RECONSTITUTION OF RHO GTPASE INTERACTIONS AT THE MEMBRANE

A Thesis

Presented to the Faculty of the Graduate School

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Doctor of Philosophy

by

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ABSTRACT

In order for fibroblasts to migrate, budding yeast to polarize, and macrophages to undergo phagocytosis, each cell must coordinate a membrane-localized signal with a robust morphological change. The Rho Family of GTPases play critically important roles in mediating these cellular processes. However, in order for the Rho GTPases to help regulate these processes in a tightly coordinated spatial and temporal manner, they need to be properly localized to specific membrane signaling sites. The mechanisms by which this precise localization is achieved are still not fully understood, but represent the subject of this thesis.

The Rho GTPases are post-translationally modified at their C-terminus by the covalent attachment of a 20 carbon lipid tail, called a geranylgeranyl moiety, which allows them to associate with membranes. To facilitate their cytosolic localization, the Rho GTPases require the assistance of a ubiquitously-expressed regulatory protein, the Rho guanine nucleotide-dissociation inhibitor (RhoGDI). The mechanism by which the Rho GTPases move between distinct locations in the cell, and in particular, how they are able to cycle on and off between different membranes, has been a challenging question. I have set out to begin to define this mechanism by reconstituting *in vitro* the interactions between the geranylgeranylated Rho GTPase Cdc42, RhoGDI, and liposomes of well-defined lipid composition. In taking advantage of the sensitivity and real-time capabilities of these reconstituted systems, some unexpected findings emerged regarding how RhoGDI influences the membrane-to-cytosol distribution of Rho GTPases like Cdc42.

One such unexpected discovery involves my finding that RhoGDI can distinguish between the signaling-inactive (GDP-bound) and signaling active (GTP-bound) forms of Cdc42 when they are associated with a lipid bilayer. In particular, despite having similar affinities for the GDP- and GTP-bound forms of Cdc42 in solution, I found that when RhoGDI interacts with Cdc42 along the membrane surface, it has a much higher affinity for GDP-bound Cdc42 compared to its GTP-bound counterpart. Moreover, the membrane-release of Cdc42-RhoGDI complexes occurs at a similar rate as the release of Cdc42 alone, with the major effect of RhoGDI then being to significantly slow the re-association of Cdc42 with membranes. These findings lead us to propose a new model for how RhoGDI influences the ability of Cdc42 to move between membranes and the cytosol. I further demonstrated that the cycling of Rho GTPases like Cdc42 and Rac1 between the membrane and cytosol can be strongly influenced by RhoGEFs and RhoGAPs, as well as RhoGDI, such that the membrane association-dissociation cycle of the Rho GTPases is directly coupled to their GTP-binding/GTPase cycle.

Most of the Rho family GTPases contain a cluster of positive-charged residues (i.e. a 'polybasic domain'), directly preceding their geranylgeranyl moiety. It has been suggested that this domain serves to fine-tune their localization among different cellular membranes. I have used reconstituted systems to examine the role of the polybasic domain of Cdc42 in its ability to bind to membranes. These studies highlight a key role for a di-arginine motif close to the C-terminus of Cdc42 in binding to phosphatidylinositol-4,5-bisphosphate (PIP_2). While this interaction does not influence the ability of activated Cdc42 to signal the necessary changes to impact the

actin cytoskeletal architecture and induce filopodia/microspikes, it is essential for the ability of an oncogenic mutant of Cdc42 to transform fibroblasts. Thus, these findings highlight the importance of Cdc42 being localized to a PIP_2-enriched membrane site to engage those signaling partners that mediate oncogenic transformation.

BIOGRAPHICAL SKETCH

The author was born and raised in rural Iowa, on a farm just outside the town of Reinbeck. His mother, Cindy, was a high school teacher and his father, Lee, was a large and small animal veterinarian who raised beef cattle on the side. Along with his two younger sisters, Janee and Brianne, Jared spent much of his time outdoors, doing field work, during the summers, and helping take care of livestock, all year round. After graduating from high school, Jared enrolled at the University of Northern Iowa, initially as a chemistry and business dual-major. However, as the semesters passed, Jared gravitated progressively towards the natural sciences, eventually dropping his business major and adding a major in molecular biology. Jared graduated *summa cum laude* with a B.A. in chemistry and molecular biology.

Jared began his graduate studies in the Biochemistry, Molecular and Cell Biology Program at Cornell University where he joined Richard Cerione's laboratory. During his graduate career, he developed *in vitro* reconstitution assays to study small G-protein signaling at the membrane. The author now looks forward to joining the laboratory of Dr. Lewis Cantley at Harvard Medical School as a postdoctoral researcher.

To my family

ACKNOWLEDGMENTS

During my first year in the BMCB program, I had fortuitously followed my girlfriend (and future wife) into a course entitled 'The Chemistry of Signal Transduction,' taught by Richard Cerione. Notwithstanding it being scheduled during the late 6-8 pm Wednesday time slot, I was captivated by this field of study and this was due in no small part to efforts of the instructor. I was convinced that I should devote my graduate career to applying physical techniques to address medically-relevant questions and, as such, Rick's laboratory seemed a good match. Now nearing completion, I have very little doubt of that choice. I am thankful for Rick's constant support and I appreciate having the opportunity to develop my scientific inquiry and critical thinking skills from Rick's very impressive example.

To my committee members: Dr. Barbara Baird and Dr. Ruth Collins. Barb, you have been a great and constant source of support throughout the years. Together, you and Dave create a very nice atmosphere, making my visits to your lab pleasurable. And, for the record, your 'enzyme kinetics' course should be part of the curriculum of all Cerione lab members!

Ruth, I greatly appreciate your enthusiasm in my work. In my remaining time in Ithaca, I look forward to extending my *in vitro* approaches to investigating the membrane association-dissociation cycling of the Rab GTPases.

Jon Erickson, had I not crossed paths with you when I did, I might still be blurting out half-baked ideas of research to those around me. My early experiences with you taught me the value of exercising appropriate restraint and having patience in the face of skepticism and opposing views. Beyond that, I enjoyed our many conversations, both scientific and nonscientific.

Sekar Ramachandran (Ram), I consider it to be a rite of passage for me once I became able to engage in kinetic discussions with you. It has been a great experience, sharing the laboratory environment with you, and it was always a delight to get a visit from Tarun during those evening hours.

It was a pleasure to have been a part of the interdisciplinary and collaborative environment of the Cerione Lab. For me, three particular collaborations stand out as being the most prominent (listed in no particular order).

I enjoyed working with Yeyun Zhou in our investigation of the Rho exchange factor DOCK7's activity on Rho GTPases at the membrane, despite that our fluorescence work often kept us in the lab well into the painfully-late hours. At least we had the pleasure of Joy Lin's company on a few of those occasions.

To the tissue transglutaminase (TG) group, Marc Antonyak, Bo Li, Jingwen Zhang, and Lindsay Burroughs, it was a lot of fun discussing the many approaches we could take in reconstituting the limiting components needed to make oncogenic 'shed' vesicles as well as investigating TG's interactions at the membrane. Beyond that, you are all a fun bunch to go outside of the lab with. And I apologize to those of you for those nights where you had to walk back home.

Makoto Endo, my *in vivo* counterpart, we have had many nice conversations on how to coordinate our studies on the brain isoform of Cdc42 and I will be interested in seeing how your PIP_3 and palmitoylation studies continue to unfold.

It would be a sin to have the remainder of those who influenced me go unnoticed. Many thanks to Kristin Wilson, Kathy Rojas, Kristin Wilson, Bill Katt, Clint Stallnecker, Joe Druso, Sungsoo Yoo, Qiyu Feng, Chenyue Wang, Garima Singh, Shawn Milano, Kai Su Green, Michael Lukey, Chengliang Zhang, Ariel Yang, and past Cerione lab members

My sister, Janee, I have you to thank for convincing (coercing) me to add chemistry as a major during my undergraduate career at UNI. As my closest childhood friend, it should not be a surprise that you knew what was best for me. I look forward to many more experiences with you and your family: Kamal, Kanzah, and Qais.

My youngest sister, Brianne, you followed in my footsteps into the natural sciences at UNI, while managing the additional responsibility of being a lead cheerleader for UNI's basketball team. I could not be more proud you.

To my mom, because of you, my departure from Iowa was indeed challenging. I am grateful for your unconditional support and love. My wife and I look forward to many more Christmas's in Florida with you and Bill.

To my dad, you practiced veterinary medicine for almost 30 years and, throughout it all, you expressed only satisfaction in your line of work, something I was deeply influenced by. The most difficult part of my Cornell experience was accepting that our time together is limited.

On my second day in Ithaca, I crossed paths with Mami Shindo. Here, a fulfilling relationship would begin, one that would take us through Thailand, Malaysia, Indonesia, Hawaii, France, Italy, Switzerland, and Japan and culminate in our wedding, here at the Cornell Plantations. Throughout all this, I was blessed with the wonderful love and support from her family. Without a doubt, the greatest part of my Ithaca experience was my time spent with her.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

RhoGDI	Rho guanine nucleotide-dissociation inhibitor
Mant-	methylanthraniloyl-
HAF	hexadecanoylaminofluorescein
GTPγS	guanosine 5'-3-O-(thio)triphosphate
GMP-PNP	guanosine-5'–[(βγ)-imido]triphosphate
PE	phosphatidylethanolamine
PS	phosphatidylserine
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
RSE	rapid solvent exchange
GEF	guanine nucleotide exchange factor
DHR2	DOCK homology region 2
GAP	GTPase activating protein
GIP	GTPase inhibitory protein
GDF	GDI displacement factor
Cdc42SS	Cdc42(KK191,192SS)
Cdc42QQ	Cdc42(RR193,194QQ)

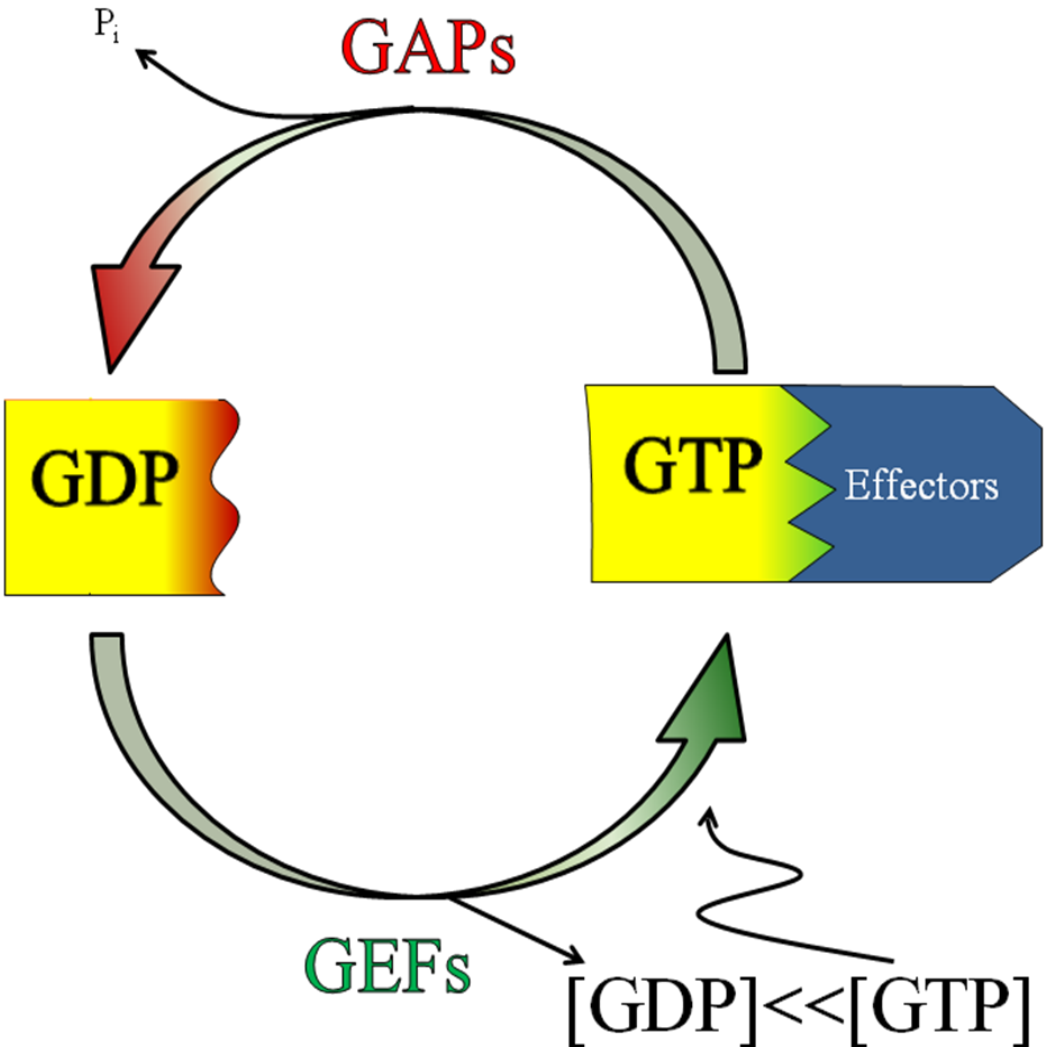
CHAPTER 1
INTRODUCTION

Overview of the small G-proteins

The Ras GTPase superfamily comprises a large and functionally diverse collection of signaling proteins that participate in an enormous range of cellular activities, which include cell growth, cell polarity, cytoskeletal remodeling, nucleocytoplasmic transport and vesicle-trafficking (1-5). Based on sequence homology, this family is divided into five major branches; these are designated the Ras, Rho, Rab, Arf, and Ran/TC4 GTPases. The functional theme which ties this family together is their ability to be tightly regulated in a switch-like fashion by the state of their bound guanine nucleotide (6) (Figure 1.1). In the signaling-active GTP-bound state, GTPases are able to initiate cellular signaling, the outcomes of which depend on the GTPase's specific array of downstream effectors and the state of its cellular environment. Over time, the bound GTP is hydrolyzed to GDP, which results in conformational changes that greatly reduce the GTPase's affinity for its effectors, thus rendering it inactive. Only upon the exchange of its bound GDP with GTP, can the GTPase return to its activated state and resume signaling. All members of the Ras GTPase superfamily undergo this cycle of regulation.

In the cell, two categories of proteins, Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs), specialize in accelerating the activating and de-activating steps in this cycle, respectively, and provide the temporal

Figure 1.1 The GTP-binding/GTPase cycle of the small G-proteins. A schematic representation of how the nucleotide state of the small G-protein signaling is regulated. The activated, GTP-bound, state of the G-protein (yellow) is positively regulated by its GEFs and negatively by its GAPs.


P_i
GAPs
GDP
GTP
Effectors
GEFs
[GDP]<<[GTP]

control necessary for proper cellular signaling activities (7). GEFs function by destabilizing the interaction between the GTPase and its bound nucleotide, causing the nucleotide to dissociate, thereby leaving the binding site open for an incoming nucleotide. The tenfold cellular excess of GTP over GDP ensures that GTP is the incoming nucleotide. GAPs, on the other hand, specifically target the GTP-bound form of the GTPase and accelerate its hydrolytic rate, switching it off. In light of their exquisite target-specificity and their diverse modes of regulation, GEFs and GAPs are major contributors to the signaling functions of all Ras Family members.

The Ras proteins, particularly H- and K-Ras, have been heavily implicated in human cancers, and a considerable proportion of cancers are driven by mutations within the ras genes (8-11). These mutations invariably drive the Ras proteins toward their signaling-active, GTP-bound states. This can be achieved in two ways: Through the activation of nucleotide exchange or through the inhibition of GTP hydrolysis. Notably, it is the latter that enabled researchers to identify the first point mutation that could drive tumor progression. In the early 1980's, the Ras subfamily gene, H-Ras, had been identified as the transforming element in human bladder tumors and colon cancers and it was shown to be capable of inducing transformation in mouse fibroblasts (NIH 3T3). Further investigation linked the oncogenicity of H-Ras to a mutation which resulted in the substitution of a glycine, at position 12 within its amino acid sequence, to a valine. Remarkably, this single amino acid change was sufficient to trap Ras in a GTP-bound signaling-active state and to commit NIH 3T3 cells to transformation, as read out by anchorage-independent growth. Biochemical and structural analysis clarified our understanding of the mechanism underlying the

functional effects of this point mutation (12-13). Ras(G12V) had lost its ability to hydrolyze GTP and was, thus, locked in a constitutively-active state.

GTPases undergo defined conformational changes upon activation. These changes occur, most notably, within two distinct regions that encircle the bound guanine nucleotide, called the switch 1 and 2 domains (6, 12). The switch domains are paramount to GTPase activation and regulation, serving as hot spots for effector-binding and signal propagation and providing the means by which GEFs and GAPs can modulate their nucleotide state. The mechanism responsible for GTP hydrolysis critically depends on residues located within these domains. Glutamine 61, located within the Switch 2 domain, is responsible for deprotonating and coordinating a water molecule for nucleophilic attack on the gamma phosphate of GTP, which results in the dissociation of inorganic phosphate, leaving behind GDP. Glycine 12, located within the Switch 1 domain, is suitable for this position simply because its small size provides enough room for the attacking water during the GTP-hydrolytic reaction. Substituting this glycine residue with valine, or any other amino acid, imposes a steric constraint that restricts access to the water molecule, thus rendering Ras(G12V) hydrolytically defective. A significant proportion of humans tumors are known to have Ras mutants that are GTP hydrolysis-defective due to mutations at position 12 (14-16). Another commonly GTP hydrolysis-defective Ras mutant involves substitution for glutamine at position 61, as this residue is essential for catalytic activity. Moreover, neurofibromatosis is a disease that arises from the disruption of the gene encoding the Ras GTPase-activating protein (GAP), neurofibromin or NF1.

Thus, the constitutive activation of Ras GTPases by mutagenesis or the absence of an intact regulator are both observed to be contributing factors in human cancers (17-19).

As might be expected, oncogenic transformation can also occur by enhancing the nucleotide exchange capability of GTPases. This appears to occur more often within the Rho subgroup of the Ras superfamily. The earliest hints of the Rho GTPase involvement in cancer came shortly after the connections made between H-Ras and malignant transformation, although the link here was not so obvious at first. Researchers at NIH had identified a genomic element in human diffuse B-cell lymphoma that was capable of transforming NIH3T3 fibroblasts, as read out by cell-cell contact inhibition. This led to the aggregate growths of cells, referred to as foci. Molecular cloning approaches identified the malignant gene, designated Dbl (for Diffuse B-cell Lymphoma), which bore no resemblance to any other oncogenic protein known at the time, but possessed 29% sequence homology to a yeast cell division cycle protein, called Cdc24, which together with the yeast Rho GTPase Cdc42, was required for budding in Saccharomyces cerevisiae (20). Then, in 1991, a major milestone occurred when the protein product of the Dbl gene was shown to be a guanine nucleotide exchange factor (GEF) for the human Cdc42 protein (21).

The Dbl oncogene was the prototype for what we now know is a large (>60 members) family of GEFs. A closer examination of the oncogenic version of Dbl, referred to as oncoDbl, revealed that it had undergone an N-terminal truncation. Indeed, the structure of Rho GEF's, in general, consists of a C-terminal catalytic domain preceded by an N-terminal regulatory domain (22-23). In this case, cells of hematopoietic lineage, which are often prone to accidental chromosomal

translocations, had most likely rearranged the gene encoding proto-Dbl in a manner that separated its GEF domain from its regulatory region. Shortly afterward, another Rho GEF, TIAM1, having undergone a similar truncation as Dbl, was implicated in invasive T-lymphoma. And, today, comprehensive genomic analyses of tumors continue to reveal Rho-specific GEF mutants that are predicted to be activated.

The Rho GTPases

The Rho GTPases are the focus of this work. Consisting of 22 members, this subfamily is central to many important cellular processes. The three most studied members are the ubiquitously-expressed Cdc42, Rac1, and RhoA proteins. As mentioned earlier, Cdc42 was discovered in a cell division cycle mutation screen in budding yeast, where its disruption led to the inability to form new buds, uncoupling cell growth from cell division and resulting in enlarged, multinucleated cells (24). We now know that Cdc42 not only needs to be present, but must be properly localized and activated within the cell, in order to initiate a successful budding event. The initiation process itself has gained a lot of interest in the systems biology field, as a tractable system for studying fundamental symmetry-disrupting events in biology (25). Many of the molecular details in this pathway have been worked out and have suggested a positive feedback loop between Cdc42, its exchange factor Cdc24, and an adaptor protein, Bem1. Cdc42 is initially recruited to a specific point on the plasma membrane and activated by Cdc24. Following this, the GTP-bound Cdc42 then can bind to Bem1. Bem1 contains an additional binding site for Cdc24, which is further recruited shortly thereafter. In sum, a localized positive feedback event takes place

where all three proteins accumulate at a particular point on the inner leaflet of the plasma membrane, which ultimately establishes where the budding site will be.

To cell biologists, Cdc42's involvement in budding foreshadowed a prominent role for it in cell polarity, one that is conserved across all eukaryotic classes. In metazoa, the establishment of cell polarity is equally as crucial as it is in yeast, but considerably more complex. The ensuing asymmetric cell divisions give rise to differential cell fates and different morphologies. By acting through the multi-subunit containing Par (partitioning defective) Complex, which includes Par6, Par3 and the atypical protein kinase Cγ, Cdc42 plays a required role in metazoan development by orchestrating normal tissue organization (26-27). Cdc42's specific involvement in this signaling process remains an active area of investigation.

Shortly after its identification in yeast, Cdc42, along with the Rho family members, Rac1 and RhoA, gained wide recognition for their direct involvement in bringing about dramatic morphological changes in mammalian cells (28-32). When the activated form of Cdc42 was introduced into Swiss 3T3 fibroblasts by microinjection, it caused the formation of thin actin-mediated plasma membranous projections, called filopodia, which radiated in all directions from the cell. Rac1 reorganized the actin cytoskeleton and plasma membrane into large ruffled protrusions, called lamellipodia. RhoA molded actin into tight bundles of microfilaments, called stress fibers and had a contracting effect on the cell, in contrast to the expanding effects seen with Cdc42 and Rac1. All three GTPases facilitated the formation of focal adhesion complexes and cell-cell junctions. These analyses were extended to the remainder of the Rho family, and, indeed, most of the members can

exert a major influence on the cell's morphology in ways that are distinct even between isoforms. Cancer biologists took an interest, particularly because filopodia, lamellipodia, and stress fibers are often observed, and even coexist, in cells treated with certain growth factors (33). Of significance to cell biologists were the connections made between membrane-localized signaling events and organized morphological changes, which could be utilized selectively during many cellular processes. Today, there is enormous interest in understanding how the various Rho GTPases are orchestrated, in time and space, during many of the most complex cases of cellular change, particularly cell migration and neuronal development (32).

