with hormones. However, when they added the membranes back, they were able to recover activity. Perhaps more important was the discovery that incubating membranes with hormone prior to heat-inactivation, could still activate phosphorylase when the membrane fraction was recombined with the cytosol. This lead these investigators to look for a heat-insensitive intermediate which resulted in their discovery of cyclic AMP. The membrane activity that converted ATP to cyclic AMP was then called Adenyl Cyclase (referred to as AC hereafter) and later changed to Adenylyl Cyclase or Adenylate Cyclase to be more chemically correct.

At this time a number of laboratories became interested in the mechanism of AC regulation. It was previously known that a number of different hormones both positively and negatively regulated AC and that the hormones all worked on a fixed pool of AC instead of each hormone activating its own AC. It was also shown that the hormone receptor and AC were two distinct entities that could be resolved and highly purified. What was less certain was how the hormone-receptor complex regulated AC or if there were more proteins involved.

The first insights into AC regulation that lead to the discovery of G proteins came from Martin Rodbell for which he, together with Alfred Gilman, was also awarded the Nobel Prize. Rodbell, Birnbaumer and their colleagues noted that in the presence of ATP, the hormone glucagon was able to bind to its receptor much faster but was also released from the receptor much more quickly (2). The major breakthrough came with the realization that the ATP being used in the AC activity assays was contaminated with other nucleotides, notably GTP. The question then arose as to what is the effect of GTP on the ability of glucagon to stimulate AC activity? Rodbell and colleagues synthesized App(NH)p (a non-hydrolyzable ATP analog) and assayed glucagon's ability to activate AC. They found that App(NH)p did not stimulate AC unless GTP was present in the buffer at concentrations comparable to what was necessary for regulating the hormone-receptor (3). This was the first real evidence of a "Transducer", as Rodbell had called it, that mediated the signal from the hormone-receptor to AC.

Rodbell and his colleague Yoram Salomon went on the show that synthesized Gpp(NH)p could induce the activation of AC, even in the absence of hormone although the hormone greatly increased the rate of activation (4). Another intriguing finding was that Gpp(NH)p showed prolonged and substantiated activation of AC, much greater than with hormone and GTP alone (5). These findings suggested that

GTP was acting at a stage after the hormone-receptor interaction and before the stimulation of AC.

In the latter half of the 1970's it was discovered that hormones like epinephrine and glucagon were acting to displace bound GDP in favor of GTP (6). This was subsequent to the finding that these hormones could increase the hydrolysis of GTP to GDP (7). From this work, Cassel and Selinger put forward the idea that the hydrolysis of GTP to GDP and P_i was the mechanism of shutting down the signaling system whereas the binding of GTP in favor of GDP was responsible for activating the system. At the time, scientists were calling the GTP-binding entity the regulatory unit of AC which was distinct from the catalytic unit but not necessarily separate. The possibility that these units represented distinct proteins emerged when investigators looked into the ability of hormones to inhibit AC activity. The discovery of adenosine receptors that required GTP to inhibit AC made it more convincing (8) and Rodbell went on to call the purported GTP-binding units Ns and Ni for nucleotide-binding stimulatory proteins respectively (9).

At about the same time, (i.e. the late 1970's), work by Gilman and colleagues was starting to show direct proof of a separate nucleotide-binding protein that was necessary for the function of the AC system. These investigators were trying to reconstitute AC activity *in vitro* by using a variant of S49 lymphoma cells called cyc- because it was thought to be deficient in AC. They found that if they prepared a detergent extract from wild-type S49 cells, and heat inactivated the AC activity at 37˚C, they could recombine it with a detergent extract from cyc- cells and restore AC activity (10). However, they noted that if they incubated the cyc- extract at 30˚C and recombined it with the wild-type extract, they lost AC activity. They postulated that the two factors being lost in the wild-type and cyc- heated extracts might be the same. In contrast, they found that if wild-type extracts were heated at 50˚C and recombined

with cyc- extracts, AC activity was lost over time. Incubation with NaF or Gpp(NH)p increased the stability of a thermostabile factor indicating its ability to bind guanine nucleotides. This led Gilman and colleagues to propose that there were two factors responsible for the activation of AC. The heat labile factors were sensitive to protease cleavage and *N*-ethylmaleimide indicating they were both proteins. Soon after this work, Gilman and his colleagues concluded that the "thermolabile" protein in both the wild-type and cyc- extracts was the catalytic domain of AC (11). Furthermore, combining extracts from cyc- cells containing the thermolabile protein with extracts that contained the thermostabile components, but lacked the β-adrenergic receptor, reconstituted the epinephrine-sensitive AC activity. Therefore, the cyc- cell lines were not deficient in AC but rather lacked the regulatory component, which interacted with guanine nucleotide.

A more direct approach of discovering the GTP-binding factor was under way at the same time by Thomas Pfeuffer. Pfeuffer was able to covalently label the GTP-binding activity in pigeon erythrocyte membranes and resolve it from the AC (12). Additionally, he was able to remove the GTP-binding fraction from the catalytic fraction by GTP-Sepharose affinity chromatography. The flow-through lacked detectable AC activity in the absence of the GTP-binding fraction. Upon elution of the GTP-binding fraction with GTP, he was then able to reconstitute AC activity by recombining the fractions.

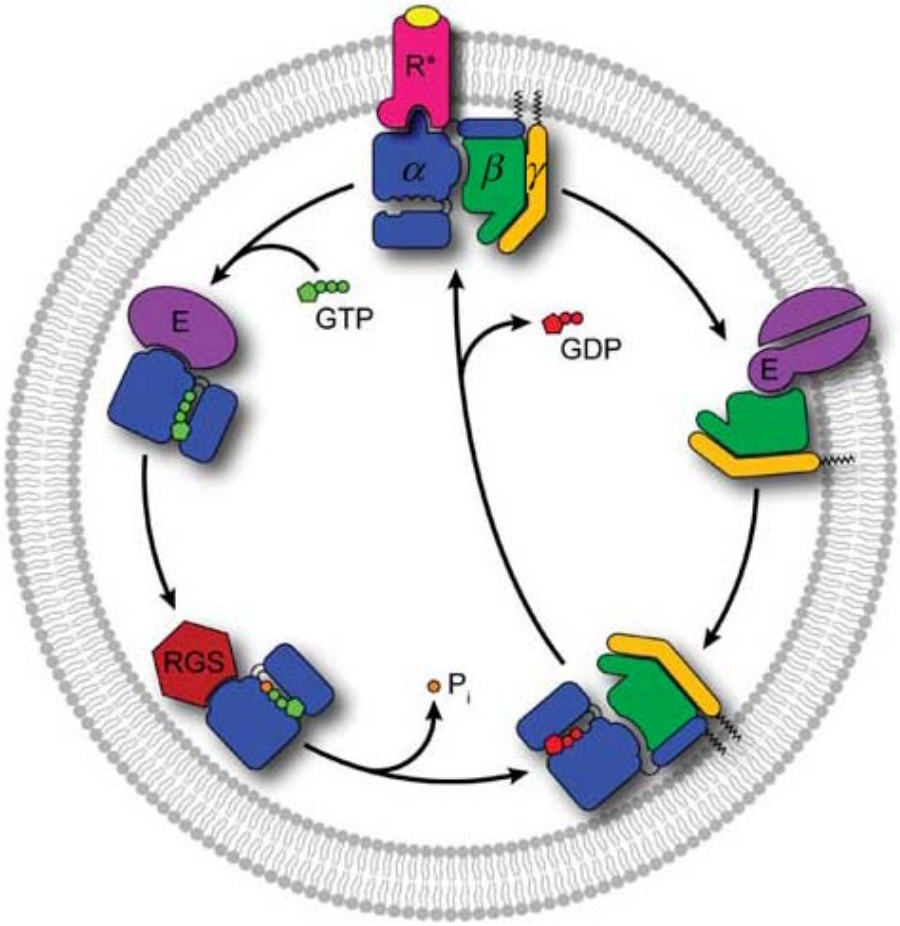
Gilman and his colleagues eventually succeeded in purifying to homogeneity the GTP-binding protein in 1980 (13). They named this GTP-binding protein Gs and originally suggested that it contained two subunits, which they named α (M_r ~ 45 kDa) and β (M_r ~ 35 kDa). The α subunit bound nucleotide and could stimulate AC activity in the cyc- cell lines as well as another cell line named unc which did not exhibit hormone-stimulated AC activity although AC in these cells could be directly activated

by G proteins. Later it was recognized that heptihelical receptor-associated G proteins are actually heterotrimeric after the discovery that the γ subunit (M_r ~ 9 kDa) remains constitutively associated with the β subunit under all non-denaturing conditions (14).

The final conclusive evidence was supplied by Rick Cerione and his colleagues who were able to purify all three components of the β-adrenergic receptor-coupled AC system and reconstitute them in lipid vesicles (15). These investigators showed that AC alone was unresponsive to activation by GTP, however, upon addition of what is now known as Gαs with βγ, the GTP-dependent activation was restored but not responsiveness to hormone. Addition of the β-adrenergic receptor then fully returned hormone sensitivity. Thus, for the first time there was indisputable evidence for a three component system being the biologically relevant signaling pathway for cAMP formation (Fig. 1.1).

Since then there have been numerous discoveries about what are now collectively called G proteins and their roles in biological systems. It is now recognized that there are roughly 1000 GPCRs (G Protein coupled receptors) encoded in the mammalian genome (16) and more than 20 heterotrimeric G protein α subunits corresponding to 16 gene products (17). Additionally, RGS (Regulators of G protein Signalling) proteins were discovered, as reviewed by Birnbaumer, that stimulate the GTPase activity of the Gα subunit, thereby shutting off the signal in a timely manner (18). Even more surprisingly, it has been shown that the Gβγ dimer can act as a signaling unit separate from the Gα subunit, in a variety of different systems (18). Heterotrimeric G proteins are involved in most vital functions in the human body such as glycogen metabolism, cardiac contractions, smell, taste, and vision just to name a few. They have also been implicated in a number of different disease states such as cancer, diabetes, and cholera (18,19). However, the story does not end with heterotrimeric G proteins. Around the same time that people were starting

Figure 1.1 Heterotrimeric G Protein Three Component System. Pulled from Hamm, HE and Oldham WM, Structural Basis of Function in Heterotrimeric G Proteins. *Quarterly Reviews of Biophysics*, 2006. An agonist (yellow) binds a Heptihelical receptor (R*) which then activates the G protein by stimulating the exchange of GTP for GDP on the Gα subunit (α). The β,γ-subunits dissociate from the Gα which is free to interact with downstream effector proteins (E). The system is turned off by hydrolysis of GTP to GDP which is facilitated by an RGS protein (Regulator of G protein signaling). Once turned off, the Gα subunit reassociates with the β,γ subunits and is able to bind receptor again. In many cases, the β,γ subunits can interact with their own effector proteins and contribute to cell signaling.


R*
α
β
γ
GTP
GDP
E
E
RGS
P_i

to recognize the importance of heterotrimeric G proteins in second messenger systems, discoveries were made that lead to the understanding of the importance of a new class of G proteins, namely the Ras-like small G proteins.

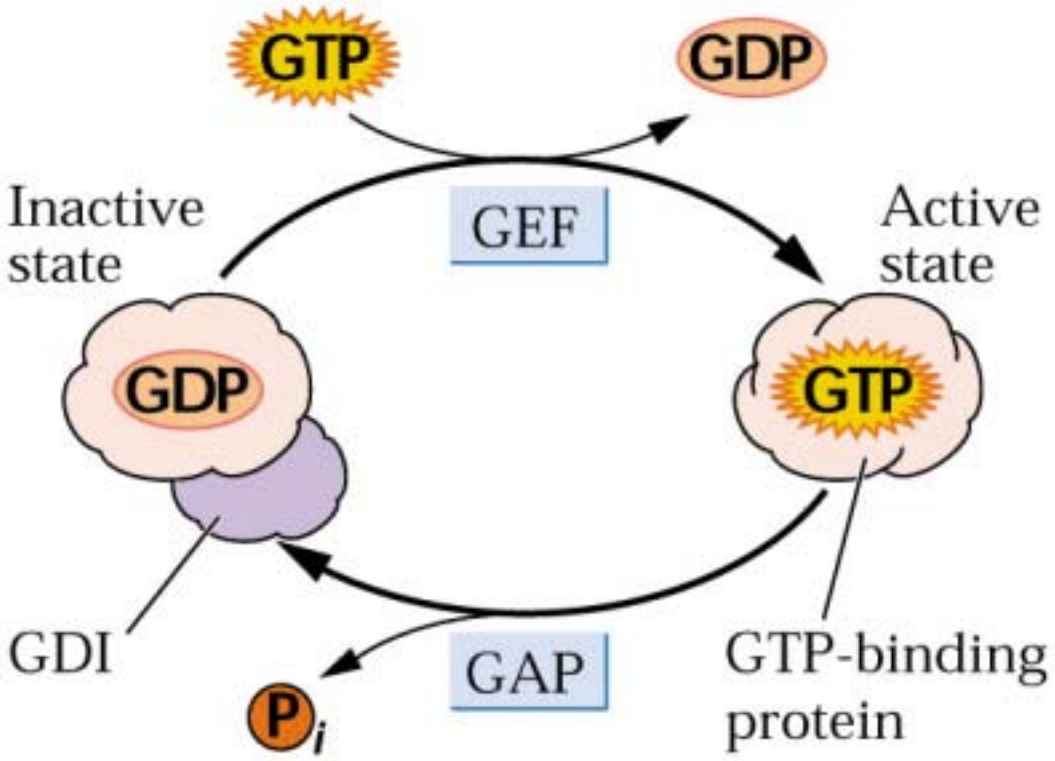
Ras discovery and function:

Small G proteins gained prominence in the early 1980s with the discovery that the transforming agent of the Harvey and Kirsten strains of rat sarcoma virus was a small G protein named Ras (rat sarcoma) (20,21). Interest in Ras intensified when it was discovered to be involved in a number of human cancers (22). It was determined that the oncogenic forms of Ras were commonly mutated at positions 12, 13, 59, and 61, with the majority of mutations occurring at position 12. Mutations at those positions were found to inhibit Ras's GTPase activity and thus rendered it constitutively active (as reviewed by Barbacid, 1987).

Since that time the family of small G proteins has grown to over 150 members with orthologs in most animal species (23). Virtually all G proteins share a similar GTPase cycle whereby exchange of GDP for GTP by a GEF protein activates the G protein and hydrolysis of GTP to GDP and P_i catalyzed by a GAP protein deactivates it (Fig. 1.2). Ras is considered the archetype of the small G protein family due to its minimal structure based on sequence and structural alignments. This minimal architecture is also homologous to the GTP-binding core of heterotrimeric Gα subunits and is considered the minimum structural unit needed to bind guanosine nucleotides (Fig 1.3).

The most studied and thus best-understood function of Ras is its involvement in mitogenic signaling which leads to cancer if unregulated. Ras is a key component in most growth factor-stimulated signaling pathways that originate at the cell surface and

Fig. 1.2 GTPase Cycle. G proteins are in an inactive "off" state when bound to GDP. Activation takes place typically through a GEF, which helps kick out the GDP in favor of GTP which is at a higher concentration in the cell. In the heterotrimeric G protein system, the GEF is the heptihelical receptor. The G protein is then able to interact with downstream targets and transduce a signal. The G protein is turned "off" by a GAP (small G proteins) or an RGS (in the case for Heterotrimeric G proteins) which accelerates the G protein's own intrinsic GTP hydrolytic activity. In a few cases, a GDI can bind to the G protein and prevent nucleotide exchange and/or hydrolysis.


GTP
GDP
Inactive state
GEF
Active state
GDP
GTP
GDI
GAP
GTP-binding protein
Pi

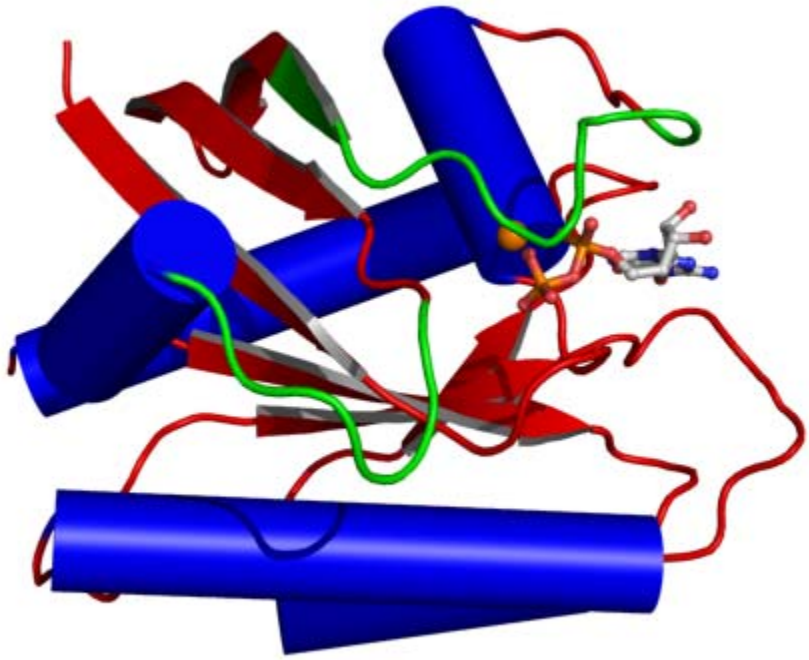
culminate in the nucleus. In fact, Ras is known to not only regulate cell proliferation, but to also participate in signaling pathways involved in apoptosis, differentiation, and morphologic rearrangements (24).

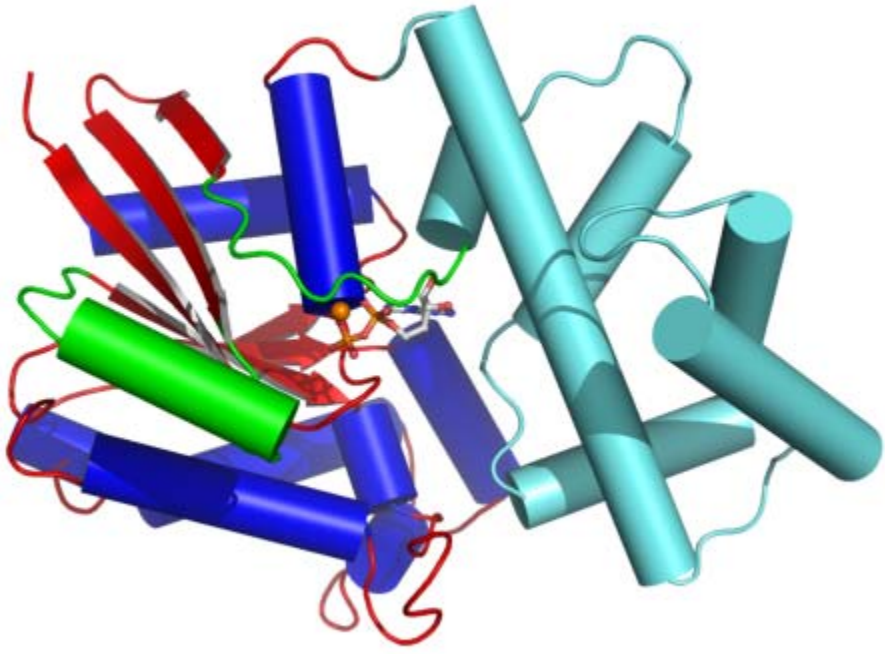
Much is now known about the activation of Ras and how this stimulates signals that are transmitted to the nucleus. The most common method for Ras activation is through receptor tyrosine kinases (RTK). Receptor tyrosine kinases are integral membrane proteins which have 3 main domains, an extracellular ligand-binding domain, a single transmembrane helical domain, and an intracellular tyrosine kinase domain. These receptors are activated by an external signal, usually a peptide, which stimulates their dimerization. This in turn results in the auto-phosphorylation of tyrosine residues within the cytoplasmic domain which recruit a number of signaling molecules to the receptor (25).

Receptor tyrosine kinase activation of Ras occurs through SOS (Son of Sevenless), which is a guanine nucleotide exchange factor (GEF) specific for Ras. SOS is recruited to the plasma membrane upon activation of RTKs through a constitutive interaction with an adaptor protein called Grb2 (25). Adaptor molecules such as Grb2 can interact with phosphorylated tyrosine residues through specialized domains such as SH2 (src homology domain) or PTB (phosphotyrosine binding) domains. Grb2 associates with the activated receptor, bringing SOS along with it, through interactions with Grb's SH2 domain.

Ras, which is attached to the plasma membrane through a farnesyl group on its carboxy terminus, is activated by SOS through a positive feedback mechanism (25). SOS resides in an autoinhibited state which is able to bind Ras – GDP at an allosteric site separate from the catalytic site (26). Binding of Ras – GDP releases some of the autoinhibition in SOS and allows for the formation of Ras – GTP. Activated Ras is then able to bind with higher affinity to the same allosteric site previously occupied by

Figure 1.3 Minimal GTP-binding domain of Ras Compared to Transducin. Ras (top) is composed of 6 β-sheets (red) and 5 α-helices (blue). Switch I and II are depicted in green and are the sites of structural rearrangement during activation of the protein. Transducin (bottom) has the same basic GTP binding domain as Ras although with 4 insertions, one of which is an N terminal helical domain depicted in aquamarine blue.





Ras – GDP and elicit full activation of SOS (27,28).

Ras has multiple targets that feed into a number of different pathways with perhaps the most important for cellular transformation being its interaction with the Raf (for Rapidly growing fibrosarcomas) kinases. Raf family members are part of a highly conserved MAPK (Mitogen activated protein kinase) pathway, which ultimately leads to regulation of transcription in the nucleus. All MAPK pathways are comprised of a MAPK which is activated by phosphorylation from a MAPKK (MAPK kinase) which itself is activated by phosphorylation from a MAPKKK (MAPK kinase kinase) (as reviewed by (29)). Raf is part of the ERK1-2 (Extracellular signal-regulated kinase) pathway which is involved in cell motility, proliferation, differentiation, and survival (30). The MAPKKK, Raf, becomes activated by an intricate process that is not yet fully understood. It then activates MEK1/2 (also known as MAPK and ERK kinase), which in turn activates ERK, the MAP kinase. ERK has a number of substrates including RSK (p90 ribosomal S6 kinase), MSK (mitogen and stress activated kinase), and MNK (MAPK interacting kinase), as well as cell attachment and migration proteins including paxillin, focal adhesion kinase and calpain, and finally the transcription factors Elk 1, c-Fos, c-Myc and Ets domain factors amongst others (30).

Activation of C-Raf (also known as Raf-1) is the best understood of the Raf homologues and appears to share a similar mechanism of activation to A-Raf and B-Raf. Briefly, Ras recruits Raf to the plasma membrane where it becomes activated by a combination of interactions with Ras and other proteins, the plasma membrane, and phosphorylation/dephosphorylation at multiple sites (31). This complex regulation of both Ras and Raf, and by extension, the ERK pathway, underscores the importance of these proteins in normal cellular functions. In fact, mutations in Ras have been found in 15% - 30% of all human cancers, ERK is hyperactivated in ~30% of cancers, and

Raf has been found to be mutated to large degrees in thyroid, ovarian, and colorectal cancers (31).

Raf is not the only Ras effector that can lead to cellular transformation however. Another Ras effector, PI3K (Phosphatidylinositol 3-kinase), is a heterodimer consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit and is responsible for generating phosphoinositides phosphorylated at the 3′ position such as the second messanger PIP3 (phoshpatidylinositol (3,4,5) P3). Ras can bind and activate the p110 catalytic subunit of PI3K which then converts PIP2 to PIP3, as reviewed by (32), at the plasma membrane. PIP3 then recruits AKT, a serine/threonine kinase, to the plasma membrane by interacting with its PH (Pleckstrin homology) domain. Once recruited, AKT becomes activated by phosphorylation at two sites by PDK1 and PDK2 (3-Phosphoinositide-dependent protein kinase-1 and 2). Subsequently, it is able to interact with downstream effectors that control cell survival, proliferation, and growth. Constitutive activation of this pathway has also been shown to lead to cancer.

Ras is not the only G protein that plays an important role in human cancers however. As mentioned previously, there are over 150 different small G proteins and many of them, if mutated, can cause cellular transformation. Small G proteins are classified into 5 different families based on sequence and functional similarities. The 5 families are the Rho, Rab, Arf, Ras, and Ran families. Each G protein family shares structural and mechanistic similarities with each other but often perform physiologically distinct functions. There is however, a lot of crosstalk between G protein pathways, which adds to the complexity of the system.

Rab and Arf cellular functions

The Rab (Ras related in brain) and Arf (ADP-ribosylation co-factor) families of G proteins are involved in anterograde and retrograde cellular trafficking from the ER through the golgi to the endosome and plasma membrane. Both Rab and Arf families are essential to eukaryotic cells and are required for cellular trafficking (33). The Arf family consists of sub-family members Arf, Arl (Arf-like), Arp (Arf-related proteins) and the less closely related Sar (Secretion-associated and Ras-related) proteins (34). The most well understood function of Arf proteins is recruitment of coat proteins required for coated vesicle trafficking (24). Arf is activated by a GEF located on the membrane compartment, and then recruits coat protein complexes such as COP (Coat proteins) I, COP II, or clathrin. After the budding of the vesicle, a cytosolic GAP (GTPase activating protein) catalyzes the hydrolysis of GTP to GDP which allows Arf to disengage from the vesicle. This leads to the eventual pinching off of the coated vesicle which is trafficked to another compartment (24).

Currently, our knowledge of Arf and its family members is rather limited. Only Arl1 and the Sar proteins have been shown to function in membrane trafficking (34). Arl4 has a possible role in the nucleus while Arf 6 is involved with cell migration and lamellipodia formation. Little is known about other Arf proteins however, leaving open the possibility for novel functions (34).

Rab G proteins are the most numerous of all the small G proteins. More than 60 mammalian Rab proteins have been identified. They are localized primarily to the golgi and endosomal compartments where they function as regulators of vesicular trafficking. Trafficking can be divided into four essential steps which include budding, transport, tethering, and fusion (35). Evidence points to a function for Rab proteins in all of these events as well as cargo sorting and vesicle formation (36). Rab proteins function by making certain that the right cargo is targeted to the right compartment.

Like all Ras-like G proteins, Rab proteins cycle between an inactive GDP-bound form and an active GTP-bound form (see Fig. 1.2). This GTP-GDP cycle is controlled by GEF and GAP proteins specific for Rab family members. In addition, Rab proteins undergo a membrane attachment and extraction cycle which is partially coupled to the nucleotide cycle (33,36). Inactive prenylated Rab proteins are sequestered in the cytosol by a GDI (Guanine-nucleotide dissociation inhibitor) that preferentially binds the inactive (GDP-bound) Rab. This is a common feature among a number of other small G proteins including members of the Rho family. Rab insertion into membranes is facilitated by a GDF (GDI displacement factor) which catalyzes the release of the Rab protein from the GDI, enabling its insertion into the membrane (37). Tight binding to membranes is imparted by a feature specific for most Rab proteins, specifically two C-terminal cysteins modified by geranylgeranylation instead of only one (33).

Ran GTPase Overview

Ran (Ras-related nuclear protein) GTPases are the only Ras-like proteins that lack post-translational lipid modifications. In addition, unlike other members of the family, they are found mostly in the nucleus. Ran is the product of only one gene in some cell types but two or more highly related Ran genes have been found in other cells (38). Even so, Ran is one of the most abundant proteins in eukaryotic cells.

Currently, Ran is known to function in two crucial cellular processes, nuclear transport, and mitosis. Nuclear transport is the most well understood function of Ran and many details of its involvement have been discovered. As reviewed in (39), molecules larger than 40 kDa are actively transported across the nuclear envelope through NPCs (Nuclear Pore Complexes). Cargo proteins containing a NLS (Nuclear Localization Signal) bind to importin-α which in turn binds to importin-β in the

cytosol. Importin-β is the carrier molecule that transports the cargo through the NPC while importin-α acts as an adapter protein. Once inside the nucleus, Ran – GTP binds importin-β which causes it to dissociate from importin-α. Importin-α then dissociates from the cargo molecule and is ready to be recycled to the cytoplasm through its nuclear export factor CAS which is also bound to Ran – GTP. Once the importin-α/CAS/Ran – GTP complex reaches the cytoplasm, RanGAP, which is found only outside the nucleus, catalyzes the hydrolysis of GTP to GDP. This causes the release of importin-α for another cycle of transport. Ran – GDP is then transported back into the nucleus by NTF2 (Nuclear Transport Factor-2) where it can be activated again by the RanGEF, RCC1 (Regulator of Chromosome Condensation), and thus the cycle is perpetuated. This spatial separation of the RanGEF and RanGAP into the nucleus and cytoplasm, respectively, are important for the proper regulation of Ran and controls the directionality of transport through the NPC.