Oncogenicity of Cdc42

Cdc42 has thus far demonstrated tangential involvement in cancer, as it lies downstream to an oncogenic event (oncoDbl) and it is capable of inducing morphological changes reminiscent of those caused by certain growth factors. Furthermore, Cdc42's presence is required in order for oncogenic Ras to transform cells (34). However, a direct connection between Cdc42 signaling and cell transformation remained to be established. In 1997, Lin et al. demonstrated that a single point mutant of Cdc42 could transform NIH3T3 cells, as read out by their growth in low serum and colony formation in soft agar (35). The mutation involved the changing of a phenyalanine at position 28, within the Switch 2 domain, to the smaller, but hydrophobic-equivalent, leucine residue. Subsequent structural and biochemical analyses clarified our understanding of this mutation. Cdc42(F28L) was able to constitutively undergo nucleotide exchange, a process that normally requires a

GEF's assistance. While being able to bind nucleotides, this mutant had been mutated so that it could spontaneously replace its bound nucleotide with any others that were introduced, be they GTP or GDP. The X-ray crystal structure of Cdc42 showed phenylalanine 28 to be positioned within only a few angstroms of the guanine base of its bound nucleotide and was quite distanced from the gamma phosphate, ruling out any influence over GTP hydrolysis. As such, it is likely to participate in pi-pi stacking interactions with the similarly aromatic guanine base, accounting for its contribution to nucleotide binding. Removing much of this contact weakened its affinity for nucleotides, but not so much to disrupt binding entirely. Because the cellular concentration of GTP is much greater than GDP, the F28L mutant readily undergoes GDP-GTP exchange in cells, and then ultimately GTP hydrolysis. Indeed, in contrast to oncogenic Ras, (G12V), Cdc42(F28L) does not remain irreversibly in the GTP-bound state, but participates in the same cycle that wild type Cdc42 undergoes, only at much faster rate of conversion between the GDP- and GTP-bound states, and hence is often referred to as a 'fast cycler.' Thus, coupling a cellular environment, rich in GTP, with a fast-cycling capability, a means of constitutive activation for the Rho GTPases has been achieved and it is also the preferred route for their oncogenic activation. Inevitably, this led to identifying which of Cdc42's downstream components are required for its transformation. In fact, the roles of Cdc42 in cell morphology and cell polarity only scratch the surface of its many cellular and biological functions (36) (Figure 1.2). From a systems biology perspective, Cdc42 can be thought of as a centralized node in a signaling network containing numerous branches that extend both upstream and downstream from the GTPase. As it turns out,

Cdc42's oncogenicity depends on the combined activities of several of its downstream targets, with many being necessary, but none sufficient. Some of these molecular events will be discussed below.

Downstream of Cdc42

The human form of Cdc42 had been originally identified in our laboratory in phosphorylation assays searching for binding partners to the epidermal growth factor (EGF) receptor (37). The EGF receptor is an epithelial tissue-specific transmembrane receptor tyrosine kinase that has shown up either mutated and/or amplified in a large number of human cancers and has consequently become a high profile target in drug development. Its growth factor ligand, EGF, induces the co-activation of two EGFR's, by docking onto their extracellular domains and steering their orientations so that their intracellular tyrosine kinase domains can phosphorylate one another. Then, begins a series of autophosphorylation events. In the end, two cytoplasmic-facing EGFR C-terminal tails have become decorated with phosphates and are now able to serve as binding platforms for a number of important signaling proteins, not the least of which is SOS, an exchange factor that activates the Ras GTPases. Another notable downstream component of EGFR is the phosphoinositide-3-kinase (PI3K). When brought within close proximity to the membrane by EGFR, PI3K phosphorylates the lipid phosphatidylinositol(4,5)-bis-phosphate (PIP_2) and converts it to the growth-stimulating and anti-apoptotic second messenger phosphatidylinositol(3,4,5)-trisphosphate (PIP_3). In recruiting a catalog of signaling proteins to the critical plasma membrane, a potent wave of growth activation is thus initiated by EGF. Given that

EGFR's activation lies upstream of numerous signaling cascades, the cell needs to keep the EGFR under constant check to avoid risking unwanted widespread signal initiation that might lead to aberrant cell proliferation. This is accomplished by two important mechanisms: the enzymatic dephosphorylation of the receptor's phosphotyrosines by tyrosine phosphatases and the endocytic uptake of receptors, removing them from the crucial plasma membrane and sorting them for either a delayed return or degradation at the lysosome. The events that lead up to EGFR endocytosis were, in fact, shown to be inhibited by Cdc42 signaling. Receptor tyrosine kinase endocytosis is initiated by the enzymatic attachment of single ubiquitin moieties (monoubiquitination) to its intracellularly-facing lysines and, in the case of EGFR, the ring-type E3 ubiquitin-ligase Cbl is largely responsible for this. Our laboratory discovered a novel means by which Cdc42 signaling sequesters, and hence neutralizes, Cbl (38). The ubiquitously-expressed Rho GTPase exchange factor p85Cool-1 was shown to stimulate nucleotide exchange in Cdc42 and then, unexpectedly, bind to the GTP-bound form of Cdc42, serving an unusual dual role as a Cdc42 regulator and effector. This heterodimer was able to bind to Cbl and prevent it from reaching and ubiquitinating the receptor tyrosine kinase, thereby extending its lifetime at the plasma membrane. Cdc42's ability to transform NIH3T3 cells was shown to be completely dependent on these events.

In 2001, Cdc42's oncogenicity was additionally connected to its newfound role as a regulator of vesicle trafficking between the Golgi and the ER(39). This all depended on a pair of lysine residues located at the distal end of Cdc42's C-terminus. Intra-Golgi trafficking and Golgi to the ER transport of vesicles are both under the

control of the COPI complex. This complex forms at the Golgi membrane by the stepwise recruitment of an Arf family GTPase and a 550 kDa cytoplasmic complex called the coatomer, which consists of COPs- α, β, β', δ, ε, λ, and γ. The γ subunit is largely responsible for the important role of cargo sorting as it specifically engages C-terminal di-lysine motifs of proteins to be transported. Our laboratory showed that the binding of Cdc42 to γCOP, via its lysine pair, is crucial for its involvement in both Golgi to ER vesicle trafficking and transformation. Importantly, this was a clear demonstration of Cdc42's involvement at multiple different locations in the cell (39).

The remodeling of the actin cytoskeleton by Cdc42, while not the primary contributor to its transformation, has been implicated in migration and chemotaxis. The reorganization of actin by Cdc42 is largely explained, at the molecular level, by its activation of the Wiscott-Aldrich Syndrome Protein (WASP) (40). WASP is activated through the binding and removal of its autoinhibitory domain, occurring through a joint effort between the GTP-bound form of Cdc42 and PIP_2 at the plasma membrane. Once both components have relieved the autoinhibition of WASP, it is able to engage and activate the Arp2/3 complex. Arp2/3 is a potent stimulator of actin polymerization and, when bound to WASP, serves to build actin filaments off of preexisting ones, producing branch-like networks of actin, similar to what is seen at the very leading edge of migrating cells (41).

Upstream of Cdc42.

At the heart of Rho family GTPase signaling is their context-dependent interplay with GEFs and GAPs (7). Given the diverse repertoire of signaling locations

for Cdc42 in the cell, the importance of spatial regulation cannot be over emphasized. Occurring simultaneously with Rho-GEF-stimulated activation of Rho GTPases is their observed recruitment to the membrane. This is particularly evident during chemotaxis in neutrophils, where heterotrimeric G-protein activation at the leading edge of the cell results in the localized recruitment and activation of the Rho-GEF Cool-1 and the subsequent recruitment of Cdc42 to that location (58). Moreover, the physical model for bud site selection in yeast also depends on an exchange factor, Cdc24, to recruit Cdc42 to the plasma membrane (25).

While Rho GTPase activation and membrane recruitment seem to go hand in hand, the specific mechanism of their membrane recruitment remains unknown. With over 80 different Rho-specific GEFs, this comprises the largest known group of positive regulators for any of the small GTPases (23). Within this category of regulatory proteins are two distinct families, Dbl and DOCK. Both are defined by a conserved module, DH-PH (Dbl homology - Plextrin homology) and DHR1-DHR2 (DOCK Homology Region 1/2), respectively (42-43). In each case, the DH and DHR2 domains, respectively, have been shown to be responsible for catalyzing nucleotide exchange, with each using a distinct mechanism for perturbing the coordination of the GTPase's magnesium ion, ultimately leading to its release, along with GDP. Equally as important are those regions not directly involved in nucleotide exchange, called the regulatory domains. These regions, in general, present an obstacle for GEF activation. Consisting mostly of protein-protein and/or protein-lipid interaction modules, they serve to occlude the active site, negatively constrain substrate binding or conceivably assist with substrate binding upon activation, in

manners not unlike the regulatory modes of the protein kinases (44). GEF activation often requires a posttranslational modification event or the entrance of a regulatory domain binding protein or lipid, all occuring under precise spatiotemporal control. The Rho-GEF's regulatory domains are, by and large, where each of the members diverge, making the compelling case that the large size of the Rho GEF family is a reflection of the diversity of upstream signaling events and locations that need to call upon the Rho members.

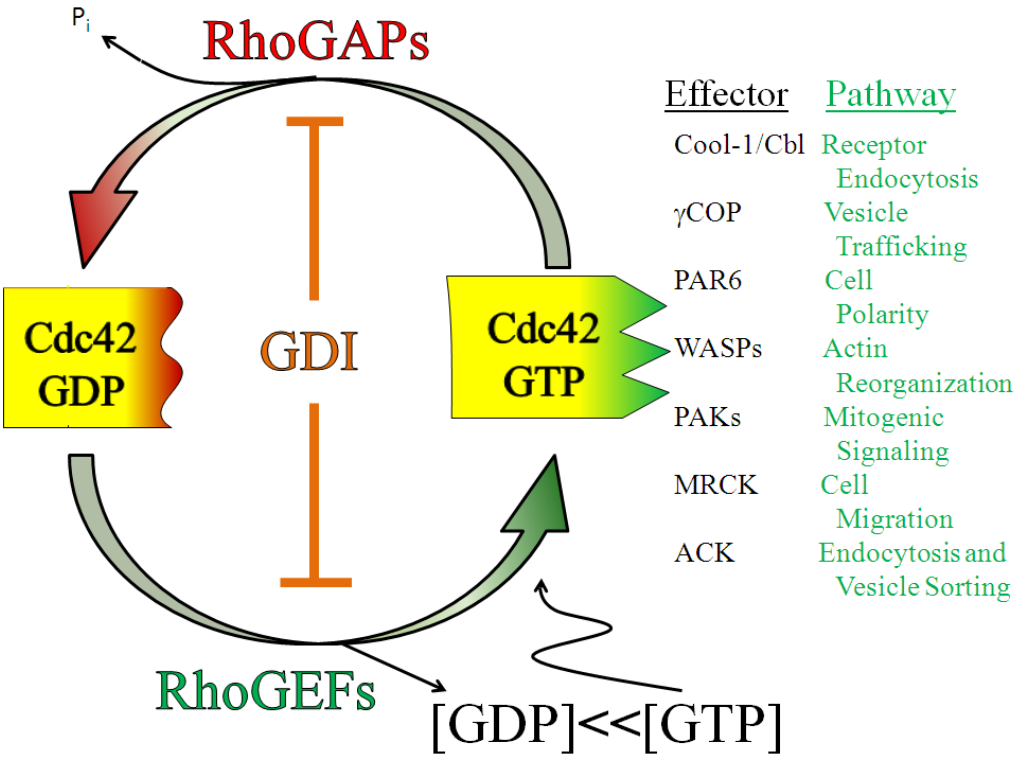
The Rho-GAP family, consisting of over 50 members, is under an exquisite regulation in its own right (7). They accelerate GTP-hydrolysis by inserting an arginine residue in trans into the active site of the GTPase and thereby stabilize the developing negative charge of the transition state. Similar to the Rho-GEFs, spatiotemporal regulation plays an important role in Rho-GAP activity. One particularly interesting example, is the RhoA-specific GAP, Deleted in Liver Cancer 1 (DLC1). DLC1 has gained recognition as a prominent tumor suppressor, where its deletion has been identified in over 90% of human hepatocellular carcinomas and it was found to be epigenetically silenced in a variety of other cancers (45). Recent biophysical work identified a putative PIP_2 interaction motif, more specifically a stretch of positively-charged residues, tangential to its catalytic domain, which was shown to be required for it to suppress Rho GTPase-stimulated transformation. PIP_2 resides in the inner leaflet of the plasma membrane and conceivably its recruitment of DLC1 may be a necessity for keeping Rho GTPase plasma membrane signaling in check in many tissues.

In spite of the enormity of both the Rho- GEF and Rho-GAP families, they can, for our purposes, be distilled down to two different sets of catalytic domains, one that activates Rho GTPases and the other that shuts down their activation, respectively. Crystallographic and kinetic analyses have made great headway in clarifying the catalytic mechanisms for each. The highly focused nature of that work, however, ignores an important broader picture of Rho GTPase signaling, one that is of much interest to us. How do GEFs and GAPs influence the spatial dynamics of the Rho Family members? Given that Cdc42 participates in signaling at multiple locations, what is the mechanism of its recruitment and departure from membranes? These questions will be further elucidated upon in the following paragraphs, as they strike right at the core of another family of regulators which are truly unique to the Rho GTPases.

RhoGDI

In addition to Rho GEFs and Rho GAPs, there is a third important class of Rho regulators, called the Guanine Nucleotide Dissociation Inhibitors (GDIs). In contrast to the GEFs and GAPs, with their clearly defined roles as positive and negative regulators, respectively, an analogous designation for GDI has not been so simple. The GDI family consists of three members: GDIα, GDIβ, and GDIγ. Among them, it is the ubiquitously-expressed GDIα that regulates most of the Rho family members. GDI was originally identified, and named, as a factor that inhibited GDP release from RhoA, in the presence of either Rho GEFs or excess EDTA (46). Consequently, GDI was regarded as a negative regulator of Rho GTPase signaling, as it stabilized the

Figure 1.2 The small G-protein regulatory cycle modified for Cdc42. A schematic representation of the regulatory factors influencing the signaling activities of Cdc42 and a selected listing of Cdc42's targets and downstream signaling pathways.


P_i
RhoGAPs
Cdc42
GDP
GDI
Cdc42
GTP
RhoGEFs
[GDP]<<[GTP]
Effector
Pathway
Cool-1/Cbl
Receptor Endocytosis
γCOP
Vesicle Trafficking
PAR6
Cell Polarity
WASPs
Actin Reorganization
PAKs
Mitogenic Signaling
MRCK
Cell Migration
ACK
Endocytosis and Vesicle Sorting

GDP-bound form of RhoA and prevented GEFs from catalyzing GDP-GTP exchange. Furthermore, the overexpression of GDI in mammalian tissue culture was shown to have an inhibitory effect on Rho family signaling activities. However, work from our laboratory demonstrated that the GDI was able to engage the activated GTP-bound form of Cdc42 and inhibit the intrinsic and GAP-stimulated GTP hydrolysis, thus extending the lifetime of Cdc42's activated state (47). Moreover, it was subsequently shown that GDI was essential for the oncogenic activity of Cdc42 (F28L) and its ability to transform NIH 3T3 cells (48). Collectively, these results indicated that GDI cannot be simply considered as a negative regulator, but instead it must be playing some type of a positive role in Cdc42 signaling.

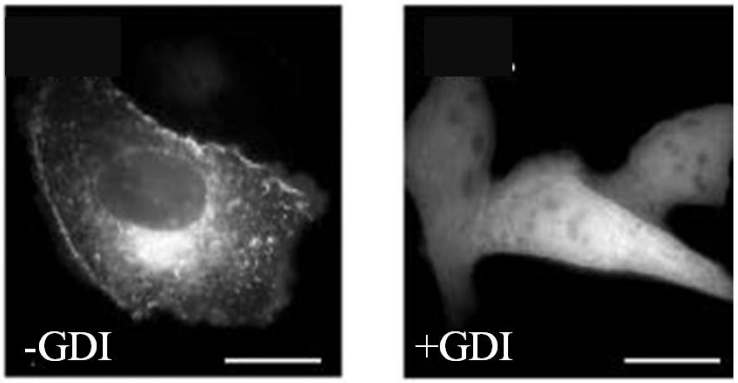
Indeed, a third and certainly an important aspect of the regulation imparted by the GDI concerns its ability to stabilize the soluble (cytoplasmic) pool of Rho GTPases (49). Overexpressing the GDI in cells profoundly affects the cellular localization of Cdc42, shifting it from the membranes to the cytosol (Figure 1.3A). For this to occur, GDI takes advantage of an important posttranslational modification on the GTPase. Virtually all Rho GTPase signaling occurs at the surface of a membrane, as this is the favored location for all of their known effectors. In order to associate with membranes, these GTPases must undergo the covalent attachment of a 20 carbon isoprenyl moiety (i.e. geranylgeranylation) which serves as a hydrophobic membrane anchor. The eukaryotic cell has a systematic way of implementing this process, which centers around the presence of a C-terminally located CAAX (C=Cys, A=aliphatic, X=any amino acid) tetrapeptide motif, present in most of the Rho family members. Following synthesis on the ribosome, these GTPases are recognized by the

cytosolic enzyme, geranylgeranyltransferase I (GGTI), which uses a coordinated zinc ion to deprotonate and position the thiol of the cysteine, within the CAAX motif, for a nucleophilic attack on the C1 carbon of geranylgeranyldiphosphate. The pyrophosphate gets displaced and the geranylgeranyl group is transferred to the cysteine. The newly geranylgeranylated GTPase then associates nonselectively with cellular membranes, primarily with the endoplasmic reticulum, owing to its dominant surface area in the cell. This is also the location where the next two enzymes in the modification process reside. The transmembrane CAAX protease cleaves off the AAX, priming it for cysteine carboxymethylation by the membrane bound methyltransferase. In the end, a C-terminal CAAX sequence is converted to a geranylgeranylated, carboxy-methylated cysteine, thereby ensuring that the Rho GTPase can associate with the lipid bilayers. Of utmost importance, GDI will only interact with the geranylgeranylated form of Rho-family GTPases. This modification is critical for the ability of the GDI to inhibit nucleotide dissociation and GTP hydrolysis, as well as for its ability to stabilize the cytoplasmic pool of Rho GTPases.

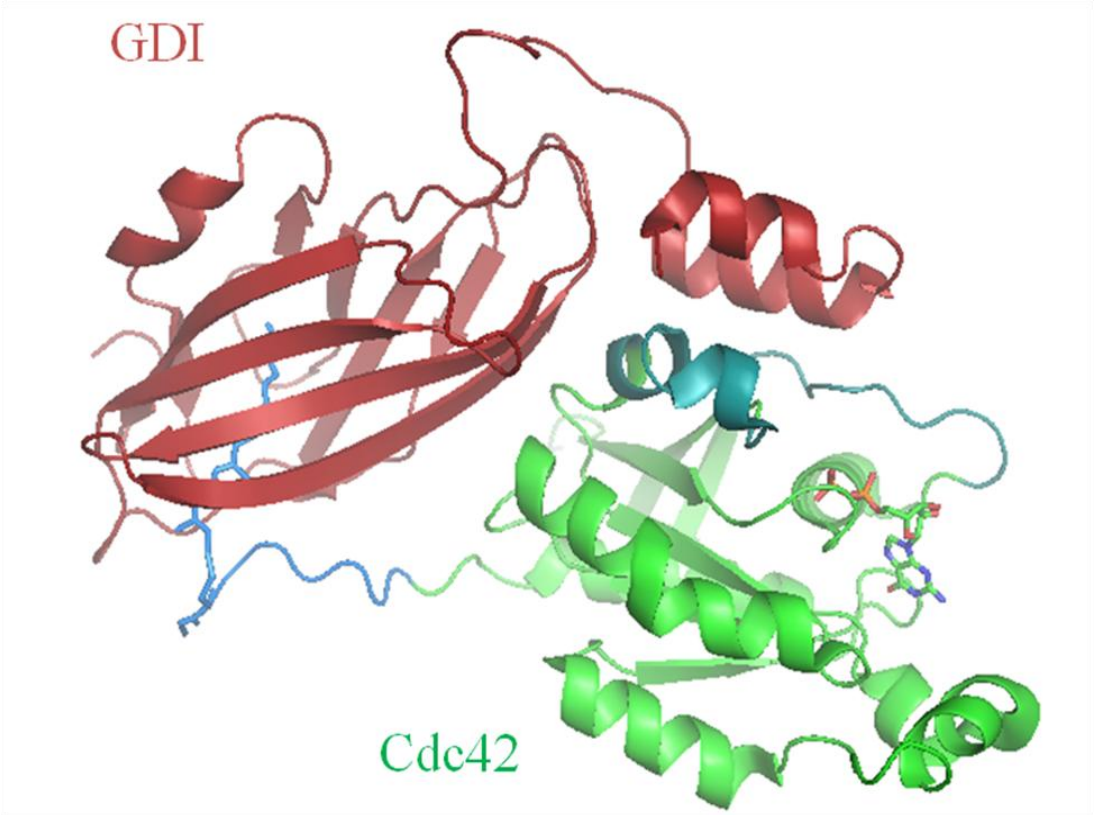
The physical basis for the interaction between Cdc42 and GDI was established in 2001, when our laboratory succeeded in solving the high resolution x-ray crystal structure of geranylgeranylated Cdc42-GDP in a complex with GDI (50) (Figure 1.3B). Two regions on the GDI were critical for its binding interaction with Cdc42, one involving the N-terminal third of the GDI molecule, which is mostly unstructured, and the other involving the C-terminal portion of the GDI, which assumes a β-immunoglobulin fold. Despite having little secondary structure, the N-terminal region of the GDI makes solid contact with both the switch 1 and 2 domains of Cdc42 and, as

Figure 1.3 Regulation of membrane binding of Cdc42 by RhoGDI. **A)** Images of MDCK cells transiently expressing GFP-tagged Cdc42, examined under digital epifluorescnce microscopy (left and right). On the right hand side, the cells were additionally transfected with untagged RhoGDI (adapted from ref 54) **B)** 2.6 Å X-ray crystallographic structure of the Cdc42(green)/RhoGDI(magenta) complex. The Switch regions of Cdc42 are labeled in turquoise and its C-terminal region, including its geranylgeranyl moiety, are labeled in blue (adapted from ref 50).

A.