As mentioned earlier, Ran is also implicated in mitotic control. Until fairly recently, the evidence was fairly controversial. The question arose as to whether Ran is involved directly in mitotic control as well as if its involvement is a distinct and separate function from nuclear transport. There is now proof that Ran functions in mitosis after the break-up of the nuclear envelope. As reviewed by Ciciarello et. al. (40), activating mutants of Ran induced the assembly of microtubule asters and spindles in M phase arrested *Xenopus* oocyte extracts, whereas a dominant negative mutant was inhibitory. Furthermore, it has been shown that activated Ran releases TPX2, a protein that is known to regulate spindle pole formation through a kinesin, from importin-α containing complexes. Similarly, activated Ran has been shown to stimulate the release of another spindle pole organizer, NuMA, from an importin-α/β complex. A model has been developed in which, before mitosis, there is an accumulation of SAFs (Spindle Activating Factors) bound to either the importin α/β

complex or importin-β alone in the nucleus. After the nuclear envelope breaks down, RCC1 associates with chromatin and causes a localized concentration of activated Ran around the chromatin that diffuses away causing a gradient. Thus inhibition of SAFs is relieved in a gradient dependent manner where the greatest activity of SAFs is around the chromatin. There is now evidence that Ran is also involved in centrosomal duplication, stabilization of microtubules, promotion of microtubule plus end directed movement, chromosomal "search and capture" by microtubules, and exiting of M phase into G1.

Rho GTPases

The Rho sub-family of Ras-like G proteins consists of at least 26 different members (41) of which Rho (Ras homologous), Rac (Ras-related C3 botulinum toxin substrate), and Cdc42 (Cell Division Cycle mutant 42) are the best characterized. Similar to their Ras-like brethren, they cycle between a GTP (active) and GDP (inactive) state that is highly regulated by GEF and GAP proteins. In addition, much like the Rabs, they can be sequestered in the cytoplasm and shuttled between membranes by RhoGDI. Rho members are involved in a variety of essential cellular processes such as cytoskeletal organization, cellular trafficking, cellular polarity, proliferation, and apoptosis. In fact, Rho, Rac, and Cdc42 are absolutely essential for cell cycle progression through the G1 to S phase transition in mammalian cells (42).

Rho family G proteins interact with over 60 downstream effectors, the largest number of effectors for any family of G proteins, indicative of their many cellular roles (43). To complicate matters, they also share many of the same targets, especially Cdc42 and Rac which are about 68% identical based on primary sequence alignments. In addition, there are over 60 GEF proteins and over 70 GAP proteins in the human

genome regulating Rho family members (44). Many GEF and GAP proteins regulate multiple Rho family members while some even contain domains which regulate other small G protein family members (44).

The first discovered function for Rho family members was their involvement in cell morphological changes related to the cytoskeleton. It was originally observed, through microinjection experiments, that Rho caused stress fiber formation in a number of cell lines (45). Later it was confirmed that endogenous Rho caused stress fiber formation as well as focal adhesion complex formation in response to LPA (lysophosphatidic acid) (46). Rac as well as Cdc42 were soon to follow as it was shown that Rac caused lamellipodia (membrane ruffles) formation as well as focal adhesion complex formation that were distinct from Rho, while Cdc42 caused filopodia (actin spikes at the leading edge) formation in Swiss 3T3 cells (47). Additionally, Cdc42 was observed to activate Rac which then activated Rho in a sequential manner in NIH 3T3 cells.

Soon after the discovery of the role of Rho family proteins in cytoskeletal rearrangements, another major function in mitogenic signaling and cellular transformation was found. Constitutively active Rac and Rho were found to cause transformation in fibroblasts (48-50). Similarly, a fast-cycling mutant of Cdc42 (F28L), which no longer required a GEF to become activated, showed hallmarks of cellular transformation when stably expressed in NIH3T3 cells (51). Indeed, it was shown that the ability of Cdc42 to cycle at an accelerated rate between its GDP- and GTP-bound states was necessary for inducing cellular transformation, whereas GTP hydrolysis defective forms of Cdc42 were toxic to cells. Finally, it was shown that Rho, Rac, and Cdc42 were each necessary for Ras induced transformation, clearly defining their importance in cellular processes (50,52-54).

In the intervening years, much has been learned about Rho family member cellular pathways. For instance, it has been shown that Rac1 and Cdc42 can regulate the stress activated p38 and JNK MAPK pathways directly which leads to the expression of genes in the nucleus (55,56). Activation of these pathways by both Rac and Cdc42 are mediated by at least two serine/threonine kinases, PAK (p21 activated kinase), and MLK3 (Mixed lineage kinase) which are substrates for both G proteins (57,58). In addition, PAK can phosphorylate MEK1 which then activates ERK in a way that seems to act synergistically with Raf-1 in ERK activation (59).

Recently, indirect routes for Cdc42-mediated regulation of MAPK signaling have been shown to be just as important. These routes involve a non-traditional Cdc42 effector named Cool (Cloned out of library) or PIX (for p21-interacting exchange factor) which will be referred to hereafter as Cool. The Cool family of proteins were first discovered through their association with PAK (60,61). Later studies found Cool proteins also associated with c-Cbl (Casitas B lymphoma), an E3-ubiquitin ligase necessary for the ubiquitination and subsequent degradation of receptor tyrosine kinases, including EGFR (62-64). Due to the presence of the tandem DH/PH motif commonly found in RhoGEF proteins, it was assumed that they functioned as GEFs for Rho family members. Interestingly, unlike most GEFs, it was discovered that Cool-1 bound preferentially to activated Cdc42 instead of the inactivated form (65). This finding was all the more intriguing due to previous data showing that by inhibiting the ability of c-Cbl to catalyze the ubiquitination of EGFRs, there was significantly enhanced EGF-dependant activation of MAPK activity (63,64). This lead to the discovery that cells transfected with fast-cycling Cdc42(F28L) had a greater number of EGF receptors at the cell surface and exhibited sustained EGF-coupled signaling activities (65). Wu and his colleagues went on to show that loss of the interaction between Cdc42 (F28L) and Cool-1, or between Cool-1 and c-Cbl, was

sufficient to abolish Cdc42 (F28L) mediated cellular transformation. A model was proposed whereby activated Cdc42 binds Cool-1 which itself is bound to c-Cbl and sequesters c-Cbl away from the EGFR. As a result, EGFR is not degraded but instead recycled back to the plasma membrane where it continues to signal to the nucleus.

Rho family members and their effectors are involved in a multitude of signaling pathways which often intersect and synergise with other pathways to perform an array of functions. The mechanisms for some of these functions have yet to be determined but there is no question as to the Rho family proteins importance in the cell.

Structural considerations

The structure of Ras was the second small G protein structure to be solved after the bacterial elongation factor EF-Tu (66,67). The GTP-binding domain was found to be nearly identical between the two structures, encompassing a domain containing 6 β-strands and 5 α-helices. It is now known that this is a common fold for all G proteins including the heterotrimeric G proteins. Ras is considered to be the minimum structural fold for G proteins and as such, is the standard by which all other small G proteins are compared.

With the advent of molecular cloning techniques during the 1980s, increasing numbers of G proteins were discovered and more sequences were available for comparisons. It was appreciated early on that there were regions in the heterotrimeric Gα subunits that were highly conserved and important for binding the guanine-nucleotide. After the previously mentioned structures of EF-Tu and Ras were published, it was noted that there were 5 regions of conservation named G1-G5 across all G protein families that were critical for GTP-binding and hydrolytic activity (68). These regions include the GXXXXGK(S/T) or P-loop, DXXG which binds to the γ-phospahte as well as the Mg^{2+} ion, N/TKXD which binds the guanine ring, the SAK

motif which confers specificity, and a region encompassing Switch I (see below) which includes Thr35 that coordinates the Mg^{2+} (69,70).

As seen in the crystal structures for Ras, the differences between signaling-active and signaling-inactive forms of G proteins reflect structural rearrangements in two loop regions encompassing amino-acids 30-38 and 60-76 named Switch I and Switch II respectively (71). It was revealed both biochemically and structurally that these regions are the sites of interaction with downstream targets. Due to the amount of sequence homology in the switch regions across all G proteins and the availability of various structures, it is widely accepted that this rearrangement is likely true for all small GTPases. In fact, this rearrangement has also been shown to be true for the heterotrimeric G proteins as well (72).

Turning G proteins on at the proper time is essential for the proper functioning of the cell. Direct activation is achieved by GEFs in the case of small G proteins, whereas heptihelical receptors serve an analogous role for the large G proteins. Structurally, GEFs do not share significant homology, however they do share a common mechanism of action. In general, GEFs bind the switch regions and perturb the Mg^{2+} as well as the phosphate-binding site (44). Often, negatively charged residues found within Switch II are brought into close proximity to the β-phosphate of GDP while an alanine, either from the GEF or found within the DXXG motif on Switch II, is used to clear the Mg^{2+}-binding site. The dissociation of Mg^{2+}, coupled with phosphate destabilization, decreases the affinity between the G protein and GDP by several orders of magnitude allowing for GDP to dissociate. Intracellular GTP, which is at a 10 fold higher concentration than GDP, is then free to bind and thus activate the G protein.

Similarly, GAPs across the different G protein families share little structural or sequence homology but often employ similar mechanisms to catalyze the GTPase

activity. Nearly all G proteins exhibit an intrinsic ability to hydrolyze GTP to GDP and P_i, albeit at a rate that is too slow to for the precise timing of signals required by the cell. In general GAPs work by helping to orient a nucleophilic water molecule, usually via a glutamine residue located within the Switch II region of the G protein, and by stabilizing the negative charge that builds up on the γ-phosphate with the help of an arginine residue, usually referred to as an "arginine finger", either supplied by the GAP in the case of small G proteins, or by the large helical domain unique to Gα subunits (44,70). The rates of GTP hydrolysis vary depending on the G protein, however heterotrimeric α-subunits typically have higher rates than Ras-like small G proteins due to the presence of the arginine residue already found in their helical domain. Additionally, there is evidence that stabilization of the switch regions by the GAP, even in the absence of the "arginine finger", may be an important part of the catalytic process (73).

Rho family structure

The first X-ray crystal structure of a Rho family member G protein, Rac1, was solved in 1997 (74). Other Rho family members Rho and Cdc42 were soon to follow. Rho family members are distinguished from other small G proteins by the presence of a helical insert comprising 13 amino acid residues (i.e. 123 – 135 in the case of Rac and Cdc42). The function of this insert is largely unknown. However, it has been shown that replacement of this region with the corresponding Ras sequence inhibits the ability of Rho-family proteins to transform cells (75,76).

Overview of thesis research:

Activated structures of Cdc42 bound to effector and regulatory molecules have been solved using both NMR and x-ray crystallography. In addition, the signaling-

inactive structures (GDP-bound) of Cdc42 have been solved both in the presence and absence of RhoGDI (Guanine-nucleotide Dissociation Inhibitor) (77,78). These structures have provided many insights into the mechanisms for Cdc42 activation, GTP hydrolysis, as well as effector protein interaction.

Upon comparison of signaling-active Cdc42 in complex with effector proteins versus the signaling-inactive structures of Cdc42 (G12V) (pdb id. 1A4R) as well as Cdc42-GDP (pdb id. 1ANO) it seems evident that Cdc42 shares a similar activation mechanism with Ras. As mentioned earlier, activation of Ras leads to the re-orientation of two loops located in the effector-binding region named Switch I and Switch II, residues 30-40 and 60-76 respectively (71). These regions serve as the binding interface between Ras and its effectors and as such, are sometimes referred to as effector loops. Effector proteins for Cdc42 containing CRIB domains bind in a similar manner. They form an intermolecular β-sheet with strand β2 from Cdc42, which is located just after Switch I. However, unlike Ras, Switch II in Cdc42 appears to be much more rigid and does not rearrange between the inactive (GDP) and active (GTP) structures. Switch I however shows a relatively large conformational transition in the activated effector-bound structures when compared to the inactive structures. This implies that the activation of Cdc42 is accompanied by the rearrangement of Switch I into an orientation that can now be recognized by effector proteins, similar to what has been shown for Ras. However, there is no structure yet available for an activated Cdc42 – GTP-analog complex to compare with the effector-bound structures. With this in mind, the focus of my thesis has been to try and understand the exact mechanism of Cdc42 activation.

To this end, I provide evidence in the following two chapters that, contrary to what was expected, signaling-active Cdc42 undergoes virtually no structural rearrangement upon binding a GTP analog when compared to Cdc42 – GDP. Instead,

coupling of an effector protein is necessary for Cdc42 to achieve a fully activated structure. Furthermore, in the final chapter, I show that the constitutively activated Cdc42 (Q61L) mutant bound to the non-hydrolyzable guanine-nucleotide analog GMP-PCP, does indeed show a structural rearrangement consistent with the effector-bound structures for Cdc42. Interestingly, direct comparisons between GTP- and GMP-PCP-bound Cdc42 (Q61L) by ^{31}P-NMR revealed that both nucleotides confer similar properties on the protein. These results imply that the Q61L point mutant alters the thermodynamics of Cdc42 so as to favor the activated structure. Additionally, these results are consistent with previously derived K_d values for both wild-type and Q61L mutant forms of Cdc42 whereby the Q61L isoform has been shown to have an order of magnitude higher affinity for effector proteins than wild-type (79,80).

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Chapter 2

EFFECTOR PROTEINS EXERT AN IMPORTANT INFLUENCE ON THE SIGNALING-ACTIVE STATE OF THE SMALL GTPASE CDC42

Abstract

GTP-binding (G) proteins regulate the flow of information in cellular signaling pathways by alternating between a GTP-bound "active" state, and a GDP-bound "inactive" state. Cdc42, a member of the Rho family of Ras-related small G-proteins, plays key roles in the regulation of cell shape, motility, and growth. Here we describe the high-resolution X-ray crystal structure for Cdc42 bound to the GTP-analog GMP-PCP (i.e. the presumed signaling-active state) and show that it is virtually identical to the structures for the signaling-inactive, GDP-bound form of the protein, contrary to what has been reported for Ras and other G-proteins. Especially surprising was that the GMP-PCP- and GDP-bound forms of Cdc42 did not show detectable differences in their Switch I and Switch II loops. Fluorescence studies using a Cdc42 mutant in which a tryptophan residue was introduced at position 32 of Switch I also showed that there was little difference in the Switch I conformation between the GDP- and GMP-PCP bound states (i.e. <10%), which again differed from Ras where much larger changes in Trp32 fluorescence were observed when comparing these two nucleotide-bound states (>30%). However, the binding of an effector protein induced significant changes in the Trp32 emission specifically from GMP-PCP-bound Cdc42, as well as in the phosphate

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resonances for GTP-analogs bound to this G-protein as indicated in NMR studies. An examination of the available structures for Cdc42 complexed to different effector proteins, versus the X-ray crystal structure for GMP-PCP-bound Cdc42, provides a possible explanation for how effectors can distinguish between the GTP- and GDP-bound forms of this G-protein and ensure that the necessary conformational changes for signal propagation occur.

Introduction

Cdc42 is a member of the Rho family of Ras-related small G-proteins and is an essential protein found in all eukaryotic organisms including yeast, flies, and mammals (1-3). Like Ras, the founding member of the small G-protein family (4), Cdc42 undergoes a GTP-binding/GTP-hydrolytic cycle that enables it to act as a molecular switch in cells. It is activated to undergo GDP-GTP exchange by members of the Dbl-family of guanine nucleotide exchange factors (GEFs) (5-7). GTP-bound Cdc42 can bind and/or activate over 20 downstream effector proteins that are responsible for mediating a diversity of cellular functions, including actin cytoskeletal remodeling, cell polarity, intracellular trafficking, EGF receptor degradation, and cell cycle progression (1-3,8). These different signals are terminated when Cdc42 is deactivated through its ability to hydrolyze GTP, a reaction that is catalyzed by GTPase-activating proteins (GAPs) (9).

Structural studies of a number of GTP-binding proteins, beginning with the bacterial elongation factor, Ef-Tu, and including H-Ras and the α subunits of various members of the family of large G-proteins, have shown that a conserved architecture exists for GTP-binding and hydrolytic activity, comprising five α-helices and six β-strands (10-14). Moreover, comparisons of the X-ray crystal structures for many of these proteins bound to GDP and GTP-analogs highlighted two regions called Switch I and Switch II that change their conformation upon GDP-GTP exchange. In the case of H-Ras and related

small G-proteins, Switch I encompasses residues 30-38 within the α1-β2 loop while Switch II is made up of residues 60-76 within β3-α2 (12,15). It has been commonly assumed that changes in these Switch regions represent the underlying basis for GTP-dependent signal propagation and indeed Switch I has been shown to be a principle site used by Ras and related small G-proteins including Cdc42 to engage their downstream effectors.

Given the shared architecture between Ras, Cdc42 and other Rho-family small G-proteins, there was every reason to expect that Cdc42 would exhibit the same types of GTP-dependent changes in Switch I and II, as originally described for H-Ras. However, surprisingly, we found that this was not the case. The high-resolution X-ray crystal structure for Cdc42 complexed to the non-hydrolyzable GTP-analog GMP-PCP was virtually identical to that for Cdc42 bound to GDP, despite the fact that only GMP-PCP-bound Cdc42 and not its GDP-bound counterpart is able to productively engage effector proteins. Likewise, we found that Cdc42 molecules containing a tryptophan residue inserted into position 32 of Switch I, in place of the normal tyrosine residue, showed little or no change in their tryptophan fluorescence, when comparing the GMP-PCP- and GDP-bound forms of the protein. These results again differed from those obtained with the corresponding Switch I mutant of H-Ras, which showed significant changes in the fluorescence of the Switch I tryptophan residue when comparing the GDP- and GMP-PCP-bound states.

Thus taken together, these findings raised the question as to how effector proteins are able to selectively recognize the GTP-analog-bound form of Cdc42, and whether effectors might be capable of inducing and/or stabilizing specific conformational transitions within a Cdc42 species that appears to start-off predominantly in a signaling-inactive conformation. Here we show that despite the GDP- and GMP-PCP-bound forms of Cdc42 sharing a similar Switch I conformational state, the tryptophan fluorescence of the GMP-PCP-bound Cdc42(Y32W) mutant undergoes a specific and

significant change upon the binding of an effector protein. NMR experiments also showed that effector proteins were able to specifically promote conformational changes within Cdc42 molecules bound to GTP-analogs. When this structure-function information is considered together with the structures for Cdc42 complexed to effectors that either use the conventional Cdc42/Rac-interactive-binding (CRIB) domain, such as is the case for Pak (for p21-activated kinase) (16), or a non-conventional Cdc42/Rac-binding domain, as occurs with Par6 (for Partitioning-defective protein-6) (17), it becomes apparent how effector proteins help to ensure that Cdc42 undergoes the necessary conformational changes for signal propagation.

Overall, our findings support the idea that there is a spectrum of possibilities regarding the conformational states that G-proteins can assume following the exchange of GDP for GTP (or GTP-analogs), i.e. what is commonly referred to as the G-protein activation event. One end of the spectrum represents cases like Cdc42 where, in the absence of an effector protein, the majority of the population of the GTP- (or GTP-analog-) bound G-protein exists in conformational states that are minimally changed from those for the GDP-bound form of the protein. Thus, Cdc42 relies heavily upon effector proteins to induce the correct conformational changes to enable signal propagation to occur. At the other end of the spectrum are the Gα subunits of heterotrimeric (large) G-proteins, and small G-proteins like H-Ras where, upon GDP-GTP exchange, the majority of the G-protein population assumes conformational states that clearly differ from the GDP-bound protein and more closely approximate the signaling-active, conformational states that are formed upon the binding of effectors.

EXPERIMENTAL PROCEDURES

Protein purification- E. coli cells expressing pET15b-his-Cdc42, pGEX-KG-Cdc42(Y32W, W97H), pGEX-KG-PBD (W98F), and pGEX-KG-PBD were grown at

37˚C until an O.D. of 0.8. Induction was initiated by the addition of IPTG (1 mM) and the cells were allowed to grow for another 3 hours. Cells were pelleted at 6000 x g for 10 minutes and frozen at -80˚C. Cell pellets were homogenized in HMA (20 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM NaN_3) buffer and lysed by sonication. Cell debris was centrifuged at 20,000 rpm for 30 minutes and the supernatant was saved. Supernatants containing 6-histidine (His)-tagged Cdc42 were incubated briefly with Chelating-Sepharose beads (Amersham) charged with Ni^{2+} and equilibrated with HMA. Beads were washed with HMA plus 20 mM imidazole before elution with HMA plus 200 mM imidazole. GST-tagged proteins were incubated with glutathione-beads (Amersham) equilibrated with HMA for 30 minutes at 4˚C. Beads were washed with HMA containing 500 mM NaCl and again with HMA. Protein was eluted with 10 mM glutathione in HMA.

The His-tagged proteins were incubated with thrombin (Haematological Technologies Inc.) at 4˚C for 3-4 hours. Clipped proteins were further purified by chromatography on a HiTrap Q column (Amersham) and protein fractions were pooled.

Nucleotide loading- Mutant and wild-type Cdc42 protein concentrations were measured using Bradford reagent (Pierce). Non-hydrolyzable nucleotide analogs were added to an ~5-fold excess relative to the protein concentration. Ammonium sulfate was added to a final concentration of 200 mM along with 100 units of alkaline phosphatase-bound acrylic beads (Sigma # P0927). After a 4 hour incubation at 4˚C, the beads were washed with HMA and the flow-through was collected. The buffer was exchanged on a PD-10 column (Amersham) equilibrated with HMA. Nucleotide content was confirmed by HPLC analysis, using a previously published protocol (18).

GST-Cdc42 pull-down assays- Equal amounts of glutathione-beads (Amersham) were saturated with GST-Cdc42 bound to GMP-PCP, GMP-PNP, GTPγS, or GDP. After incubation for 1 hour at 4˚C, the beads were washed 3x with HMA and an equivalent amount of the limit Cdc42/Rac-binding domain from Pak3 (PBD) was added

to a 10-fold molar excess of Cdc42. Beads were washed 3 x with HMA after a 3 hour incubation at 4˚C. Equal amounts of protein from each assay were boiled and loaded on a 4%-20% gradient gel (Invitrogen).

X-Ray crystallography- Cdc42-GMP-PCP (80 mg/ml) crystals were grown in 12% PEG 6K, 100 mM ammonium sulfate, 100 mM Na acetate and 50 mM MES, pH 6.0, at 18˚C. Data was collected at the Cornell MacCHESS beamline A1 using an ADSC Quantum-210 CCD Detector. Data processing was performed using Mosfilm and initial phases were solved by molecular replacement using MolRep from the CCP4 suite (19). The model structure used to find the initial phases was Cdc42-GDP (PDB ID 1AN0) without nucleotide. Structure refinement was completed using CNS (20) and validated with Procheck (21).

^{31}P-NMR- Samples were prepared by dissolving Cdc42 (1 mM) pre-exchanged with GMP-PNP or GMP-PCP, either with or without the PBD (1 mM), in HMA containing 10% D_2O. ^{31}P spectra were obtained at 5˚C on a Varian INOVA spectrometer operating at 202.37 MHz using a 5 mm Varian DBG probehead. ^{1}H broadband decoupling was applied throughout the acquisition. A relaxation delay of 8 seconds was used between scans, with 3000 - 4000 scans summed prior to analysis. Data was zero-filled to 128k points and an exponential multiplication (5 - 10 Hz) was performed prior to Fourier Transform. Spectra were referenced externally to 85% phosphoric acid (0 ppm).

Fluorescence spectroscopy- All experiments were performed on a Varian Cary Eclipse fluorimeter. Excitation and emission slit widths were ±5 nm and ±10 nm, respectively. Cdc42 and H-Ras emission-scan experiments were performed in HMA at 30˚C, using an excitation wavelength of 295 nm in HMA. Excitation and emission wavelengths for the binding assays of Cdc42(Y32W, W97H) with PBD(W98F) were 295 nm and 353 nm, respectively.

RESULTS

The X-ray crystal structure for GMP-PCP-bound Cdc42- The original X-ray crystal structures for the signaling-active (GTP-bound) form of H-Ras were obtained using the non-hydrolyzable GTP-analogs GMP-PNP and GMP-PCP (11,12), because GTP as well as GTPγS are hydrolyzed during the crystallization procedure. As is the case for Ras, these two non-hydrolyzable GTP-analogs enable Cdc42 to interact with its downstream effector proteins. An example is presented in Figure 2.1 that shows the selective ability of GST-Cdc42, when bound to different GTP-analogs, to pull-down the limit Cdc42/Rac-binding domain from Pak3 (i.e. the PBD), whereas the GDP-bound form of GST-Cdc42 was unable to bind to the effector. We therefore used the Cdc42-GMP-PCP complex for high-resolution structural analysis of the signaling-active form of this G-protein.

Figure 2.2A shows the ribbon diagram for the X-ray crystal structure for GMP-PCP-bound Cdc42 solved to 2.4 Å resolution (see Table 1 below for statistics on data collection and refinement). The structure conforms to an α/β fold comprised of six β-strands, six α-helices and one 3/10 helix, and in general displays an architecture that is characteristic of other small G-proteins. Stabilization of the GMP-PCP molecule inside the nucleotide-binding pocket is achieved through hydrogen bonds between the guanine ring and surrounding residues, π-π stacking interactions with Phe28, and hydrogen bonds with the phosphate oxygen atoms. Figure 2.2B shows a representative electron density for a portion of the GMP-PCP-binding site.

There are two molecules of GMP-PCP-bound Cdc42 within the asymmetric unit. Crystal contacts involving Switch I from chain A help to stabilize the loop, specifically, at residues Tyr32 and Phe37. A cleft is formed by Met1, Pro50, and Met45 in a symmetry-related molecule which provides a hydrophobic interface for the phenyl group of Tyr32 in Switch I. The formation of a hydrogen bond between Tyr32 and

FIGURE 2.1. **Interaction between signaling-active forms of Cdc42 and the p21-binding domain of Pak3.** Glutathione-beads were saturated with GST-Cdc42 (700 μg) bound to GDP, GMP-PCP, GMP-PNP, or GTPγS. Washed beads were incubated with a 10-fold molar excess of Pak3-PBD (p21-binding domain) for 3 hours and washed. Lane 1 shows signaling inactive Cdc42-GDP is ineffective at binding target. Lanes 2-4 show signaling-active Cdc42 is able to interact with PBD, which is detected as a doublet of 8 kDa – 10 kDa. PBD appears as a doublet most likely due to non-specific proteolytic cleavage from the bacterial host's proteosome.

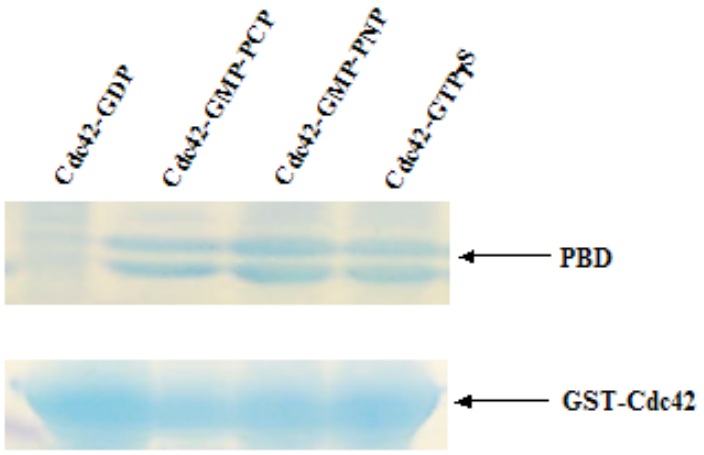

Cdc42-GDP
Cdc42-GMP-PCP
Cdc42-GMP-PNP
Cdc42-GTPγS
PBD
GST-Cdc42

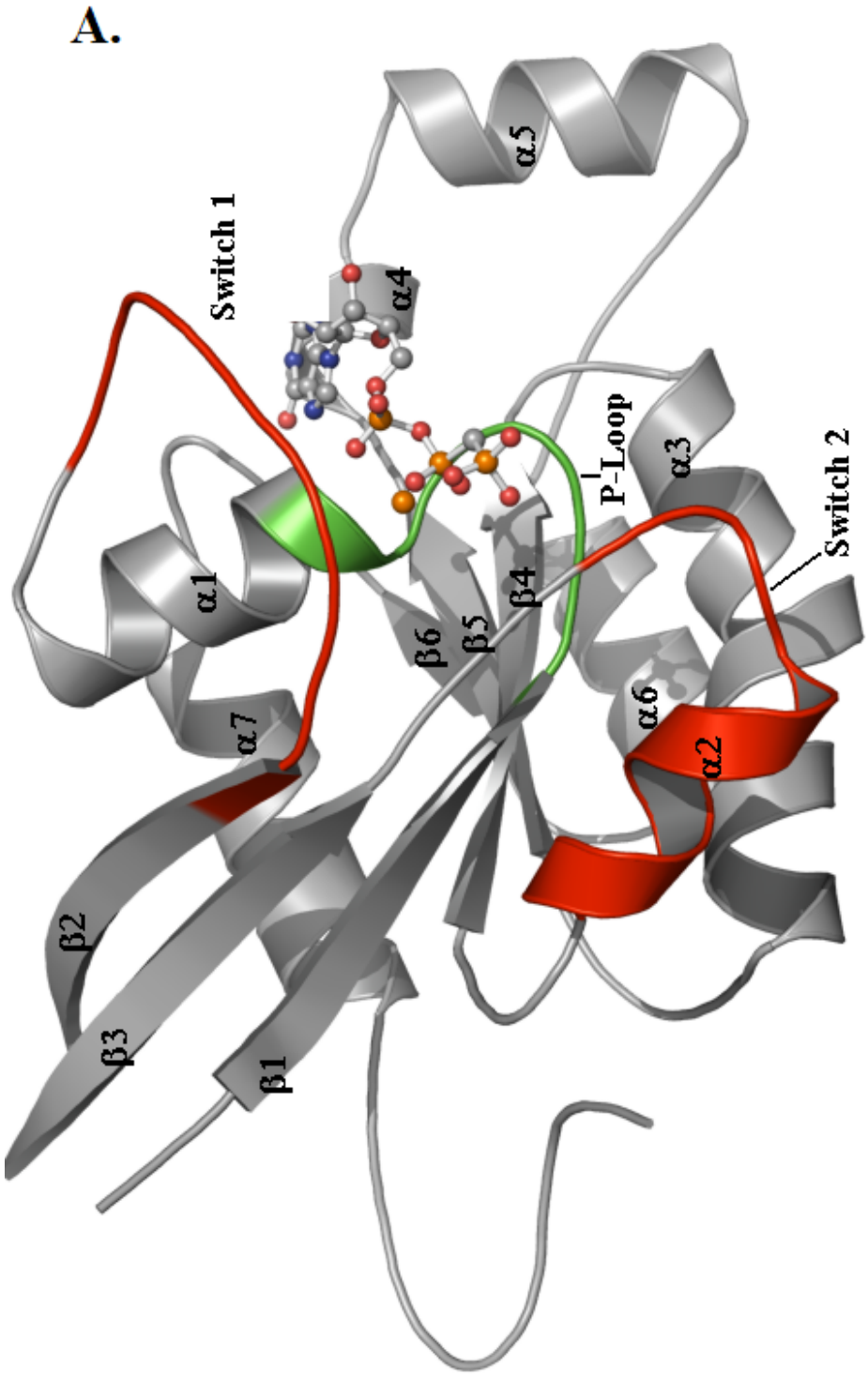
TABLE 1

a $R_{sym} = \Sigma_{hkl} \Sigma_i[I_{i(hkl)} - \langle I_{(hkl)} \rangle]/\Sigma_{hkl}\Sigma_{i(hkl)}$

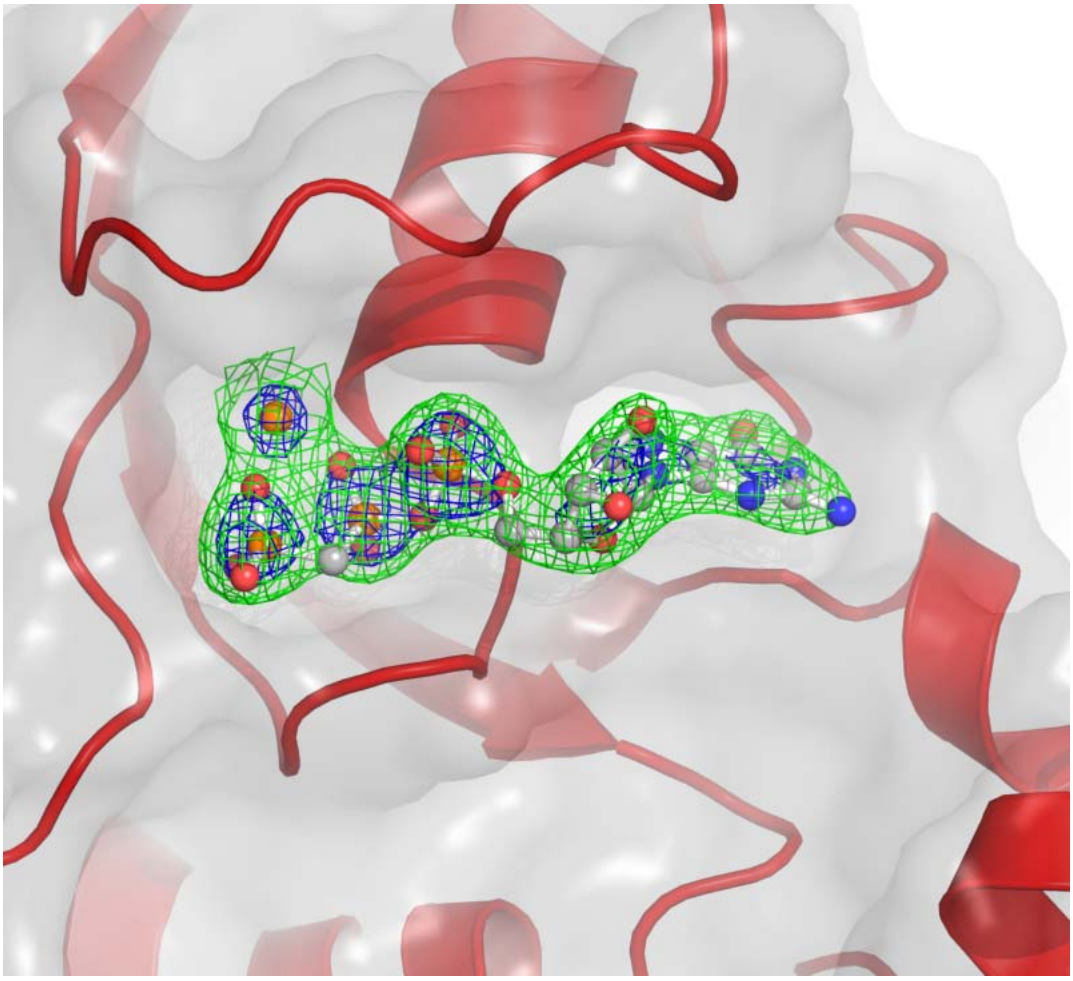
b R_{free} and $R_f = \Sigma|Fobs - Fcalc|/ \Sigma Fobs$

Data Collection and refinement statistics for Cdc42-GMPPCP	
Space Group	$P4_12_12$
Unit Cell (Å)	98.5, 98.5, 102.4
Resolution (Å)	2.4
Average Redundancy (highest resolution)	4.1 (2.8)
I/óI	6.8 (2)
R_{sym} [a] (highest resolution)(%)	6.8 (39)
Refinement Statistics	
Completeness %, (highest resolution)	95.5 (91.4)
Refinement resolution range (Å)	31 – 2.4
Number of reflections in working set	18448 (95%)
Number of reflections in test set	991 (5%)
R_{free} [b] (%)	26
R_f [b] (%)	23
Ramachandran	
Most favored regions	87.3 %
Allowed regions	12.3 %
rms deviation from ideal bondlength (Å)	0.010
rms deviation from ideal bond angle (¢[a])	1.4

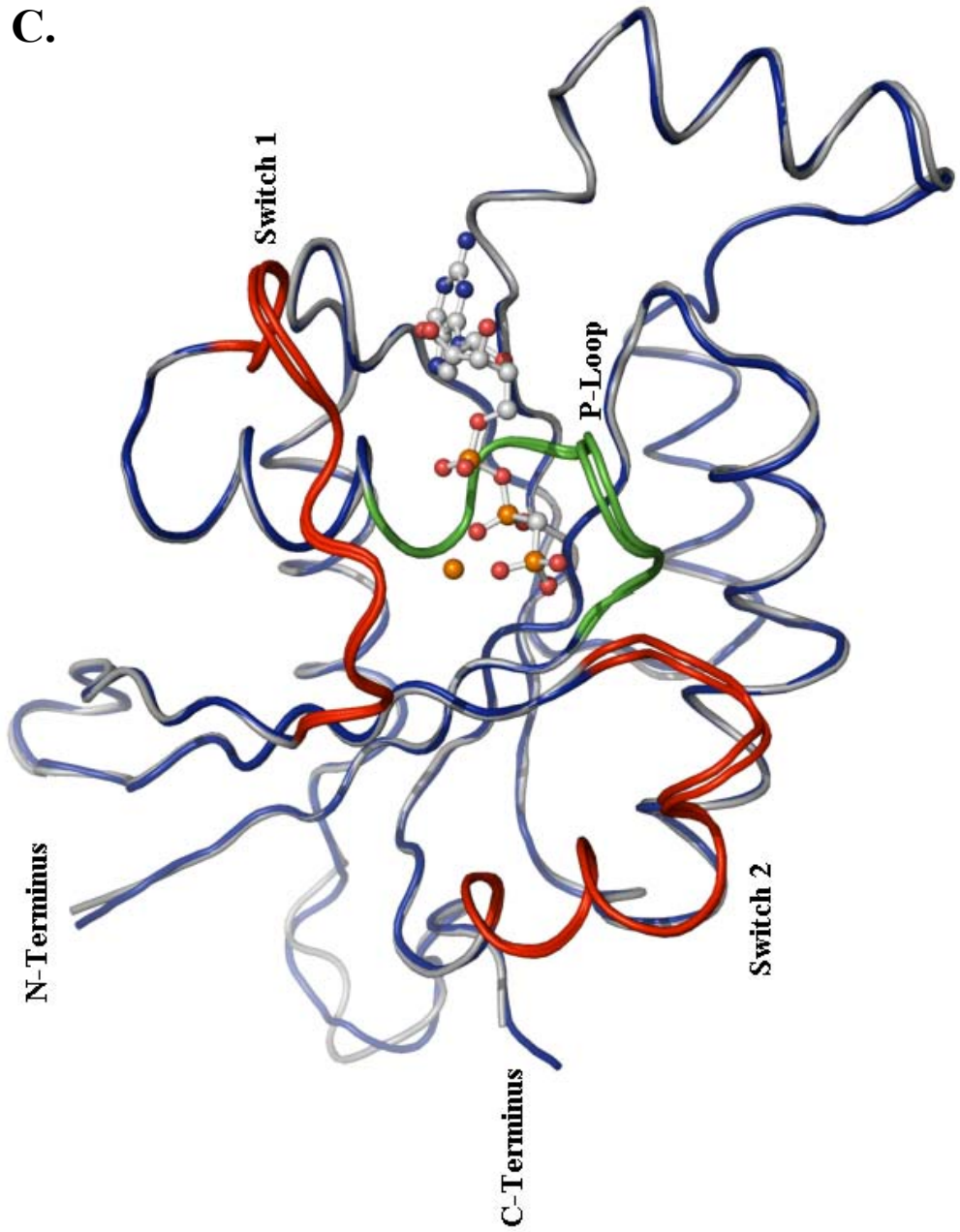
FIGURE 2.2 A-C X-ray crystal structure of GMP-PCP bound Cdc42 at 2.4 Å. *A,* Overall fold of GMP-PCP-bound Cdc42 is a classic G domain encompassing six β-sheets and five α-helices with a short two-helical insertion after the β-strand 5 known as the Rho-insert region. The α-helix 4 is a short 3-10 helix. Switch I and II are colored in red encompassing residues 30-37 and 60-70 respectively. P-loop residues, which are important for binding the phosphates, are colored green. *B,* Electron density around the GMP-PCP is contoured at 1.5σ (green) and 4σ (blue) from a $2f_o$-f_c map. Clear density is present for the γ-phosphate even at 4σ. *C,* Structural alignment of the signaling-active Cdc42-GMP-PCP complex (grey) with the signaling-inactive Cdc42 (G12V)-GDP (blue). Switch regions and the P-loop are colored red and green, respectively. There is an overall r.m.s.d. of 0.75 Å for all Cα atoms between the two structures. *D and E,* Close-up views of the Mg^{2+} coordination between the Cdc42 (G12V)-GDP and Cdc42-GMP-PCP structures, respectively. Phosphates and magnesium are shown in orange, whereas water and nitrogen molecules are colored blue. Threonine 35 does not coordinate to the γ-phosphate in the Cdc42-GMP-PCP structure as it does in other signaling active structures. It is replaced by a water molecule in the Cdc42 structures, while all other contacts are conserved. Figures 2.2 and 2.3 were created with Pymol (50).


A.
Switch 1
α5
α4
α3
P-Loop
Switch 2
α1
β6
β5
β4
α6
α2
α7
β2
β3
β1

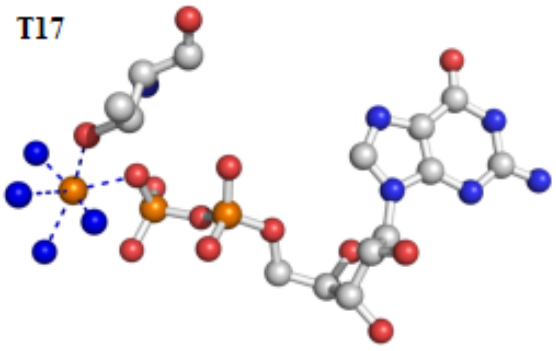
B.



C.



D.



Cdc42 (G12V)-GDP

E.

Cdc42 GMP-PCP

Thr35 from chain A helps to prevent Thr35 from coordinating the Mg^{2+} (also see below), while Phe37 is stabilized by π-π interactions with Tyr64 from a symmetry-related molecule. The individual temperature factors for residues within Switch I are higher than those found in adjacent areas of the protein where secondary structure stabilizes the residues. Moreover, the temperature factors for Switch I residues in chain B are higher than those for the corresponding residues in chain A. This is probably due to the fact that Phe37 in chain B does not undergo π-π interactions with a symmetry-related molecule.

Comparisons of the structures for the GMP-PCP- and GDP-bound forms of Cdc42- Based on the X-ray crystallographic studies of H-Ras (11,12) and various Gα subunits of the family of heterotrimeric or large G-proteins (13,14), as well as the corresponding structures for the elongation factor Ef-Tu (10), it has been suggested that there are two conserved regions designated as Switch I and Switch II that undergo conformational changes as an outcome of GDP-GTP exchange (often referred to as the G-protein-activation event). Thus, it has been generally assumed that changes occurring in Switch I and Switch II underlie the molecular switch function of both small aqnd large G-proteins, enabling them to selectively engage their downstream signaling effector proteins. However, the X-ray crystal structure for the GMP-PCP-bound form of Cdc42 was immediately intriguing because when it was compared to signaling-inactive forms of Cdc42, these structural differences were not evident. The overall topology of the Cdc42-GMP-PCP complex, and its Switch I and II conformations in particular, were virtually identical to both the X-ray crystal structure that we had earlier solved for the GDP-bound form of Cdc42 (PDB ID 1ANO), as well as the reported structure for a signaling-inactive Cdc42(G12V)-GDP complex (22). Figure 2.2C compares the Cdc42-GMP-PCP and Cdc42(G12V)-GDP complexes. A structural alignment of all the Cα

atoms between these two complexes yielded an r.m.s deviation of approximately 0.75 Å.

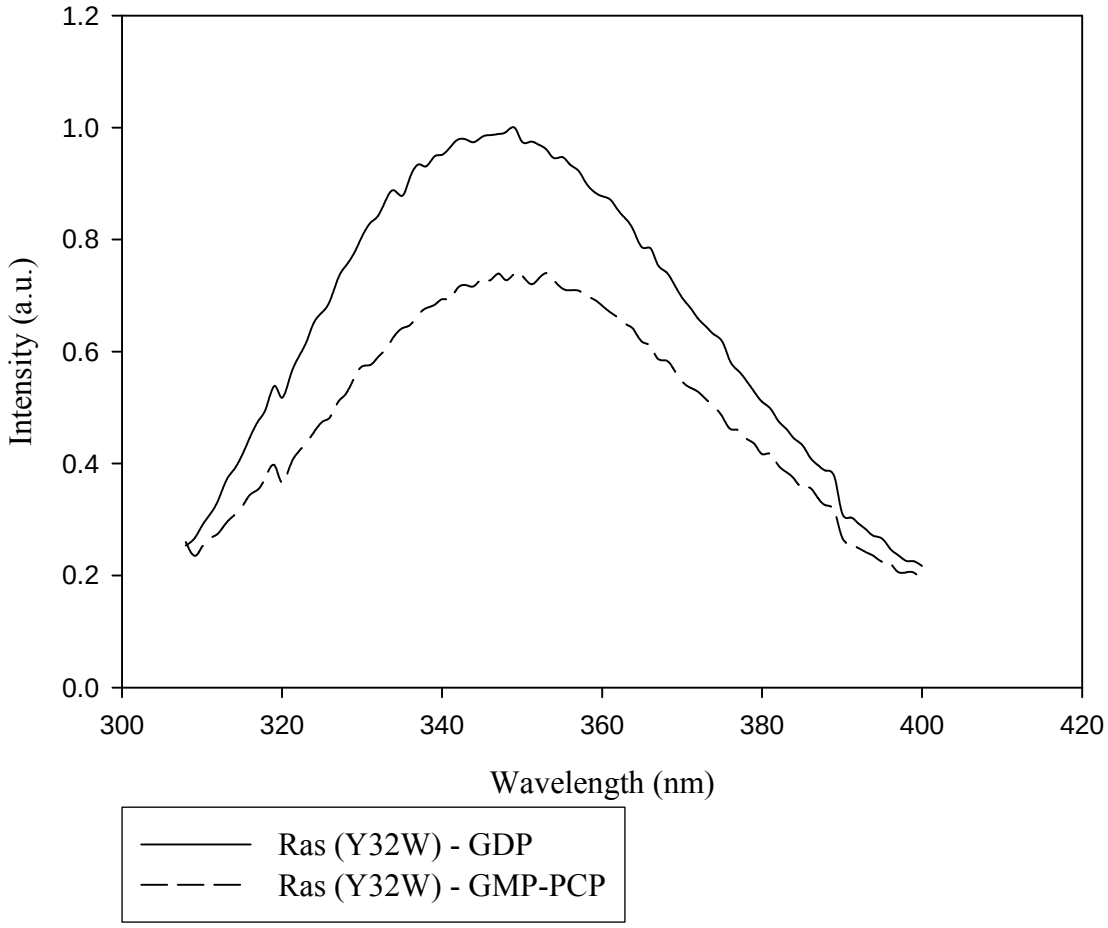
The coordination of the Mg^{2+} ion is also virtually identical in the structures for the signaling-inactive GDP-bound form of Cdc42 versus the Cdc42-GMP-PCP complex (Figures 2.2D and 2.2E, respectively). It is interesting that in GMP-PCP-bound Cdc42, Thr35 does not participate in Mg^{2+} coordination, given that it has been suggested to be critical for the structural change imparted by GTP-analogs within the Switch I loop of H-Ras (23,24). Specifically, Thr35 appears to interact with the γ-phosphate of GTP in Ras with this interaction being lost upon GTP hydrolysis and thereby possibly accounting for the observed change in the orientation of Switch I. In the structure for the Cdc42-GMP-PCP complex, Thr35 is not in position to coordinate the Mg^{2+} ion; however, a water molecule is visible and substitutes for the hydroxyl group of the threonine residue. The same is true for the structure for the GDP-bound form of Cdc42, as well as for the Cdc42(G12V)-GDP complex (22).

The Cdc42-GMP-PCP complex crystallized in the same space group and unit cell dimensions as the Cdc42(G12V)-GDP complex. As a result, both the GDP- and GMP-PCP-bound Cdc42 structures share similar crystal contacts that help to stabilize Switch I. Many of these crystal contacts are absent in the second Cdc42-GMP-PCP molecule that is present within the asymmetric unit for the GTP-analog-bound form of the G-protein. While the electron density for Switch I from the second Cdc42 molecule shares a similar backbone conformation with Switch I from the first Cdc42 molecule within the asymmetric unit, it possesses elevated B factors. This leads us to suspect that Switch I is probably highly mobile in both the GDP- and GMP-PCP-bound forms of Cdc42, an idea supported by NMR findings (25,26). Thus, crystal contacts have probably enabled us to view one conformational state from possibly a number of states that may be shared by these two nucleotide-bound forms of Cdc42.

Fluorescence studies of GDP- versus GMP-PCP-bound Cdc42- We have taken advantage of the sensitivity of fluorescence spectroscopy to further examine the Switch I conformations for the GDP- and GMP-PCP-bound forms of Cdc42. The basic strategy was to first eliminate any background tryptophan fluorescence from Cdc42 by changing the tryptophan at position 97 to a histidine, and then to introduce a tryptophan residue at position 32 of Switch I, in place of the usual tyrosine residue. The expectation was that the Trp32 residue would serve as a conformational probe for Switch I in Cdc42. This approach has been successfully used with H-Ras to detect conformational changes within Switch I upon GDP-GTP exchange (although in the case of H-Ras there are no tryptophan residues in the wild-type protein and so only the single substitution at position 32 was necessary) (27). Indeed, Figure 2.3A shows that there is a significant difference in the intrinsic fluorescence measured for the H-Ras(Y32W) mutant when comparing its GDP- and GMP-PCP-bound states, such that the binding of the GTP-analog results in an ~30% quenching in the Trp32 fluorescence. On the other hand, the differences in the intrinsic fluorescence for the corresponding forms of the Cdc42(Y32W, W97H) mutant were much more subtle with GMP-PCP-bound Cdc42 showing at most a 5-10% decrease in Trp97 fluorescence compared to its signaling-inactive, GDP-bound counterpart (Figure 2.3B). Thus, these findings corroborated the results from X-ray crystal studies that showed the differences between the GDP- and GTP-analog-bound forms of Ras were much more pronounced than the differences for the corresponding forms of Cdc42. Nonetheless, effector proteins are still able to distinguish between the GDP- and GMP-PCP-bound forms of Cdc42, as we observed a significant change in the fluorescence of the GMP-PCP-bound Cdc42(Y32W, W97H) protein (Figure 2.4A), but not in the GDP-bound form of the protein (Figure 2.4B), upon the addition of a recombinant form of the PBD in which its sole tryptophan residue was changed to a phenylalanine [PBD(W98F)]. Successive additions of PBD(W98F) resulted in a dose-dependent quenching of the fluorescence from GMP-

FIGURE 2.3 A-B Fuorescent spectra of the signaling-active and inactive forms of Ras (Y32W) and Cdc42 (Y32W, W97H). *A*, H-Ras(Y32W) (1 μM) bound to GDP (top) or GMP-PCP (bottom) was scanned at an excitation wavelength of 295 nm. A decrease in quantum yield of nearly 30% is observed upon binding of GMP-PCP. *B*, The same experiments performed with the Cdc42(Y32W, W97H) mutant show that only a minor difference in quantum yield is observed between the GDP- and GMP-PCP-bound forms of the protein.

A.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity (a.u.)
300
320
340
360
380
400
420
Wavelength (nm)
Ras (Y32W) - GDP
Ras (Y32W) - GMP-PCP

B.

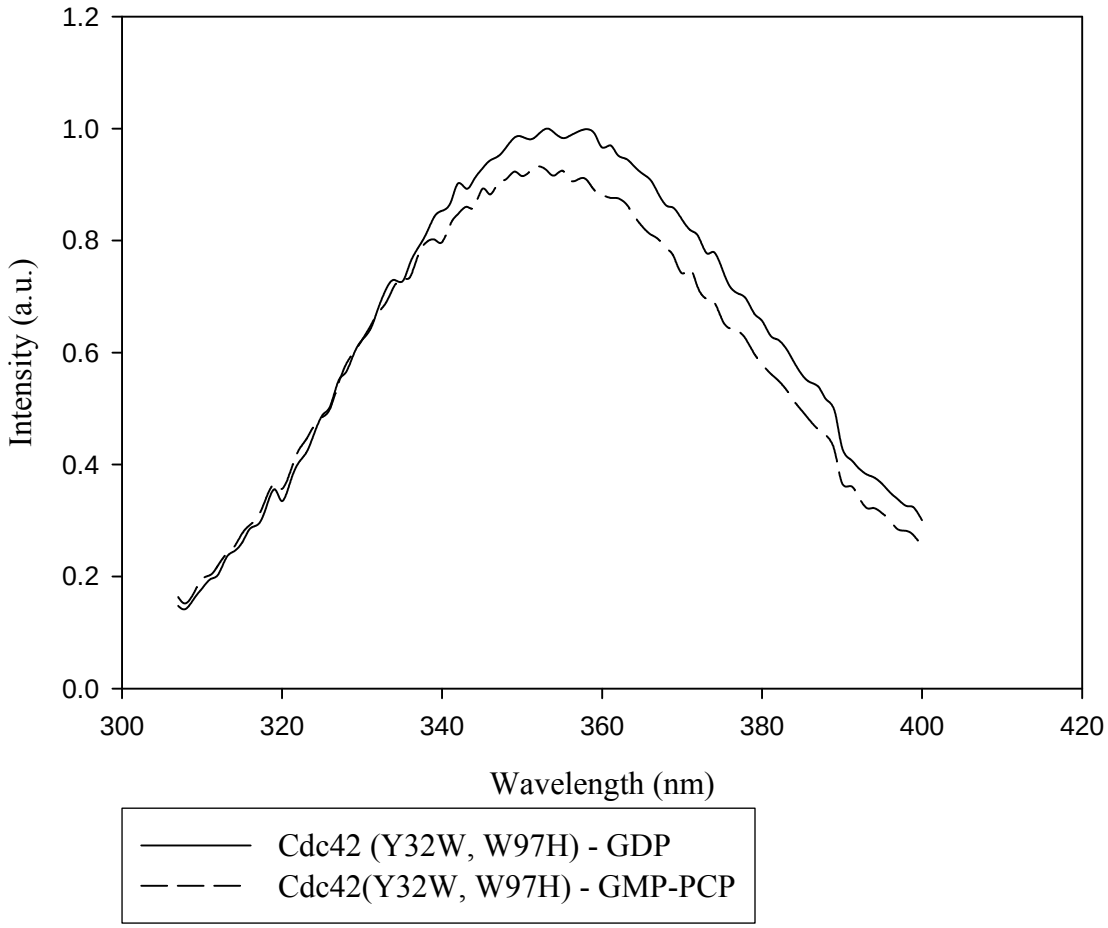
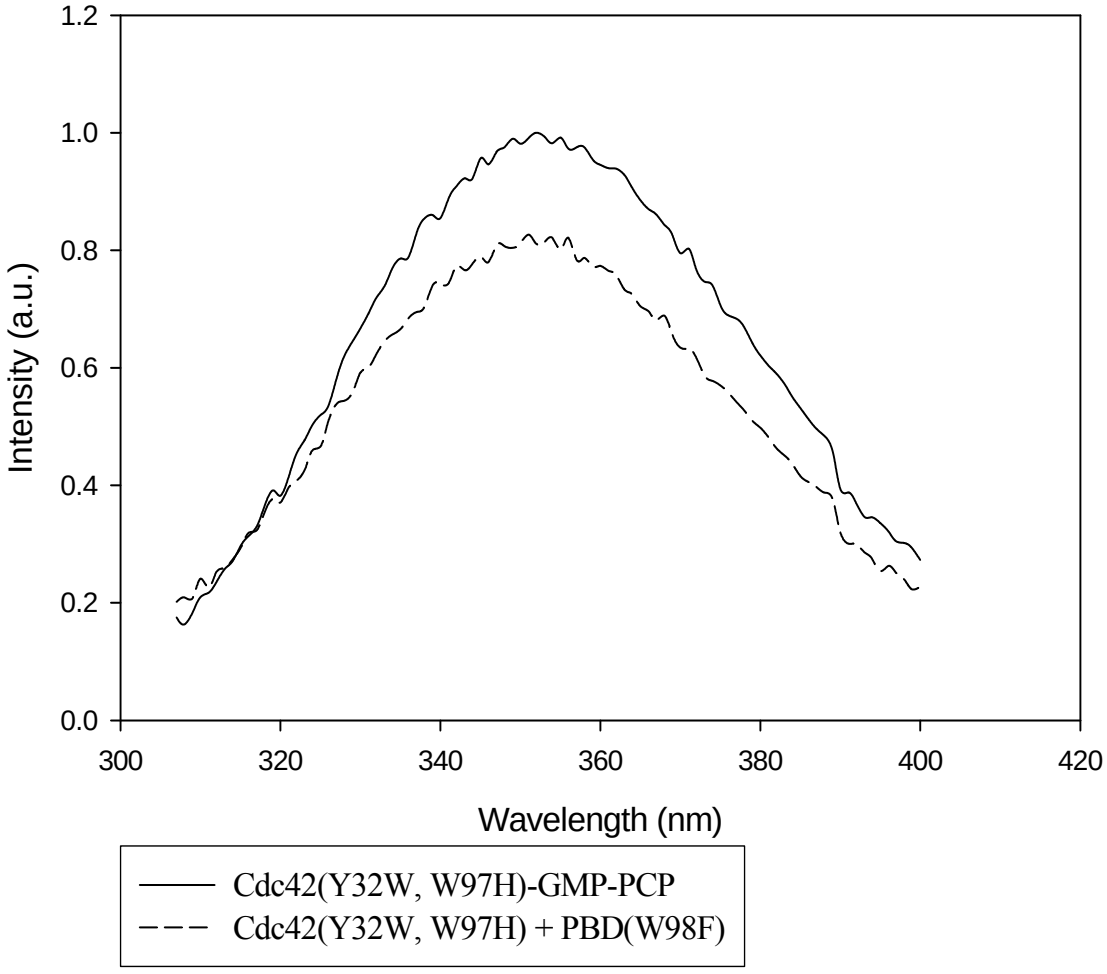
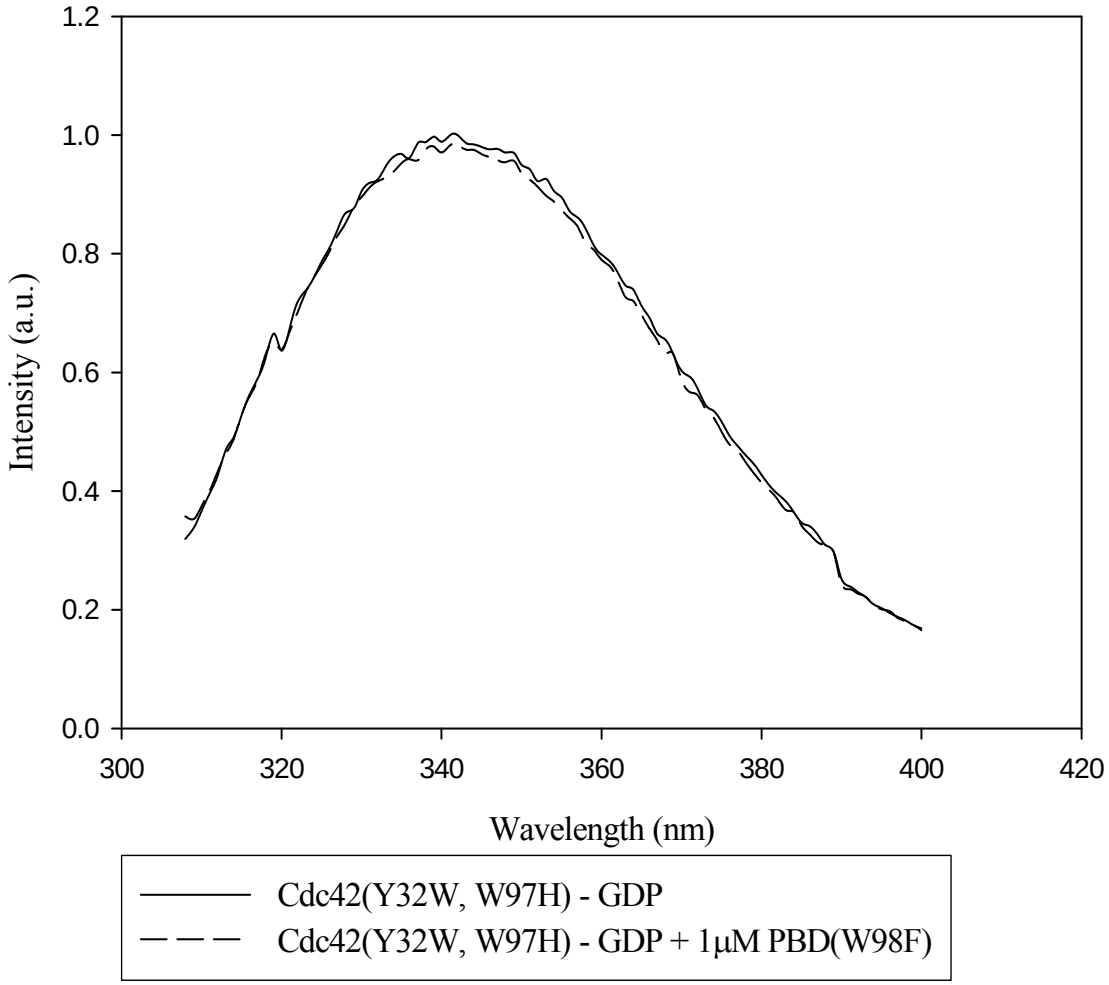