B.



such, is responsible for regulating the nucleotide state of Cdc42. Indeed, it has been shown biochemically, that N-terminal truncation mutants of GDI lose their ability to inhibit nucleotide dissociation and GTP hydrolysis, even at saturating concentrations (51). It appears that the N-terminal domain of GDI stabilizes the switch domains of Cdc42 in a conformation optimal for coordinating Mg^{2+}. Since GEFs and EDTA perturb the binding of Mg^{2+}, this can account for the ability of the GDI to block the GEF-stimulated GDP-GTP exchange reaction. Furthermore, in interacting with both of Cdc42's switch regions, the GDI's binding interface largely overlaps with that of the GEFs, thus implying a competitive inhibition. A high resolution complex of GDI with GTP-bound Cdc42 would be of interest for many important reasons, as it would give physical insight into the ability of GDI to inhibit GTP hydrolysis. Aside from an obvious direct binding competition with GAPs, GDI must be influencing the activated conformation of Cdc42so as to inhibit its intrinsic GTP hydrolysis. Additionally, a side-by-side comparison with Cdc42 in its GDP-bound state could provide an intriguing rationale for how a Rho regulator is able to accommodate the switch domain conformations of both the active and inactive states of Cdc42.

The third function of the GDI, its ability to help promote the translocation of Rho GTPases from the membranes to the cytosol, is largely explained by the interaction of the C-terminal portion of the GDI with the isoprenoid moieties of these GTPases. After undergoing geranylgeranylation and carboxy-methylation, Cdc42 is essentially fixed to the membrane surface. In order for Cdc42 to dissociate from the membrane and remain in the cytosol, the GDI must help to compensate for the entropic loss that comes with introducing a hydrophobic carbon chain into an aqueous

environment. GDI accomplishes this by providing a binding pocket for the geranylgeranyl moiety through its β-immunoglobulin C-terminal fold. This is essentially a water-soluble globular domain with an internal pocket, lined with hydrophobic residues, ideal for binding the geranylgeranyl moiety of Cdc42. This pocket is optimal for specifically binding prenyl chains, as acylated C-terminal mutants of Cdc42 fail to interact with GDI. Thus, GDI is able to help Cdc42 make the transition from the membranes into the cytosol by providing a hydrophobic pocket custom fit for the geranylgeranyl moiety.

The Polybasic Domain

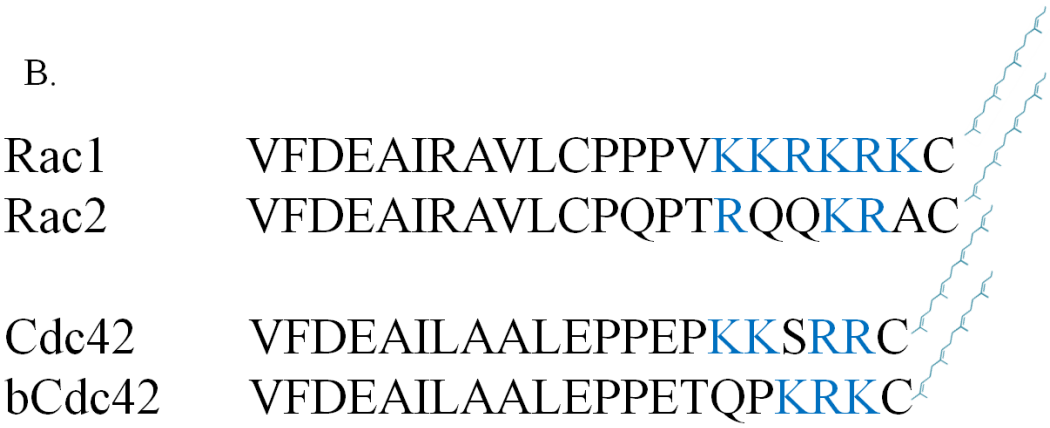
Many Rho GTPases contain regions that electrostatically strengthen their interaction with membranes, synergizing with their geranylgeranyl modification (Figure 1.4). At Cdc42's C-terminus, directly preceding its geranylgeranyl, are two pairs of positively-charged residues (KKSRR-C-geranylgeranyl). These include the lysine pair, mentioned earlier, that is involved in Cdc42's regulation of vesicle trafficking. Rac1 and RhoA also contain positively-charged residues upstream of their geranylgeranyl moieties. This region has been referred to as the polybasic domain, emphasizing its high frequency of basic residues, as well as the hypervariable region, because it has the greatest sequence diversity among the Rho family. Indeed, there are isoforms of Cdc42, Rac1, and RhoA that differ essentially within their respective hypervariable regions. The brain-specific isoformof Cdc42, Cdc42b, is a splice variant of Cdc42 with a unique exon incorporated into its C-terminus, which

Figure 1.4 The polybasic domain of the Rho GTPases. **A)** Illustration of the common domain organization of the Rho GTPases. The GTPase domain is labeled in green and the membrane binding (polybasic + geranylgeranyl moiety) domain is labeled in blue. **B)** Alignment of the C-terminal amino acid sequences of the Rac and Cdc42 isoforms (basic residues are highlighted in blue).

A.


+ + + +
N
Signaling
Membrane Docking
C

B.


Rac1 VFDEAIRAVLCPPPVKKRKRKC
Rac2 VFDEAIRAVLCPQPTRQQKRAC
Cdc42 VFDEAILAALEPPEPKKSRRC
bCdc42 VFDEAILAALEPPETQPKRKC

introduces a new hypervariable region, while leaving the remaining 97% of the sequence unchanged. Our laboratory has shown, as a consequence, that Cdc42b is capable of inducing striking filopodia at the axonal ends of cultured neurons, and that this effect is significantly enhanced, relative to what is observed with the ubiquitous form of Cdc42 (M. Endo, unpublished). Furthermore, Cdc42b is believed to engage a different repertoire of effector proteins than the ubiquitously-expressed Cdc42, as it was found to initiate a distinct subset of signaling cascades in neurons (M. Endo, unpublished). The polybasic domain also confers distinguishing traits onto the Rac isoforms, Rac1 and Rac2. The polybasic domain of Rac1 contains a stretch of 6 consecutive basic residues, whereas Rac2's contains half the amount of basic residues. Arguably, the most glaring consequence is the vastly different localization profiles seen between these two Rac isoforms. Rac1 is found exclusively at the plasma membrane and endosomes, while Rac2 largely populates the more internally-localized Golgi, ER, and nuclear membranes (57). Similar to the Cdc42 isoforms, these disparate subcellular locations set boundaries to which effectors each isoform can encounter. It is likely that for this reason, Rac1 and Rac2 make distinct contributions to the differentiation of hematopoeitic cells.

Prenyl moieties will associate with nearly all membranes in the cell, with the possible exception of lipid raft domains. To achieve membrane-binding specificity, many Rho GTPases utilize their polybasic domain to strengthen their binding to negatively-charged membranes. In the cell, the organellar membranes are stratified such that as you move outward from the nuclear membrane, the membrane surfaces become progressively more negatively-charged, with the inner leaflet of the plasma

membrane encompassing the most negative surface in the cell. This is due largely to the increasing peripheral concentrations of phosphatidylserine and phosphatidylinositol-4,5-bisphosphate (PIP_2) (56-57). The highly-positive polybasic domain of Rac1 is charge-matched with the highly negative plasma membrane and endosomal membranes, explaining its presence at those locations, while the less positive polybasic domain of Rac2 is relatively uncommitted to any particular membrane and as a result remains internally localized on the outer surfaces of the ER. Cdc42 is found to localize at both the plasma membrane and the endomembranes (specifically the Golgi).

Reconstitution of Cdc42's interactions at the membrane

The GDI gene has accompanied Cdc42 ever since their origin in single-celled eukaryotes. GDIα, in particular, has remained structurally conserved throughout metazoa and, apart from the emergence of two tissue-specific isoforms, GDIβ and GDIγ, is largely self-sufficient in its regulation of the Rho family members. Its ubiquity notwithstanding, the cellular function for GDI still remains uncertain. The fact that it is required for Cdc42 transformation makes a strong case for its role as a positive regulator of Rho signaling (48). Unfortunately, providing a cellular rationale for this positive regulation has never been straightforward. In order to approach these questions, we believe one must first understand the underlying mechanisms by which GDI regulates the binding of Cdc42 to membranes. To tackle these questions, we developed *in vitro* reconstitution systems capable of sensitive and time-resolved measurements of geranylgeranylated Cdc42's interaction and interplay with GDI and

membranes. This work, described in Chapter 2, provides a detailed analysis of Rho GTPase-membrane interactions (52). In utilizing these approaches, many unexpected characteristics of these interactions were revealed. Although GDI binds with equally high affinity to both the active and inactive states of Cdc42 in solution (53), their interaction becomes nucleotide-specific at the membrane. GDI is selective for the inactive GDP-bound form of Cdc42 with an affinity tenfold greater than the activated GTP-bound form. Here, a newfound appreciation was gained for the role of the membrane in Cdc42 signaling.

Despite having a highly hydrophobic geranylgeranyl tail at its carboxy terminal, Cdc42 spends a much shorter time at the membrane than expected before its intrinsic dissociation. In particular, we found the intrinsic rate of release of Cdc42 from liposomes to be nearly identical to the rate for membrane dissociation measured in the presence of GDI, leading us to reevaluate our mechanistic understanding of how GDI influences the membrane-to-cytosol partitioning of Cdc42.

Upon revising our model of the interaction of GDI with Cdc42, we next examined how RhoGAPs and RhoGEFs influence the partitioning of Cdc42 between GDI and membranes, which is the focus of Appendix 1. Here, we show that the membrane association-dissociation cycle is directly coupled to its GTP-binding/GTPase cycle. The data in Chapter 2 and Appendix 1 were organized into a comprehensive model depicting how Cdc42 is able to cycle on and off the membrane.

In Chapter 3, we set out to see if certain phospholipids are more effective than others in stabilizing the association of Cdc42 with membranes. Our *in vitro* reconstitution assays are ideal for exploring this, as they allow us to tweeze apart the

contributions made by specific phospholipids in binding to Cdc42. As will be described, these studies led us to appreciate an important role for PIP_2 in binding to Cdc42 and influencing its ability to send signals that are important for cell growth and oncogenic transformation.

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CHAPTER 2

NEW INSIGHTS INTO HOW THE RHO-GUANINE NUCLEOTIDE DISSOCIATION INHIBITOR REGULATES THE INTERACTION OF CDC42 WITH MEMBRANES*

2.1 Introduction

The Rho-family GTPases are a tightly regulated class of signaling proteins that control a number of important cellular processes. Known most prominently for their ability to remodel the actin cytoskeleton in mammalian cells (1-3), members of this GTPase family have been shown to play essential roles in cell migration, epithelial cell polarization, phagocytosis, and cell cycle progression (4-11). The Rho-family member Cdc42 was discovered for its essential role in bud formation in *Saccharomyces cerevisiae* (12). However, following its identification in higher organisms (13), Cdc42 has been implicated in a diverse array of signaling pathways including those involved in the regulation of cell growth and in the induction of malignant transformation (14). Indeed, point mutations which enable Cdc42 to undergo the spontaneous exchange of GDP for GTP cause NIH3T3 cells to form colonies in soft agar and grow in low serum, two hallmarks of cellular transformation (15). The introduction of activated Cdc42 mutants into nude mice gives rise to tumor formation (16). Moreover, cellular transformation by oncogenic Ras, one of the most commonly mutated proteins in human cancers, requires the activation of Cdc42 (17). [1]

* Chapter 2 is adapted from Johnson, et al., (2009) *J. Biol. Chem.* 284, 23861-71

At the molecular level, there are a number of mechanisms that possibly contribute to the roles played by Cdc42 in cell growth control and cellular transformation. These include the ability of Cdc42 to activate the JNK and p38/Mpk2 signaling pathways (18-20), as well as spatially regulate proteins implicated in the establishment of microtubule-dependent cell polarity including GSK-3β and APC (21), extend the lifetime of epidermal growth factor receptor-signaling activities by sequestering Cbl, an ubiquitin E3 ligase (22), and influence intracellular trafficking events (23,24). In order to mediate such a wide range of cellular responses, two parameters must be properly regulated: the activation state of Cdc42 and its subcellular localization. As is the case with other GTPases, the activation of Cdc42 occurs as an outcome of GDP-GTP exchange, which then enables it to undergo high affinity interactions with effector proteins (25-27). Upon the hydrolysis of GTP to GDP, Cdc42 is converted back to a signaling-inactive state. Two families of proteins work in opposing fashion to regulate the GTP-binding/GTPase cycle of Cdc42. GAPs (GTPase-Activating Proteins) recognize the GTP-bound form of Cdc42 and accelerate the hydrolysis of GTP to GDP, rendering Cdc42 inactive (28,29). GEFs (Guanine nucleotide Exchange Factors) stimulate the dissociation of GDP from Cdc42, thereby promoting the formation of its signaling-active, GTP-bound state (29,30).

Of equal importance to its activation status is the spatial regulation of Cdc42. This is highly contingent on the particular cellular membranes that serve as sites of binding and/or recruitment of Cdc42 (31-33). The vast majority of *in vitro* studies performed on Cdc42 have been carried out in the absence of lipids, which is an important omission, considering that virtually all of the physiological functions of Cdc42 occur

on a membrane surface (34). Cdc42, along with most other Rho-family GTPases, undergoes a series of carboxy-terminal modifications which result in the covalent attachment of a 20-carbon geranylgeranyl lipid anchor (35-37). Directly preceding this lipid tail is a sequence of basic residues that further stabilizes the association of Cdc42 with the membrane surface (31,33,38). A ubiquitously expressed 22 kDa protein called RhoGDI (Rho Guanine nucleotide Dissociation Inhibitor) was found to form a soluble (cytosolic) complex with Cdc42 and other Rho GTPases and to apparently promote their release from membranes (39,40). RhoGDI was originally discovered and named for its ability to block the GEF- and EDTA-stimulated nucleotide exchange activity of Rho-family GTPases (39,41,42), and then subsequently shown to inhibit the GTP-hydrolytic activity of Cdc42 (43) and to be capable of interacting with the GDP- and GTP-bound forms of Cdc42 in solution with equal affinity (44). The x-ray crystal structure of a complex between RhoGDI and Cdc42-GDP revealed two types of binding interactions (45). An amino-terminal regulatory arm of RhoGDI was shown to form a helix-loop-helix motif that binds to both of the switch domains of Cdc42, leading to the inhibition of GTP hydrolysis and GDP dissociation (45,46). The carboxy-terminal two-thirds of RhoGDI assumes an immunoglobulin-like domain, forming a hydrophobic pocket that in effect provides a membrane-substitute for the geranylgeranyl moiety of Cdc42. Following release from membranes, the lipid anchor of Cdc42 binds in the hydrophobic pocket of RhoGDI, thereby helping to maintain Cdc42 in solution (45-47).

Prior work from our laboratory has demonstrated an essential role for RhoGDI in Cdc42-mediated cellular transformation. Based on the x-ray crystal structure for the

Cdc42-RhoGDI complex, Arg66 of Cdc42 makes multiple contacts with RhoGDI. When this residue was changed to alanine, Cdc42 was unable to bind to RhoGDI, but was still capable of interacting with its other regulatory and effector proteins. Interestingly, when the R66A mutant of Cdc42 was examined in the constitutively active Cdc42(F28L) background, the resulting Cdc42 double-mutant was no longer able to transform cells (48). Knocking-down RhoGDI by siRNA also blocked transformation by Cdc42. These findings highlighted a key role for RhoGDI in the ability of Cdc42 to stimulate signaling pathways of importance to cellular transformation, presumably by influencing the membrane association of Cdc42 and ensuring its proper cellular localization.

In the present study, we have set out to better understand how RhoGDI regulates the signaling functions of Cdc42, and in particular, how RhoGDI affects the association of Cdc42 with membranes. We show how the membrane plays a previously unappreciated role in allowing RhoGDI to distinguish between the signaling-inactive (GDP-bound) and signaling-active (GTP-bound) forms of Cdc42. By assaying the binding of Cdc42 to insect cell membranes and compositionally-defined liposomes through different approaches including a sensitive, real-time FRET read-out, we have been able to establish how RhoGDI influences the ability of Cdc42 to transition between a membrane-bound and soluble species. This has led us to propose a new mechanism describing how RhoGDI performs its important regulatory function.

2.2 Methods

Preparation of insect cell-expressed Cdc42

Cdc42 was purified as a His_6-tagged protein by baculovirus-mediated expression in *S. frugiperda* (Sf21) insect cells. All purification steps were performed at 4°C. One-liter stirred cultures of Sf21 cells underwent baculoviral infection for 48 hours as carried out at Kinnakeet Biotechnology (Midlothian, VA). Cell pellets were resuspended in 40 mL of hypotonic buffer (20 mM sodium borate, pH 10.2, 5 mM $MgCl_2$, 200 μM PMSF, and 1 μg/mL aprotinin and leupeptin) and disrupted by Dounce homogenization. The membrane-containing components of the lysate were spun down at 150,000 × *g* in a Ti70 rotor (Beckman Coulter) for 20 minutes, after which the supernatant containing non-prenylated Cdc42 was discarded and the pellet was resuspended in 50 mL of TBS-containing magnesium (TBSM; 50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$). The procedure was repeated twice and the resulting pellet was resuspended in TBSM that contained 1% Triton-X 100. The lysate was further homogenized and agitated for 30 minutes on a rotisserie, resulting in the solubilization of the geranylgeranylated Cdc42. The remaining insoluble fraction was pelleted in a tabletop centrifuge at 9,000 × *g* for 20 minutes at 4°C and discarded. The supernatant containing detergent-solubilized, isoprenylated His_6-tagged Cdc42 was incubated for 30 minutes with chelating Sepharose-beads (QIAGEN) charged with Ni^{2+}. The beads were washed with 400 mL of a high salt buffer (50 mM Tris, pH 7.5, 700 mM NaCl, 5 mM $MgCl_2$, 0.1% CHAPS, and 20 mM imidazole) and protein was eluted with 10 mL of elution buffer (50 mM Tris, pH 7.5,

150 mM NaCl, 5 mM $MgCl_2$, 0.1% CHAPS, and 500 mM imidazole). The fractions containing Cdc42 were pooled and concentrated to a volume of 2 mL.

Preparation of E. coli-expressed Cdc42 and RhoGDI

Bacterial cells harboring plasmids encoding His_6-Cdc42 or GST-RhoGDI were grown at 37°C until an OD of 0.8 was reached. Induction was initiated by the addition of isopropyl 1-thio-β-D-galactopyranoside (1 mM), and the cells were allowed to grow for another 3 hours before pelleting at 6,000 $\times$ *g* for 10 minutes. Cell pellets were homogenized in TBSM and lysed by sonication. Cell debris was centrifuged at 20,000 $\times$ *g* for 30 minutes, and the supernatant was used for purification. His_6-tagged Cdc42 was purified using Ni^{2+}-charged Sepharose beads as described above. Supernatants containing GST-tagged RhoGDI were incubated with glutathione-beads (Amersham Biosciences) and equilibrated with TEDA buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1 mM sodium azide) for 30 minutes at 4°C. The beads were then washed with several column volumes of TEDA-containing 500 mM NaCl. After a final rinse with TBSM, the protein was eluted with 10 mM glutathione in TBSM. All eluents were concentrated in a 10 MWC Amicon Ultra concentrator (Fisher). Protein concentrations were determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

Preparation of membrane vesicles from insect cells

Membranes were prepared from uninfected Sf21 cells, using a modification of the Thom procedure (49). Briefly, 10 mL of stationary phase cells were incubated in 1 mL of hypotonic buffer (20 mM sodium borate, pH 10.2) at 4°C and then subjected to Dounce homogenization. The cellular debris was pelleted by centrifugation at 900 × *g* for 10 minutes and the supernatant was transferred to a new tube and centrifuged at 16,000 × *g* for 20 minutes, separating the membranes from the soluble components. The membrane-rich pellet was resuspended in 1 mL of TBSM.

Preparation of liposomes

Two approaches were used to prepare liposomes. For large liposomes (i.e. several microns in diameter) that can be pelleted by low-speed centrifugation, rapid solvent exchange was utilized (50). For fluorescence spectroscopy experiments, smaller lipid vesicles were prepared by extrusion (Avanti mini-extruder). All lipids used in these experiments were obtained from Avanti Polar Lipids, unless stated otherwise. The standard lipid composition in molar percentages was 35% PE, 25% PS, 5% PI, and 35% cholesterol (Nu Chek Preps).

Liposome/insect cell membrane centrifugation assays

To assay the binding of Cdc42 or RhoGDI to liposomes, 1 μg of *E. coli*-expressed Cdc42, insect cell-expressed Cdc42, or *E. coli*-expressed RhoGDI was incubated in 200 μL of 1 mg/mL lipids, prepared by rapid solvent exchange, for 10 minutes at room temperature and centrifuged at maximum speed for 20 minutes. Supernatants and lipid pellets were examined by SDS-PAGE. For radioactive assays measuring the dissociation of Cdc42 from liposomes or insect cell membranes, Cdc42 (100 pmol) was preloaded with [^{35}S]GTPγS (1400 cpm/pmol) or α[^{32}P]GTP (1500 cpm/pmol) by EDTA-stimulated nucleotide exchange. The α[^{32}P]GTP bound to Cdc42 was allowed to hydrolyze to α[^{32}P]GDP by performing a 30 minute incubation on ice in the presence of excess magnesium. The protein was then mixed with 500 μL of insect cell membranes (or with liposomes prepared from 1 mg/mL lipids by rapid solvent exchange) for 10 minutes, and pelleted for 20 minutes at 16,000 × *g* in a microfuge. The lipids were resuspended in TBSM buffer containing different concentrations of RhoGDI and incubated for 10 minutes, subjected to a final centrifugation, and then radioactivity was measured in the supernatant and lipid pellet. The degree of release of the Cdc42-RhoGDI complex from the liposome/membrane preparations as a function of RhoGDI concentration was fit to the following equation:

$$[\text{Cdc42} : \text{GDI}] = \frac{[\text{Cdc42}]_{\text{TOTAL}}[\text{GDI}]}{K_D + [\text{GDI}]} \qquad (1)$$

where K_D is the dissociation constant describing the interaction between Cdc42 and RhoGDI, as reflected by the transition between the membrane- and soluble-forms of Cdc42, and [GDI] is the concentration of free RhoGDI where [GDI]~$[GDI]_{total}$.

Fluorescence assays for the interaction of Cdc42 with liposomes

Fluorescence measurements were made using a Varian Cary Eclipse fluorimeter in the counting mode. Excitation and emission wavelengths were 365 and 440 nm, respectively. Samples were stirred continuously at 25°C in TBSM. To prepare HAF-(Hexdecanoylaminofluorescein)-labeled lipids for FRET assays, 1.25 nmol of HAF (Molecular Probes) was vortexed in 50 μL of lipids (1 mg/mL).