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity (a.u.)
300
320
340
360
380
400
420
Wavelength (nm)
Cdc42 (Y32W, W97H) - GDP
Cdc42(Y32W, W97H) - GMP-PCP

FIGURE 2.4 A-C Effector-induced changes in the Switch I conformation of GMP-PCP-bound Cdc42. *A*, The addition of the limit Cdc42/Rac-binding domain of Pak3 (PBD) (1 μM) to GMP-PCP-bound Cdc42(Y32W, W97H) (1 μM) causes a quenching of Trp32 fluorescence. *B*, The addition of the PBD to GDP-bound Cdc42(Y32W, W97H) has no effect on Trp32 fluorescence. Titration (0.2-4 μM) of GMP-PCP-bound Cdc42(Y32W, W97H) with increasing amounts of PAK3-PBD (W98F). *C*, Addition of PBD (W98F) decreases the quantum yield of the Cdc42 mutant until it reaches saturation at roughly 80% of the original intensity.

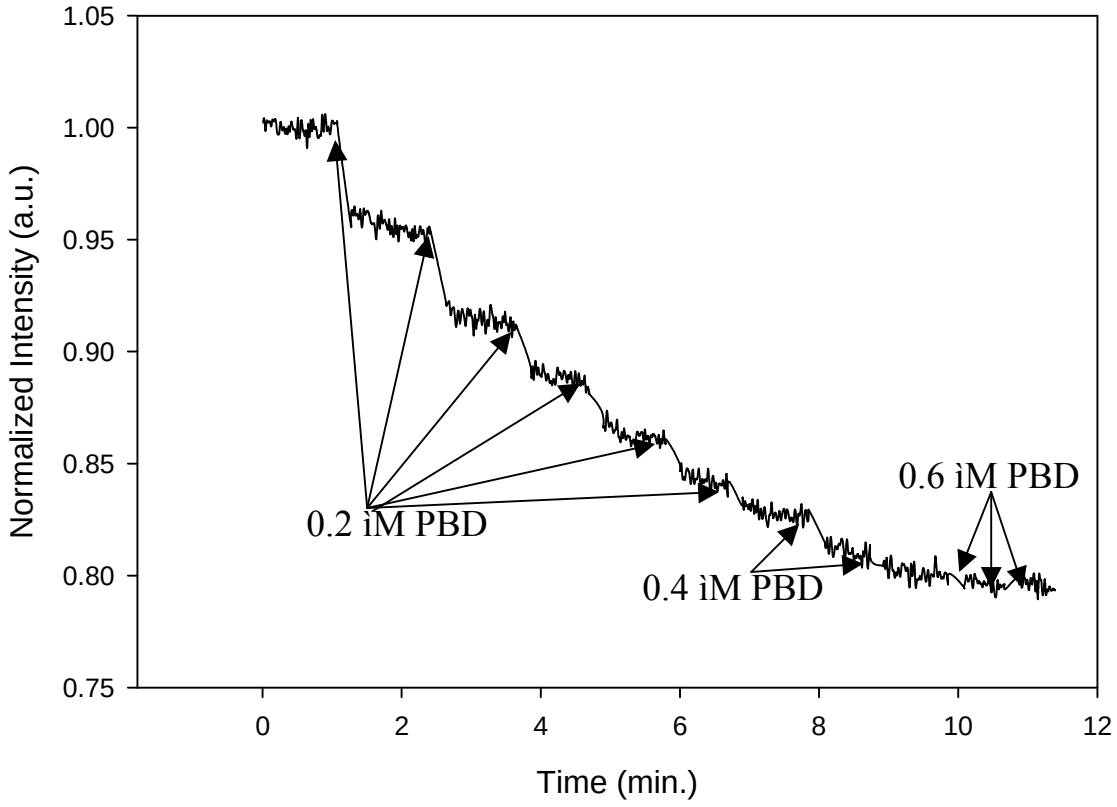
A.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity (a.u.)
300
320
340
360
380
400
420
Wavelength (nm)
Cdc42(Y32W, W97H)-GMP-PCP
Cdc42(Y32W, W97H) + PBD(W98F)

B.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity (a.u.)
300
320
340
360
380
400
420
Wavelength (nm)
Cdc42(Y32W, W97H) - GDP
Cdc42(Y32W, W97H) - GDP + 1μM PBD(W98F)

C.


1.05
1.00
0.95
0.90
0.85
0.80
0.75
Normalized Intensity (a.u.)
0
2
4
6
8
10
12
Time (min.)
0.2 ìM PBD
0.4 ìM PBD
0.6 ìM PBD

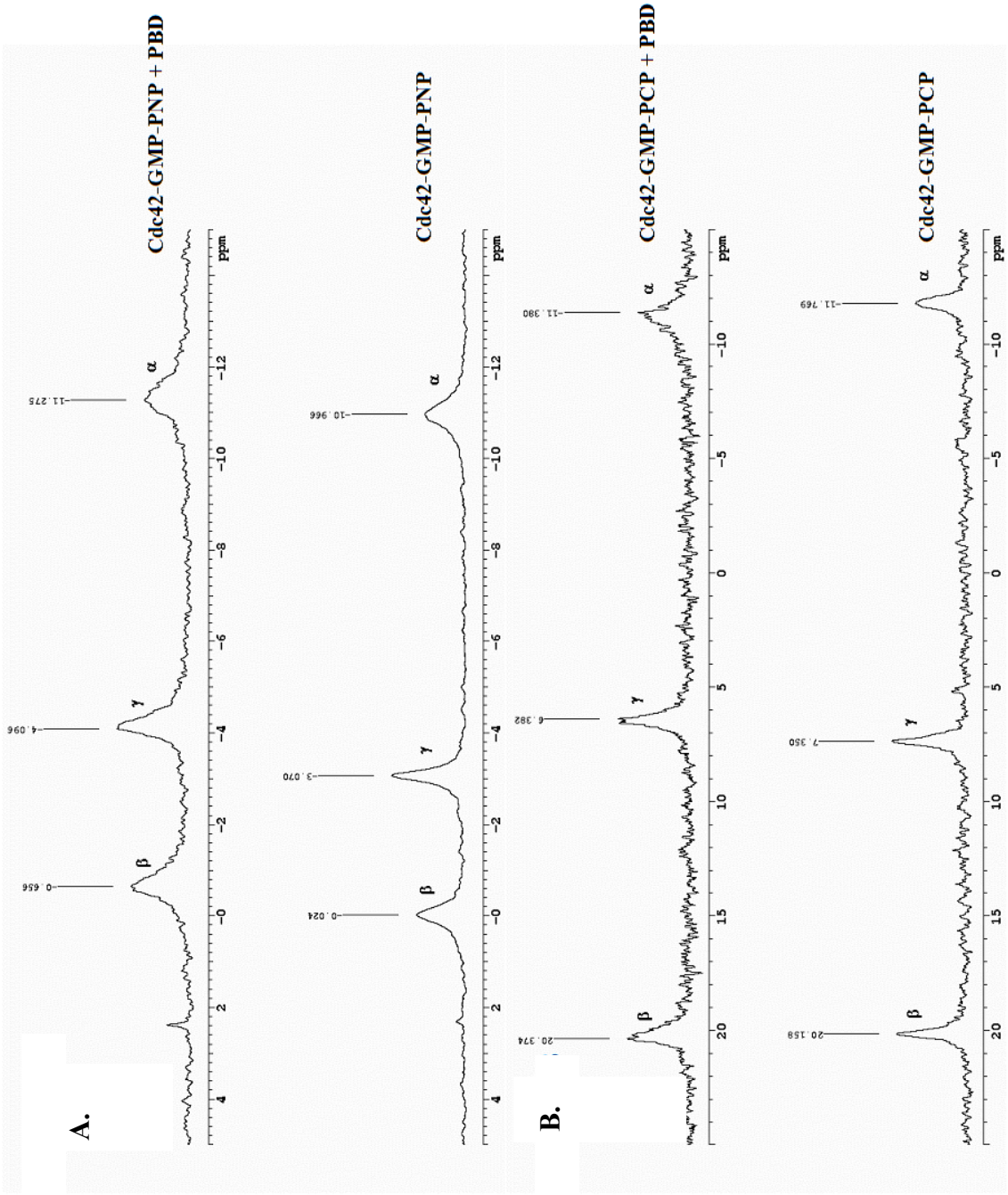
PCP-bound Cdc42(Y32W, W97H) that saturated at about 20% (Figure 4C). Identical experiments were carried out with GMP-PNP-bound Cdc42(Y32W, W97H) with similar results (not shown). The NMR structure for GMP-PNP-bound Cdc42 complexed to the CRIB domain of Pak1 shows that the nearest effector residue to position 32 in Switch I is approximately 12 Å away (16). The same is true when examining the coordinates from the recently determined X-ray structure for Cdc42-GMP-PCP bound to its limit-binding domain on Pak6 (PDB ID 2ODB). Thus, the fluorescence changes seen upon the addition of the PBD construct are likely a direct reflection of effector-induced conformational changes that are specific for the GTP-analog-bound forms of Cdc42.

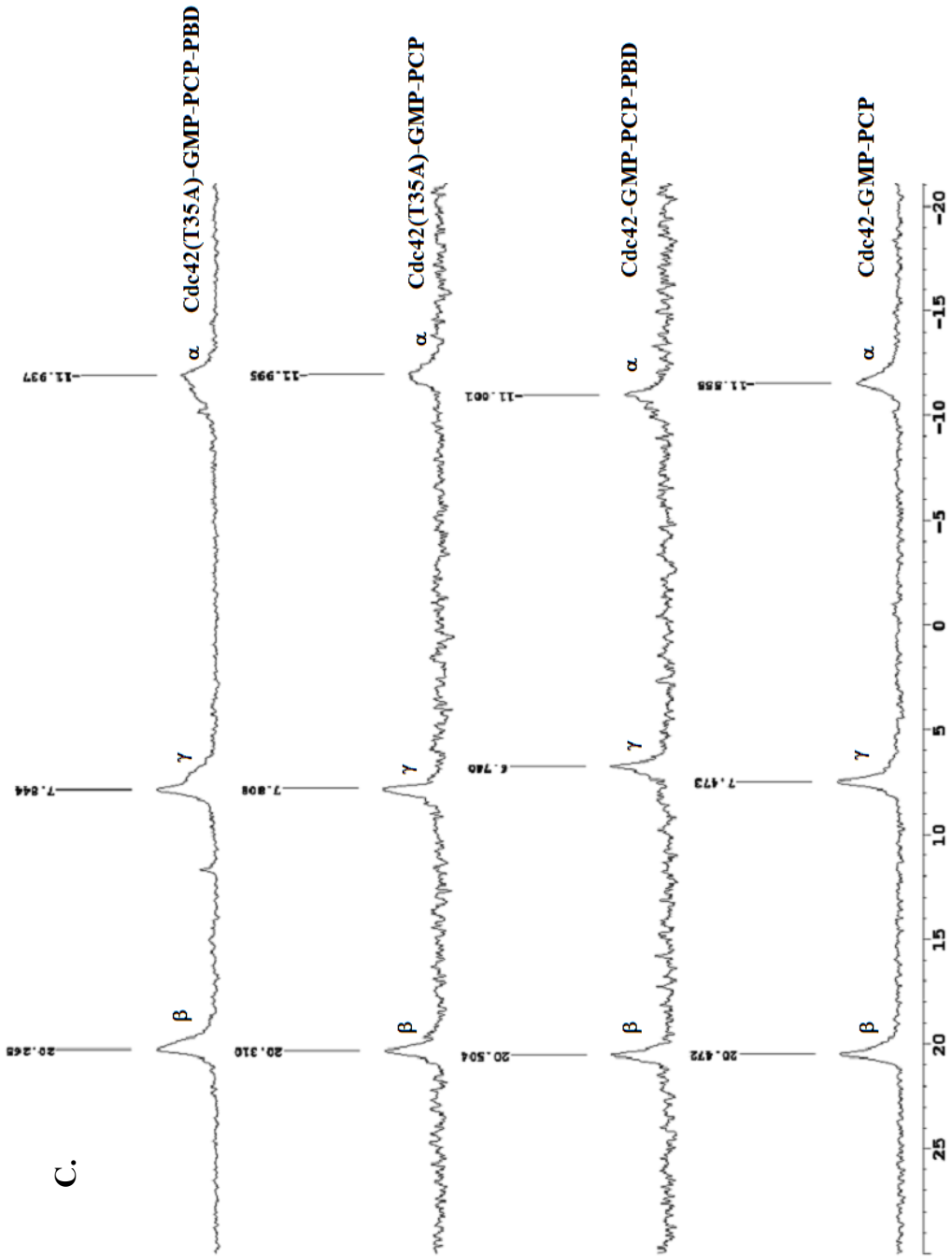
NMR studies of effector-induced changes in GTP-analog-bound forms of Cdc42- ^{31}P-NMR spectroscopy has proven to be very useful in studying the GTP-dependent activation of H-Ras in solution (23,24). In particular, NMR studies led to the suggestion that the Ras-GMP-PNP complex exists in at least two conformational states (denoted as state 1 and state 2), as indicated by a split in the resonances of the β- and γ-phosphates of the GTP-analog (23). More recently, a similar split in the phosphate resonances was detected when NMR analysis was applied to H-Ras bound to GMP-PCP (24). The addition of the limit Ras-binding domain (RBD) from either of two Ras-effectors, namely the Raf kinase or Ral-GDS, then pushed the equilibrium toward state 2. Therefore, state 2 for H-Ras is considered to represent the signaling-active species as seen in the X-ray crystal structures for the GTP-analog-bound forms of the protein. Based on the crystal structure for the signaling-inactive Ras (T35S) – GMP-PNP complex, which lacks electron density for switch I, as well as ^{31}P-NMR experiments for other inactivating mutations within Ras, state 1 is thought to represent an equilibrium of sub-states that are in fast exchange on the NMR-time scale that most likely are more closely related to the structure for GDP-bound Ras than GTP-bound Ras (28,29). Given

these findings with H-Ras, we were interested in performing a similar analysis of Cdc42. As was done with H-Ras, we performed these experiments at 5˚C in order to slow-down any inter-conversion that might occur between different conformational states of Cdc42, so that they could be detected on the NMR time-scale. However, unlike the case for H-Ras, when we analyzed the complexes of Cdc42 bound to either GMP-PNP or GMP-PCP, we detected only a single peak for each phosphate (Figures 2.5A and 2.5B, respectively). These results indicated that the GTP-analog-bound forms of Cdc42 assume only a single detectable conformational state in solution. Upon the addition of an equivalent molar amount of the PBD, conformational transitions were then detected in both the Cdc42-GMP-PNP (Figure 2.5A) and Cdc42-GMP-PCP (Figure 2.5B) complexes as indicated by shifts in the resonances for the γ-phosphate and to lesser extents for the β- and α-phosphates.

^{31}P-NMR studies of the H-Ras Switch I mutant, H-Ras(T35A), suggested that this mutant resides exclusively in the signaling-defective state 1, even after the addition of an effector protein (24,28,29). It seemed likely that the same would be true for Cdc42, given that the yeast Cdc42(T35A) mutant was shown to be unable to sustain cell proliferation, polarization or budding, and to be defective in binding to all known yeast Cdc42-effectors (30), and so we examined the phosphate resonances for the human Cdc42(T35A) mutant, in the presence and absence of the PBD. Figure 2.5C shows that the resonances for the Cdc42(T35A)-GMP-PCP complex were slightly shifted when compared to those for GMP-PCP bound to wild-type Cdc42, suggesting that these two Cdc42 species may start-off in very similar, albeit subtly different, conformational states (Figure 2.5C). However, importantly, the addition of the PBD did not cause a clearly detectable change in the positions of the peaks for the phosphate resonances for the GMP-PCP-bound Cdc42(T35A) mutant but only an overall broadening of the lines, whereas in the same experiment, the addition of the PBD caused obvious changes in the peak positions for the phosphate resonances for the GMP-PCP-bound wild-type Cdc42.

FIGURE 2.5 A-C ^{31}P-NMR reveals that Cdc42 exists only in one conformation in solution but assumes another upon addition of an effector. *A,* Cdc42-GMP-PNP (0.75 mM) with (top) or without (bottom) Pak3-PBD (0.75 mM) at 5˚C. *B,* Cdc42-GMP-PCP (1 mM) with (top) or without (bottom) Pak3-PBD (1 mM) at 5˚C. A chemical shift of 1 ppm is seen for the γ-phosphate upon addition of the effector. Peaks are not split in any of the experiments implying Cdc42 has only one conformational state before binding the effector. *C,* Comparison between 1 mM Cdc42(T35A)-GMP-PCP (top) and 1 mM Cdc42-GMP-PCP (bottom) with or without 1 mM PBD.

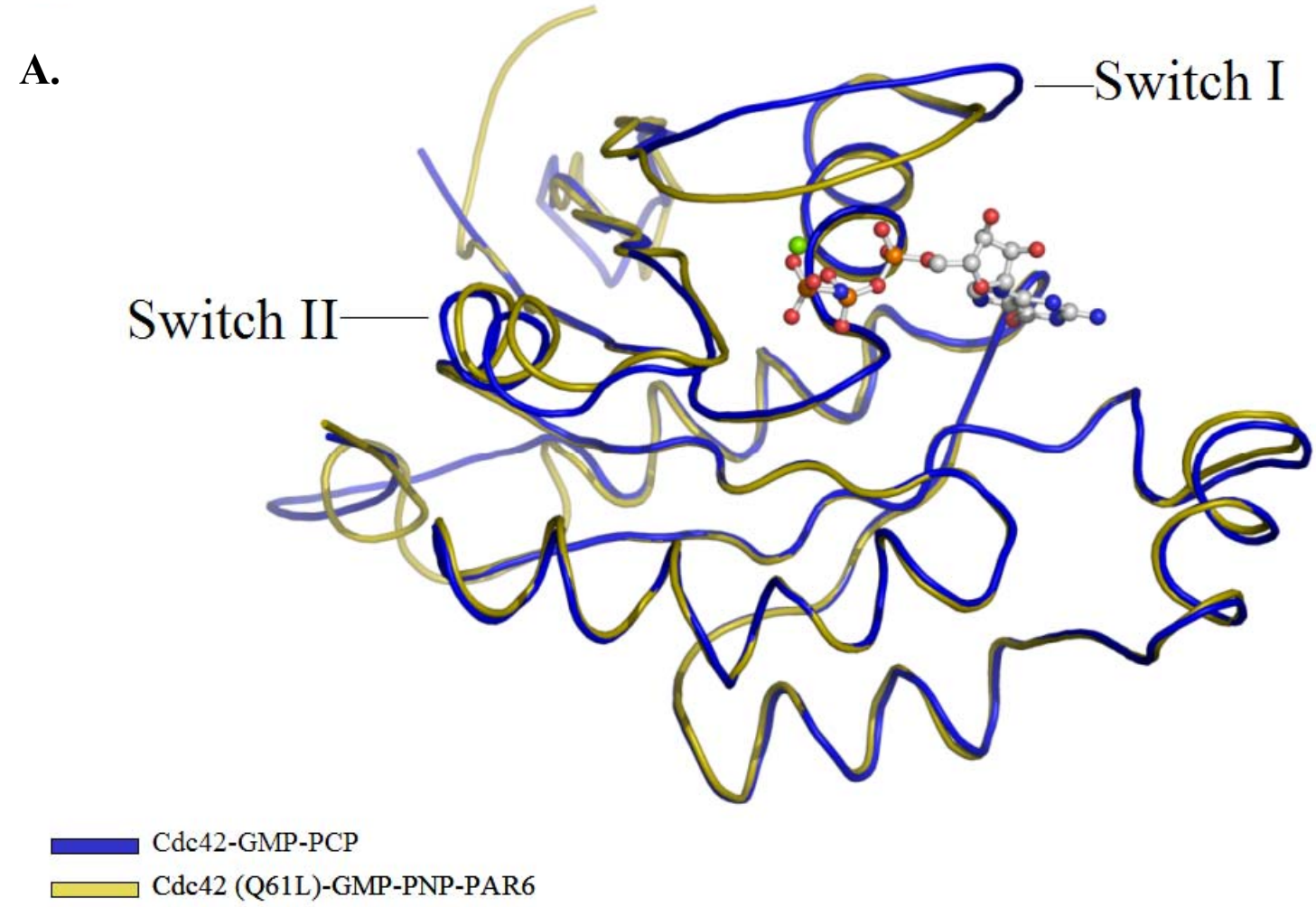

A.
Cdc42-GMP-PNP + PBD
-0.656
-4.096
-11.275
β
γ
α
ppm
Cdc42-GMP-PNP
-0.024
-3.070
-10.966
β
γ
α
ppm
B.
Cdc42-GMP-PCP + PBD
-20.374
-6.382
-11.380
β
γ
α
ppm
Cdc42-GMP-PCP
-20.158
-7.350
-11.769
β
γ
α
ppm


C.
Cdc42(T35A)-GMP-PCP-PBD
Cdc42(T35A)-GMP-PCP
Cdc42-GMP-PCP-PBD
Cdc42-GMP-PCP
α
β
γ
-11.937
7.844
-20.268
-11.995
7.808
-20.310
-11.061
6.740
-20.504
-11.858
7.473
-20.472
25
20
15
10
5
0
-5
-10
-15
-20

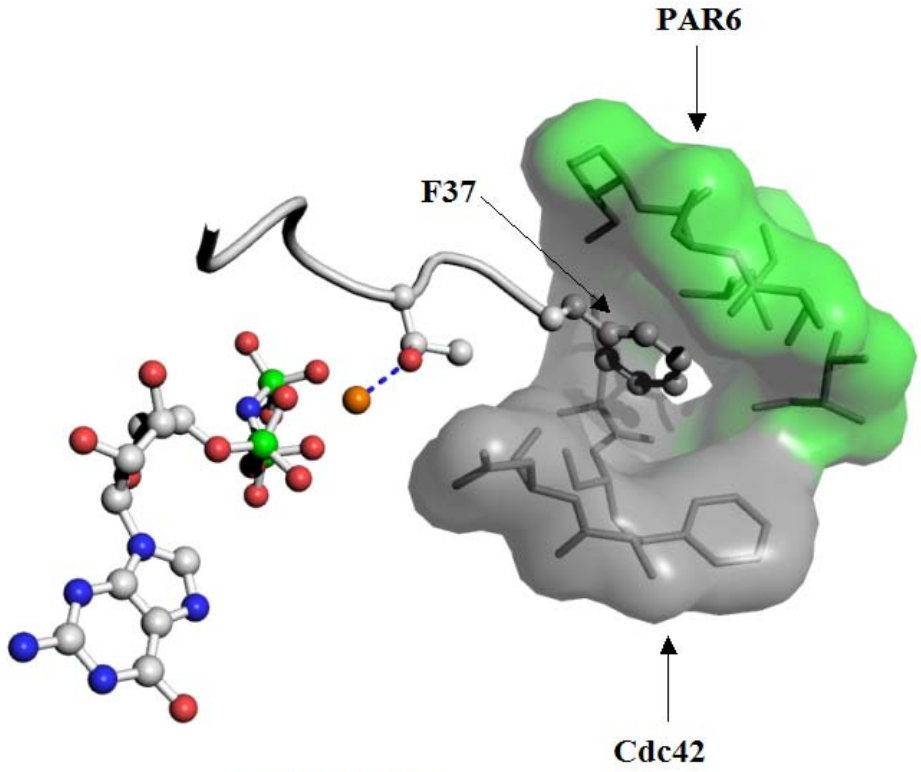
These findings suggest that the GMP-PCP-bound Cdc42(T35A) mutant has a weaker affinity for the PBD due to the loss of stabilizing interactions from Thr35 to the Mg^{2+}.

Insights into the mechanism by which activated Cdc42 propagates signals to its downstream effector proteins- Based on the results from our fluorescence and NMR studies, it would appear that effector proteins play a major role in selectively inducing and/or stabilizing the signaling-active conformational states of Cdc42. Various lines of evidence also indicate that both the signaling-inactive (GDP-bound) and signaling-active (GMP-PCP-bound) forms of Cdc42 have Switch regions that are highly mobile and likely encompass very similar, or at least overlapping, conformational equilibria (25,26). This then raises a key question, namely, how are effectors able to distinguish between the GDP- and GTP-analog-bound forms of Cdc42, such that they interact with the latter Cdc42 species with high affinity? Given the plasticity of Switch I, it is possible that key residues such as Val36 or Phe37 may be oriented in such a way as to be recognizable to effectors, when Cdc42 is bound to either GDP or GTP-analogs. However, presumably the effector would then only be able to engage the GTP-analog-bound form of Cdc42 in a manner that results in a high affinity, signaling-competent interaction. Careful comparisons of the structure for Cdc42-GMP-PCP with those for different Cdc42-effector complexes (16,17,31,32) suggest how this might occur. An example is shown in Figure 2.6A which compares the overall topology for the Cdc42-GMP-PCP complex with that determined from the X-ray structure for the complex between GMP-PNP-bound Cdc42(Q61L) and the effector protein Par6. Although the two structures show little differences in Switch II, they highlight an obvious change in the Switch I effector loop. These differences, when considered together with the available X-ray and NMR structures for other complexes between Cdc42 and effector proteins, suggest the following sequence of events for how effectors are able to selectively bind with high affinity to the GTP-bound state of Cdc42. The first step involves the formation of a small hydrophobic pocket that buries either Switch I residue Phe37 or Val36, or both. Figure 2.6B depicts this step for Par6, while Figure 2.6C shows another example for one

FIGURE 2.6 A-D Effector proteins stabilize Switch I in an "active" conformation. *A*, Comparisons of the overall fold of GMP-PCP-bound Cdc42 versus GMP-PNP-bound Cdc42(Q61L) complexed to Par6 (PDB ID 1NF3). *B,* Switch I residues Thr35 and Phe37 for Cdc42(Q61L)-GMP-PNP bound to the non-conventional Cdc42/Rac-interactive-binding domain of Par6 are shown in grey. Par6 residues (in green) form the lid to a hydrophobic pocket for Phe37, with the bottom of the pocket being contributed by residues from Cdc42 (grey). Phe37 acts as a fulcrum to flip Thr35 into position to coordinate the Mg^{2+}. An overlay of Thr35 and Phe37 from Cdc42-GMP-PCP is displayed in blue to illustrate the lever action and stabilization of Phe37 by Pak. *C,* The same view of Cdc42-GMP-PNP bound to the CRIB domain of Pak1 based on the NMR structure for this complex (PDB ID 1E0A). Phe37 is embedded in a hydrophobic pocket created by both Pak1 (green) and Cdc42(Q61L) (grey). *D,* Overlay of Switch I from the NMR structure for the Cdc42-GMP-PNP-Pak1 complex (PDB ID 1E0A) and the X-ray structure for the Cdc42-GMP-PCP complex. Notice that in the Pak1 complex, Switch I residues from Cdc42 are rotated 180° from their position in the Cdc42-GMP-PCP structure and are stabilized by the interaction of Phe37 with the effector protein.

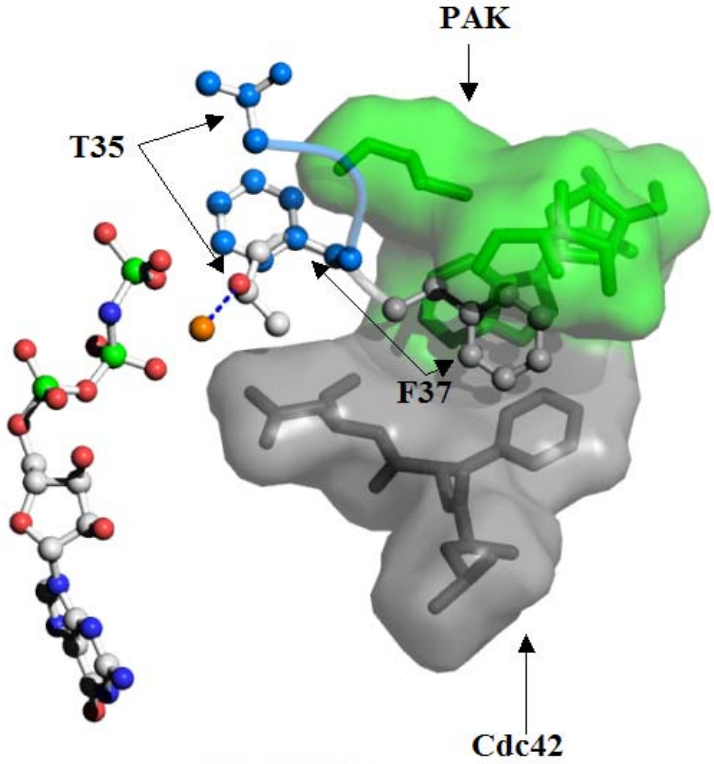

A.
Switch I
Switch II
Cdc42-GMP-PCP
Cdc42 (Q61L)-GMP-PNP-PAR6

B.



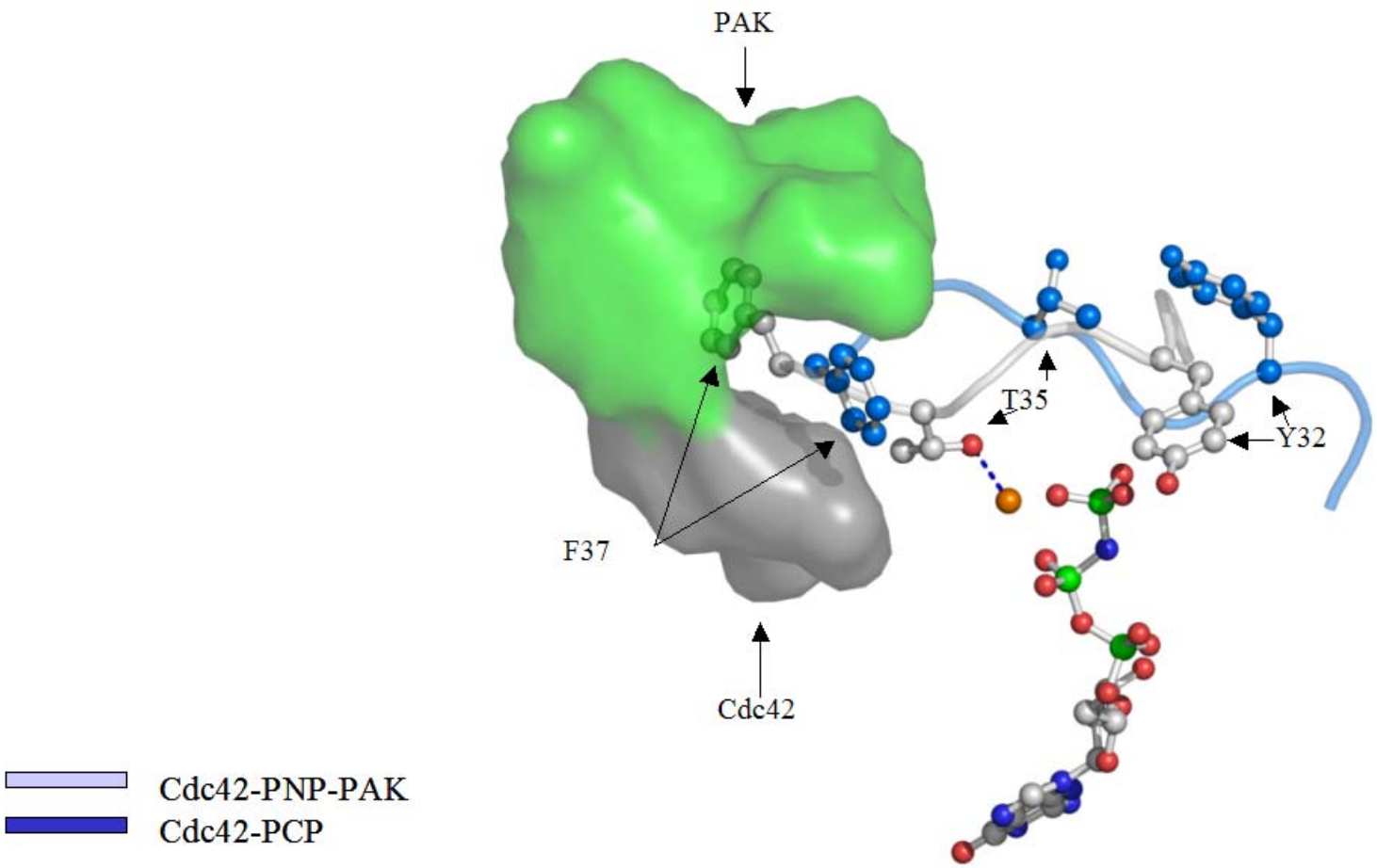
Cdc42-PAR6

C.



Cdc42-PAK

D.



of the better-known effectors for Cdc42, Pak1 (p21-activated kinase-1). In both of these cases, residues from the effector proteins (shown in green) contribute to the lid of the binding-pocket, while residues from Switch II of Cdc42 (shown in gray) form the bottom of the pocket. The potential role for the hydrophobic pocket is to stabilize Switch I residues, and in particular, to enable Phe37 to act as a lever to flip Switch I, effectively reversing the orientations of key residues when compared with the signaling-inactive (GDP-bound) forms of the protein. This rearrangement, which is depicted in Figure 2.6D for the case of Cdc42 binding to Pak1, allows Tyr32 as well as Thr35 to coordinate the γ-phosphate and Mg^{2+} ion, respectively, and to lock the signaling-active conformation in place. When the nucleotide in the binding pocket is GDP (instead of GTP), the absence of the γ-phosphate would not allow these stabilizing interactions with Switch I residues, and as a result, the effector is not able to bind with high affinity. Likewise, the Cdc42(T35A) mutant, which lacks the critical Switch I threonine residue, is defective in its ability to assume a stable activated conformational state in the presence of effector proteins (Figure 2.5C).

It should be noted that this pattern of recognition of the GTP-bound state of Cdc42 is also used by the Cdc42-GAP (33). In this case, residues from the GAP together with Switch II form a pocket that buries Phe37, with a network of interactions involving Arg305 from the GAP (i.e. the 'arginine finger') linking Switch I and Switch II residues with the γ-phosphate.

DISCUSSION

Structural studies performed on H-Ras and various members of the large heterotrimeric G-protein family demonstrated that there were two conserved regions (Switch I and Switch II) that changed conformation upon GDP-GTP exchange (11-14). These conformational “hot-spots” have been assumed to represent the basis by which

G-proteins act as molecular switches in cellular signaling pathways, by binding and regulating the activities of their biological effectors. GMP-PCP-bound Cdc42 is able to recognize effector proteins similar to other signaling-active forms of Cdc42 that contain bound GTP or other GTP-analogs, and in a manner distinct from the signaling-inactive GDP-bound Cdc42. Thus, it was surprising when we determined a high-resolution X-ray crystal structure for Cdc42 bound to the GTP-analog GMP-PCP and found that it was virtually identical, including its Switch I and Switch II regions, to the corresponding structures for signaling-inactive, GDP-bound forms of the protein [i.e. both wild-type Cdc42-GDP and Cdc42(G12V)-GDP].

Studies performed in solution further supported the conclusions that we reached from an analysis of the X-ray structures. We introduced a tryptophan residue at position 32 within Switch I, in order to use its intrinsic fluorescence properties as a conformational monitor for changes that occur within this region of Cdc42 following GDP-GTP exchange, as well as upon the ensuing binding of an effector protein. In these studies, we saw little if any change in the Switch I conformation of Cdc42 when comparing the GDP- and GMP-PCP-bound states of the protein (i.e. on the order of at most 5-10%). Here again this differed from the case for H-Ras, as we and others have been able to detect more significant differences in the Switch I conformation (i.e. $\geq$ 30%) when using a similar fluorescence read-out to compare the GDP- and GTP-analog-bound states of the protein. We then showed by using ^{31}P-NMR spectroscopy that unlike the case for H-Ras, where two distinct states were detected for GTP-analog-bound forms of the G-protein (24,28,34), with one of these apparently representing the signaling-active state, both the GMP-PNP- and GMP-PCP-bound forms of Cdc42 exhibited only a single (non-activated) conformational state.

Despite the structural similarities between the GDP- and GMP-PCP-bound forms of Cdc42, effector proteins are able to distinguish between these two species. When the limit-binding domain of Pak3 was added to the GMP-PCP-bound form of the

Cdc42(Y32W, W97H) protein, a clear change in the fluorescence emission from the Switch I tryptophan residue was observed, whereas, the effector protein had no effect on the fluorescence emission of the GDP-bound Cdc42(Y32W, W97H) mutant. The addition of the effector protein also caused shifts in the phosphate resonances for the GMP-PCP- and GMP-PNP-bound forms of Cdc42, as read-out by NMR spectroscopy. The latter findings are consistent with various other lines of evidence from NMR studies. The NMR-derived structures for different activated forms of Cdc42 bound to WASP (Wiscott-Aldrich Syndrome Protein), Pak, and Ack (Activated Cdc42-associated kinase), showed no resonances for either the Switch I or Switch II regions of Cdc42, prior to the addition of the effector protein (16,31,32,35). This was attributed to the dynamic nature of these regions. However, upon the addition of a Cdc42-effector protein, the resonances for these regions then became clearly defined (16,31,32,35). Additionally, Oswald and colleagues demonstrated in NMR studies that Switch I and Switch II contained significant flexibility and exhibited rapid conformational exchange in both the GDP- and GMP-PCP-bound forms of Cdc42, and that this flexibility was reduced in the Cdc42-GMP-PCP species upon the binding of an effector (26).

Taken together, these findings highlight the strong influence exerted by effector proteins on Cdc42 and its ability to assume signaling-active conformational states. However, the role of effector proteins in influencing such conformational transitions is not restricted to Cdc42, as fluorescence and NMR studies have shown that effectors also affect the signaling-active states of H-Ras (24,28,29,36-38). As alluded to above, the results from NMR experiments showed that upon binding GTP-analogs, H-Ras can exist in two stable conformational states. The majority of the GTP-analog-bound H-Ras population appeared to assume what was felt to be a signaling-active conformational state (state 2), with the remainder of the H-Ras molecules being in a signaling-inactive conformation (state 1). The binding of the limit-functional domain from a Ras-effector (e.g. the Raf serine/threonine kinase) then promoted and/or stabilized the signaling-

active state 2 conformation, such that the total H-Ras population assumed this state in the presence of the effector (24). Interestingly, the NMR results reported for M-Ras were similar to what we have seen for Cdc42; specifically, GMP-PNP-bound M-Ras showed only a single stable conformational state as detected by ^{31}P-NMR, which was then altered upon the addition of the effector protein, Raf (36). Still, the X-ray crystal structures for the GDP- and GMP-PNP-bound forms of M-Ras showed changes in Switch I. The authors concluded that upon binding GTP-analogs, essentially the entire pool of M-Ras adopts the signaling-inactive state-1 conformation originally described for H-Ras, and that the binding of effector proteins then drives the GTP-analog-bound forms of M-Ras entirely to the signaling-active state-2 conformation. What distinguishes the findings that we report here for Cdc42 is that both the state-1 and state-2 conformations for the GTP-analog-bound Ras proteins can be structurally distinguished from GDP-bound Ras, whereas we are able to detect little if any structural differences between the GDP- and GMP-PCP-bound forms of Cdc42. Nonetheless, effector proteins are able to distinguish between these two forms of Cdc42.

An important question concerns whether the apparent strong reliance exhibited by Cdc42 for effector-induced conformational changes occurs in cells with the physiologically relevant, activating nucleotide GTP. In fact, it has been shown that the equilibrium constants for the inter-conversion between the inactive- and active-states of H-Ras, as measured by ^{31}P-NMR (i.e. states 1 and 2, respectively), are lower for H-Ras bound to GMP-PCP or GMP-PNP when compared to GTP (24). It was postulated that the increased amount of GTP-analog-bound H-Ras molecules that are in the signaling-inactive state 1 conformation, compared to when H-Ras is bound to GTP, might be due to a loss of an important interaction between the P-loop and the bridging oxygen from GTP, or the result of the increased pKa on the γ- and β-phosphate groups in the non-hydrolyzable analogs. The latter could have an effect on the ability to form hydrogen bonds to key residues on Switch I. We have tried to use fluorescence spectroscopy to

see whether Cdc42 might behave differently when bound to GTP, compared to the non-hydrolyzable analogs GMP-PCP or GMP-PNP. Thus far, these experiments have been inconclusive. Similar to what we have seen with the non-hydrolyzable GTP analogs, we have been unable to detect a significant difference in the fluorescence emission from Trp32 between the GDP-bound and GTP-bound Cdc42(Y32W, W97H) species. However, as yet we have not been able to observe reliable differences in the Trp32 fluorescence upon the addition of the limit-binding domain of Pak3. At least part of the challenge in making interpretations from these experiments comes from the fact that Cdc42 shows a significantly higher intrinsic GTP hydrolytic activity compared to H-Ras (39), which may obscure differences, such as those induced by effector proteins. Still, the fact that effectors have a clear and substantial influence on the final conformational state that Cdc42 is able to assume in the presence of non-hydrolyzable GTP-analogs that are signaling-competent, leads us to believe that effector proteins will play a similarly important role for GTP-bound Cdc42 in the cell.

All of this leads to yet another interesting question, namely how do effector proteins select between the GDP- and GTP-bound forms of Cdc42, so as to ensure that signals are only transmitted when this G-protein is in the GTP-bound state? We obtained some clues toward answering this question when considering the available structures for GTP-analog-bound forms of Cdc42 in complexes with the limit domains of different effector proteins. For each of these cases, the effector provides a binding pocket for Phe37 of Switch I of Cdc42, which then leads to an interaction between Thr35 from Switch I and the γ-phosphate of the GTP-analog. In solution, the Switch I loop of Cdc42 is highly mobile and is likely able to interconvert between different conformations representing local minima on the energy landscape. Some of these minima may represent signaling conformations that can be reached by both the GDP- and GMP-PCP-bound proteins and enable the proper presentation of Phe37 to be "captured" by the effector protein. Once this occurs, it creates a domino effect whereby

important residues such as Thr35 as well as Tyr32 are brought into close contact with the nucleotide. The net outcome is that Cdc42 is locked into a complex with the effector, but only when it contains the γ-phosphate of GTP.

The role played by Phe37 in forming a high affinity complex with effectors probably explains our earlier observations that this residue is essential for Cdc42-coupled signaling events linked to cell growth and cellular transformation (40). Also in support of this model, we showed in NMR studies that the GMP-PCP-bound Cdc42(T35A) mutant was not able to respond to effectors in the same way as the wild-type Cdc42-GMP-PCP complex. Specifically, the addition of the PBD to GMP-PCP-bound Cdc42(T35A) did not cause distinct shifts in the phosphate resonances, unlike the case for the wild-type Cdc42-GMP-PCP species, but did cause some line broadening. This suggests that the effector is able to interact with the Cdc42(T35A)-GMP-PCP complex but with a much weaker affinity compared to GMP-PCP-bound wild-type Cdc42. Therefore, in the absence of the critical Switch I Thr35 residue, the GMP-PCP-bound Cdc42 mutant was unable to be locked into a high affinity, signaling-active conformational state by the effector. The fact that we observed slightly different peak positions for the phosphate resonances from the Cdc42(T35A)-GMP-PCP complex versus the Cdc42-GMP-PCP complex would suggest that these two Cdc42 species do not share identical conformational profiles, even though they both exist primarily in signaling-inactive states. What nonetheless seems clear from comparisons of the X-ray structures for the GDP- and GMP-PCP-bound forms of Cdc42, as well as from fluorescence and NMR studies, is that the spectrum of conformational states for these different nucleotide states of Cdc42 must overlap and that the Switch conformations for the wild-type Cdc42-GMP-PCP complex and the GDP-bound Cdc42 species are more closely related than those for GMP-PCP-bound Cdc42 before and after the binding of effectors.

The results that we have obtained regarding the conformational status of the Switch regions of Cdc42 in response to GDP-GTP exchange, versus the changes induced by biological effectors, are consistent with the biochemical and structural analyses of the interactions of Cdc42 with RhoGDI. Both the GDP- and GTP-bound states of Cdc42 are regulated by RhoGDI, such that the GDI significantly slows their rates of GDP dissociation and GTP hydrolysis (41,42). Fluorescence measurements have shown that RhoGDI binds to the GDP- and GTP-bound forms of Cdc42 with identical affinities (43). This can be explained by the fact that Switch II looks identical when comparing the X-ray crystal structure for the Cdc42-GDP-RhoGDI complex (44) with that for GMP-PCP-bound Cdc42. Switch II represents the initial site of contact for RhoGDI and so the lack of any detectable change in this region when comparing GDP- and GTP-bound forms of Cdc42 would be consistent with the inability of the GDI to distinguish between these different states of the G-protein. What is especially interesting is that Switch II also shows little or no change when comparing the GDP- and GTP-bound forms of Cdc42 with any of the reported X-ray crystal structures for Cdc42 complexed to effector proteins.

Given these findings, it becomes interesting to consider whether G-proteins related to Cdc42 might show a similar behavior with regard to their Switch domains, and thus rely heavily on effector proteins to induce the necessary changes to ensure signal propagation. One such related G-protein is Rac1, which is 68% identical to Cdc42 and shares some of the same effector proteins. In the case of the X-ray crystal structure for GMP-PNP-bound Rac1, Switch I residues Tyr32 through Val36 exhibited poor electron density and consequently this region was modeled in the completed structure by using data from the related *Dictyostelium discoideum* protein Rac1a (45). This makes it difficult to know with certainty whether Switch I changes in the X-ray crystal structures for the GTP-analog- and GDP-bound forms of Rac1. However, it is interesting that the X-ray structures for the GDP- and GMP-PNP-bound forms of Rac1, when complexed to

the putative Rac-effector Arfaptin, are nearly identical (46). Moreover, Thr35 from Switch I does not participate in coordinating the Mg^{2+} ion in either the GDP- or GMP-PNP-bound Rac1-Arfaptin structures, which is in contrast to what is typically seen in the structures of all small G-proteins when they are in their signaling-active states. These results would seem to suggest that GDP-GTP exchange on Rac1 is not sufficient to induce significant conformational changes within the Switch I region of the protein.

The X-ray crystal structures for the GDP- and GTP-analog-bound forms of RhoA show that changes in the Switch I loop do occur within this G-protein as an outcome of GDP-GTP exchange (47). On the other hand, our recent studies with RhoC suggest that it can exist in multiple activated-states, and that the effector protein again influences the final conformational states that are reached and necessary for signal propagation (48).

Thus, it is becoming increasingly clear that a spectrum of possibilities exist regarding how G- proteins reach the conformational states that are necessary for signal propagation. At one end of the spectrum are the α subunits of large G-proteins (Gα subunits), for which the conformational changes necessary for signal propagation may be entirely driven by GDP-GTP exchange. Indeed, the ability of Gα subunits to undergo such conformational transitions is necessary for their signaling function, as the GDP-bound Gα subunits are tightly associated with their partner Gβγ complexes and movements in Switch II that accompany GDP-GTP exchange are thought to be necessary to reduce the affinity of Gβγ and enable the GTP-bound Gα subunit to engage its downstream signaling effector. For the case of H-Ras, GDP-GTP exchange may be sufficient to induce differences in Switch I and Switch II within a significant population of the G-protein molecules that can be recognized by many of their effector proteins. A number of these effectors (e.g. Raf, PI 3-kinase, Ral-GDS) appear to use a very similar mode of binding and so each might even be able to recognize the same GTP-induced conformational state within the G-protein (49). Still, the principle site for effector-binding in Ras (Switch I) is a highly flexible region, given that two conformational

states have been detected for GTP-analog-bound forms of H-Ras from NMR studies, such that the binding of effectors can induce and/or stabilize one of these states and thereby ensure that the vast majority of GTP-analog-bound H-Ras molecules are in a signaling-active conformation (24).

Cdc42 may represent an example of a G-protein that is at the other end of the spectrum, as its GTP-bound state appears to be strongly receptive to effector-induced conformational changes. It may be especially important that the effector-binding site on Cdc42 shows significant plasticity, given that the GTP-bound form of this protein engages a number of different cellular effectors. Moreover, Cdc42 needs to move between distinct cellular compartments to activate specific effector proteins (e.g. between the plasma membrane and Golgi), and this movement may be mediated by RhoGDI, which, as alluded to above, binds to both the signaling-inactive and signaling-active forms of the G-protein (3). Thus, the ability of individual effector proteins to mold Cdc42 into a favorable conformational state for different signaling events, would be highly advantageous by providing maximum flexibility for a G-protein that needs to switch on a number of effector activities located at different sites within the cell.

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Chapter 3

THE CONSTITUTIVELY ACTIVATED MUTANT CDC42 (Q61L) DOES NOT REQUIRE AN EFFECTOR PROTEIN TO INDUCE THE FULLY ACTIVATED CONFORMATION

Introduction

G proteins are one of the most important families of proteins in the human genome. They are often referred to as molecular switches, turning specific cellular signaling pathways on and/or off at the appropriate time. G proteins are involved in nearly every major cellular process including, but not limited to, cellular growth, differentiation, trafficking, and apoptosis. G proteins are activated by binding cellular GTP and deactivated by hydrolyzing GTP to GDP and P_i. Once bound to GTP, the G protein is able to interact with downstream effectors which usually starts a signaling cascade whose overall outcome is determined by the pathway. As might be expected, G proteins themselves are highly regulated, both temporally and spatially, by GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins). Deregulation of many G proteins either by mutation or gene expression, can lead to varied disease states including cancer. In fact, one of the first known oncogenes discovered was the small G protein Ras (1,2).

Ras became the subject of intense research once its role in human cancers was established and, as a result, it was one of the first G protein structures to be solved by x-ray crystallography (3). Since that time, numerous G protein structures have been solved from both the large and small G protein families, providing us with invaluable mechanistic information. One of the mechanistic paradigms that came from this work is the structural rearrangement of two "switch" regions named switch I and II (loops 2

and 4 respectively in Ras nomenclature) upon activation by GTP (4). These structural rearrangements were later proven to be present in all G protein families for which structures are available. It was assumed that these structural rearrangements were the basis for cell signaling as the G protein would cycle between an effector-recognizable, signaling-competent GTP-bound state and a signaling-defective GDP-bound state. Supporting evidence came in the form of numerous structures for activated G proteins bound to the limit binding-domains of various effectors where the main binding interface was in fact the switch regions.

With this as a backdrop, we previously reported the first known structure of a signaling-active G protein, Cdc42-GMP-PCP, which was virtually identical to its signaling-inactive counterpart, Cdc42-GDP (81). We showed conclusively that this protein was biologically active by its ability to complex with the Cdc42 limit binding-domain (CBD) of its effector Pak3 *in vitro*. These findings were surprising and led us to the natural question of how targets for Cdc42 are able to distinguish between the GTP-bound and GDP-bound forms of the protein. One seemingly plausible explanation was the theory of induced-fit first proposed for the action of enzymes in the late 1950s (5). Simply stated, the theory asserts that a substrate can induce conformational rearrangements in its binding partner that drives the final structure of the enzyme-substrate complex toward a fully catalytic state. It seems likely that this is also the case for Cdc42 where in the absence of a target/effector protein, the switch regions in both the GDP- (inactive) and GMP-PCP-bound (active) forms assume similar, if not identical, conformations. Under such conditions, Cdc42 can be thought of as existing in an "open" state denoted by the absence of contacts between the γ-phosphate of GTP and key switch I residues, specifically Tyr32 and Thr35. After the addition of an effector protein, a conformational rearrangement is induced and/or stabilized which is characterized by switch I assuming a "closed" state as seen in the

x-ray structure for Cdc42 complexed with the Cdc42 binding-domain from Par6 (6). In this structure, as well as a number of NMR structures of Cdc42 bound to the limit-binding domains from various effectors, the "closed" state is characterized by interactions between switch I residues Tyr32 and Thr35 with the γ-phosphate and Mg^{2+}, respectively, and is very similar to the structures of activated Ras and other small G proteins (7-9).

Through the use of fluorescence spectroscopy and ^{31}P-NMR, we were able to show that GTP-analog-bound forms of Cdc42 undergo an effector-induced conversion from a GDP-like conformational state to a signaling-active state. In addition, we found that in solution, activated Cdc42 adopts only one discernable conformation which is thought to resemble the "open" GDP-like state. However, upon the addition of an effector protein, the total population of Cdc42 molecules shifted to the activated state as described in the "closed" conformation.