The association of insect cell Cdc42 with liposomes was assayed as follows. A Cdc42-Mant-nucleotide complex (50 nM) was mixed with liposomes prepared by extrusion that contained different concentrations of HAF-labeled lipids, resulting in the quenching of Mant-fluorescence. For comparison, the association curves were fit to a single exponential equation of the form:

$$F(t) = F_o e^{-k_{obs} t} \qquad (2a)$$

or in the case of increasing fluorescence (i.e. when monitoring the release of Cdc42-Mant-nucleotide complexes from liposomes containing HAF):

$$F(t) = F_o(1 - e^{-k_{obs}t}) \qquad (2b)$$

where F(t) represents the relative fluorescence as a function of time, F_o represents the initial fluorescence, and k_{obs} is the rate constant describing the fluorescence change. A rate constant (k_{off}) characterizing the dissociation of Cdc42 from liposomes was estimated by applying the following equation:

$$k_{obs} = k_{off} + k_{on}[Lipids] \qquad (3)$$

To monitor the release of Cdc42 from liposomes, Cdc42 was preloaded with Mant-nucleotide (GDP or GMP-PNP) and incubated with 30 μL of HAF-containing liposomes at room temperature for 5 minutes. The mixture was added to the cuvette and at the designated time-point, RhoGDI was added with stirring. Typically, the initial 2.5 minutes were recorded, generating traces that monitored the changes in Mant fluorescence due to changes in FRET between Mant-nucleotide-bound Cdc42 and liposomes containing HAF.

The FRET assay used in these studies provides a real-time read-out of the formation of the Cdc42-RhoGDI complex as a function of time. The interaction between Cdc42 and RhoGDI is proposed to consist of two steps, an initial binding step and a rate-limiting membrane-dissociation step, with k_1 and k_{-1} representing the

forward and reverse rate constants, respectively, for step 1, while k_2 and k_{-2} are the forward and reverse rate constants, respectively, for step 2.

$$\text{Cdc42 + RhoGDI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(membrane-associated complex)}}{\text{Cdc42:RhoGDI}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(soluble complex)}}{\text{Cdc42:RhoGDI}}$$

By assuming the initial binding step is in rapid equilibrium, and that the second step is rate-limiting, we arrive at the following rate equation for the association of Cdc42 with RhoGDI:

$$\frac{[\text{Cdc42 : GDI}]}{[\text{Cdc42 : GDI}]_{max}} = 1 - e^{-k_{obs}t}$$

$$\text{where } k_{obs} = \left[\frac{k_2[\text{GDI}]}{K_{D1} + [\text{GDI}]} \right] + k_{-2} \qquad (4)$$

The plots of k_{obs} versus [GDI] were fit with equation (4) where $K_{D1} = \frac{k_{-1}}{k_1}$.

Derivations of equation 4:

Starting with the following two-step binding interaction,

$$\text{Cdc42 + RhoGDI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(Membrane-Associated)}}{\text{Cdc42•RhoGDI}}^{*} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(Soluble)}}{\text{Cdc42•RhoGDI}}$$

and designating the second step as rate-limiting, we have the following rate equation:

$$\frac{d[\text{Cdc42•GDI}]}{dt} = k_2[\text{Cdc42•GDI*}] - k_{-2}[\text{Cdc42•GDI}] \qquad (5)$$

It is assumed that the initial binding step is in rapid equilibrium relative to the second step (k_1[GDI], $k_1 >> k_2, k_{-2}$), so $\frac{k_1}{k_{-1}}$ is assumed to be at steady state:

$$K_{D1} = \frac{k_{-1}}{k_1} = \frac{[Cdc42][GDI]}{[Cdc42 \bullet GDI^*]} \tag{6}$$

Substituting for $[Cdc42]_{free}$ and rearranging equation 6 yields equation 7:

$$[Cdc42 \bullet GDI^*] = \frac{[Cdc42]_{total}[GDI] - [Cdc42 \bullet GDI][GDI]}{K_{D1} + [GDI]} \tag{7}$$

Substituting equation 7 into equation 5 yields equation 8:

$$\frac{d[Cdc42 \bullet GDI]}{dt} = k_2\left(\frac{[Cdc42]_{total}[GDI] - [Cdc42 \bullet GDI][GDI]}{K_{D1} + [GDI]}\right) - k_{-2}[Cdc42 \bullet GDI] \tag{8}$$

Combining all [Cdc42•GDI] terms and integrating with respect to time yields equation 5:

$$[Cdc42 \bullet GDI] = \left(\frac{k_2[Cdc42]_{total}[GDI]}{k_2[GDI] + k_{-2}K_{D1} + k_{-2}[GDI]}\right) \quad \left(1 - e^{-\left(\frac{k_2[GDI] + k_{-2}K_{D1} + k_{-2}[GDI]}{K_{D1} + [GDI]}\right)t}\right)$$

$$\left(\frac{k_2[GDI] + k_{-2}K_{D1} + k_{-2}[GDI]}{K_{D1} + [GDI]}\right) = k_{obs}$$

$$\left(\frac{k_2[Cdc42]_{total}[GDI]}{k_2[GDI] + k_{-2}K_{D1} + k_{-2}[GDI]}\right) = [Cdc42 \bullet GDI]_{max}$$

$$\frac{[Cdc42 \bullet GDI]}{[Cdc42 \bullet GDI]_{max}} = 1 - e^{-(k_{obs})t}$$

$$k_{obs} = \left(\frac{k_2[GDI] + k_{-2}K_{D1} + k_{-2}[GDI]}{K_{D1} + [GDI]}\right) \tag{9}$$

Rearranging equation 9 yields equation 4:

$$k_{obs} = \left(\frac{k_2[GDI]}{K_{D1} + [GDI]}\right) + k_{-2} \tag{4}$$

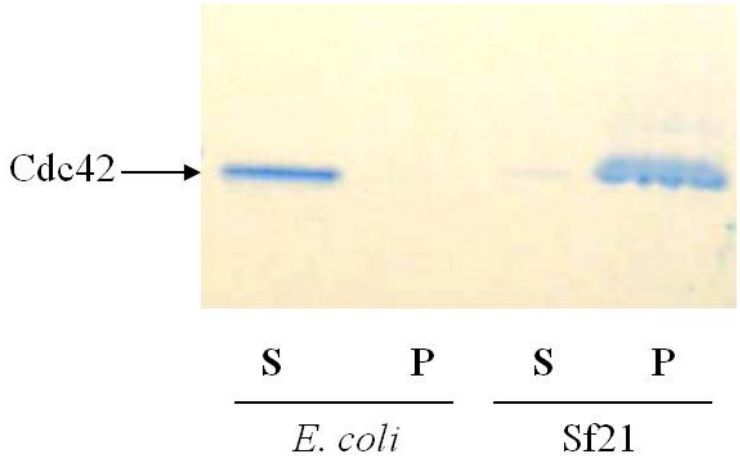
2.3 Results

2.3.1 Rho-GDI distinguishes between membrane-associated forms of GDP- and GTP-bound Cdc42- As an initial step toward determining how RhoGDI helps to increase the amount of soluble (non-membrane-associated) Cdc42, as well as examining whether RhoGDI interacts preferentially with a specific nucleotide-bound state of the membrane-associated GTPase, we set out to establish experimental systems for monitoring the binding of Cdc42 to membranes. We first verified that insect cell (Sf21)-expressed, recombinant Cdc42, by virtue of its C-terminal geranylgeranyl moiety, was capable of associating with lipid bilayers. The results presented in Figure 2.1A show that when the insect cell-expressed Cdc42 protein was briefly incubated with liposomes prepared by rapid solvent exchange (50), pelleted by centrifugation and analyzed by SDS-PAGE, virtually all of the Cdc42 was recovered in the membrane pellet. In contrast, when *E. coli*–expressed recombinant Cdc42, which lacks the C-terminal geranylgeranyl tail, was used, the entire pool of Cdc42 was detected in the soluble fraction.

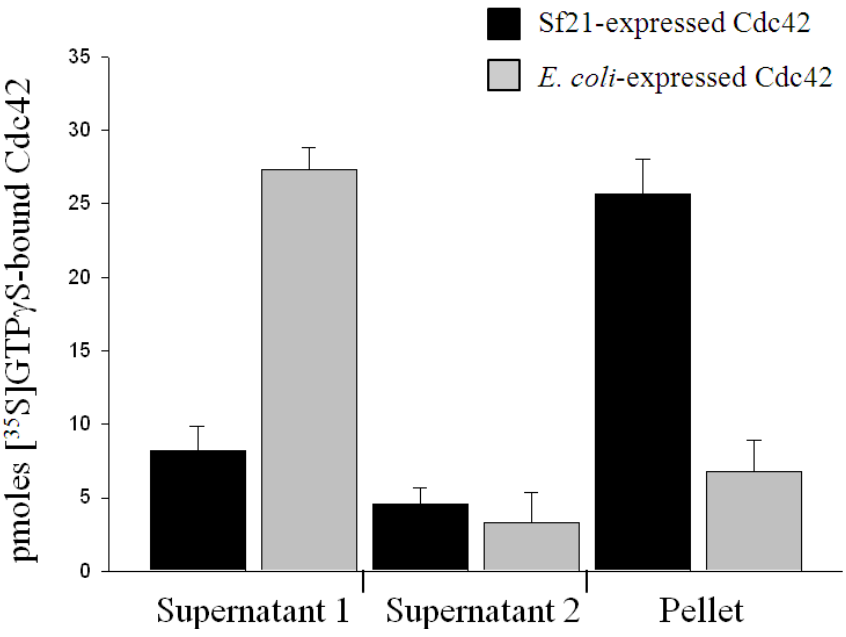
We then confirmed these results by examining the ability of the different recombinant Cdc42 proteins, bound to radio-labeled guanine nucleotides, to associate with membranes derived from insect cells. Recombinant Cdc42 purified from insect cells, when bound to either $\alpha[^{32}P]$GDP or $[^{35}S]$GTPγS, was able to associate with insect cell membranes. An example for $[^{35}S]$GTPγS-bound Cdc42 is shown in Figure 2.1B, where the majority of the insect cell Cdc42 protein was present in the membrane pellet. Again, the converse was true for *E. coli* recombinant Cdc42, as the

Figure 2.1 Binding of Cdc42 to lipid bilayers. **A)** *E. coli* or insect cell recombinant Cdc42 (1 μg) was incubated in 200 μL of liposomes containing 1 mg/mL lipids, prepared by rapid solvent exchange, for 10 minutes. The lipids were centrifuged at maximum speed for 20 minutes and the supernatant and pellet were analyzed by SDS-PAGE. **B)** *E. coli* or insect cell recombinant Cdc42 (40 pmol) was loaded with [^{35}S]GTPγS and incubated with 100 μL of insect cell membranes for 5 minutes. The membranes were pelleted by centrifugation, then resuspended in fresh buffer and pelleted once more. Radioactivity was measured in both supernatants and the lipid pellet.

A.


Cdc42
S
P
S
P
E. coli
Sf21

B.


Sf21-expressed Cdc42
E. coli-expressed Cdc42
pmoles [35S]GTPγS-bound Cdc42
35
30
25
20
15
10
5
0
Supernatant 1
Supernatant 2
Pellet

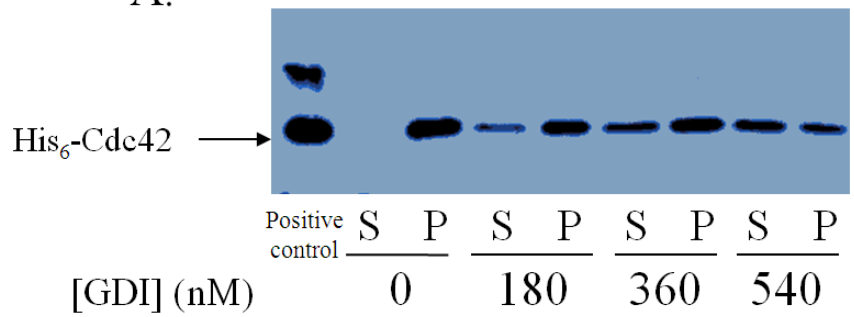
[^{35}S]GTPγS-bound protein was mainly present in the soluble (supernatant) fraction.

We then examined the ability of RhoGDI to increase the amount of insect cell Cdc42 in the soluble fraction, and in particular, set out to determine whether there were differences in the effectiveness of RhoGDI depending upon whether Cdc42 was bound to GDP or GTP. Figure 2.2A shows that the *E. coli* recombinant RhoGDI, in a dose-dependent manner, was able to increase the amount of GTPγS-bound, His_6-Cdc42 in the soluble (supernatant) fraction relative to the membrane fraction. We then compared the effects of RhoGDI on the dissociation of GDP- versus GTPγS-bound Cdc42 from insect cell membranes. Figure 2.2B shows that the decrease in the amount of α[^{32}P]GDP-bound Cdc42 associated with membrane pellets, as a function of RhoGDI concentration, closely mirrored the appearance of GDP-bound Cdc42 in the soluble supernatant fraction. The apparent K_D values (equation 1 in "Methods") estimated for the interaction of α[^{32}P]GDP-Cdc42 with RhoGDI from these two dose-response curves were similar (i.e. ranging from ~60-70 nM).

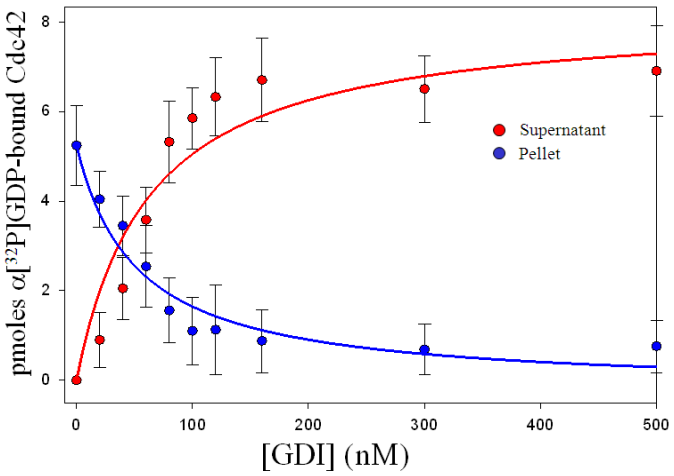
Figure 2.2C shows the corresponding set of assays for the effects of RhoGDI on membrane-associated, [^{35}S]GTPγS-bound Cdc42. A significantly greater amount of RhoGDI was necessary to achieve similar effects with GTPγS-bound Cdc42, compared to the GDP-bound form of the protein. In particular, apparent K_D values ranging from ~440 nM to 465 nM were obtained for the ability of RhoGDI to bind and increase the amount of GTPγS-bound Cdc42 detected in the soluble fraction. Thus, these results provided us with our first indication that RhoGDI exhibited a binding

Figure 2.2 Influence of RhoGDI on the translocation of Cdc42 from lipid membranes to the soluble fraction. **A)** Insect cell recombinant His_6-tagged Cdc42 (1 μg) was loaded with GTPγS and incubated with 300 μL of uninfected insect cell membranes. The membranes were pelleted by centrifugation and exposed to the indicated concentrations of RhoGDI. After a final centrifugation, the lipid pellets and supernatants were subjected to SDS-PAGE and the recovery of Cdc42 was analyzed by Western blotting using a polyhistidine-specific primary antibody. Insect cell recombinant Cdc42 (100 pmol) bound to α[^{32}P]GDP **(B)** or [^{35}S]GTPγS **(C)** was incubated with 500 μL of uninfected insect cell membranes, divided into equal fractions, and exposed to the indicated concentrations of RhoGDI. The membranes were pelleted by centrifugation. Radioactivity was measured in the pellets (blue) and supernatants (red) and plotted with respect to the RhoGDI concentration. The mean (± s.e.) values from three independent experiments are shown. Solid lines show the least-squares fit to equation 1 (red) or its inverse (blue) (see "Methods").

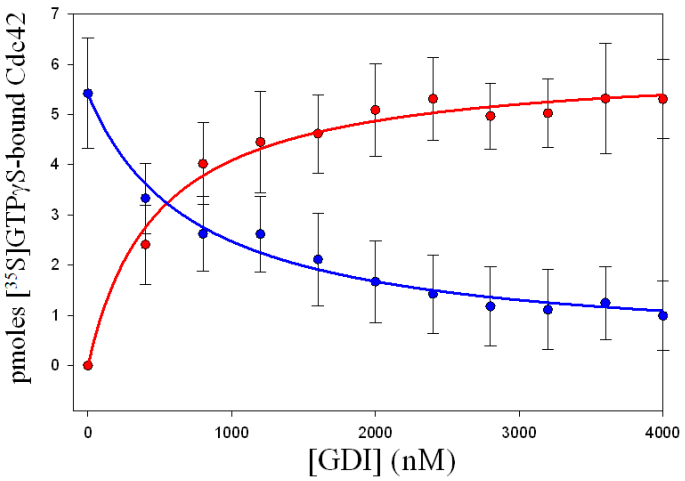
A.


His6-Cdc42
Positive control
S P S P S P S P
[GDI] (nM)
0 180 360 540

B.


pmoles α[32P]GDP-bound Cdc42
[GDI] (nM)
Supernatant
Pellet
0 2 4 6 8
0 100 200 300 400 500

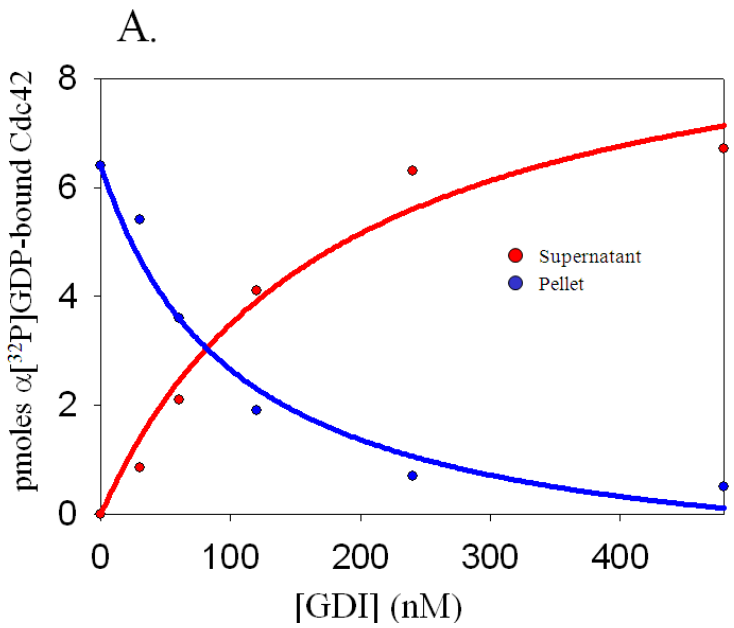
C.

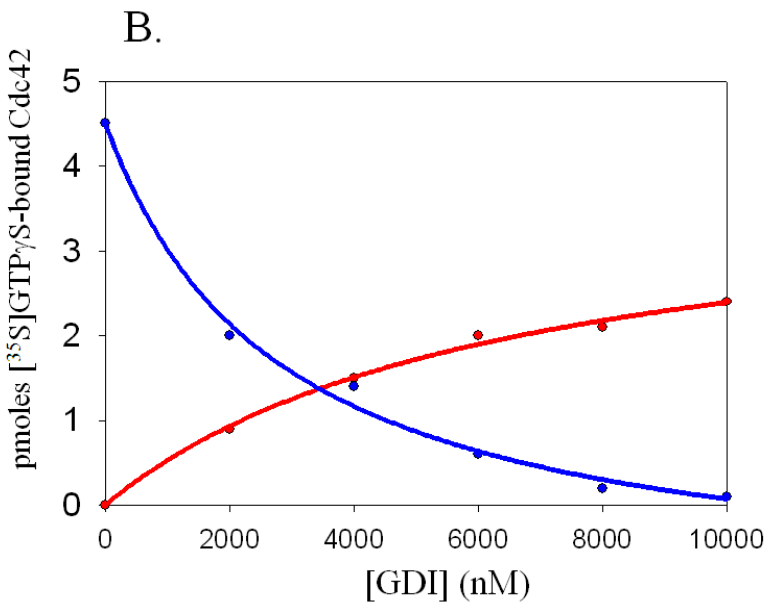

pmoles [35S]GTPγS-bound Cdc42
[GDI] (nM)
0 1 2 3 4 5 6 7
0 1000 2000 3000 4000

preference for membrane-associated GDP-bound Cdc42, compared to the GTPγS-bound form of the protein.

We went on to further verify these findings by using compositionally-defined liposomes, since we were ultimately interested in applying a real-time spectroscopic assay to model membrane systems in order to more closely analyze the kinetics of the interactions of Cdc42 with membranes and the effects of RhoGDI (see below). The results presented in Figures 2.3A and 2.3B show that the ability of RhoGDI to distinguish between membrane-associated GDP- versus GTPγS-bound Cdc42 was also observed in liposomes, although there was a general shift in the dose-response profiles for the effects of RhoGDI. In the case of liposome-associated, α[^{32}P]GDP-bound Cdc42, we determined apparent K_D values ranging from ~100 nM to 180 nM for its interaction with RhoGDI, compared to values of ~2.8-6 μM for the corresponding interaction of [^{35}S]GTPγS-bound Cdc42 with RhoGDI. The shift in the dose-response profiles for the effects of RhoGDI on liposome-associated Cdc42, compared to those obtained with insect cell membranes, is likely due to the large excess of synthetic liposomes, relative to the concentrations of Cdc42 and RhoGDI used in these experiments. Because both Cdc42 and RhoGDI are able to bind independently to lipid bilayers (Figure 2.8), this results in higher concentrations of RhoGDI being required to achieve the same degree of Cdc42-RhoGDI complex formation on the surfaces of liposomes, compared to the case for insect cell membranes.

Figure 2.3 Influence of RhoGDI on the translocation of Cdc42 from liposomes to the soluble fraction. Insect cell recombinant Cdc42 (100 pmol) bound to $\alpha[^{32}P]$GDP **(A)** or $[^{35}S]$GTPγS **(B)** was incubated with liposomes containing 1 mg/mL of lipids, prepared by rapid solvent exchange, divided into equal fractions, and exposed to the indicated concentrations of RhoGDI. The data were fit as described in Figure 2.2.