Two of the questions that arose from our structural studies for Cdc42 bound to GMP-PCP were; does an activated mutant of Cdc42 (i.e. Cdc42 (Q61L)), when bound to GMP-PCP, assume a signaling-inactive ("open") or signaling-active ("closed") conformation and what are the effects of the physiologically relevant nucleotide GTP on Cdc42 activation? The second question arises from the fact that there has been evidence in the literature to suggest that different nucleotide analogs may activate G proteins differently depending on the bridging atom between the β-γ-phosphate (10-12). To this end, we provide structural evidence that Cdc42 (Q61L) – GMP-PCP, unlike wild-type, assumes a classic activated switch I architecture where Thr35 and Tyr32 are in close contact to the Mg^{2+} and γ-phosphate respectively. We further show that GTP behaves like its non-hydrolyzable GTP-analogs GMP-PCP and GMP-PNP, since it shows little capability, in the absence of effector proteins, to induce a signaling-active conformational state in Cdc42.

Experimental Procudures

Protein Purification- Cdc42 (Q61L) was expressed in BL-21 bacterial cells from a pET-15b plasmid. IPTG (Isopropyl-β-D-1-thiogalactopyranoside) was added to a final concentration of 1 mM when the cells reached an O.D. of 0.6 at 600 nm and cells were allowed to grow for another 3 hours. Cells were lysed in 1 x HMA (20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 0.1 mM Na-Azide) supplemented with ~2 μM benzamidine, ~20 μM PMSF (phenylmethanesulphonylfluoride), ~50 ng/μl aprotinin and leupeptin, by sonication and cleared lysates were briefly incubated with nickel beads. The nickel beads were washed with 250 ml of 1 x HMA with 20 mM imidazole and 500 mM NaCl and then with 1 x HMA alone. Protein was eluted in 70 ml of 1 x HMA supplemented with 200 mM imidazole. The eluent was concentrated to ~ 30 ml, incubated at 4˚C overnight with thrombin (Haematological Technologies Inc.), and then chromatographed on a 5 ml Hi-Trap Q column (Amersham) eqilibrated with 1 x HMA. The flow-through was collected and concentrated. Nucleotide exchange was carried out by incubating the G protein with a 5-fold molar excess of GMP-PCP in the presence of 200 mM ammonium sulfate and 100 U of alkaline phosphatase beads (Sigma # P0927) for ~4 hours at 4˚C. The flow-through from the reaction was collected and the beads were washed 3 times with 1 x HMA. The eluent was then chromatographed on a G-75 gel filtration column (Amersham) and concentrated to 80 mg/ml. Purified proteins were snap-frozen in liquid nitrogen and stored at – 80˚C.

Crystallization conditions were found by use of a PEG ion screen (Hampton Research) and further pH refinement. Optimal crystallization conditions required 5% PEG 4K, 100 mM citric acid, pH 3.0. Crystals were obtained by the hanging drop

method. Prior to data collection, crystals were soaked in mother liquor containing 20% glycerol and frozen in liquid nitrogen.

Data Collection and Processing- Data was collected with a rotating anode x-ray generator using a Rigaku R-AXIS IV image plate detector with a 0.5˚ oscillation step, and a 15 minute exposure time. Data was processed using d*Trek (13). Rotation and translation searches were performed using CNS as well as structure refinement (14). Cdc42 (1ANO) with the GDP removed was used as the search model and correct phases were assessed by the presence of clear electron density for the GMP-PCP moiety. The final structure was validated with Procheck (15). The final statistics are presented in Table 2.

^{31}P-NMR- Samples were prepared by dissolving Cdc42 (1 mM) pre-exchanged with GMP-PNP or GMP-PCP, either with or without the limit Cdc42-binding domain from PAK3 (PBD) (1 mM), in HMA-containing 10% D_2O. ^{31}P spectra were obtained at 5˚C on a Varian INOVA spectrometer operating at 202.37 MHz using a 5 mm Varian DBG probehead. ^{1}H broadband decoupling was applied throughout the acquisition. A relaxation delay of 8 seconds was used between scans, with 3000 - 4000 scans summed prior to analysis. Data was zero-filled to 128k points and an exponential multiplication (5 - 10 Hz) was performed prior to Fourier Transform. Spectra were referenced externally to 85% phosphoric acid (0 ppm).

Results

Structure of the Cdc42 (Q61L) – GMP-PCP complex

The x-ray crystal structure for the Cdc42 (Q61L) – GMP-PCP complex conforms to the same structural fold as that exhibited by GMP-PCP-bound, wild-type Cdc42 (16) as well as those for other Rho family members in general. It is composed of 6

TABLE 2

a $R_{sym} = \Sigma_{hkl} \Sigma_i[I_i(hkl) - \langle I(hkl) \rangle]/\Sigma_{hkl}\Sigma_i(hkl)$

b R_{free} and $R_{crys} = \Sigma|F_{obs} - F_{calc}|/ \Sigma F_{obs}$

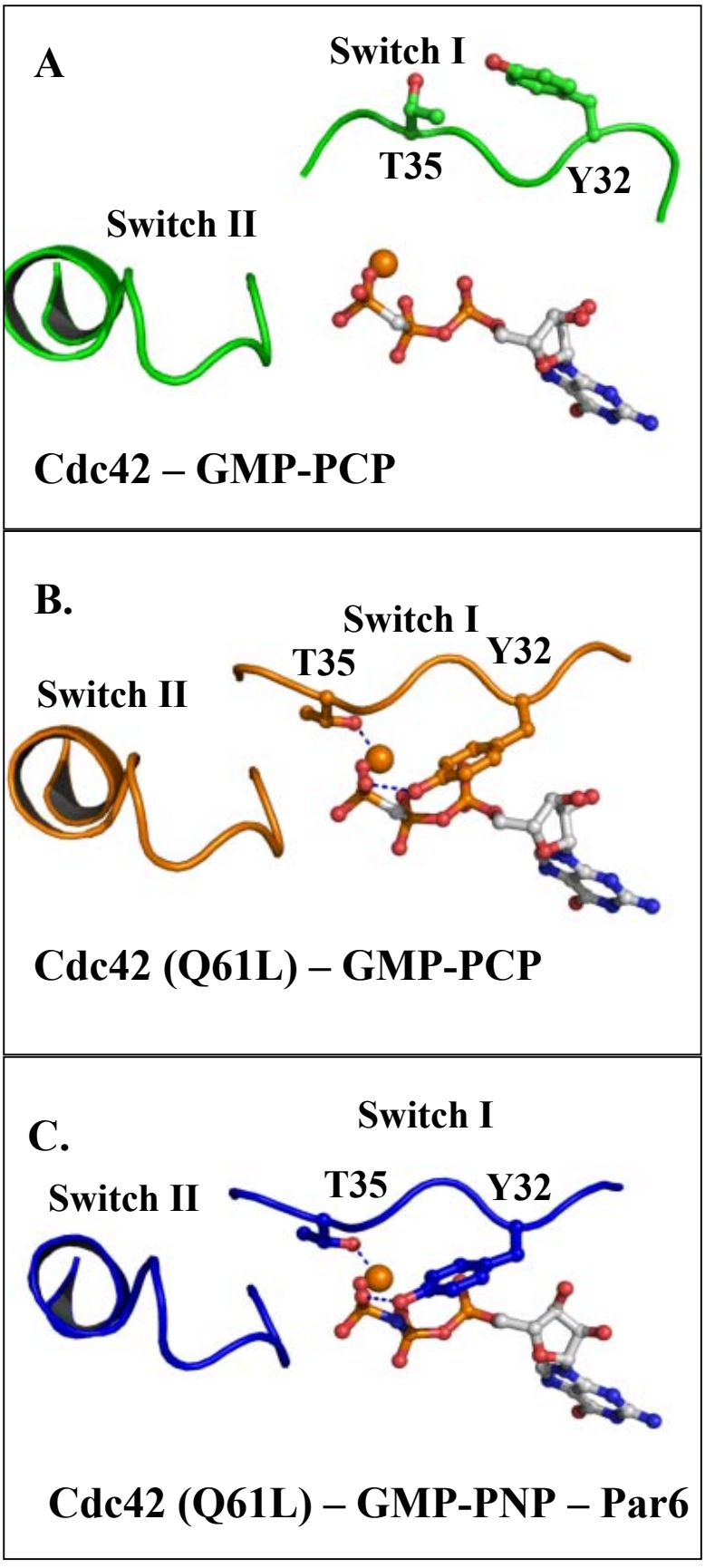
Data Statistics	
Resolution Range (Å)	51 - 2.0
R_{sym} [a], % overall (last shell)	4.4 (26)
Completeness (%), overall (last shell)	98.4 (87.8)
Multiplicity, overall (last shell)	6.12 (4.2)
Total no. of refections	363873
No. of unique reflections	59471
I/sigI, overall (last shell)	17.1 (3.8)
Refinement Statistics	
R_{free} [b], (%), overall (highest shell)	22 (48)
R_{cryst} [b] (%), overall (highest shell)	21 (45)
Ramachandran Plot	
Residues in most favored (%)	93.5
Residues in allowed regions (%)	6.2
Residues in generously allowed regions (%)	0.3

β-sheets arranged in a β-sandwich motif surrounded by 6 α-helices and a small 3/10 helix just before the Rho - insert region (residues 120-132). The guanine-nucleotide is bound to Cdc42 (Q61L) in a similar fashion to nearly all other G proteins. The tri-phosphate tail is stabilized by an extensive hydrogen bond network to key residues in both switch I and II (residues 30-38 and 60-76 respectively), as well as residues in the P-loop (residues 10-18). Specific to activated forms of most G proteins, the γ-phosphate is hydrogen bonded to the backbone amides from Thr35 as well as Gly60, in addition to making hydrogen bonds to the hydroxyl group from the side-chains of Tyr32, Thr35, as well as the ε-amino group from Lys16. These interactions with switch I residues Tyr32 and Thr35 appear to be most important for stabilization of the activated conformation of the protein. The guanine base is stabilized by contacts with the carboxyl side-chain of Asp118, which is part of the TQID motif (NKXD in Ras), as well as with the backbone amides from Ala159 and Leu160. The guanine ring is further stabilized through π-stacking interactions with Phe28.

Comparison between Cdc42 – GMP-PCP and Cdc42 (Q61L) – GMP-PCP

The x-ray crystal structure for the Cdc42 – GMP-PCP complex revealed a switch I architecture identical to previous structures for the signaling-inactive Cdc42 – GDP complex (1ANO), as well as Cdc42 (G12V) – GDP (1A4R). In comparison with these structures, the Cdc42 (Q61L) – GMP-PCP complex is significantly different in one very important region. Switch I in Cdc42 (Q61L) – GMP-PCP has now rearranged to cover the nucleotide-binding pocket and coordinate to the Mg^{2+} and γ-phosphate in a similar manner to what has been reported for the effector-bound structures for Cdc42 (Fig 3.1A-C). Differences between the Cdc42 – GMP-PCP and Cdc42 (Q61L) – GMP-PCP structures occur between Pro29 and Asn39 encompassing all of switch I. Phenylalanine 37, which is important for the binding of many effectors, is observed to

Figure 3.1 X-ray crystal structures of signaling active Cdc42 complexes. *A*, Switch I and II from the Cdc42 – GMP-PCP complex reveal an inactive GDP-like structure. Threonine 35 and tyrosine 32 are positioned toward the solvent and are unable to interact with the nucleotide. *B*, Switch I and II from the constitutively activated Cdc42 (Q61L) – GMP-PCP x-ray crystal structure reveal an activated structure. Threonine 35 and tyrosine 32 are coordinated to the Mg^{2+} and γ-phosphate respectively, which is a hallmark of G protein activation. *C*, Switch I and II for an activated, effector-bound Cdc42 (Q61L) structure. Comparison of the Switch regions with the Cdc42 (Q61L) – GMP-PCP complex alone (3.1B) reveals a virtually identical architecture.


A
Switch I
T35
Y32
Switch II
Cdc42 – GMP-PCP
B.
Switch I
T35
Y32
Switch II
Cdc42 (Q61L) – GMP-PCP
C.
Switch I
T35
Y32
Switch II
Cdc42 (Q61L) – GMP-PNP – Par6

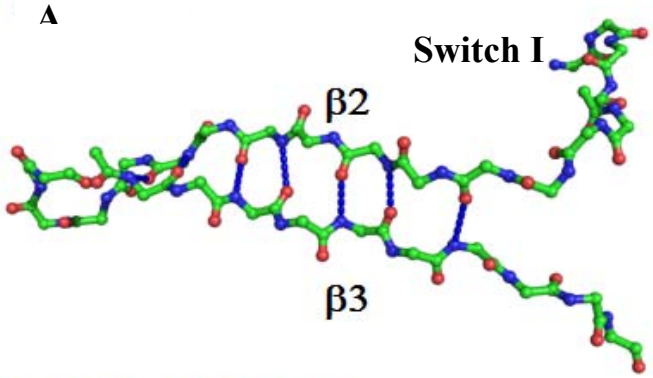
be rearranged so as to now face toward the solvent as opposed to facing toward the nucleotide-binding pocket as is the case for the Cdc42 – GMP-PCP complex. As a consequence of this rearrangement, switch I has shifted its position by not only folding over the guanine nucleotide-binding pocket, but also by translating roughly one residue toward the C-terminal end. This has the effect of extending the intra-molecular β-sheet between β strands 2 and 3 by two hydrogen bonds. Specifically, hydrogen bonds form between the main-chain carbonyl and amide group from Asp38 to the main-chain amide and carbonyl group from Asp57, respectively (Fig 3.2A-C). An additional hydrogen bond is formed between the main-chain carbonyl oxygen from Val36 to the amide nitrogen from Ala59. These hydrogen bonds connect switch I with the highly conserved switch II $D^{57}XXG^{60}$ motif. The net effect of this is to create a larger binding platform for effector proteins. All known structures for Cdc42 complexed with effectors share a common structural binding motif whereby an intermolecular β-sheet is formed between the effector and β2 from Cdc42, which includes the extended area.

Switch II reveals subtle differences between the two structures. A slight shift of ~0.5 Å is observed for the $D^{57}XXG^{60}$ residues that have been implicated in the binding of guanine nucleotides, i.e. Ala59 and Gly60. Gly60 is hydrogen bonded to the γ-phosphate in both structures. As a result of the shift, Gly60 in the Cdc42 (Q61L) – GMP-PCP complex is closer to the γ-phosphate compared to its position in the wild-type complex (i.e. 2.71 Å versus 3.28 Å) and thus has a stronger interaction with the γ-phosphate.

Perhaps the most notable difference between the structures for switch II in the Cdc42 (Q61L) – GMP-PCP and Cdc42 – GMP-PCP complexes resides in the orientation of the side-chain of Leu61 from Cdc42 (Q61L) – GMP-PCP. In the Cdc42 – GMP-PCP complex, Gln61 faces toward the nucleotide and makes a hydrogen bond

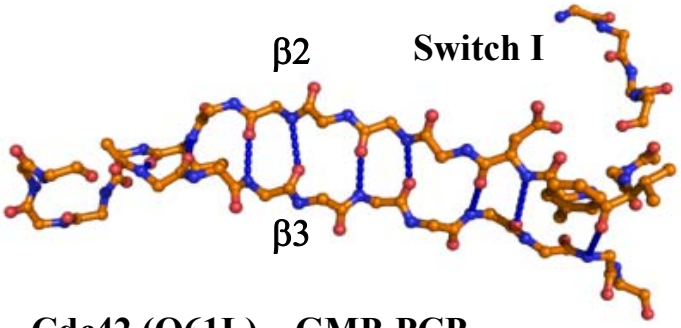
Figure 3.2 A-C Activation of Cdc42 (Q61L) provides a larger binding platform for interactions with effector proteins. The inter-switch region between switch I and II is composed of β-strands 2 and 3 which form a β-sheet. *A*, The signaling-active structure for Cdc42 – GMP-PCP (green) reveals a break in the β-sheet as it approaches switch I. This break is attributable to the lack of switch I interactions with the GTP-analog which does occur in the activated Cdc42 (Q61L) – GMP-PCP structures. *B*, The Cdc42 (Q61L) – GMP-PCP complex reveals a β-sheet which is extended by two hydrogen bonds when compared to the Cdc42 – GMP-PCP complex (A). This extension creates an important binding platform for effector proteins. *C*, X-ray crystal structure for Cdc42 (Q61L) – GMP-PNP bound to the limit binding domain of Par6 (pbd i.d. 1NF3) is an example of how effector proteins bind. All structures for effector proteins bound to Cdc42 reveal an intermolecular β-sheet between the effector protein and Cdc42 in the region of the extended β-sheet.

A



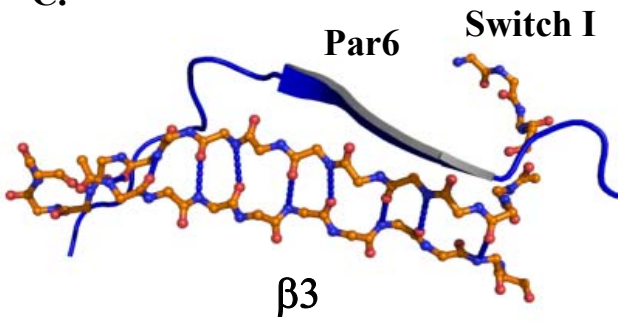
Cdc42 - GMP-PCP

B.



Cdc42 (Q61L) – GMP-PCP

C.



Cdc42 (Q61L) – GMP-PNP – Par6

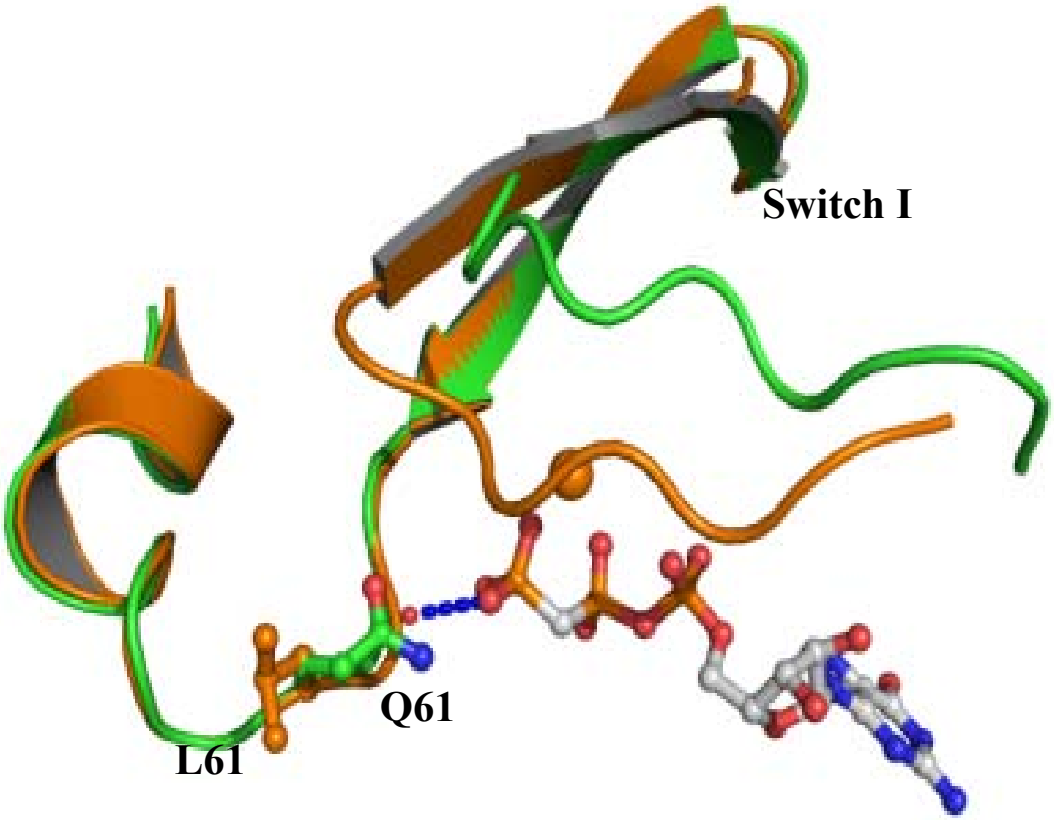
with the γ-phosphate. In contrast, Leu61 in the Cdc42 (Q61L) – GMP-PCP complex faces away from the negatively charged γ-phosphate (Fig. 3.3). However, a water molecule in the Cdc42 (Q61L) – GMP-PCP structure is seen to occupy the same position as the Gln61 side-chain in the wild-type complex and is hydrogen bonded to the γ-phosphate.

The temperature factor for Leu61 in the Cdc42 (Q61L) – GMP-PCP complex is not significantly higher than the temperature factors for neighboring residues and is in fact lower than the average overall temperature factor for the total structure, indicating that this conformation is quite stable. Looking carefully, this stability is imparted by the fact that Leu61 is situated as the first residue of a β-turn and as such, its main-chain carbonyl group is hydrogen bonded to the main-chain amide from Tyr64 as well as to the side-chain guanidinium group from Arg68. In addition, the aliphatic side-chain from Leu61 makes van der Waals contacts with the polar side-chains of other residues within the β-turn and is therefore sandwiched between the highly charged γ-phosphate on one side and the polar side-chains from the other β-turn residues on the other. This point is illustrated by the fact that although residue Leu61 in the Cdc42 (Q61L) – GMP-PCP complex is facing in the opposite direction from Gln61 in the Cdc42 – GMP-PCP structure, the difference between the solvent accessibilities of the two residues is only 5%, (i.e. 22% versus 17%, respectively).

Comparison of the structure of the Cdc42 (Q61L) – GMP-PCP complex with those for activated Cdc42-effector complexes

Comparisons of the rotomeric conformational state of residue 61 between the x-ray crystal structures for Cdc42-effector complexes versus the Cdc42 (Q61L) – GMP-PCP complex, reveals a multitude of conformations that range from Gln61

Figure 3.3 Comparison of Gln61 and Leu61 between Cdc42 – GMP-PCP and Cdc42 (Q61L) – GMP-PCP respectively. Cdc42 – GMP-PCP is depicted in green and Cdc42 (Q61L) – GMP-PCP is depicted in orange. Glutamine 61 is an important residue for GTP hydrolysis. It is thought to function by helping to orient a nucleophillic water molecule for an in-line attack on the γ-phosphate during the hydrolysis reactions transition state. In the Cdc42 – GMP-PCP complex, it is seen to hydrogen bond to the γ-phosphate allowing it to be in a more favorable position for the hydrolysis reaction. Mutation of Gln61 to leucine results in a reorientation of the leucine side-chain away from the nucleotide binding pocket. However, a water molecule (red) from the Cdc42 (Q61L) – GMP-PCP complex is observed to replace the lost hydrogen bond from the Cdc42 – GMP-PCP complex.

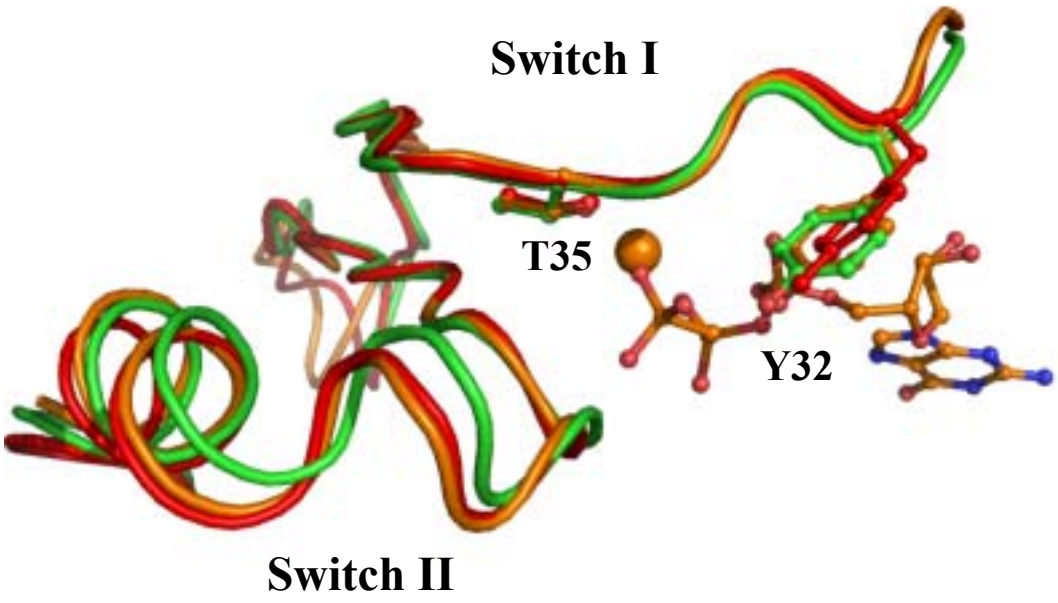

Switch I
Q61
L61

facing towards the γ-phosphate, as seen in the Cdc42 – GMP-PCP complex, to the extreme where the side-chain of Leu61 is facing in the opposite direction in the Cdc42 (Q61L) – GMP-PCP complex. In agreement with the x-ray and NMR structures of activated forms of Cdc42 in complex with different effectors and regulators (6-8,17-19), switch I within the Cdc42 (Q61L) – GMP-PCP complex is rearranged to coordinate to the Mg^{2+} as well as to the γ-phosphate from the GTP-analog (Fig. 3.4). The side-chain hydroxyl groups from switch I residues Thr35 and Tyr32 are flipped away from the solvent and make hydrogen bonds with the Mg^{2+} and the γ-phosphate, respectively. The Mg^{2+} ion has a hexavalent coordination geometry composed of the β- and γ-phosphates, as well as the Thr17 and Thr35 side-chain hydroxyl groups which provide four corners of a coordination plane with Mg^{2+} in the center while a water molecule is bonded to it on either side.

As is the case with the structures for activated forms of Cdc42 bound to effectors, Phe37 within the Cdc42 (Q61L) – GMP-PCP complex has re-oriented in such a way as to allow for Thr35 to flip into position to coordinate the Mg^{2+} ion. Coordination of the Mg^{2+} by Thr35 is a hallmark of nearly all activated G protein structures. However, unlike the x-ray structures for Cdc42 (Q61L) – GMP-PNP bound to the limit-binding domain from Par6 (1NF3) or Cdc42 – GMP-PCP bound to the limit-binding domain of Pak6 (2ODB), Phe37 in the Cdc42 (Q61L) – GMP-PCP complex is in a different rotomeric conformation. These differences in conformations arise from specific interactions between Phe37 and the various effector proteins. In both the Cdc42 (Q61L) – GMP-PNP – Par6 (1NF3) and Cdc42 – GMP-PCP – Pak6 (2ODB) structures, Phe37 is buried in a hydrophobic pocket composed of residues from the effector molecule as well as switch II residues from Cdc42 itself.

Subtle differences in switch II are also observed between the ground-state Cdc42 (Q61L) – GMP-PCP complex and the available x-ray structures for Cdc42

Figure 3.4 Switch I for the Cdc42 (Q61L) – GMP-PCP complex is in an identical conformation with those for Cdc42 bound to effector proteins. The hallmark of G protein activation is the movement of switch I toward the guanine-nucleotide where highly conserved switch I residues, Thr35 and Tyr32, can coordinate to the Mg^{2+} and γ-phosphate respectively. A structural alignment of the Cdc42 (Q61L) – GMP-PCP complex with the structures for the GTP-analog-bound form of Cdc42 in complex with the effector proteins Par6 and Pak6 reveals an identical switch I conformation. switch II between the Cdc42 (Q61L) – GMP-PCP and the Cdc42 – GMP-PCP – Pak6 complexes is identical as well. Switch II in the Cdc42 (Q61L) – GMP-PNP – Par6 complex is slightly shifted due to specific interactions with the effector protein.