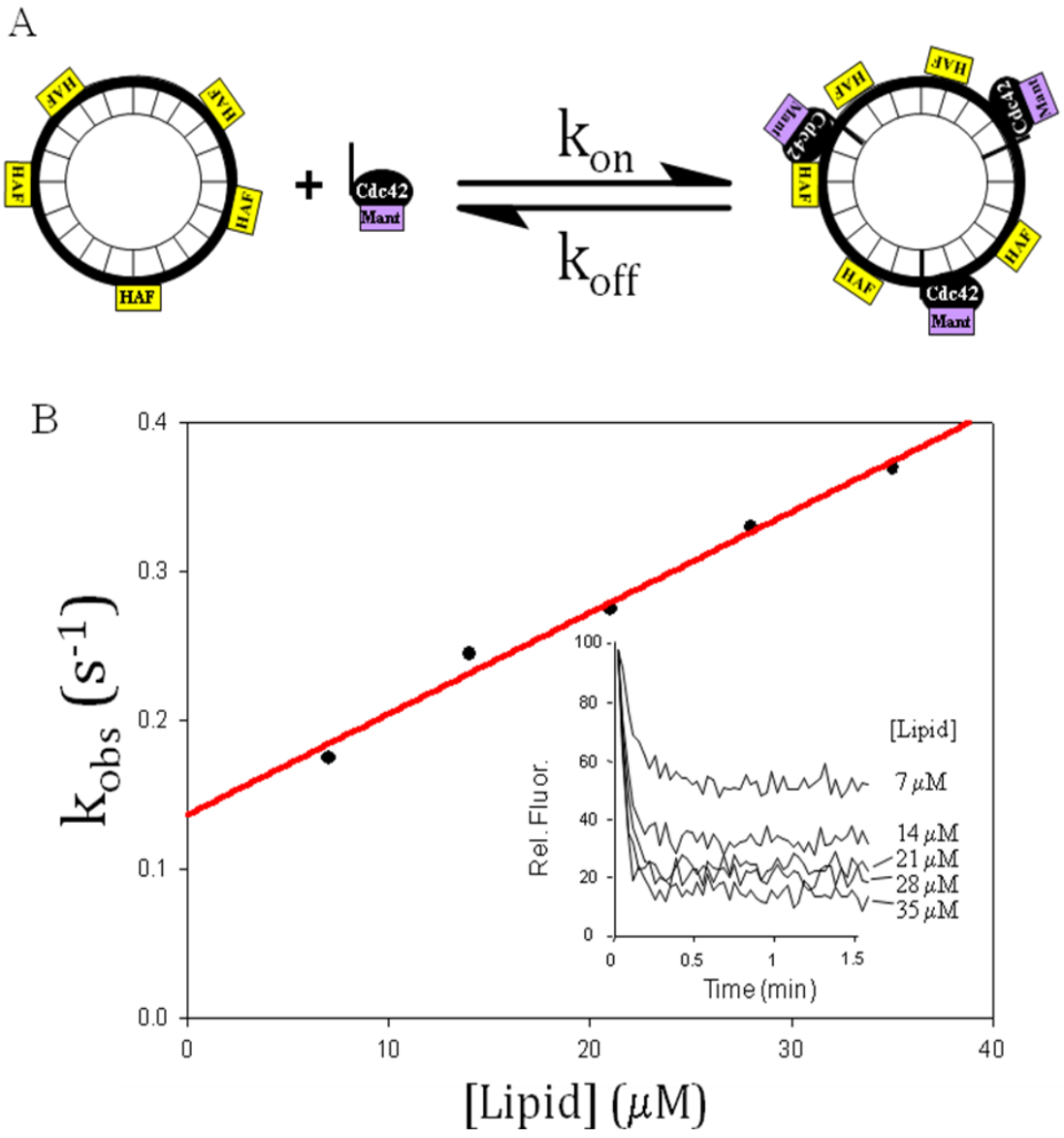

A.
pmoles α[32P]GDP-bound Cdc42
8
6
4
2
0
0
100
200
300
400
[GDI] (nM)
Supernatant
Pellet


B.
pmoles [35S]GTPγS-bound Cdc42
5
4
3
2
1
0
0
2000
4000
6000
8000
10000
[GDI] (nM)

2.3.2 A real-time spectroscopic read-out for the association of Cdc42 with lipid bilayers- Figure 2.4A depicts the fluorescence read-out that we used to examine the interactions of Cdc42 with liposomes in real-time. This assay takes advantage of the fluorescence resonance energy transfer (FRET) that occurs between Mant-nucleotide-bound Cdc42 (designated Mant-Cdc42 in Figure 2.4A) and liposomes containing the lipid molecule HAF, as an outcome of the membrane association of Cdc42 (47). Mant-labeled guanine nucleotides, when bound to the nucleotide-binding site of Cdc42, exhibit an increased fluorescence emission at 440 nm. Because the emission spectrum for Mant partially overlaps the excitation spectrum of fluorescein, Mant-nucleotides serve as appropriate FRET donors for fluorescein. Thus, the experimental strategy involves monitoring the changes in the fluorescence emission of Mant-nucleotide-bound Cdc42 upon its association with liposomes containing HAF.

Titration experiments were performed where we varied the bulk lipid concentrations in preparing the liposomes, and then examined the association of these lipid vesicles with either Cdc42-Mant-GDP or Cdc42-Mant-GMP-PNP (Figure 2.4B). The fluorescence quenching curves that were obtained as an outcome of the association of Mant-nucleotide-bound Cdc42 with liposomes containing HAF (Figure 2.4B, inset) were fit with equation 2a ("Methods") in order to obtain apparent rate-constants (k_{obs}). When the observed rate constants were plotted against lipid concentration, a linear relationship was obtained. Interestingly, the rate of dissociation for both Mant-GMP-PNP-bound Cdc42 and Mant-GDP-bound Cdc42 from liposomes (k_{off}), obtained by fitting the data to equation 3 ("Methods"), was ~0.1 s^{-1}.

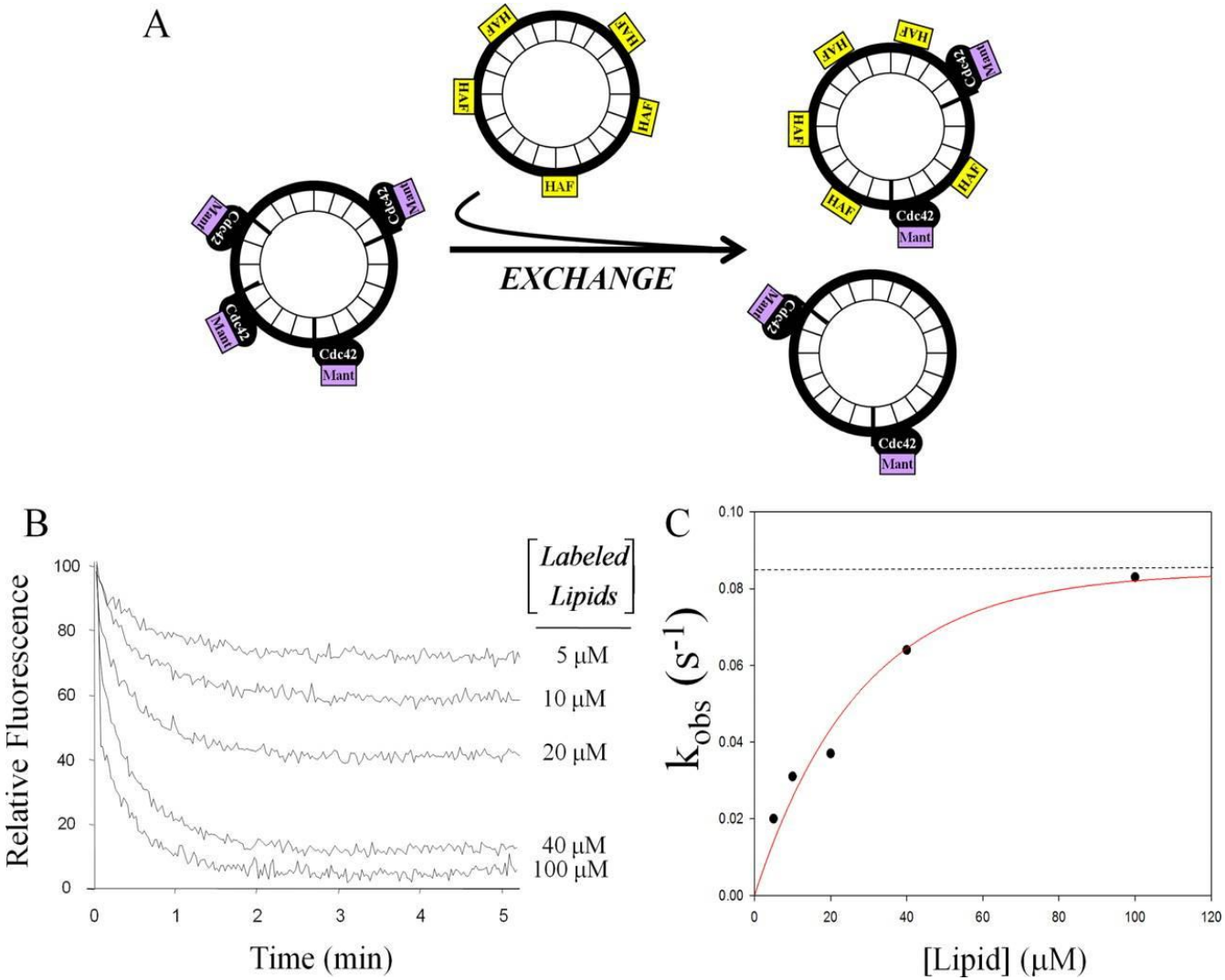
Figure 2.4 Fluorescence analysis of the binding of Cdc42 to liposomes. **A)** Schematic of the liposome-binding assay where Cdc42, bound to Mant-guanine nucleotide, associates with liposomes containing HAF. **B)** Cdc42-Mant-GMP-PNP (50 nM) was incubated with liposomes prepared by extrusion as described in "Methods", from different concentrations of HAF-labeled lipids. Individual measurements are shown in the inset, using the indicated concentrations of labeled lipids. The curves were fit to equation 2a in "Methods". The k_{obs} values obtained were plotted as a function of lipid concentration, showing a linear dependence that was fit with equation 3.


A
HAF
HAF
HAF
HAF
+
Cdc42
Mant
k_{on}
k_{off}
Cdc42
Mant
B
k_{obs} (s^{-1})
[Lipid] (μM)
Rel. Fluor.
Time (min)
[Lipid]
7 μM
14 μM
21 μM
28 μM
35 μM

This indicates that Cdc42 is undergoing rapid translocation between the membrane surface and solution even in the absence of RhoGDI, and that the GDP- and GTP-bound forms of Cdc42 dissociate from liposomes at essentially the same rate.

Further verification for the rapid equilibration of Cdc42 on and off the membrane surface was obtained by assaying the exchange of Cdc42 between different populations of liposomes. A depiction of how this liposome-exchange assay was carried out is shown in Figure 2.5A. In these experiments, Mant-GMP-PNP-bound Cdc42 was initially incubated with one population of liposomes that lacked HAF. A second population of liposomes containing HAF was then added to the mixture. The exchange of Cdc42 between the initial vesicle population and the vesicles containing HAF was monitored in real-time by the changes in FRET that accompany the association of Mant-GMP-PNP-bound Cdc42 with the HAF-containing liposomes. These liposome-exchange assays were performed with varying amounts of labeled lipids (Figure 2.5B), yielding the plot that shows the half-time for the re-distribution of Mant-GMP-PNP-bound Cdc42 between the different liposome populations (k_{obs}), as a function of the concentration of labeled lipids (Figure 2.5C). Note that the maximum value for the rate constant describing the exchange of Mant-GMP-PNP-bound Cdc42 between the two liposome populations (dashed line in Figure 2.5C) approaches the value of the rate constant measured for the dissociation of Cdc42 from liposomes, as obtained from the experiments described in Figures 2.4A and 2.4B, above.

Figure 2.5 Cdc42 translocates between liposomes in the absence of RhoGDI. **A)** Schematic of the inter-vesicle transfer of Cdc42 between the surfaces of unlabeled liposomes, or between unlabeled liposomes and liposomes containing HAF. **B)** Insect cell recombinant Cdc42-Mant-GMP-PNP (50 nM) was bound to unlabeled liposomes at a lipid concentration of 20 μM, and mixed with increasing amounts of HAF-containing liposomes, and the Mant-fluorescence was monitored. The plotted values for k_{obs} were determined by measuring the time at which the quenching of Mant-fluorescence was halfway completed. The raw data obtained are shown with the indicated concentrations of labeled lipids. **C)** The k_{obs} values obtained from the fluorescence data in Figure 2.5B were plotted as a function of the labeled lipid concentration. The dashed line shows that the k_{obs} values reach a value that is similar to the rate-constant for the dissociation of Cdc42 from liposomes, obtained from the data in Figure 2.4B.

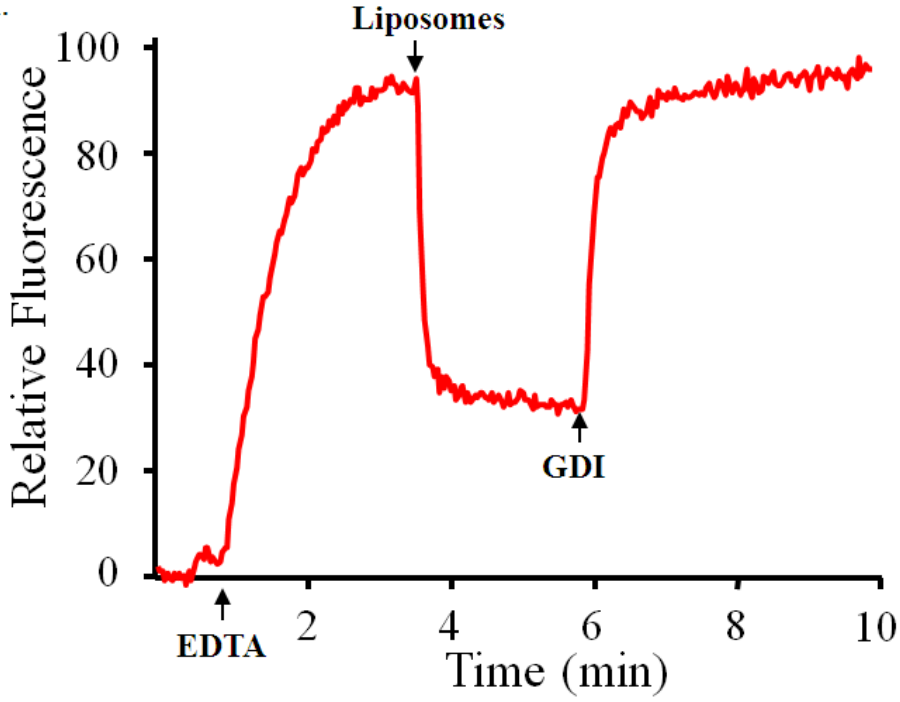

A
HAF
Cdc42
Mant
EXCHANGE
B
Relative Fluorescence
Time (min)
[Labeled Lipids]
5 μM
10 μM
20 μM
40 μM
100 μM
C
k_{obs} (s^{-1})
[Lipid] (μM)

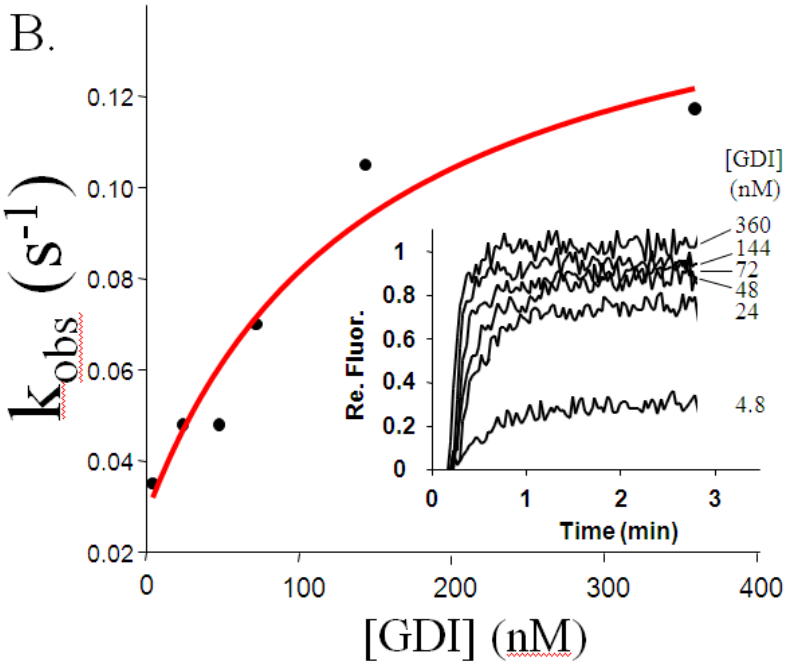
2.3.3 Spectroscopic assay for the release of Cdc42 from lipid bilayers in the presence of RhoGDI- We then used the FRET assay to examine the nucleotide-dependent kinetics for the release of Cdc42 from liposomes in the presence of RhoGDI. Figure 2.6A shows the results of an experiment where insect cell-expressed Cdc42 was bound with Mant-GDP, as an outcome of the EDTA-catalyzed dissociation of GDP and its exchange with the Mant-nucleotide, and then mixed with liposomes containing HAF at the indicated time-point. A partial quenching of the Mant-fluorescence was observed over a time-period of two minutes. Upon the addition of RhoGDI, there was a complete recovery of the Mant-fluorescence (i.e. the component of the fluorescence emission that was originally quenched by the labeled lipids). These results are consistent with the interpretation that the initial quenching of the Mant-nucleotide fluorescence is due to the FRET that occurs between the Mant-nucleotide-bound Cdc42 and HAF, upon the association of Cdc42 with the HAF-containing liposomes, and that the subsequent recovery of fluorescence is due to the release of Cdc42 from the liposome surface that occurs in the presence of an excess of RhoGDI.

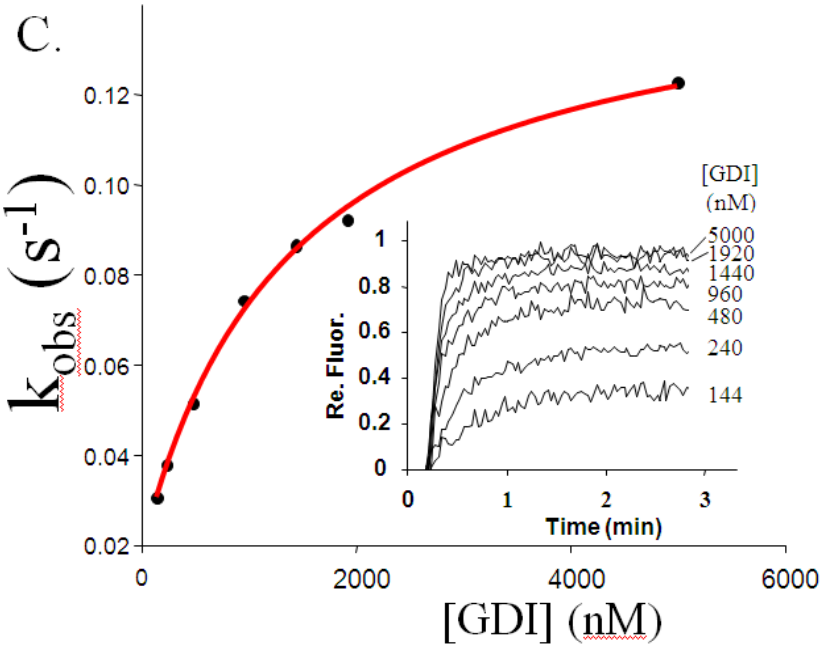
The insets to Figures 2.6B and 2.6C show the results of FRET experiments in which we monitored the rates of dissociation of Mant-GDP-bound Cdc42 and Mant-GMP-PNP-bound Cdc42, respectively, from liposomes containing HAF, in the presence of different concentrations of RhoGDI. In these experiments, the release of the different guanine nucleotide-bound forms of Cdc42 was monitored in real-time by the increase in Mant-emission. Significantly higher concentrations of RhoGDI were required to increase the amount of Mant-GMP-PNP-bound Cdc42 in the soluble

Figure 2.6 Influence of RhoGDI on the translocation of Cdc42-Mant-nucleotide from HAF-containing liposomes to the soluble fraction. **A)** Insect cell recombinant Cdc42 (60 nM) was loaded with 250 nM Mant-GDP in 5 mM EDTA at the indicated time. After 10 mM $MgCl_2$ was added, liposomes prepared with 30 μg/mL of HAF-labeled lipids were then added. At the 6 minute time-point, 1 mM RhoGDI was added, restoring Mant fluorescence. Insect cell recombinant Cdc42-Mant-GDP (13 nM) **(B)** or Cdc42-Mant-GMP-PNP (48 nM) **(C)** was bound to liposomes prepared from 30 μg/mL of labeled lipids and incubated with the indicated concentrations of RhoGDI. Mant-fluorescence was measured (λ_{ex} = 365 nm, λ_{em} = 440 nm). The raw data are shown in the insets, where the concentration of labeled lipids is indicated. The curves were fit to equation 2b in "Methods." The k_{obs} values obtained were plotted as a function of RhoGDI concentration, showing a hyperbolic dependence that was fit to equation 4 in "Methods".

A.


Liposomes
GDI
EDTA
Relative Fluorescence
100
80
60
40
20
0
2
4
6
8
10
Time (min)

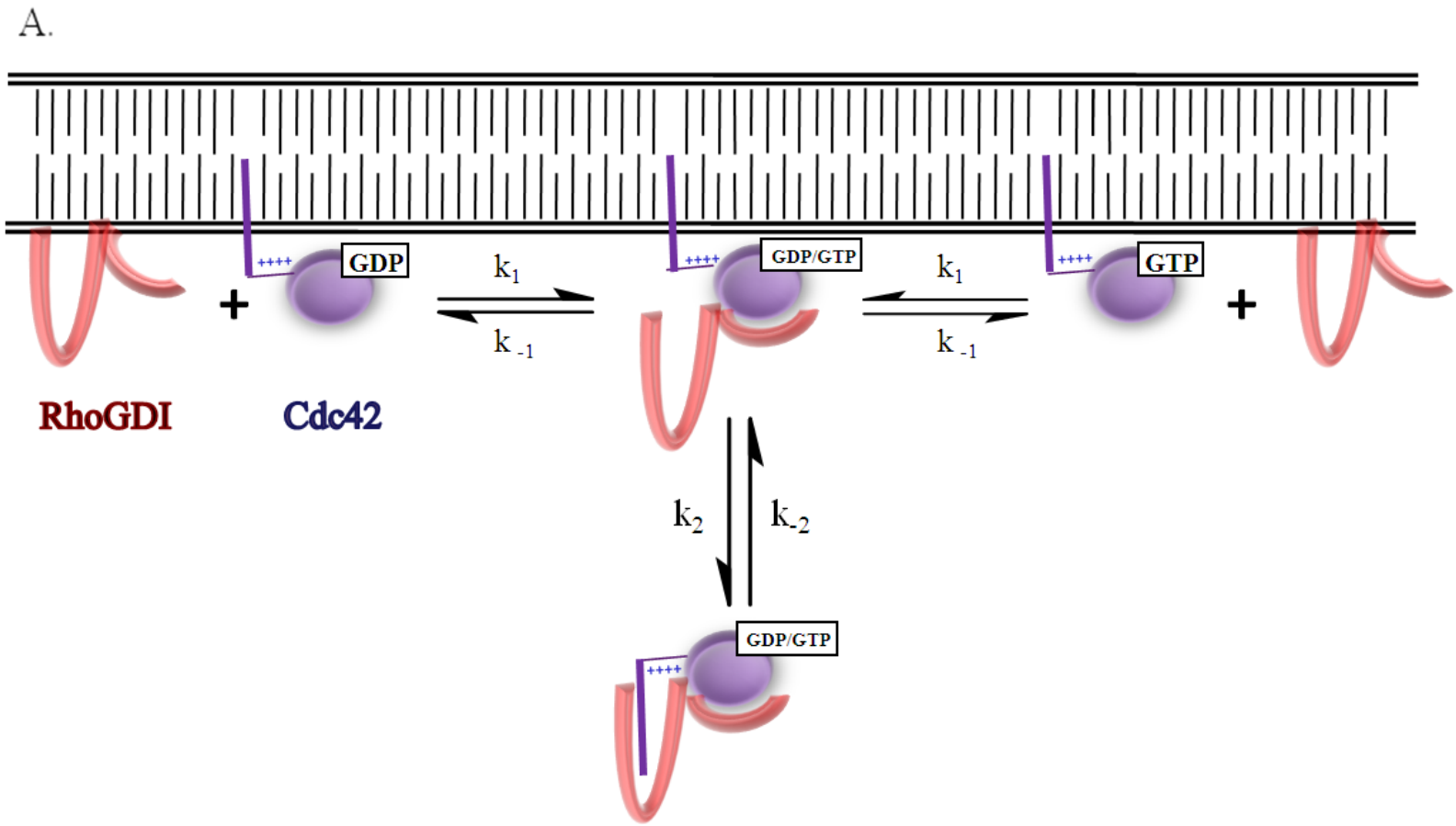

B.
k_{obs} (s^{-1})
0.12
0.10
0.08
0.06
0.04
0.02
0
100
200
300
400
[GDI] (nM)
[GDI]
(nM)
360
144
72
48
24
4.8
Re. Fluor.
1
0.8
0.6
0.4
0.2
0
0
1
2
3
Time (min)


C.
k_{obs} (s^{-1})
0.12
0.10
0.08
0.06
0.04
0.02
0
2000
4000
6000
[GDI] (nM)
[GDI]
(nM)
5000
1920
1440
960
480
240
144
Re. Fluor.
1
0.8
0.6
0.4
0.2
0
0
1
2
3
Time (min)

fraction (i.e. released from HAF-containing liposomes), compared to the amount of RhoGDI necessary to cause a similar increase in soluble Mant-GDP-bound Cdc42. This is consistent with what we had observed when using radio-labeled guanine nucleotides bound to Cdc42 (Figures 2.3A and 2.3B). The recovery of Mant-fluorescence that accompanied the dissociation of Mant-nucleotide-bound Cdc42 from the HAF-containing liposomes, at each level of RhoGDI, could be described by a single exponential equation. Figures 2.6B and 2.6C show plots of the rate constants (k_{obs}) for the release of Mant-GDP- and Mant-GMP-PNP-bound Cdc42 from liposomes, as a function of RhoGDI concentration. When these plots were fit to equation 4 ("Methods"), they yielded the same maximum value for k_{obs}. These findings suggested that despite RhoGDI being able to distinguish between the GDP- and GTP-bound forms of Cdc42 when they are associated with membranes, a common rate-limiting step exists for the membrane-release of both nucleotide-bound forms of Cdc42.

2.3.4 The mechanism by which RhoGDI promotes the transition of Cdc42 from a membrane-associated state to a soluble species- The findings described above are consistent with the model presented in Figure 2.7A, which depicts how RhoGDI influences the transition of Cdc42 between a membrane-associated state, and a soluble species. The first step represents the initial binding of RhoGDI to Cdc42 along the surface of the membrane and is assumed to be in rapid equilibrium. The large differences in the apparent affinities of RhoGDI for the GDP- and GTP-analog-bound forms of Cdc42 are reflected in this first step; for example, the apparent K_D

Figure 2.7 Model depicting the interaction of RhoGDI with Cdc42. **A)** Initially, both RhoGDI and Cdc42 are at the membrane surface, where RhoGDI engages Cdc42 in a step that is regulated by its nucleotide-bound state such that Rho-GDI binds to GDP-bound Cdc42 with a higher affinity compared to GTP-bound Cdc42. The Cdc42-RhoGDI complex then dissociates from the membrane surface. The binding of the isoprenoid tail of Cdc42 in the hydrophobic pocket of RhoGDI stabilizes the Cdc42-RhoGDI complex in the cytosol and slows the rate at which Cdc42 reassociates with the membranes. **B)** The table includes the values for constants obtained from the fits to the real-time FRET measurements for the dissociation of Mant-GDP-bound Cdc42 and Mant-GMP-PNP-bound Cdc42 from HAF-containing liposomes in the presence of RhoGDI. The dissociation constants for the first step, K_{D1}, represent the initial binding between the different nucleotide-bound forms of Cdc42 and RhoGDI at the membrane. The second step, with rate constants k_2 and k_{-2}, describes the translocation of the Cdc42-RhoGDI complexes from the membrane surface to the cytosol.

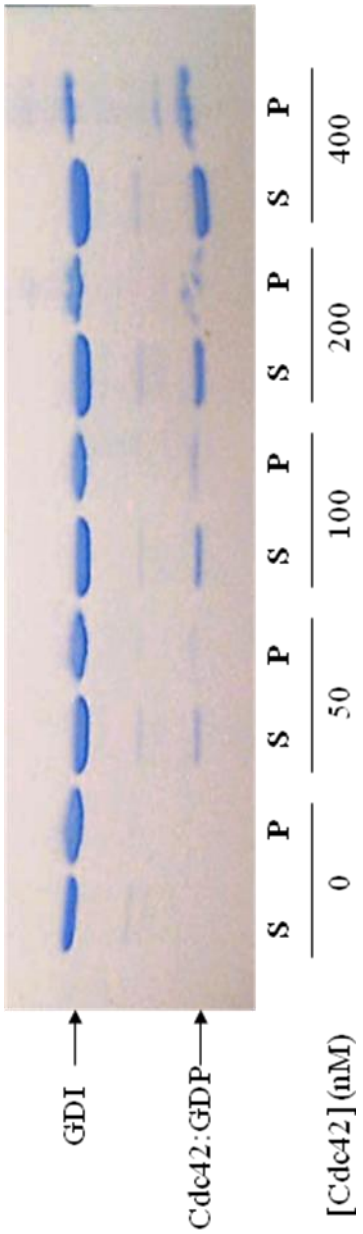

A.
GDP
GDP/GTP
GTP
k1
k-1
k1
k-1
RhoGDI
Cdc42
k2
k-2
GDP/GTP

B.

	GDP	GMP-PNP
$K_{D1} = k_1/k_1$	180 nM	1400 nM
k_2	0.14 s^{-1}	0.13 s^{-1}
k_2	0.03 s^{-1}	0.02 s^{-1}

value for Mant-GDP-bound Cdc42 is approximately 8-fold lower than that for Mant-GMP-PNP-bound Cdc42 (i.e. 180 nM versus 1400 nM; see Figure 2.7B). RhoGDI is depicted as being at the membrane surface during its initial binding to Cdc42, based on experiments that show it associates with liposomes (Figure 2.8). This is also consistent with the findings that Rho GTPase-binding-defective mutants of RhoGDI localize predominantly at the plasma membrane (51). The addition of increasing amounts of Cdc42 to mixtures of RhoGDI and a fixed concentration of lipids resulted in shifting RhoGDI from the membrane-associated pellet fraction to the soluble fraction (Figure 2.8). This indicates that both Cdc42 and RhoGDI have the capability of increasing the amount of their respective binding partner in the soluble fraction, presumably as an outcome of their forming a complex that is subsequently released from the membrane. The rate-limiting step that represents the actual dissociation of the Cdc42-RhoGDI complex from the membrane is reflected experimentally by the changes in Mant fluorescence shown in Figures 2.6A-C. The measured rate constants for this step (k_2 and k_{-2} in Figures 2.7A and 2.7B) are virtually the same for the two nucleotide-bound forms of Cdc42. Thus, as alluded to in the previous section, the release of Cdc42 from the membrane is not influenced by its nucleotide-bound state. Importantly, the release of Cdc42 from liposomes in the presence of RhoGDI is described by a rate constant that is very close in value to the rate constant measured for the RhoGDI-independent dissociation of Cdc42 from liposomes. Therefore, RhoGDI is not actively extracting Cdc42 from the membrane. However, when Cdc42 dissociates from membranes in a complex with RhoGDI, the geranylgeranyl moiety of Cdc42 binds within the carboxyl-terminal, isoprenoid-binding pocket of RhoGDI, thus

Figure 2.8 Association of RhoGDI with the lipid bilayer surface. GST-tagged GDI (1 µg) was incubated with 100 µL of liposomes (1 mg/mL lipids) prepared by rapid solvent exchange and the indicated concentrations of insect cell recombinant Cdc42 for 5 minutes. The lipids were pelleted by centrifugation at maximum speed for 20 minutes. The supernatant and the pellet fractions containing Cdc42 and RhoGDI were analyzed by SDS-PAGE and Coomassie-blue staining.


GDI
Cdc42:GDP
S P S P S P S P S P
[Cdc42] (nM)
0 50 100 200 400

stabilizing the Cdc42-RhoGDI complex in solution and reducing the rate at which Cdc42 re-associates with membranes. This could then account for previous observations that RhoGDI appears to promote the release of Cdc42 from membranes (39,42).

2.4 Discussion

2.4.1 RhoGDI discriminates between the GDP- and GTP-bound forms of Cdc42 in membranes- Previous work by our group and others has shown that RhoGDI can bind to both the GDP- and GTP-bound forms of Cdc42 in solution. Using Mant-labeled guanine nucleotides bound to Cdc42 as fluorescent reporter groups, we found that RhoGDI was able to interact with the GDP- and GTP-bound forms of the GTPase with essentially equal affinities (44). Likewise, Hall and colleagues showed that equivalent amounts of RhoGDI were precipitated with GST-Cdc42 bound to either GDP or GTPγS (52). Moreover, we demonstrated that the binding of RhoGDI to GTP-bound forms of Cdc42 had functional consequences, as RhoGDI inhibited both the intrinsic and GAP-stimulated GTP-hydrolytic activity of Cdc42 (43). Taken together, these findings seemed to be consistent with recent structure-function studies where through a combination of x-ray crystallography, NMR experiments, and fluorescence spectroscopy, we concluded that the GDP- and GTP-analog-bound forms of Cdc42, when analyzed in the absence of effector proteins, showed little if any differences in the conformations of their switch I and switch II domains (53). Thus, the inability of RhoGDI to distinguish between the GDP- and GTP-bound forms of Cdc42 in solution (44) seemed to be explained by the idea that

the overall orientation and/or arrangement of the primary interaction sites for RhoGDI on these two guanine nucleotide-bound forms of Cdc42 were virtually identical.

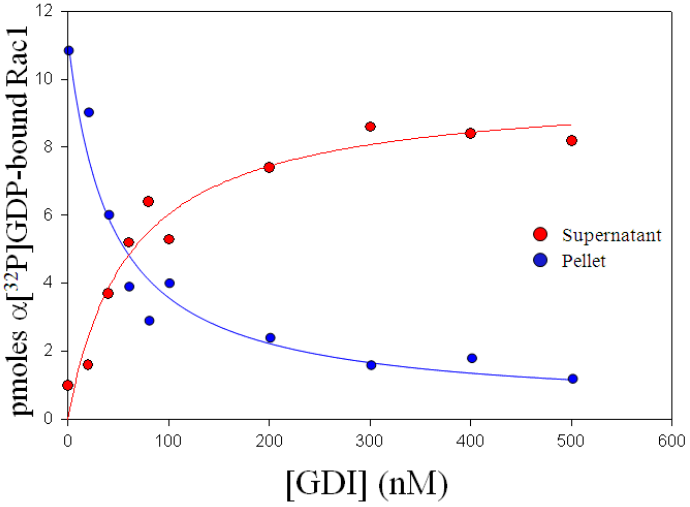
However, in the present study we now show that when Cdc42 is docked onto a membrane surface, its GDP- and GTP-bound states become distinguishable to RhoGDI, to an extent where their respective dissociation constants differ by approximately one order of magnitude. These findings hold some potentially important implications regarding the role of the membrane in Cdc42-signaling activities. They offer a possible mechanism by which GTP-bound (activated) forms of Cdc42 accumulate in membranes, given that RhoGDI works in a preferential manner to increase the amount of soluble GDP-bound Cdc42. These findings also raise some interesting possibilities regarding whether the ability of RhoGDI to distinguish between the GDP- and GTP-bound states of Cdc42, and/or the ability of Cdc42 to dissociate from membranes, might vary for different intracellular membranes depending on their lipid composition. Indeed, we have found that when Cdc42 is associated with liposomes containing PIP_2, it shows a significantly weaker interaction with RhoGDI such that there is very little release of GTP-bound Cdc42 from these membranes (Chapter 3), consistent with the idea that activated Cdc42 molecules preferentially accumulate and/or signal within specific membrane locations (e.g. the plasma membrane) in the cell.

Another potentially important implication has to do with the fundamental role of the membrane in facilitating structural changes in Cdc42 that accompany GDP-GTP exchange. The cell membrane, rather than simply serving as an inert docking platform for Cdc42 to initiate its signaling activities, may interact with Cdc42 in a manner that

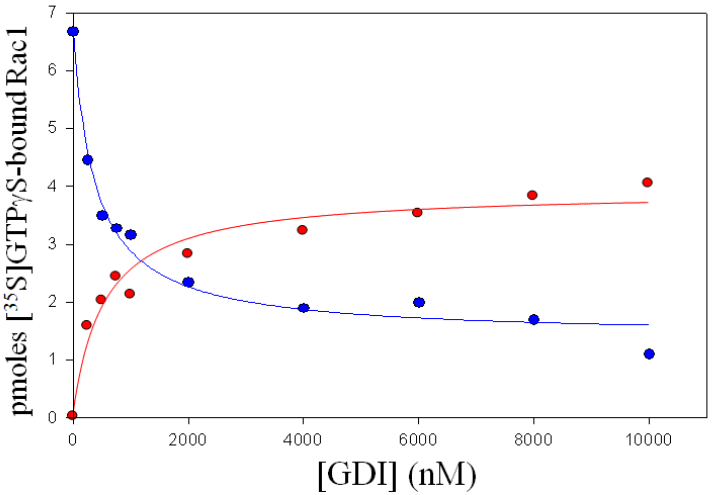
significantly influences its ability to assume an activated conformational state. Based on our inability to detect significant differences in the switch I and switch II conformations when comparing Cdc42 molecules bound to GDP versus GTP both in solution and in x-ray crystal structures, we proposed that effector proteins were able to lock GTP-bound Cdc42 into a signaling-competent conformational state whereas GDP-bound Cdc42 was not susceptible to such effector-induced changes (53). In light of our findings with RhoGDI in the present study, we can now revise that working model by adding the provision that the membrane may help GTP-bound Cdc42 to assume a conformational state that is more readily distinguishable from the GDP-bound GTPase, and thus more receptive to binding target/effector proteins. It will be interesting to see whether this is also the case for other Rho-family GTPases that respond to RhoGDI. We have in fact found that Rac1 behaves in a very similar manner to Cdc42, such that membrane-associated GDP-bound Rac1 shows a much stronger affinity (~10-fold greater) for RhoGDI compared to its GTP-bound counterpart, either when assaying membrane association by FRET (data not shown) or using radio-labeled nucleotides (Figures 2.9A and 2.9B). However, interestingly, we have not observed the same marked differences when comparing the ability of RhoGDI to associate with GDP-versus GTP-bound forms of RhoA (data not shown). This would appear to be consistent with studies performed in cells, which showed that the over-expression of RhoGDI had little effect on the localization of GFP-tagged RhoA, while leading to a complete cytosolic partitioning of Cdc42 and Rac1 (40). Thus, it will be interesting in the future to better understand what might be the

Figure 2.9 RhoGDI-facilitated removal of Rac1 from lipid membranes. Insect cell recombinant His6-tagged Rac1 (100 pmol) loaded with [^{35}S]GTPγS **(A)** or with α[^{32}P]GTP **(B)** was incubated with 500 μL of uninfected insect cell membranes, divided into equal fractions, and exposed to the indicated concentrations of RhoGDI. The membranes were pelleted by centrifugation. Radioactivity was measured in the pellets (blue) and supernatants (red) and plotted with respect to the GDI concentration. The mean (±s.e.) values from three independent experiments are shown. Solid lines show the least-squares fit to equation 1 (see "Methods").

A


pmoles α[32P]GDP-bound Rac1
[GDI] (nM)
Supernatant
Pellet
0
2
4
6
8
10
12
0
100
200
300
400
500
600

B


pmoles [35S]GTPγS-bound Rac1
[GDI] (nM)
0
1
2
3
4
5
6
7
0
2000
4000
6000
8000
10000

molecular basis for these differences between Cdc42/Rac1 and RhoA, and what consequences this might hold for their respective signaling capabilities.

3.4.2 Consequences of the mechanism by which RhoGDI influences the release of Cdc42 from membranes- Using real-time FRET assays to monitor the binding of Cdc42 to liposomes, we made the surprising finding that Cdc42 dissociated from these lipid vesicles on a time-scale of approximately 5-10 seconds, which appeared to correspond to the rate for the release of Cdc42 from liposomes in the presence of RhoGDI. Thus rather than playing an active role in stimulating the release of Cdc42 from lipid bilayers as originally assumed, RhoGDI instead takes advantage of the intrinsic ability of Cdc42 to dissociate from membranes on a time-scale of seconds and then, by providing a hydrophobic pocket for the isoprenoid moiety of the GTPase, ensures that Cdc42 remains in the soluble fraction. This idea is consistent with studies examining the interactions of the small GTPase Rac with the plasma membrane in cells, where it was suggested that RhoGDI surprisingly had little effect on the rate at which Rac dissociated from the membrane surface (54).

Therefore, these findings indicate that the principle regulatory role played by RhoGDI is to significantly reduce the rate at which soluble (cytosolic) Cdc42 is able to re-bind to lipid bilayers. What might be the physiological relevance for lowering the rate at which Cdc42 associates with membranes? One attractive possibility comes from a recent study examining how Cdc42 is able to concentrate in small, local regions on the plasma membrane, as occurs during bud-site formation in yeast (55). Indeed, the recruitment and local concentration of Cdc42 at membrane sites is required

for many of its biological functions (56,57). It has further been suggested that Cdc42-signaling responses may sometimes involve a positive feedback loop in which membrane-bound Cdc42 stimulates the recruitment and co-localization of additional Cdc42 molecules. Altschuler et al. put forward a model based on this idea, consisting of three distinct events: membrane-association, membrane-release, and membrane-recruitment. It was initially shown through computational simulations, and subsequently verified by experiments, that Cdc42 could effectively accumulate at small regions on the membrane surface, provided that its rate of association with membranes in general was low relative to the rate at which it was recruited to specific membrane-signaling sites. RhoGDI might indirectly contribute to the recruitment of Cdc42 to specific signaling sites by reducing its ability to bind indiscriminantly to membrane surfaces throughout the cell. Consistent with this idea, RhoGDI-deletion experiments in *Candida albicans* resulted in reduced polarized growth (58).

In light of these findings, it is also interesting to consider that the recruitment of the Cdc42(F28L) mutant, that is capable of constitutive GDP-GTP exchange, to specific sites at the plasma membrane may be required for its ability to transform cells. This could then explain the essential role played by RhoGDI in Cdc42(F28L)-induced cellular transformation (48). For example, RhoGDI might prevent GDP-bound Cdc42(F28L) in the cytosol from indiscriminately associating with membranes that lack its important signaling-effectors. Following GDP-GTP exchange, the activated Cdc42(F28L) molecules might then be able to 'escape' from RhoGDI by binding to specific membrane-associated effector proteins and thereby accumulate at those

membrane sites from which the necessary signals for cellular transformation are propagated.

This then raises the question of how wild-type, GDP-bound Cdc42 disengages from RhoGDI to become activated through GDP-GTP exchange, so as to initiate its signaling activities. The phosphorylation of RhoGDI has been suggested as one mechanism by which GTPases might be released from RhoGDI at the membrane so that they can undergo GEF-catalyzed nucleotide exchange (59). However, in some circumstances or cellular contexts, a membrane-localized GEF may be all that is required to effectively free Cdc42 from the actions of RhoGDI. Upon GEF-stimulated nucleotide exchange, the membrane-bound Cdc42-GTP species would have a reduced affinity for RhoGDI and an increased affinity for effector proteins, most of which are also membrane-bound. This would have the effect of slowing the release of Cdc42 from membranes and shifting the population of Cdc42 from the cytosol to the designated membrane surface. Support for this idea was provided by a recent study of the interaction between the small GTPase Rab1 and RabGDI (60). Here, the authors studied the *Legionella pneumophilia* protein, SidM, and showed that it can act as a RabGDI-displacement factor for Rab1. Interestingly, SidM was also found to catalyze nucleotide exchange on Rab1, with the region responsible for this activity overlapping the site on SidM that was shown to be necessary for RabGDI-displacement activity. The authors hypothesized that eukaryotic RabGEFs may be sufficient to dissociate Rab GTPases from RabGDI and recruit them to their target membrane sites. Thus, it is attractive to consider that functional parallels exist between the Rho and Rab

families, as Rho GTPases may also depend on GEFs for their recruitment to membrane-signaling sites.

Given that we now have a new appreciation of the role of the membrane surface in helping Cdc42 to assume a signaling-active conformational state, as well as a greater understanding of how RhoGDI influences the dynamics of the membrane-cytosol partitioning of this GTPase, we should be able to better evaluate what regulatory mechanisms the cell may utilize to properly localize Cdc42 and how this ultimately influences its signaling activities and transforming potential.

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FOOTNOTES

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The abbreviations used are: RhoGDI, Rho guanine nucleotide-dissociation inhibitor; Mant-, methylanthraniloyl-; HAF, hexadecanoylaminofluorescein; GTPγS, guanosine 5'-3-O-(thio)triphosphate; GMP-PNP, guanosine-5'-[(βγ)-imido]triphosphate; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

CHAPTER 3

THE C-TERMINAL DI-ARGININE MOTIF OF CDC42 IS ESSENTIAL FOR BINDING TO PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE-CONTAINING MEMBRANES AND INDUCING CELL TRANSFORMATION

3.1 Introduction

Members of the family of Rho GTPases regulate a variety of cellular processes that are dependent on the proper spatial orientation of proteins including cell polarity, vesicle trafficking, and migration (1-5). Despite the importance of the proper cellular localization of Rho GTPases for their cellular functions, a complete understanding of how they are targeted to the membrane locations that contain their specific signaling partners is still lacking. The ability of Rho GTPases to bind to membranes is largely mediated through their isoprenylation, which in most cases involves the geranylgeranylation of a carboxyl-terminal cysteine residue (5). This modification enables them to interact with either lipid membranes or RhoGDI (Rho Guanine Nucleotide Dissociation Inhibitor), the latter being a key regulatory protein that influences the membrane versus cytosolic distribution of Rho GTPases including Cdc42, Rac1, and RhoA (6). RhoGDI stabilizes the soluble (cytosolic) form of these GTPases, such that its over-expression in mammalian cells has been shown to result in a dramatic shift in the population of Cdc42 from membranes to the cytosol (7).