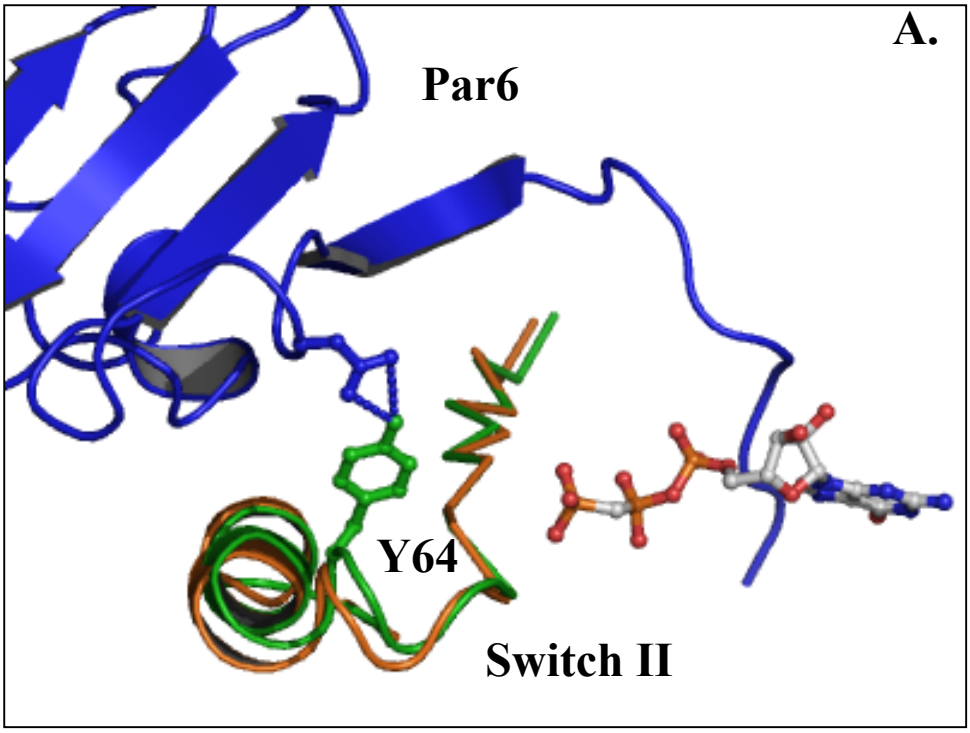
Cdc42 (Q61L) – GMP-PCP
Cdc42 – GMP-PCP – Pak6
Cdc42 (Q61L) – GMP-PNP – Par6

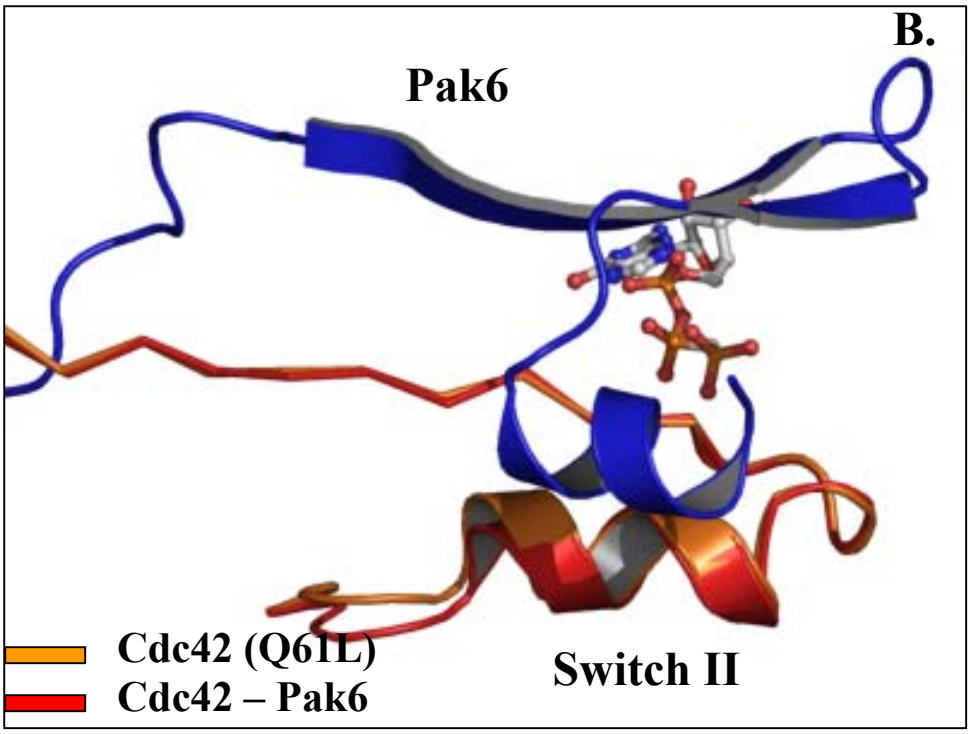
bound to its effectors Par6 and Pak6 (1NF3, 2ODB). The overall fold of switch II amongst all three x-ray structures is virtually identical (i.e. a loop region (loop4) followed by a β-turn and helix α2). However, when compared to the structure for Cdc42 (Q61L) – GMP-PCP, the α2 helix in the Cdc42 (Q61L) – GMP-PNP – Par6 complex is shifted by an average of ~1 Å overall with a maximum shift of 1.4 Å at Arg66 (Fig. 3.5A). The bulk of the changes occur between residues 60 – 75. Movement of α2 is mostly toward the bound effector protein which is hydrogen bonded to the switch II residue Tyr64 by the effector residue Asp149. The C_{α}s from Leu61 are 0.7 Å apart between the two structures with the residue from the Cdc42 – GMP-PNP – Par6-CBD complex closest to the nucleotide-binding pocket. Additionally, the side-chains are in opposite rotomeric conformations, having been rotated 180º about χ1.

Likewise, comparisons between the Cdc42 (Q61L) – GMP-PCP and Cdc42 – GMP-PCP – Pak6 complexes reveal a slight shift of helix α2 with a maximum distance occurring at Gln74 of 1.54 Å (Fig. 3.5B). However, unlike the case for the Cdc42 (Q61L) – GMP-PNP – Par6 complex, Gly60 is not involved in any detectable position shift. Additionally, the β-turn, which is composed of residues $G^{61}EDY^{64}$, is nearly unchanged. Once again, the side-chain orientation of Gln61 from the Cdc42 – GMP-PCP – Pak6 complex is slightly altered relative to its position in the Cdc42 (Q61L) – GMP-PCP complex, having been rotated about the χ1 bond by nearly 180º. Although the Cdc42 – GMP-PCP – Pak6 structure has the catalytically important Gln61, which is in a slightly different conformation compared to Leu61 in the Cdc42 (Q61L) – GMP-PCP complex, it is not in position to hydrogen bond to the γ-phosphate nor to any potential catalytic water molecule.

Interestingly, one similarity between all three structures (i.e. Cdc42 (Q61L) – GMP-PCP, Cdc42 (Q61L) – GMP-PCP – Par6, and Cdc42 – GMP-PNP – Pak6) is the

Figure 3.5. Comparison of switch II between the effector-bound complexes for Cdc42 and the Cdc42 (Q61L) – GMP-PCP complex. *A*, Switch II for Cdc42 (Q61L) – GMP-PNP (green) bound to Par6 is slightly shifted toward the effector protein when compared to the ground-state conformation depicted in the Cdc42 (Q61L) – GMP-PCP complex (orange). The bulk of the shift is caused by an interaction between switch II residue Tyr64 and an arginine residue from Par6. *B*, The interaction with Pak6 causes a slight shift in the C-terminal portion of the switch II helix when compared to the Cdc42 (Q61L) – GMP-PCP complex. Overall, these results indicate that these shifts in switch II are a result of specific interactions with the effector proteins and not the result of differences between the activated ground-states between the Cdc42 molecules.


A.
Par6
Y64
Switch II

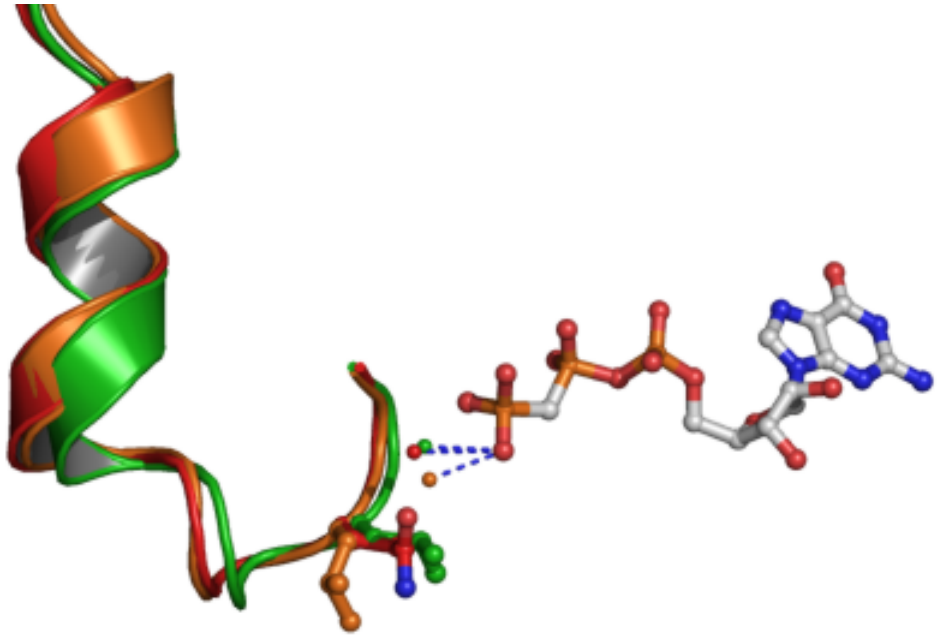

B.
Pak6
Cdc42 (Q61L)
Cdc42 – Pak6
Switch II

presence of a water molecule hydrogen bonded to the γ-phosphate of the nucleotide analog (Fig. 3.6A). This water molecule (Wat26, Wat511, Wat248 in the -Pak6, -Par6, and Cdc42 (Q61L) – GMP-PCP complexes respectively), occupies nearly the same coordinates and overlaps nicely with the position of the Gln61 carbamoyl group from the Cdc42 - GDP•AlF_3 – Cdc42GAP complex (1GRN) (Fig. 3.6B). In the Cdc42GAP complex, Gln61 is observed making contacts to both the γ-phosphate and the attacking water molecule which is in position for an in-line S_N2 type nucleophilic attack on the γ-phosphate. This is an important feature as it has been speculated that proper positioning of the water molecule by Gln61 is an important step in the hydrolysis mechanism of most G proteins (20-22). It is believed that the inability of G protein Q61L mutants to coordinate the attacking water molecule underlies their inability to hydrolyze GTP and to show constitutive signaling activity.

Comparisons between Cdc42 (Q61L) – GMP-PCP and Cdc42 - GDP•AlF_3 – Cdc42GAP

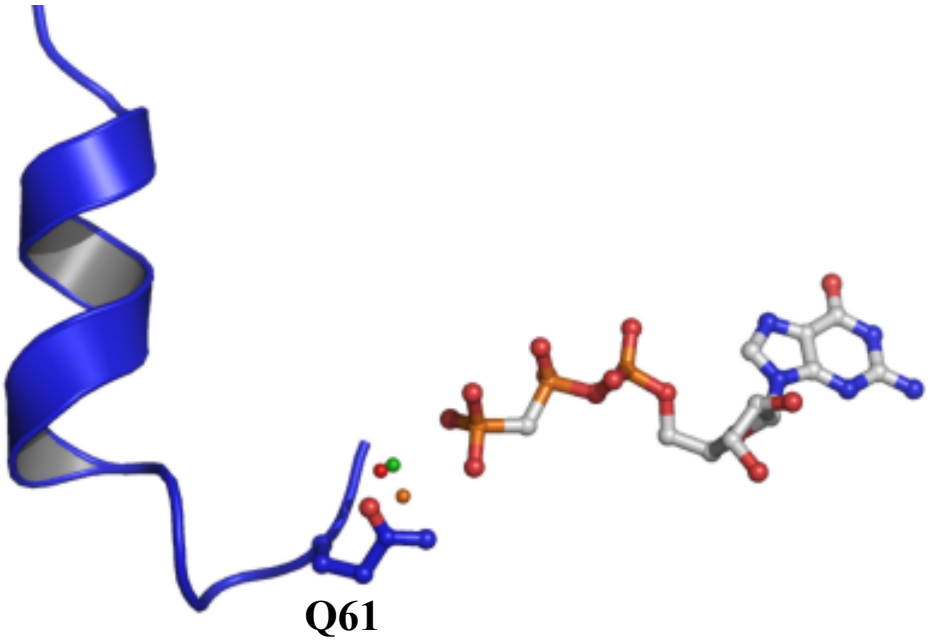
There are slight differences in the switch I conformation in the Cdc42 - GDP•AlF_3 – Cdc42GAP complex (1GRN) versus the Cdc42 (Q61L) – GMP-PCP complex. These differences arise from the distinct interactions that occur between the GAP and switch I. Notably, Tyr32, which is in direct contact with the γ-phosphate in the Cdc42 (Q61L) – GMP-PCP complex, is pushed away from the phosphate and toward the solvent in the GAP structure (Fig. 3.7). This displacement is a consequence of the interaction between the so-called arginine finger (Arg305) from Cdc42GAP and the β and γ-phosphates from the nucleotide bound to Cdc42. Arginine 305, from Cdc42GAP, occupies the same position as Tyr32 in the Cdc42 (Q61L) – GMP-PCP complex. As a result of the reorientation of Tyr32 in the Cdc42 – GDP•AlF_3 – Cdc42GAP complex, switch I residues upstream of Tyr32 have been slightly

Figure 3.6 A-B. A water molecule is hydrogen bonded to the γ-phosphate in the Cdc42 (Q61L) – GMP-PCP complex as well as the Par6- and Pak6-bound Cdc42 x-ray crystal structures. *A*, X-ray crystal structures for Cdc42 bound to either Par6 (green), Pak6 (red), compared to the structure for the Cdc42 (Q61L) – GMP-PCP (orange) complex shows an equivalent water molecule hydrogen bonded to the γ-phosphate in all cases. *B*, The water molecules from the three previous x-ray crystal structures occupy similar positions to Gln61 from the Cdc42 – RhoGAP complex (blue). Glutamine 61 is thought to help align a nucleophilic water molecule for an in-line S_N2 attack on the γ-phosphate during the transition state for GTP hydrloysis. The equivalent water molecules may represent the position of catalytic water prior to the transition state.



A.

Q61

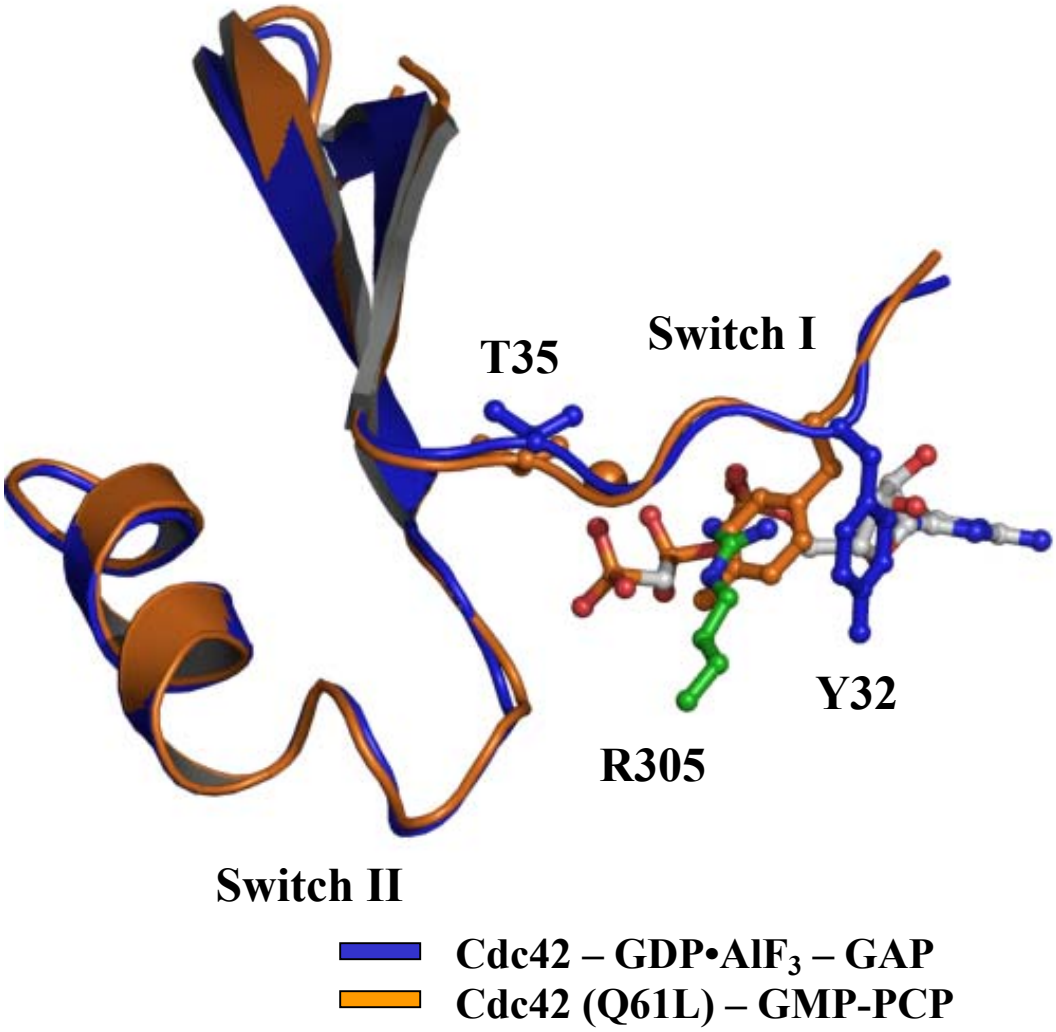


B.

Q61

Cdc42 - RhoGAP

Figure 3.7. Switch I is slightly shifted in the x-ray crystal structure for the Cdc42 – GDP●AlF_3 – GAP complex versus the Cdc42 (Q61L) – GMP-PCP complex. Arginine 305 from the GAP protein pushes Tyr32 away from the γ-phosphate which causes slight disruptions to switch I at positions N-terminal to Tyr32 when compared to Switch I from the Cdc42 (Q61L) – GMP-PCP complex. However, Thr35 is still coordinated to the Mg^{2+} ion similarly in both structures. Additionally, switch II between the two structures is virtually identical.


T35
Switch I
Y32
R305
Switch II
Cdc42 – GDP•AlF3 – GAP
Cdc42 (Q61L) – GMP-PCP

reoriented as well. Although the main-chain atoms for Phe28, which is conserved in the Rho family of small G proteins, have been shifted along with other switch I residues, the side-chain aligns well and is able to provide a π-π orbital stabilization to the guanine-ring of GDP. The side-chain of Phe37 in the Cdc42 - GDP•AlF_3 – Cdc42GAP complex is in a different rotomeric conformation than the one observed in the Cdc42 (Q61L) – GMP-PCP complex. As described earlier, the precise orientation of Phe37 can be specifically influenced by each individual effector, and in this case, by the regulator Cdc42GAP.

Surprisingly, switch II of the Cdc42 – GDP•AlF_3 – Cdc42GAP complex is mostly unchanged when compared to the structure for the Cdc42 (Q61L) – GMP-PCP complex. One notable exception is the side-chain orientation of Gln61 in the Cdc42 – GDP•AlF_3 – Cdc42GAP complex versus Leu61 in the Cdc42 (Q61L) – GMP-PCP complex. As mentioned above, Gln61 is observed facing toward the AlF_3 moiety in the Cdc42GAP complex, and actually forms hydrogen bonds with AlF_3 as well as with the purported catalytic water molecule which is in position for an in-line S_N2 nucleophilic attack, whereas glutamine 61 in the Cdc42 (Q61L) – GMP-PCP complex faces in the opposite direction (See Fig. 3.6B).

^{31}P-NMR studies of Cdc42-GMP-PCP and Cdc42 (Q61L) – GMP-PCP in the presence and absence of effector interactions

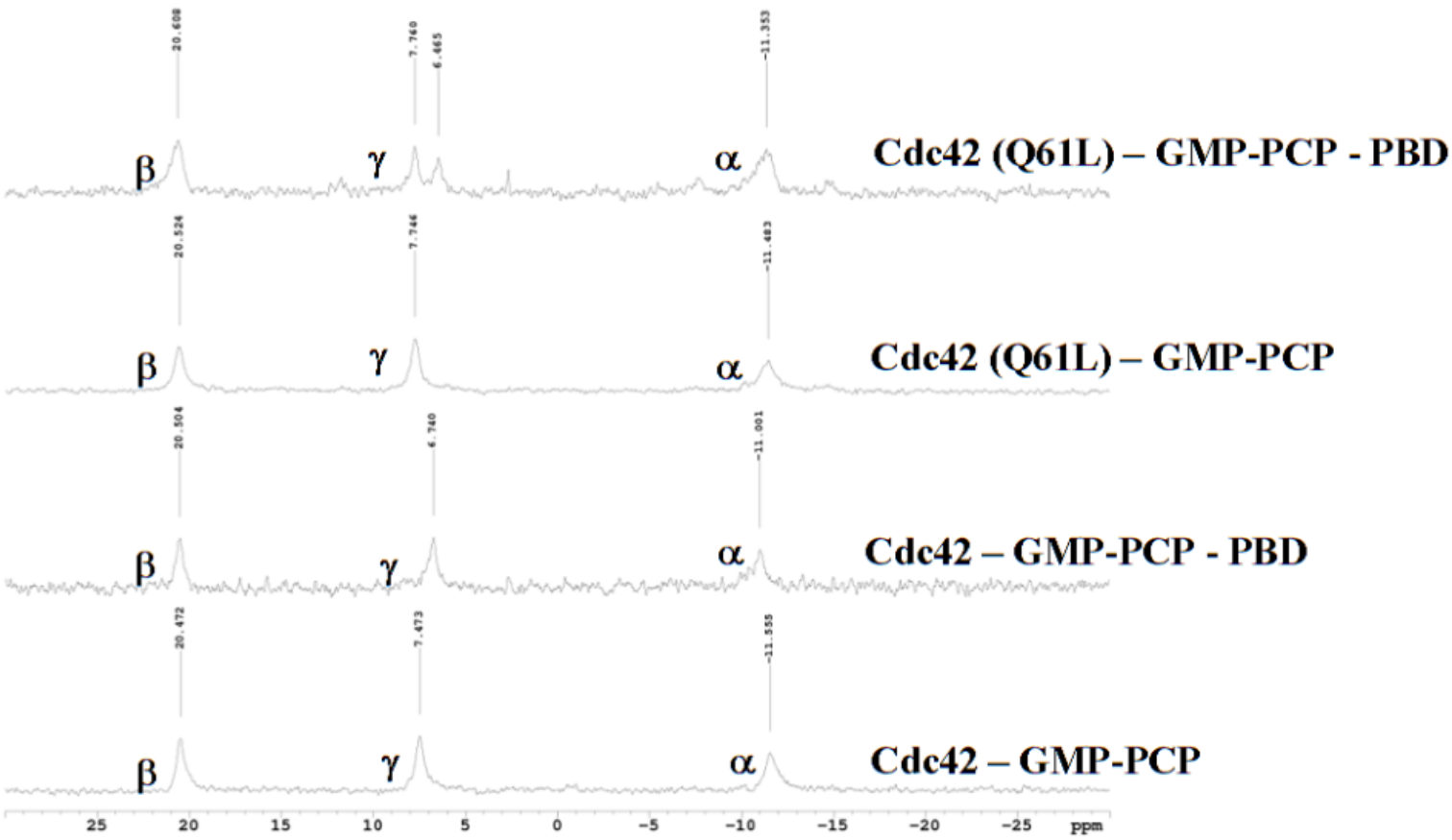
^{31}P-NMR analysis of the Cdc42 – GMP-PCP complex showed that it existed in only one conformational state. Upon addition of an effector protein (the limit-binding domain from Pak3 called Pak3-PBD), there was an apparent transition to a distinct conformational state as depicted by a chemical shift in phosphate resonances from the nucleotide. This new conformational state most likely resembles the signaling-active state as represented in the effector-bound structures for Cdc42. The initial

conformational state (representing that seen in the absence of an effector protein), most likely is an intermediate state which closely resembles the signaling-inactive (GDP-bound) state.

In an attempt to see if there are any structural differences between the wild-type Cdc42 – GMP-PCP and constitutively activated Cdc42 (Q61L) – GMP-PCP in solution, we again used ^{31}P-NMR spectroscopy. Interestingly, the ^{31}P-NMR spectra obtained for these two Cdc42 complexes were very similar. The resonance frequencies obtained for all three phosphate residues were virtually identical between the two complexes with the largest frequency shift (about 0.3 ppm) occurring at the γ-phosphate (Fig. 3.8). After the addition of the effector protein (Pak-PBD), the wild-type Cdc42 complex showed the characteristic up-field and down-field resonance shifts for the α- and γ-phosphates respectively, as reported previously (81). Surprisingly, however, the spectra for the Cdc42 (Q61L) – GMP-PCP – PBD complex revealed that the γ-phosphate peak had split into two peaks. The first and larger peak corresponded to the effector-free peak whereas the second smaller peak represented an unknown state. The α- and β-phosphate frequencies remained relatively unchanged compared to the effector-free spectra.

The splitting of the γ-phosphate peak implies an equilibrium of two states that interconvert slowly compared to the NMR time-scale. The first state, which is associated with the effector-free Cdc42 (Q61L) – GMP-PCP complex, likely represents the signaling-active structure that was observed in the x-ray crystal structure for the complex. This notion is borne out by the observation that the first γ-phosphate peak is identical to the γ-phosphate peak in the effector-free spectra for Cdc42 (Q61L) – GMP-PCP. The second peak may represent a new conformational state, perhaps unseen in the x-ray crystallographic structures, that is most likely an outcome of the Q61L substitution.

Figure 3.8. The Cdc42 (Q61L) – GMP-PCP complex is in an activated conformation in solution as seen by comparison of the ^{31}P-NMR spectras for the effector-free and effector-bound complexes. The spectra between Cdc42 – GMP-PCP and Cdc42 (Q61L) – GMP-PCP are very similar. Only one peak for each phosphate is apparent indicating only one detectable conformation in solution. However, after addition of saturating amounts of an effector protein to the Cdc42 (Q61L) – GMP-PCP complex, the γ-phosphate peak splits into two while the Cdc42 – GMP-PCP peak completely shifts upfield. The first peak for the Cdc42 (Q61L) – effector complex corresponds exactly to the peak position for the effector-free protein and is more populated than the second peak. The first peak most likely represents the activated structure as seen in the x-ray crystal structure while the second peak may represent an unknown intermediate.

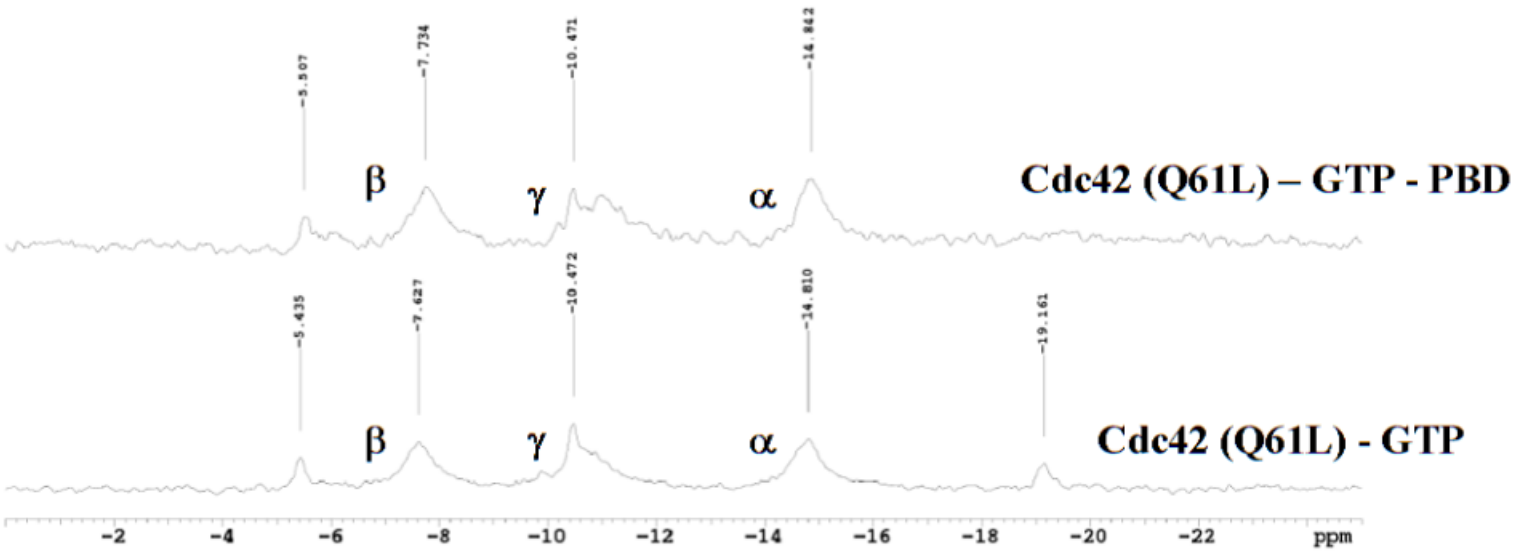

20.608
7.740
6.465
-11.353
β
γ
α
Cdc42 (Q61L) – GMP-PCP - PBD
20.524
7.744
-11.483
β
γ
α
Cdc42 (Q61L) – GMP-PCP
20.504
6.740
-11.001
β
γ
α
Cdc42 – GMP-PCP - PBD
20.472
7.473
-11.555
β
γ
α
Cdc42 – GMP-PCP
25
20
15
10
5
0
-5
-10
-15
-20
-25
ppm

^{31}P-NMR Spectra for the Cdc42 (Q61L) – GTP complex

It has been shown that different nucleotide analogs can sometimes affect the activation of G proteins in a distinct manner (10-12,23). In the case of Ras, the different nucleotide effects are observable mainly as differences in the ^{31}P-NMR-derived equilibria between two conformational states which are thought to correspond to an intermediate activated structure and a fully activated structure (12). In order to view what effect the physiologically relevant nucleotide (GTP) might have on structural activation, it was necessary to use the (Q61L) constitutively activated mutant form of Cdc42 to prevent hydrolysis over the course of the experiment.