Recently, we examined how RhoGDI influences the cellular localization of Cdc42 and gained new insights into the mechanism by which it increases the soluble pool of this GTPase (8). In particular, the association of Cdc42 with lipid membranes was

shown to be a dynamic process, such that it has an intrinsic capability to dissociate from membranes within a time-scale of seconds. RhoGDI initially engages Cdc42 while it is bound to membranes and is subsequently released from membranes in a complex with Cdc42. The ability of RhoGDI to bind to the geranylgeranyl tail of Cdc42 helps to maintain the GTPase in the cytosol by slowing its re-association with the membrane surface. We have proposed that this may have important biological consequences, as it prevents Cdc42 from binding indiscriminately to membrane surfaces within cells, ensuring that Cdc42 associates with membranes that contain specific signaling partners.

While this model for RhoGDI function provides some intriguing clues regarding how the cellular localization of Cdc42, as well as perhaps other Rho GTPases, is regulated, it is not the complete picture. The fact that clear differences are observed in the cellular localization patterns of different Rho GTPases (i.e. RhoA, Rac1, Cdc42), even when considering individual isoforms of a particular GTPase (e.g., Rac1 versus Rac2 (10-11)), would suggest that the distinct carboxyl-terminal ends of these proteins have important roles in determining the membrane locations from which these GTPases initiate signaling activities. In this regard, the carboxyl-terminal 'polybasic region' of Rho GTPases could play an important part in localizing and positioning these GTPases at the appropriate cellular membrane site for signal propagation. Most Rho GTPases contain a cluster of positively charged residues directly preceding their geranylgeranyl moiety. Rac1 and Rac2, which differ by only 12 residues (with five of these residues being located within the polybasic region), show significantly different subcellular localizations (11). The same appears to be true for the two splice variants

of Cdc42 (i.e. the ubiquitous form of the protein and the brain-specific isoform) that differ only in their ten carboxyl-terminal residues (12-13). The polybasic domain of Cdc42 contains a pair of lysine residues and a pair of arginine residues (with these two sets of charged residues being separated by a serine). The exact positioning of the two arginine residues is conserved from yeast to humans. Our laboratory has previously demonstrated a role for the carboxyl-terminal di-lysine motif of Cdc42, via its interaction with the γCOP subunit of the COPI complex, in regulating intracellular trafficking as well as cell growth and transformation (14). However, thus far, the role of the conserved carboxyl-terminal di-arginine pair in Cdc42-signaling has not been determined.

Here we have examined the importance of the di-arginine pair in the membrane-association of Cdc42 and in its ability, when hyper-activated, to propagate signals and induce the transformation of fibroblasts. We show that the carboxyl-terminal arginine residues are necessary for the association of Cdc42 with PIP_2-containing membrane sites, whereas the pair of di-lysine residues located just upstream from the di-arginine sequence does not significantly influence binding to PIP_2. Conversely, only the di-lysine pair and not the di-arginine residues is essential for the binding of γCOP. Substituting glutamine residues for the pair of carboxyl-terminal di-arginine residues has no effect on the ability of the consititutively active, fast-cycling Cdc42(F28L) mutant to induce the generation of microspikes from the cell surface. However, it completely blocks the transforming capability of Cdc42(F28L). These findings demonstrate the importance of the carboxyl-terminal di-arginine residues of Cdc42

and their ability to associate with PIP_2-containing membrane sites for the transforming activity of this GTPase.

3.2 Methods

Preparation of insect cell-expressed Cdc42

Wild-type Cdc42 and mutants were purified as a His_6-tagged protein following its baculovirus-mediated expression in *S. frugiperda* (Sf21) insect cells using the procedure described in the Methods section of Chapter 2.

Preparation of E. coli-expressed RhoGDI

RhoGDI was purified as a GST-tagged protein out of *E. coli* cells, as described in the Methods section of Chapter 2

Preparation of liposomes

All lipid vesicles were prepared by extrusion (Avanti mini-extruder). The control lipid composition in molar percentages was 35% PE, 25% PS, 5% PI, and 35% cholesterol (Nu Chek Preps). Unless otherwise specified, all lipids used in these studies were obtained from Avanti Polar Lipids. In liposomes containing PIP_2, the molar percentage of PIP_2 included was replacing an equal molar percentage of PI. In liposomes containing a higher percentage of PS, the additional PS replaced an equal molar percentage of PE.

Fluorescence assays for the interaction of Cdc42 with liposomes

Fluorescence measurements were made using a Varian Cary Eclipse fluorimeter using the procedures described in the Methods section of Chapter 2.

Cell culture and transfection

NIH 3T3 cells were cultured in DMEM plus 10% calf serum (CS) at 37°C with 5% CO_2. COS-7 cells were cultured in DMEM plus 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The cells were split at 2×10^5 in a 60 mm dish, 18 hours before transfection. For the production of stable cell lines, the selection of G418-resistant NIH 3T3 colonies was carried out 48 hours after transfection by adding 5 μg/mL geneticin to the culture medium. The cell colonies resistant to G418 were selected and subcultured in DMEM plus 10% CS and 5 μg/ml geneticin.

For effector binding assays, COS-7 cells were transiently transfected and harvested 48 hours later. Purified mutants of polyhistidine-tagged Cdc42 were immobilized on nickel agarose and exposed to COS-7 cell lysates expressing either Myc-tagged PAK, Myc-tagged WASP, or HA-tagged γCOP. The beads were extensively washed and then added to SDS-loading buffer, prior to performing SDS-PAGE and Western blot analysis using primary antibodies against the epitope tags of the effector proteins.

Immunofluorescent staining

The lipofectamine transfection kit (Invitrogene) was used for transiently transfecting NIH 3T3 and COS-7 cells with Myc-tagged Cdc42 mutants. Cells were plated on chamber slides (Falcon) 24 hours following transfection. After subculture for 24 hours, the cells were fixed and then immunostained. The GFP fluorescence of

the cells was visualized with a Leica DM1RE2 inverted confocal microscope, and the images were captured and analyzed with Leica confocal Software. Immunofluorescence experiments were performed on NIH 3T3 cells stably expressing different Cdc42 constructs as previously described (ref).

Transformation assays

The transforming activities of the Cdc42(F28L) and Cdc42(F28L,R193Q,R194Q) mutants were assessed by assaying growth in 1% serum and colony formation in soft agar. For growth in low serum, stable cell lines expressing the different Cdc42 mutants were plated and cells were trypsinized and counted at two-day intervals. For colony formation in soft agar, the stable cell lines were suspended in 0.3% agarose in DMEM.

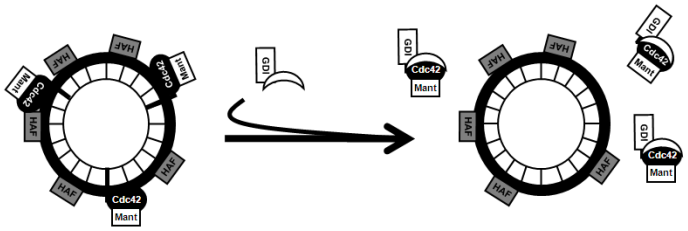
3.3 Results

3.3.1 Cdc42 shows enhanced binding to liposomes containing PIP_2 -We were interested in examining the role of the polybasic region of Cdc42 in its association with membranes and its potential influence on the ability of this GTPase to bind to specific lipids. We were especially interested in seeing how the physiologically relevant lipids phosphatidylserine (PS) and PIP_2 might influence the membrane binding of Cdc42. Figure 3.1A describes the real-time FRET assay that we used to examine the dissociation of Cdc42 from liposomes of different defined phospholipid compositions in the presence of RhoGDI. In this assay, the fluorescence of Mant-labeled guanine nucleotides bound to Cdc42 was used to read-out the association of Cdc42 with fluorescein-labeled lipids, i.e. as a result of the quenching of Mant

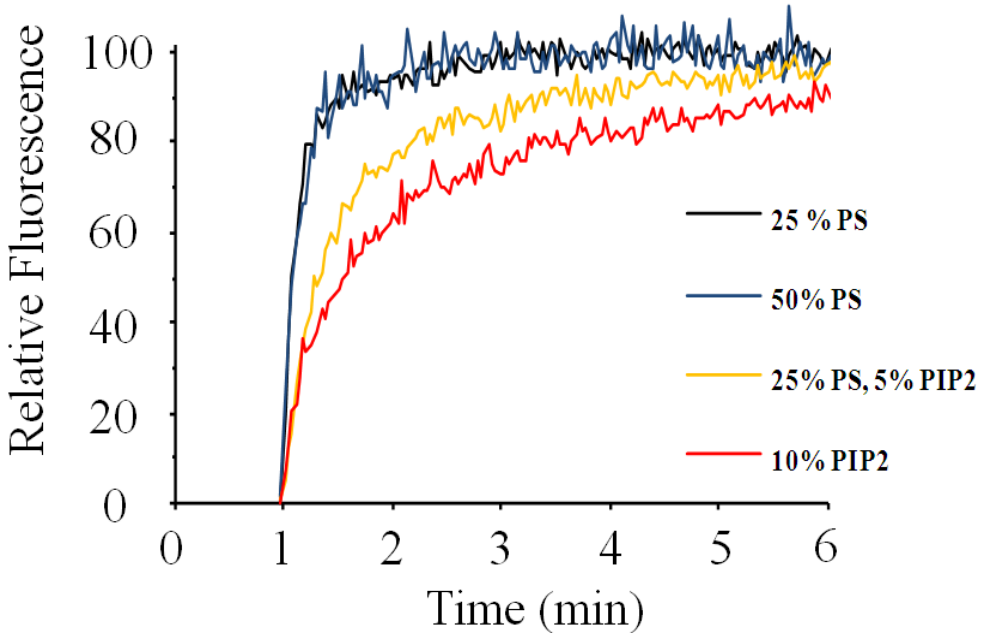
fluorescence due to FRET. As a first step, recombinant GDP-bound Cdc42, expressed in insect cells and purified as a His-tagged protein, was exchanged with Mant-GMPPNP by treatment with EDTA. The Mant-GMPPNP-loaded Cdc42 was incubated with hexadecanoyl fluorescein (HAF)-labeled liposomes for 5 minutes. This resulted in an approximate 50% quenching of the Mant fluorescence as an outcome of the binding of Mant-GMPPNP-loaded Cdc42 to the HAF-containing liposomes. RhoGDI was added and then the dissociation of Mant-GMPPNP-Cdc42 from the liposomes was followed over time, as monitored by the increased Mant fluorescence due to the loss of FRET (Figure 3.1B). Our standard lipid composition for the liposomes was 35% PE, 25% PS, 5% PI, and 35% cholesterol (here on referred to as control liposomes). We found that doubling the percentage of PS in the liposomes (i.e. 10% PE, 50% PS, 5% PI, and 35% cholesterol) had no effect on the rate or extent of Cdc42 dissociation from the lipid vesicles. In contrast, when using liposomes that contained 5% PIP_2 (i.e. 35% PE, 25% PS, 5% PIP_2, and 35% cholesterol), the dissociation of Cdc42 from the vesicles in the presence of RhoGDI was delayed by approximately 3-fold. This effect was dose-dependent as further increases in the molar percentage of PIP_2 up to 10% (i.e. 30% PE, 25% PS, 10% PIP_2, and 35% cholesterol) showed an even greater reduction in the dissociation rate of

Figure 3.1 Cdc42 exhibits a dose dependent increase in affinity for PIP_2 containing membranes. **A)** Schematic of GDI-mediated release of Mant-guanine nucleotide-bound Cdc42 from liposomes, containing HAF. **B)** Insect cell recombinant Cdc42 (50 nM) was preloaded with MantGDP and mixed with 30 uM liposomes, containing HAF, with the indicated molar percentages of PIP_2 and PS. The base lipid composition was 35% cholesterol, 25% PE, 25% PS, and 5% PI, where PIP_2 replaced an equal molar percentage of PI, and additional PS or PIP_2, beyond 5%, replaced an equal molar percentage of PE. At the 1-min time point, 1 uM RhoGDI was added and their release from the membranes was monitored by Mant/HAF de-quenching. **C)** Schematic of the inter-vesicle transfer of geranylgeranylated Cdc42 between the surfaces of unlabeled liposomes and liposomes containing HAF. **D)** Mant-GDP bound Cdc42 (30nM) was incubated in 20 uM unlabeled liposomes. At the zero time point, 20 uM liposomes, containing HAF labeled lipids, ± 5% PIP_2, were added and Cdc42's equilibration was monitored through the quenching of Mant.

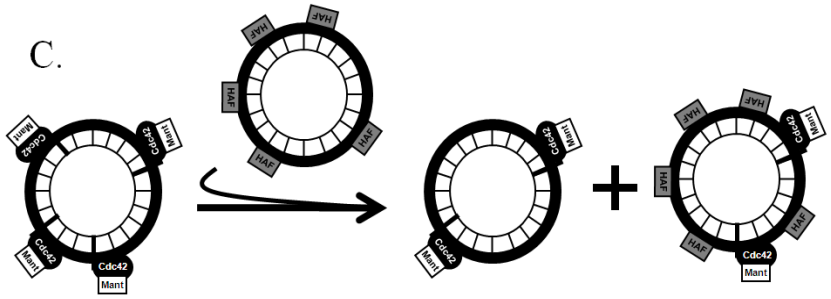
A.


HAF
Cdc42
Mant
GDI

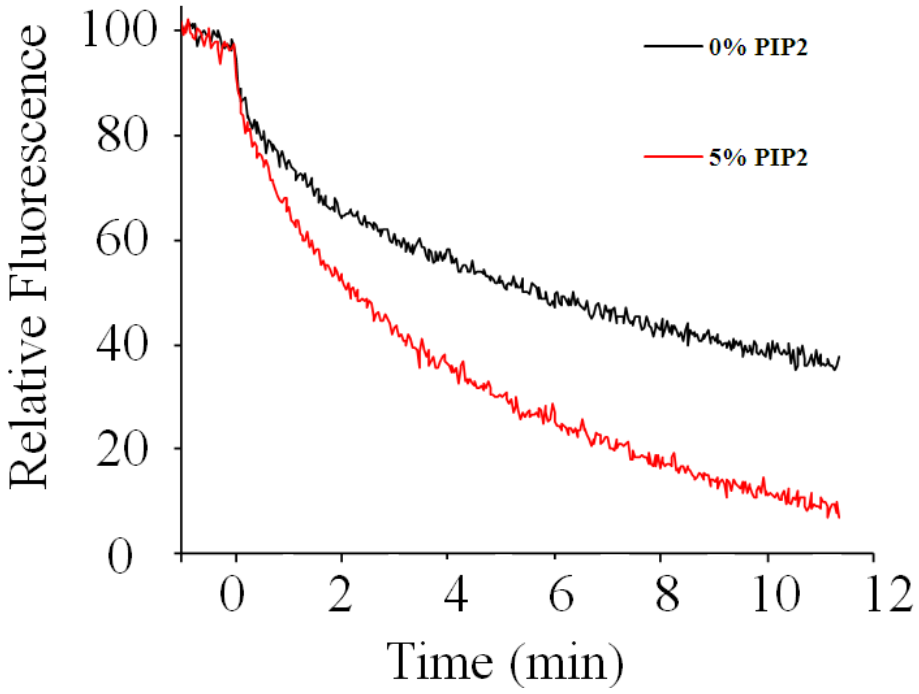
B.


Relative Fluorescence
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (min)
25 % PS
50% PS
25% PS, 5% PIP2
10% PIP2

C.


HAF
Cdc42
Mant

D.

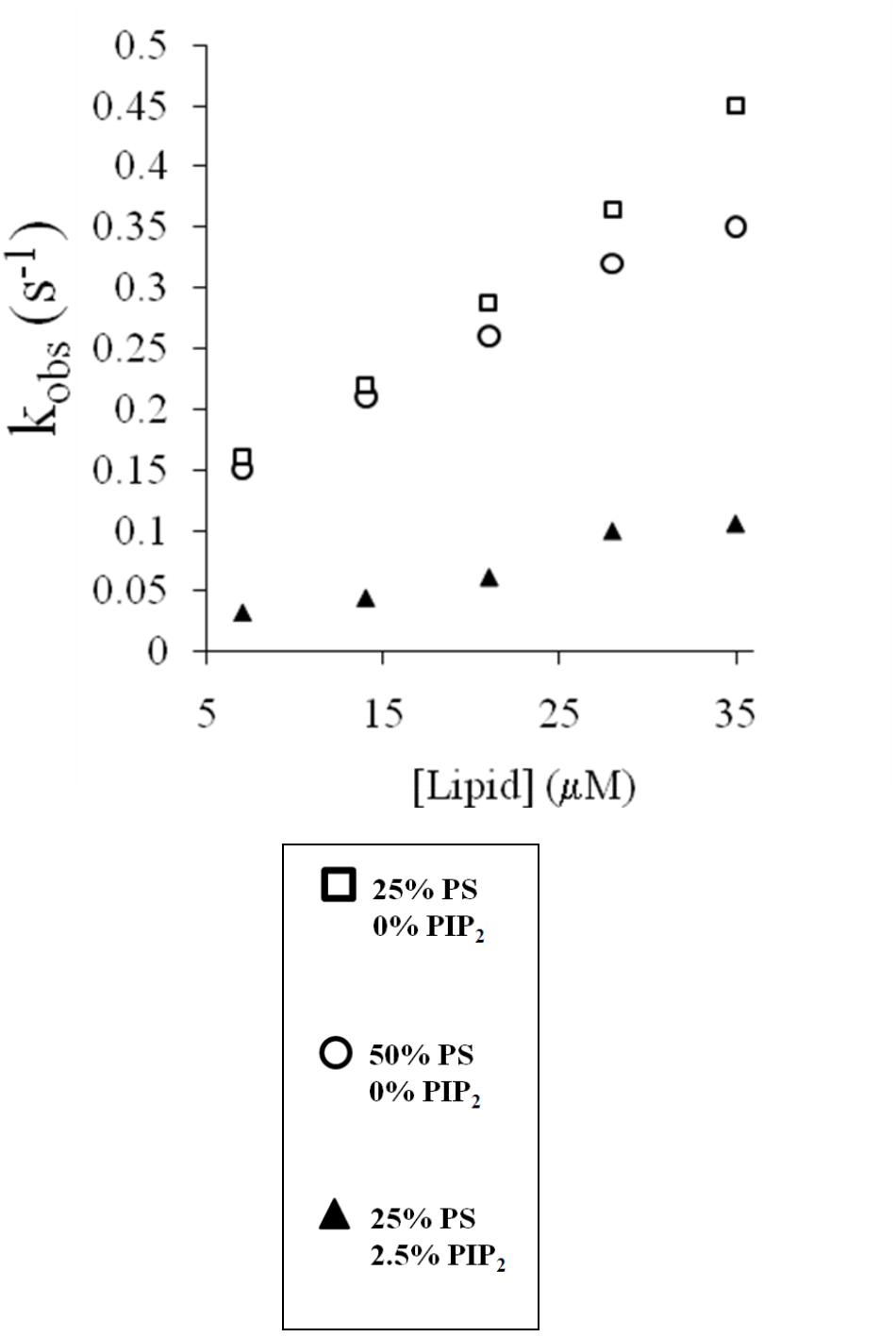

Relative Fluorescence
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (min)
0% PIP2
5% PIP2

Cdc42 from liposomes such that it was about 6-fold slower compared to liposomes lacking PIP_2.

As a complimentary approach, we used a FRET assay to monitor the exchange of Cdc42 between two different populations of liposomes (Figure 3.1C). Specifically, Mant-GMPPNP-loaded Cdc42 was initially associated with control liposomes lacking PIP_2 and then following its intrinsic capability to dissociate from these vesicles, it was free to re-associate with either control liposomes labeled with HAF (designated - PIP_2 in Figure 3.1D), or HAF-labeled liposomes containing 5% PIP_2 (designated + PIP_2). The faster rate of quenching of Mant fluorescence depicts a case where the Mant-GMPPNP-loaded Cdc42 binds to the PIP_2-containing, HAF-labeled liposomes and remains associated with the vesicles for a longer period of time compared to vesicles lacking PIP_2 (i.e. see the black trace versus the red trace in Figure 3.1D). Using this read-out, we found that Mant-GMPPNP-Cdc42 was exchanged between the two populations of liposomes at identical rates when comparing control liposomes with liposomes containing a 2-fold greater amount of PS (i.e. 50% PS instead of 25% PS) (not shown). However, when we examined the exchange of Mant-GMPPNP-Cdc42 between control liposomes and HAF-labeled liposomes that contained 5% PIP_2, there was an approximate 30% increase in the amount of Cdc42 that partitioned into the PIP_2-containing vesicles (Figure 3.1D).

In Chapter 2, we showed that the interaction between Cdc42 and membranes can be described by a bimolecular interaction, in accordance with the following equation: [Cdc42]+[Lipids] $\leftrightharpoons$ [Cdc42 : Lipids]. As described in the preceding section, PIP_2 appears to exert its effects by decreasing the rate at which Cdc42 dissociates from

Figure 3.2 PIP_2's influence on the membrane binding kinetics of geranylgeranylated Cdc42. Cdc42-Mant-GMP-PNP (50 nM) was mixed with increasing concentrations of HAF-labeled liposomes prepared by extrusion, with the indicated molar percentages of PIP_2 and PS. The base lipid composition was 35% cholesterol, 25% PE, 25% PS, and 5% PI, where PIP_2 replaced an equal molar percentage of PI, and additional PS replaced an equal molar percentage of PE. The curves were fit to equation 2a in the "Methods" of Chapter 2. The k_{obs} values obtained were plotted as a function of lipid concentration, showing a linear dependence that was fit with equation 3 in the "Methods" of Chapter 2. The membrane dissociation rate (k_{off}) of Cdc42 was obtained from the y-intercept.