Spectra for Cdc42 (Q61L) – GTP revealed only a single peak for each phosphate residue which is in agreement with the GMP-PCP data (Fig. 3.9). However, the peaks are much broader in the Cdc42 (Q61L) – GTP spectra compared to those for the Cdc42 – GMP-PCP spectra, indicating more flexibility in the protein's conformation. The addition of Pak3-PBD at levels well above the K_d value for the interaction between Pak-PBD and Cdc42 (Q61L) – GTP revealed a similar splitting of the γ-phosphate peak as observed in the ^{31}P-NMR spectra for the Cdc42 (Q61L) – GMP-PCP – PBD complex. The positions for the largest peaks were virtually unchanged compared to the effector-free spectra, indicating that the effector-free protein was in an activated conformation. Identical to the Cdc42 (Q61L) – GMP-PCP data, the second peak may represent a new conformational state distinctive to the Cdc42 (Q61L) mutant, which is in equilibrium with the activated conformation observed in the x-ray crystal structure for the Cdc42 (Q61L) – GMP-PCP complex.

Figure 3.9. GTP-bound versus GMP-PCP-bound Cdc42 (Q61L) reveals similar structural characteristics indicating the guanine-nucleotide-analog is a good mimic for cellular GTP. Comparisons between the ^{31}P-NMR spectra for effector-bound- and effector-free-Cdc42 (Q61L) – GTP reveal a similar pattern to the spectra for Cdc42 (Q61L) – GMP-PCP. The spectra for the GTP-bound protein is broader than the GMP-PCP-bound spectra which may indicate more flexibility in its conformation. Addition of an effector protein produces the γ-phosphate peak splitting observed for the GMP-PCP-bound Cdc42 (Q61L) spectra indicating both nucleotides behave similarly. Small errant peaks in both spectra most likely represent contaminating GDP and GMP moieties from the exchange process.


-5.507
-7.734
-10.471
-14.842
β
γ
α
Cdc42 (Q61L) – GTP - PBD
-5.435
-7.627
-10.472
-14.810
-19.161
β
γ
α
Cdc42 (Q61L) - GTP
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
ppm

Discussion

Comparing the x-ray crystal structure of the Cdc42 (Q61L) – GMP-PCP versus the Cdc42 – GMP-PCP complex

Previously, it was observed that the x-ray structure for the Cdc42 – GMP-PCP complex revealed a signaling-active conformation that resembled the signaling-inactive state for the Cdc42 – GDP (1ANO) complex (16). Additional information was obtained from both fluorescence spectroscopic analysis as well as ^{31}P-NMR studies which supported the x-ray structural findings. These results lead to the conclusion that Cdc42 – GMP-PCP is able to be molded by its effectors into a conformation that most likely resembles the conformational states seen in the NMR and x-ray structures of Cdc42 bound to effector proteins as well as to its regulator Cdc42GAP (6-8,18).

In light of these new findings, the question arose as to how the constitutively active Cdc42 (Q61L) mutant behaves and whether it would also require effector binding to mold it into a signaling-active conformational state. Thus we set out to solve the x-ray structure for the Cdc42 (Q61L) – GMP-PCP complex, and once again, obtained some rather unexpected results.

Switch I within the Cdc42 (Q61L) – GMP-PCP complex was rearranged to cover the nucleotide-binding pocket in much the same way as was observed in the Cdc42 – effector complexes. Unlike the structure for the Cdc42 – GMP-PCP complex, Tyr32 within the Cdc42 (Q61L) – GMP-PCP complex is hydrogen bonded to the γ-phosphate while the side chain for Thr35 is in position to coordinate to the Mg^{2+} (Fig. 3.1A-C). The remainder of the Cdc42 (Q61L) – GMP-PCP complex is virtually identical to the structure for Cdc42 – GMP-PCP, with only a slight movement in the position of some of the main-chain atoms in switch II.

This fundamental difference in switch I between the two structures has interesting implications. However, it should first be noted that the switch I conformation, as observed in the x-ray structure for the Cdc42 – GMP-PCP complex, is most likely only visible due to crystal contacts that stabilize this conformation. In solution, it is likely that switch I can interchange between a number of conformational intermediates, some of which may closely resemble, but never actually assume the signaling-active conformational states exhibited by Cdc42 bound to its effectors as well as by the Cdc42 (Q61L) – GMP-PCP complex.

Mutation of Gln61 to a leucine has apparently changed the energy landscape for Cdc42 (Q61L) – GMP-PCP so as to favor a conformation for switch I which is similar to that observed when Cdc42 is complexed with its effector proteins. The mechanism for this is unclear from the x-ray crystal structure. However, the answer most likely comes from a change in the thermodynamics of folding for the protein. One qualitative argument for how the Q61L mutation might work is by altering the free energy associated with the transition state of the switching mechanism so that the energy barrier required to reach a truly signaling-active conformational state has been reduced. This is exactly the same way an enzyme reduces the free energy associated with the transition state in a chemical reaction without altering the free energies of the reactants or products. A second possibility is that the transition state, as well as any intermediates along the conformational pathway, has been changed to a completely new set of conformations, so as to allow for the activated structure, as seen in the Cdc42 (Q61L) – GMP-PCP complex, to be energetically favored. These two possibilities are not mutually exclusive and in fact, the true mechanism may be a combination of the two.

There is some evidence from the x-ray structures of Ras switch II mutants to support the idea that a point mutation can alter the final signaling-active structure. In

the x-ray structure for the Ras (G60A) – GMP-PNP complex, a previously undocumented rearrangement of switch II, beginning at Ala60 and ending at Arg68, is observed (24). While this finding may not have been very surprising, what was unexpected was the finding that switch I had undergone a significant rearrangement so that it was no longer coordinated to the nucleotide. In fact, Phe28, which in many small G proteins makes π-π stacking interactions with the guanine ring of the bound GDP molecule, was shifted by 13 Å toward the solvent. Threonine-35, which coordinates to the Mg^{2+} as well as to the γ-phosphate in nearly all GTP-bound G protein structures, was shifted away from the nucleotide and no longer interacted with either of its ligands. The switch I orientation for the Ras (G60A) mutant was most similar to that observed in the structure for nucleotide-free Ras complexed to its guanine nucleotide exchange factor SOS (son of sevenless) (25).

Additionally, the x-ray structure for Ras (A59G) – GMP-PNP reveals a structure that has been suggested to represent an intermediate between the GDP- and GTP-bound forms of wild-type Ras (26). Interestingly, the φ,ψ dihedral angles for residue 59 are identical in both the Ras – GMP-PNP (5P21) and Ras (A59G) – GMP-PNP complexes. The main differences between the two structures are focused around switch II, specifically beginning at Gly60 and ending at Ser65. Switch II in the GMP-PNP-bound Ras (A59G) mutant appears to assume a conformation that closely resembles an intermediate state between the GDP- and GTP-bound Ras structures as predicted by molecular dynamics simulations (27). Switch I reveals only subtle changes to the side chains of Tyr32 and Glu37 in the Ras (A59G) – GMP-PNP complex. Tyrosine-32 is observed to move closer to the nucleotide and to make a water-mediated hydrogen bond to the γ-phosphate while Glu37 is less solvent exposed due to the close proximity of Tyr71.

The idea behind the two Ras mutants described previously, (G60A) and (A59G), was to change the conformational freedom at a position long thought to be critical for the structural rearrangements that occur during the transition from GTP-bound Ras to the GDP-bound Ras. However, in both cases, the structure that emerged was altered from what has been observed in wild-type and other mutant structures for Ras (i.e. Ras (G12V), Ras (Q61L)) (9,28). In the case of the A59G mutant, the structural rearrangement was somewhat predictable in that it affected mostly just the switch II region. In the case of the G60A mutation however, switch I was unpredictably altered. What can be learned from these two structures is that a point mutation, within switch II, affected the conformational rearrangements that take place during the activation event to an extent that altered the final activated structure. These final structures were different from any known structures for activated Ras and as such, were easily distinguishable as being caused by the point mutation. In a similar manner, the Q61L point mutant may be affecting the final activated conformation but in a manner favoring a structure that is virtually identical to activated structures for Cdc42 bound to effector proteins.

^{31}P-NMR studies show that GMP-PCP induces similar conformational states within wild-type Cdc42 and the Cdc42 (Q61L) mutant

^{31}P-NMR has proven to be a useful tool for investigating conformational equilibria of activated Ras in solution (12,29,30). Interestingly, crystal structures for activated forms of Ras have repeatedly shown a closed switch I conformation, denoted by Thr35 and Tyr32 being brought into close contact with the Mg^{2+} ion and the γ-phophate group, respectively (3,4,28,31). However, the ^{31}P-NMR spectra for Ras bound to GTP and various GTP analogs (GMP-PNP, GMP-PCP) in solution have revealed two conformational states in fast exchange, designated as states 1 and 2

(12,29). State 2 is slightly upfield of state 1 and has been shown to represent the activated structure of Ras as seen in the x-ray crystal structures. Addition of the Ras-binding domain (RBD) of the Ras effector Raf –1 completely shifted the population of state 1 to state 2, thus supporting the idea that state 2 represents the fully activated structure. State 1 is thought to be an intermediate state that is characterized by the lack of key interactions between Thr35 and Tyr32, and Mg^{2+} and the γ-phosphate moiety, respectively. As indicated above, these interactions are the hallmarks of activated G protein structures and as such, it is likely that switch I is in a more open state, perhaps similar to the GDP-bound state. This has been borne out by experiments with the partial loss of function T35S mutant and the complete loss of function T35A mutant (30). ^{31}P-NMR spectra for both mutants revealed only one conformation in solution corresponding to state 1 for wild-type Ras. However, upon addition of Raf-RBD, the Ras (T35S) mutant showed a second peak corresponding to state 2 whereas the Ras (T35A) mutant did not, implying a role for the methyl group from Thr35 in signaling.

In an attempt to identify the structure of state 1, the x-ray crystal structure of the Ras (T35S) – GMP-PNP complex was solved (30). The structure revealed a lack of electron density for both switch regions, indicating a high level of mobility. Since state 1 is shared by the Ras (T35A) mutant, the Ras (T35S) mutant, and wild-type Ras, it seems reasonable to assume that state 1 must be highly dynamic, and therefore lacks contacts to Mg^{2+} and the γ-phosphate of GTP or GTP-analogs.

Previously, we demonstrated that Cdc42, when bound to the non-hydrolyzable GTP-analogs GMP-PCP or GMP-PNP, exhibited only one conformational state in solution (16). This together with the x-ray crystal structure for the Cdc42 – GMP-PCP complex as well as the results from fluorescence studies performed in solution, suggests that, unlike the case for Ras, Cdc42 resides in state 1 almost exclusively. Furthermore, state 1 for GTP-bound forms of Cdc42 is most likely very similar, if not

identical, to the conformation for the GDP-bound state of the protein. Since the x-ray structure for the Cdc42 (Q61L) – GMP-PCP complex revealed a similar architecture to those determined from the structures for activated Cdc42 in complex with effector proteins, we were curious to see if the Cdc42 (Q61L) – GMP-PCP complex exhibited similar or different behavior in solution. Like the case for the Cdc42 – GMP-PCP complex, the ^{31}P-NMR spectra for the Cdc42 (Q61L) – GMP-PCP complex revealed only one conformational state in solution (Fig. 3.8). Surprisingly, the addition of an effector caused a splitting of the γ-phosphate peak. One peak, the downfield peak at -7.7 ppm, was identical to the peak observed for the Cdc42 (Q61L) – GMP-PCP complex alone and will be referred to as state 1 hereafter. Additionally, a qualitative inspection of the relative areas under the two γ-phosphate peaks reveals that state 1 appears to be more populated than state 2 (the upfield peak) indicating a greater proportion of the protein is in state 1, even after addition of the effector protein.

One possible explanation for the appearance of the two γ-phosphate peaks is that the Cdc42 (Q61L) – GMP-PCP – PBD complex was not at saturation. This would imply that upon saturation, the γ-phosphate peak would be completely shifted to the upfield position and thus disappear, similar to what occurred when the PBD was added to GMP-PNP bound to wild-type Cdc42. However, there are two pieces of evidence that argue against this. First, the spectra for the Cdc42 – GMP-PCP – PBD and Cdc42 (Q61L) – GMP-PCP – PBD complexes were collected on the same day, in the same molar quantities, which were far in excess of their equilibrium dissociation constants (32,33). Since only one γ-phosphate peak was evident in the spectra for the Cdc42 – GMP-PCP – PBD complex, it seems likely that this complex had reached saturation. The same PAK-PBD protein stock was used in all of the experiments, which makes it unlikely that a significant portion of the protein had degraded in the intervening time between experiments.

Another possible explanation is that the affinities between the effector and the different forms of Cdc42 are different whereby Cdc42 (Q61L) has a lower affinity than Cdc42 wild-type for effector proteins. The apparent equilibrium dissociation constants (K_d) have been calculated for the interaction between PAK-PBD and GMP-PNP bound Cdc42 and have been shown to be between 0.2 and 1 μM (32,33). Using the higher K_d of 1 μM, it can be calculated that the Cdc42 – GMP-PNP-PBD complex should be at saturation at the concentrations and conditions used in the experiment (i.e. 1 μM protein each, pH 8.0). Additionally, it has been shown that the Cdc42 (Q61L) – GMP-PNP complex has a ten-fold higher affinity for PAK-PBD compared to GMP-PNP-bound wild-type Cdc42, thus negating any difference in affinity between the two Cdc42 constructs as a possible explanation (32,33). In fact, the difference in affinity between the wild-type Cdc42 and the Cdc42 (Q61L) mutant supports our findings that GMP-PCP-bound wild-type Cdc42 requires interactions with effectors in order to achieve a fully activated conformation whereas the Cdc42 (Q61L) mutant does not. Conversely, our findings provide an explanation for the differences in affinities.

Differences in the affinities between wild-type Cdc42 and the Cdc42 (Q61L) mutant for multiple effectors and regulators such as ACK, WASP, PAK, and RhoGAP have been shown to exist (34). In all cases, the Cdc42 (Q61L) mutant had a greater than ten-fold increase in binding affinity when compared to wild-type Cdc42. To see how our data helps to explain these differences between Cdc42 (Q61L) and wild-type Cdc42, it is helpful to look at the kinetic model that may describe the binding. In the simplest case, the bi-molecular interaction can be described as in equation 1 where CBD is the Cdc42 limit-binding domain for any effector and Cdc42* represents state 2 as seen in the ^{31}P-NMR spectra.

$$Cdc42^* + CBD \xleftrightarrow{K} Cdc42^* CBD \qquad (1)$$

In this case, Cdc42 is already in an activated conformation (Cdc42*) and the apparent K_d is defined only by the rates at which the two proteins associate and dissociate. However, based on our data, the model is slightly more complicated as depicted in equation 2.

$$\begin{array}{ccc} Cdc42 + CBD & \xleftrightarrow{K_1} & Cdc42CBD \\ {\scriptstyle K_4}\downarrow\uparrow & & {\scriptstyle K_2}\downarrow\uparrow \\ Cdc42^* + CBD & \xleftrightarrow{K_3} & Cdc42^*CBD \end{array} \qquad (2)$$

In this model, we have added in the isomerization step for going from state 1 to state 2. Since these experiments are performed under equilibrium conditions, all steps in the model are at equilibrium. Assuming that the isomerization step for the Cdc42 – GTP-analog complex, when not bound to the effector protein, is extremely slow, we find that the reaction procedes almost exclusively through K_1 and K_2. Therefore the apparent K_d can be written explicitly as $K_1 * K_2 * K_3$ which reveals that the overall affinity between Cdc42 and its effector protein has contributions from multiple steps in the pathway. Relating this model to the model used in the literature to calculate the apparent K_d (see equation 1) (32-34), we see that there is only one equilibrium step which contributes to the overall affinity.

However, according to our data, the case for Cdc42 (Q61L) is slightly different. The model for Cdc42 (Q61L) binding to effector may be expressed in a very similar manner to wild-type Cdc42 as in equation 3 below.

$$\begin{array}{ccc} Cdc42(Q61L) + CBD & \xleftrightarrow{K_1} & Cdc42(Q61L)CBD \\ {\scriptstyle K_4}\downarrow\uparrow & & {\scriptstyle K_2}\downarrow\uparrow \\ Cdc42(Q61L)^* + CBD & \xleftrightarrow{K_3} & Cdc42(Q61L)^*CBD \end{array} \qquad (3)$$

In this case, the equilibrium between state 2 and state 1 for activated Cdc42 (Q61L) is shifted greatly towards state 2 as denoted by the larger arrow pointing towards Cdc42 (Q61L)*+CBD (represented by K_4). In fact, it is almost a certainty that the equilibrium between states 1 and 2 (K_4), before the binding of an effector, is significantly driven toward state 2 such that virtually the entire protein population exists in this state. In this case, equation 3 can be simplified to equation 4 which is the same bi-molecular binding model as seen in equation 1.

$$Cdc42(Q61L)^{*} + CBD \xleftrightarrow{K} Cdc42(Q61L)^{*}CBD \qquad (4)$$

By these new models, borne out of our data, it can be seen that the apparent K_d for wild-type Cdc42 binding to an effector protein would contain contributions from at least two additional steps, which is absent in the K_d calculated for Cdc42 (Q61L). It is likely that these extra steps account for the differences in the affinities between the Cdc42 isoforms and their effector proteins.

Overall, our findings highlight a fundamental difference between the constitutively active Cdc42 (Q61L) mutant protein and the wild-type Cdc42 protein. Based on the x-ray crystal structure for the Cdc42 (Q61L) – GMP-PCP complex we see that, unlike Cdc42 – GMP-PCP, the Q61L point mutation alters the conformational freedom of the protein so as to allow the Cdc42 (Q61L) mutant protein to assume a fully activated conformation in the absence of effector protein. Additionally, ^{31}P-NMR experiments revealed that GMP-PCP-bound Cdc42 (Q61L) exists in only one state in solution. However, addition of Pak3-PBD revealed two distinct states. State 1 corresponds to the same state observed for the effector-free Cdc42 (Q61L) – GMP-PCP complex while the second state represents an unknown

activated state. Since state 1 is identical for both the effector-bound and effector-free complexes, we can deduce that state 1 is the activated state as seen in the x-ray crystal structure. Furthermore, the physiologically relevant nucleotide, GTP, yielded similar results implying that the GTP-analog GMP-PCP does mimic GTP. Therefore, the structural effects we observe are not due to a difference in bound nucleotide.

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Chapter 4

Conclusions

Over the last 50 years, the field of cell signaling has made enormous strides towards understanding the basic principles which underlie how a cell interacts with its environment. One of the seminal discoveries in this field was the discovery of G proteins by Martin Rodbell and Alfred Gilman in the 1970s. At the very core of the problem Rodbell and Gilman were addressing was the question of how a hormone, on the outside of a cell, can induce a reaction across the membrane and affect intra-cellular function. Thanks to their pioneering efforts, and those of their colleagues, we now know that many extracellular signals are transduced across the cellular membrane by a three component system comprised of a G protein coupled receptor (GPCR), a heterotrimeric G protein, and an effector protein. The effector protein typically changes the concentration of a second messenger molecule such as cAMP, as in the case for the adenylyl cyclase system, which is able to magnify the signal by regulating the functions of various downstream proteins.

However, GPCRs are not the only cell surface receptors able to activate G proteins and initiate signaling cascades. Receptor tyrosine kinases (RTK) are also able to bind extra-cellular ligands and activate small G proteins, although indirectly. These small G proteins can control many of the major signaling pathways in the cell such as proliferation, apoptosis, differentiation, and cell motility. The most famous example of small G protein signaling involves the actions of Ras, a small G protein which is mutated in ~ 30% of all human cancers.

Common to all G proteins is the fact that they are switched "on" by binding GTP and switched "off" by hydrolyzing GTP to GDP and P_i. This G protein GTP binding and hydrolytic cycle is typically tightly regulated by both GEF and GAP proteins, for the small G proteins, and GPCR and RGS proteins for the heterotrimeric G proteins.

It was revealed through x-ray crystallographic studies on Ras, as well as the heterotrimeric Gα subunit transducin, that activation of the G protein by binding GTP caused a structural rearrangement in two loop regions designated Switch I and II (1,2). Subsequent x-ray crystal structures for various other G protein family members confirmed that indeed, all G proteins thus far investigated undergo some form of structural rearrangement in both Switch I and II upon activation by binding to GTP.

Members of the Rho family of small G proteins, at first glance, appear to behave in a similar manner to all other G proteins. Numerous x-ray crystal and NMR structures for RhoA, Rac1, as well as Cdc42 have revealed significant rearrangements of the switch regions when comparing between the active and inactive forms of the proteins (3-7). However, activated structures for Cdc42 have only been reported in the presence of effector or regulator proteins (5,6,8,9). Considering these structures along with the activated structures for virtually all other G proteins thus far known, it would seem reasonable to assume that the structure for Cdc42 bound to a GTP-analog complex would be identical to the structures for GTP-bound Cdc42 in complex with an effector protein. In fact this is not the case at all. Surprisingly, as we show in Chapter 2, the structure for Cdc42 bound to the GTP-analog GMP-PCP is virtually identical to that for the signaling-inactive Cdc42 – GDP complex (1ANO).

One of the structural hallmarks of G protein activation is the coordination of the Mg^{2+} ion by a highly conserved threonine residue located within Switch I. A second hallmark is the movement of another highly conserved Switch I tyrosine residue into

close proximity to the γ-phosphate of the GTP moiety. The interaction of these two Switch I residues with the nucleotide lock the protein into the active conformation. In the signaling-inactive GDP-bound conformation (pdb id 1ANO), both Thr35 and Tyr32 face away from the nucleotide into solvent. As reported in Chapter 2, this is also the case for the signaling-active Cdc42 – GMP-PCP complex.

This conformation presents an interesting question; how does an effector protein recognize and bind with high specificity to the GTP-bound state and not the GDP-bound state? The first clue to answering this question comes from the structures of activated Cdc42 in complex with its effector proteins (5,6). These complexes reveal Switch I in Cdc42 to be in an activated state as noted by Thr35 and Tyr32 being brought into close proximity to the Mg^{2+} and γ-phosphate respectively. This indicates that binding of an effector protein drives the conformation of Cdc42 toward the active structure in a similar manner to the theory of induced fit put forward for enzyme-substrate reactions (10).

To test this hypothesis, we used a combination of fluorescence and ^{31}P-NMR to view changes in Cdc42 before and after the activation event as well as before and after its binding to an effector protein. Fluorescence studies, whereby Tyr32 was replaced by a tryptophan residue, revealed that upon binding GTP, Cdc42 undergoes a relatively small change (an ~10% reduction) in its intrinsic tryptophan fluorescence when compared to the case for Ras. However, addition of the limit-binding domain for the Cdc42 effector Pak3, caused a more significant reduction in tryptophan fluorescence (between 20-30%) indicating the effector protein is able to drive the GTP-analog-bound Cdc42 into a different structural state. In agreement with the fluorescence data, ^{31}P-NMR revealed that GTP-analog-bound forms of Cdc42 resided in only one detectable conformational state in solution at 5ºC. After addition of an equal molar amount of PBD, the first state was completely shifted to a second

conformational state which likely corresponds to the signaling-active conformation for Cdc42 as exhibited by various x-ray and NMR structures for Cdc42-effector complexes. When compared to ^{31}P-NMR data for Ras, a clear difference is observed. Activated Ras exists in two states in solution, that have been designated states 1 and 2. In the absence of an effector protein, state 2 is typically more populated than state 1. After addition of an effector protein, the population of state 1 is completely shifted to state 2 which corresponds to the signaling-active structure for Ras. Based on these results, it is clear that in solution, GTP-bound forms of Ras exist predominately in an activated state (state 2) which is in equilibrium with another state (state 1) that is thought to resemble a signaling-inactive state. Addition of an effector protein stabilizes the activated state so that state 1 effectively disappears. Conversely, GTP-bound forms of Cdc42 reside exclusively in a single state that, based on the x-ray crystal structure for the Cdc42 – GMP-PCP complex, is identical to the signaling-inactive GDP-bound state. Addition of effector proteins then drives the formation of the signaling-active conformational state as depicted in the effector-bound structures for Cdc42.

Overall, these findings suggest that rather than a single universal model for G protein activation, where GDP-GTP exchange, alone, is sufficient to induce the signaling-active state, there instead exists a spectrum of nucleotide-dependent conformational states for different G proteins ranging from a signaling-inactive state to a fully signaling-active conformation. Cdc42 represents one extreme of this spectrum, such that GDP-GTP exchange, alone, is not capable of inducing the signaling-active state, but rather the binding of effector proteins are required to achieve a fully activated conformation. Ras, on the other hand, represents the other extreme whereby the binding of GTP is sufficient to drive the structural rearrangements associated with the G protein activation event.

In Chapter 3 we address the question of whether the constitutively activated form of Cdc42 (Q61L) also requires an effector protein to drive it to the fully activated state. Glutamine 61 (Cdc42 numbering) is a highly conserved Switch II residue that is critical for GTP hydrolysis, even in the presence of a GAP (11). Similar to the case for GTP-analog bound forms of wild-type Cdc42, until now there has not been a high resolution structure for the GTP-analog-bound forms of Cdc42 (Q61L). This raises the possibility that the Q61L version of Cdc42 also requires effector protein interactions to achieve the fully activated state. Surprisingly, we found that the x-ray crystal structure for the Cdc42 (Q61L) – GMP-PCP complex was infact in a fully activated conformation, unlike the wild-type Cdc42 – GMP-PCP complex. Alignment of the Cdc42 (Q61L) – GMP-PCP complex with the structures for GTP-analog bound forms of Cdc42 complexed to effector proteins reveals a Switch I architecture which is virtually identical. In particular, Switch I within the Cdc42 (Q61L) – GMP-PCP structure has shifted so as to cover the nucleotide binding pocket, allowing Thr35 and Tyr32 to coordinate directly to the Mg^{2+} and γ-phosphate.

Subsequently, ^{31}P-NMR experiments of Cdc42 (Q61L) – GMP-PCP in the absence of an effector revealed only one conformational state in solution at 5ºC, similar to what we had observed for GTP-analogs bound to wild-type Cdc42. However, upon addition of saturating amounts of an effector (PBD), two conformational states were observed. The first state (state 1) corresponded exactly with the chemical shifts for Cdc42 (Q61L) – GMP-PCP alone. Furthermore, state 1 was more populated than state 2 indicating that state 1 most likely represents the fully activated conformation observed in the x-ray crystal structure. State 2 on the other hand, may represent a distinct conformational state that is either specific to the Q61L point mutant, or because the interconversion between this state and state 1 is sufficiently slowed for us to observe it.

These findings are interesting because they offer an explanation to an observation made in the Lowe lab at Cambridge regarding the differences in binding affinities for wild-type Cdc42 and the Cdc42 (Q61L) mutant (12,13). Lowe and colleagues observed that Cdc42 (Q61L) had at least a 10-fold higher affinity for effector and GAP proteins than wild-type Cdc42. Based on our observations, these differences in affinity can be explained by the fact that wild-type Cdc42 needs first to bind to an effector protein and then isomerize into a fully activated structure. The binding constant for wild-type Cdc42 would contain contributions from multiple steps whereas Cdc42 (Q61L), which is already in a fully activated state, can be described by a single binding step.

Cdc42 illustrates the importance for thorough examination of all G proteins individually. It is highly unlikely that Cdc42 is the only G protein to utilize a slightly different activation mechanism than Ras and provides evidence for a spectrum of activating mechanisms for G proteins. In fact, recently, an activated structure for M-Ras in which Switch I has moved further away from the nucleotide binding pocket instead of closer to it, has recently been published (14). The results obtained with Cdc42 may require a new way of thinking about the activation of small G proteins, whereby the binding of GTP may or may not be sufficient to drive a G protein toward a fully activated structure. In the future, it will be interesting to see whether small G proteins related to Cdc42, such as Rac and TC10, behave in a similar manner and rely heavily on effector proteins to reach their fully activated conformational state. It also will be of interest to see why the Q61L mutation leads to a fully activated state and if other mutations that result in constitutive activation, like G12V and F28L, do the same.

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