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
k_{obs} (s^{-1})
5
15
25
35
[Lipid] (μM)
25% PS
0% PIP_2
50% PS
0% PIP_2
25% PS
2.5% PIP_2

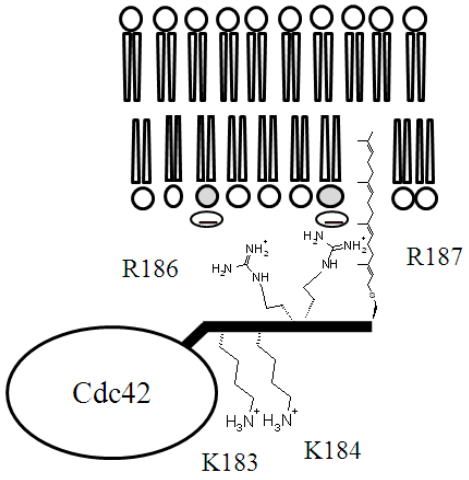
membranes. The rate constants for these different steps can be estimated using the FRET assay described above. Cdc42-Mant-GMPPNP was incubated with varying concentrations of HAF-labeled liposomes that contained either 25% PS (i.e. standard control liposomes), 50% PS, or 25% PS plus 2.5% PIP_2. The resulting profiles for the quenching of Mant fluorescence due to the FRET between Cdc42-Mant-GMPPNP and HAF-labeled liposomes, as a function of time of incubation of Cdc42 with the liposomes, were fit to the exponential equation: $F(t) = F_o(1 - e^{-k_{obs}t})$. The value for k_{obs} is related to lipid concentration through the following equation: $k_{obs} = k_{off} + k_{on}[\text{Lipids}]$. Thus, a linear plot of k_{obs} as a function of lipid concentration can yield estimates for both the rate of dissociation of Cdc42 from liposomes (y-intercept) and its rate of association with the membrane (slope). As shown in Figure 3.2, liposomes containing 2.5% PIP_2 significantly reduced the rate of dissociation of Cdc42 from the liposomes while only modestly affecting its rate of association with the membrane, when compared to the same experiments performed with liposomes lacking PIP_2. Increasing the concentration of PS within the liposomes to 50% had little effect on either the rate of membrane-association or membrane-dissociation of Cdc42, thus consistent with our findings that PIP_2 exerts a specific influence on the membrane-binding of Cdc42.

3.3.2 The di-arginine pair located at the carboxyl-terminus of Cdc42 is required for the effects of PIP_2 on membrane-binding - Given the likely importance of the polybasic region of Cdc42 for its ability to bind to membranes, we set out to examine whether it might account for the regulatory effects of PIP_2. The polybasic region of Cdc42 consists of a pair of lysine residues in tandem with a pair of arginine residues.

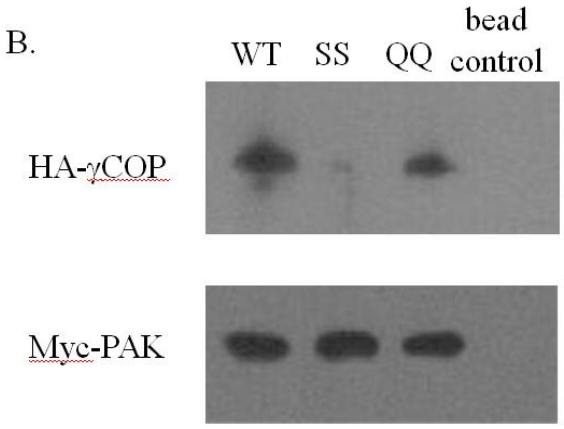
We had previously shown that the di-lysine motif was required for the binding of Cdc42 to the γCOP subunit of the COPI complex and that this interaction was both important for intracellular trafficking and for the ability of constitutively active (fast-cycling) Cdc42 to transform cells (Wu et al., 2000; also, Stamnes refs). As depicted in Figure 3.3A, this implies that the carboxyl-terminal di-lysine motif of Cdc42 is pointing-out from the membrane-surface (i.e. in order to be accessible for binding to γCOP). Assuming that the proximal di-arginine motif is not necessary for binding to γCOP, it then would be free to be positioned toward the membrane-surface, so that it could aid in the membrane binding of Cdc42. We tested this idea by examining the ability of wild-type, GTPγS-bound Cdc42 and different Cdc42 molecules containing substitutions within the di-arginine and di-lysine motifs, to bind to γCOP. HA-tagged γCOP was expressed in COS-7 cells, and then the lysates were incubated with insect cell-expressed, recombinant His_6-tagged wild-type Cdc42, or with either the His-Cdc42(RR193,194QQ) double-mutant (i.e. designated the di-lysine mutant) or His-Cdc42(KK191,192SS) (designated the di-arginine mutant), bound to nickel beads. The beads were then washed and pelleted and examined by Western blotting. Figure 3.3B shows that γCOP associates with wild-type Cdc42 and the Cdc42 di-arginine mutant, but is incapable of binding to the Cdc42 di-lysine mutant, consistent with the idea that it is the di-lysine pair that is required for binding to the COPI complex. Importantly, the Cdc42 di-arginine motif is not only able to bind to γCOP, but also interacts with other Cdc42-target/effector proteins such as PAK (for p21-activated

Figure 3.3 A C-terminal di-arginine motif mediates Cdc42's binding to PIP_2 containing membranes. **A)** Depiction of Cdc42's binding interface with the membrane surface and the proposed orientation of its C-terminus. **B)** 1 ug of insect-expressed polyhistidine-tagged Cdc42WT and C-terminus mutants, SS (K183S, K184S) and QQ (R186Q, R187Q), were pre-bound to Nickel affinity beads and incubated with lysates from COS7 cells, transiently transfected with vectors expressing the HA-tagged COPI γ subunit and the Myc-tagged PAK3. Eluents were analyzed by Western blotting with HA and Myc antibodies to assess for γ-COP- and CRIB domain-mediated interactions, respectively. **C)** Insect cell recombinant Cdc42 (50 nM), wild type and QQ, was preloaded with MantGDP and mixed with 30 uM liposomes, ± 10% PIP_2. At the 1-min time point, 1 uM RhoGDI was added and their release from the membranes was monitored by Mant/HAF de-quenching. **D)** Inter-vesicle transfer of MantGDP–bound Cdc42 (30 nM), WT and QQ, from 20 uM unlabeled liposomes to 20 uM liposomes containing HAF, ± 5% PIP_2.

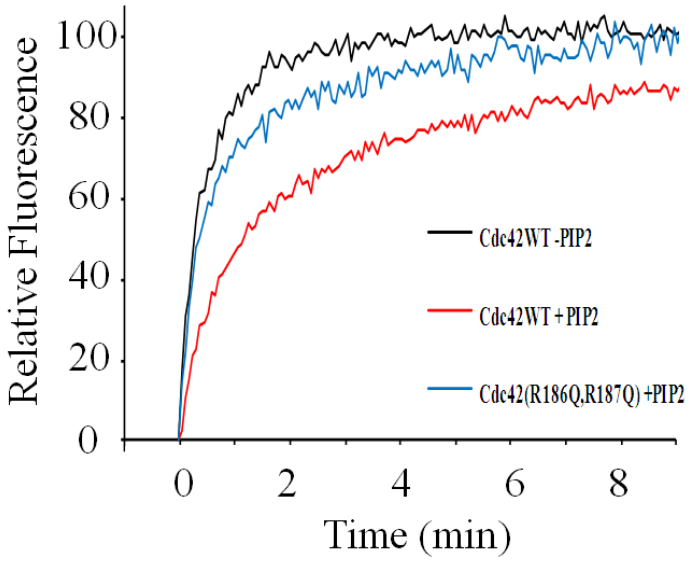
A.


R186
R187
Cdc42
K183
K184

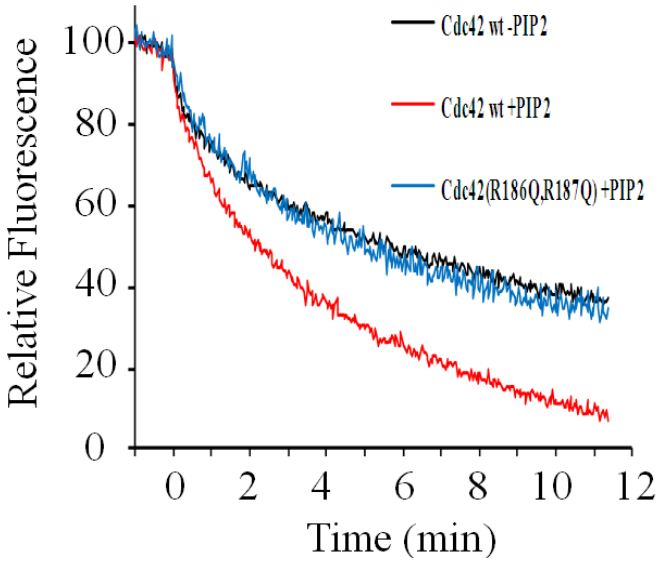
B.


WT
SS
QQ
bead control
HA-γCOP
Myc-PAK

C.


Relative Fluorescence
100
80
60
40
20
0
0
2
4
6
8
Time (min)
Cdc42WT -PIP2
Cdc42WT + PIP2
Cdc42(R186Q,R187Q) +PIP2

D.


Relative Fluorescence
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (min)
Cdc42 wt -PIP2
Cdc42 wt +PIP2
Cdc42(R186Q,R187Q) +PIP2

kinase) that bind in the classical manner through the 'effector loop' (Switch I domain) (Figure 3.3B).

We then examined how the Cdc42 di-lysine and di-arginine mutants interacted with PIP_2-containing liposomes, using two different approaches. One approach involved examining the ability of Mant-GMPPNP-loaded wild-type Cdc42 and the different Cdc42 mutants to dissociate either from HAF-labeled control liposomes, or HAF-labeled liposomes containing 2.5% PIP_2, in the presence of RhoGDI, as read-out by an increase in Mant fluorescence (Figure 3.3C). Similar to the results shown in Figure 3.1B, the presence of PIP_2 in the liposomes significantly slowed the dissociation of Cdc42-Mant-GMPPNP from the HAF-labeled vesicles. The Cdc42 di-lysine mutant exhibited a similar reduced rate of dissociation from the PIP_2-containing liposomes (not shown), whereas the Mant-GMPPNP-loaded Cdc42 di-arginine mutant exhibited a rate of dissociation from the PIP_2-containing vesicles that was similar to the rate of dissociation of wild-type Cdc42 from the control vesicles lacking PIP_2.

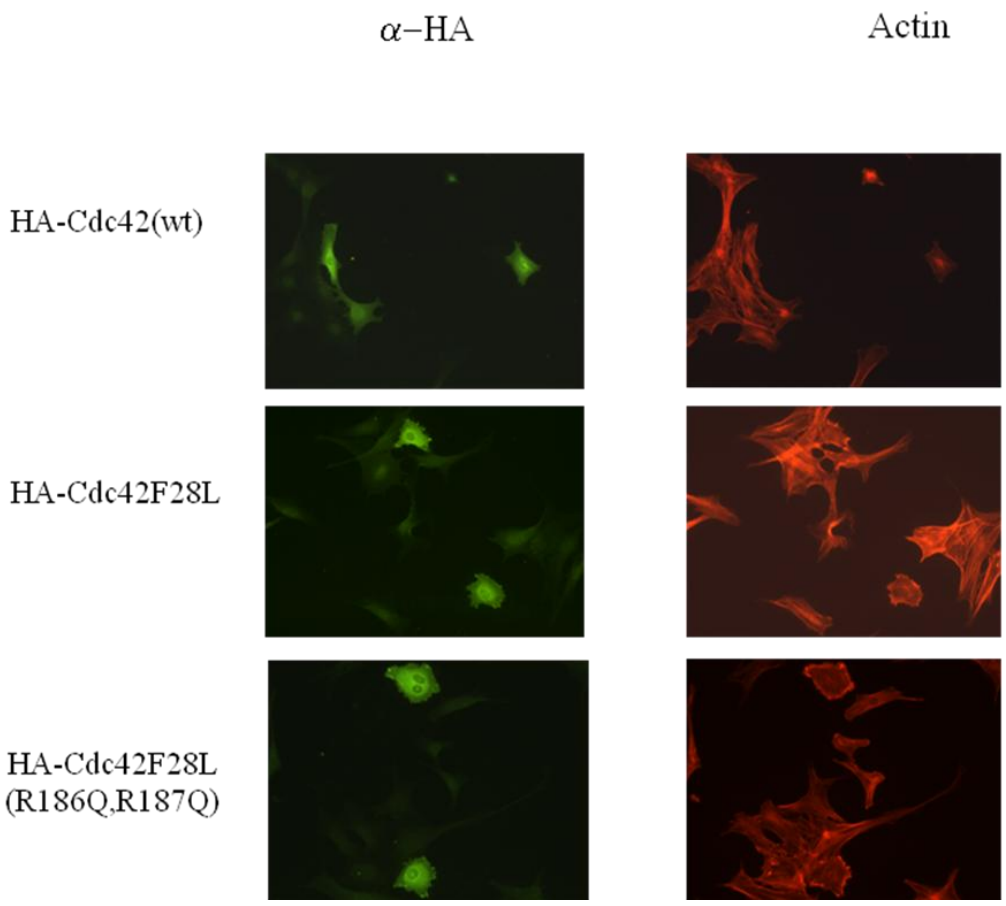
In a second set of experiments, we examined the ability of wild-type Cdc42, versus the Cdc42 di-lysine and di-arginine mutants, to exchange between control liposomes and liposomes containing 5% PIP_2, as monitored in real-time by the quenching of Mant fluorescence that accompanies the dissociation of Cdc42 from unlabeled liposomes and its subsequent binding to HAF-labeled vesicles. As shown in Figure 3.3D, the Cdc42 di-arginine mutant shows a significantly slower rate of exchange, compared to wild-type Cdc42, between control liposomes and liposomes containing PIP_2 (compare blue and red plots, respectively), but instead showed a rate of exchange that was similar to what we have measured for the exchange of wild-type Cdc42

between control liposomes lacking PIP_2 (black plot). Therefore, these results indicate that it is the di-arginine pair within the carboxyl-terminal region of Cdc42 that is essential for the high affinity membrane-binding conferred by PIP_2.

3.3.3 The interaction of Cdc42 with PIP_2 influences its cellular localization and ability to induce the transformation of fibroblasts- In light of the role played by the di-arginine pair within the carboxyl-terminal domain of Cdc42 in binding to PIP_2–containing membranes, we were interested in examining the cellular consequences of mutating these residues. First, we set out to see whether there might be gross changes in the overall cellular localization of Cdc42 containing substitutions at this site. Based on immunofluorescence experiments, we thus far have not detected significant differences in the overall cellular localization for Cdc42 that can be attributed to substitutions for the carboxyl-terminal di-arginine pair. Some examples are shown in Figure 3.4 where we have compared the localization of the HA-tagged wild-type Cdc42, the constitutively active HA-Cdc42(F28L) mutant, the HA-Cdc42(F28L,R193A,R194A) triple mutant. Each of these Cdc42 constructs showed plasma membrane-staining and Golgi-staining as typically observed for this GTPase.

We then set out to probe for the functional consequences of substituting for the di-arginine pair on Cdc42. We first examined whether these changes influenced the ability of the constitutively active Cdc42(F28L) molecule to stimulate microspike formation, i.e. one of the classical read-outs for Cdc42 cellular function (ref). However, as shown in Figure 3.4, we did not detect significant differences in the

Figure 3.4 The subcellular localization of Cdc42 and its microspike formation were not detectably altered by disrupting its interaction with PIP_2. NIH 3T3 cells were transiently transfected with HA epitope-tagged Cdc42, Cdc42(F28L) or Cdc42(F28L,R186Q,R187Q). The subcellular localization was examined by anti-HA staining (left panels). Actin cytoskeletal morphology was examined by rhodamine-conjugated phalloidin staining (right panels).


α−HA
Actin
HA-Cdc42(wt)
HA-Cdc42F28L
HA-Cdc42F28L
(R186Q,R187Q)

ability of the activated Cdc42(F28L) mutant, versus the Cdc42(F28L,R193A,R194A) triple mutant, to induce the microspike phenotype in NIH 3T3 cells.

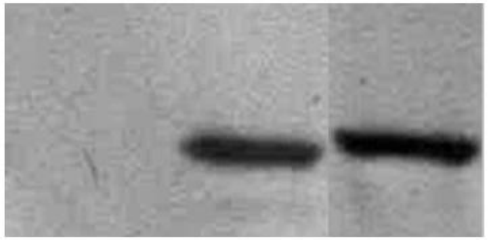
We next generated NIH 3T3 cell lines stably expressing the Cdc42(F28L) mutant and a Cdc42(F28L,R193A,R194A) triple mutant in order to see whether substitutions for the carboxyl-terminal di-arginine pair might impact the ability of activated Cdc42(F28L) to induce cellular transformation. Here, we obtained some very interesting and striking results (Figures 3.5A-D). A number of previous studies have shown that the Cdc42(F28L) mutant is able to induce the transformation of fibroblasts, as read-out by growth in low serum or by colony formation in soft-agar. This is further demonstrated in Figures 3.5B-D. However, the Cdc42(F28L,R193A,R194A) triple mutant, when stably expressed in NIH 3T3 cells at levels comparable to Cdc42(F28L) (Figure 3.5A), showed no ability to enable fibroblasts to grow in low serum (Figure 3.5B) or to exhibit anchorage independent growth (Figures 3.5C and 3.5D). Collectively, these results highlight two important points. First, they show that it is possible to uncouple the ability of an activated Cdc42 mutant to induce actin cytoskeletal changes and microspike formation from its ability to drive cellular transformation. Secondly, they demonstrate the ability of constitutively active Cdc42 to associate with PIP_2 is essential for its transforming activity.

3.4 Discussion

3.4.1 The carboxyl-terminal di-arginine motif of Cdc42 targets it to membranes containing PIP_2- The Rho family GTPase, Cdc42, helps to regulate a broad array of cellular events ranging from actin cytoskeletal remodeling and polarity-dependent processes to cell growth and differentiation. It has been commonly assumed that most of these activities are dependent upon the interactions of activated Cdc42 with its signaling targets and effector proteins along the surfaces of cellular membranes. Thus, in this study, we set out to better understand how the membrane binding of Cdc42 is mediated, and in particular, the role played by a stretch of basic amino acid residues located within its carboxyl-terminal end. As a first step toward addressing these questions, we used synthetic liposomes as model membranes with lipid compositions consistent with the inner leaflet of the plasma membrane. The negative-charged lipids PS and PIP_2, which are both major components of the inner leaflet of the plasma membrane (17, 11), have been shown to be involved in targeting polybasic domain-containing proteins to the plasma membrane. We examined the contributions from each of these anionic lipids by selectively including one or the other in liposomes. When Cdc42 was bound to liposomes containing PIP_2, its rate of dissociation from the membrane surface in the presence of RhoGDI was significantly reduced, compared to its dissociation from control lipid vesicles. No such effect was seen for Cdc42 in liposomes containing excess concentrations of PS. Furthermore, Cdc42 was more efficient at partitioning into liposomes containing PIP_2, compared to control vesicles. Collectively, these results that PIP_2 plays a specific role by

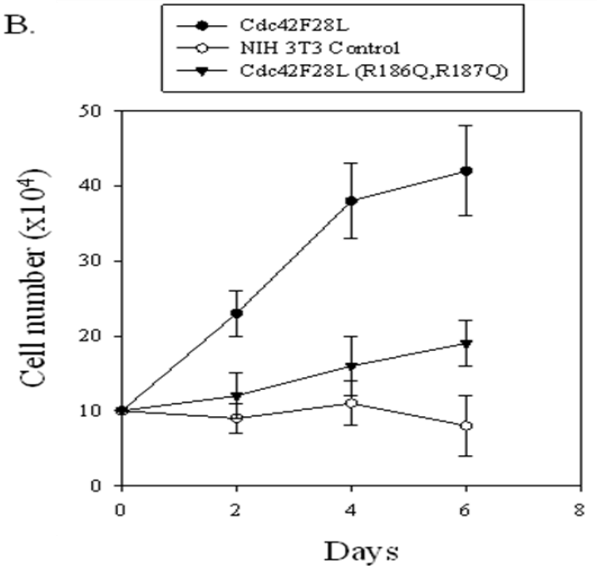
Figure 3.5 Binding to PIP_2 is required for Cdc42-stimulated cell growth and transformation. **A)** NIH 3T3 lines that stably expressed with HA epitope-tagged Cdc42(F28L) or Cdc42(F28L,R186Q,R187Q) were generated. Cdc42 protein was detected by immunoblotting with an anti-HA antibody. **B)** NIH 3T3 cells that stably expressed Cdc42(F28L) or Cdc42(F28L,R186Q,R187Q), were cultured at a low concentration (1%) of calf serum, and harvested and counted in 2 day increments over a one week period. **C)** Anchorage-independent growth of Cdc42-, Cdc42(F28L)- and Cdc42(F28L,R186Q,R187Q)-expressing NIH 3T3 cells was determined by colony formation in soft agar. After 4 weeks, plates were examined and photographed. **D)** The colonies from the soft agar plates shown in 5C were scored. In each experiment four duplicate 35 mm plates were counted at four randomly chosen areas.

A.

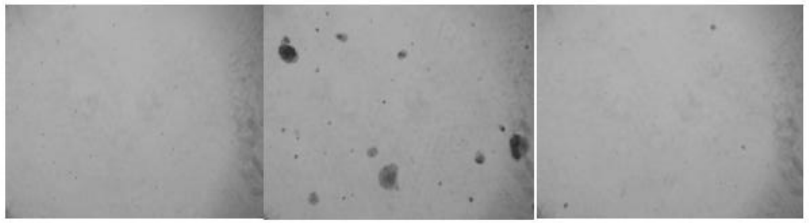


NIH3T3 Cdc42F28L Cdc42F28L (R186Q,R187Q)

B.

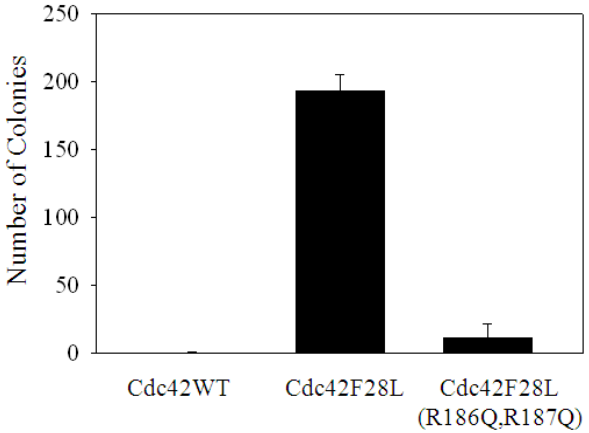


C.



Cdc42WT Cdc42F28L Cdc42F28L (R186Q,R187Q)

D.



enhancing the binding of Cdc42 to lipid bilayers that cannot be simply accounted for by its negative charge.

Cdc42 contains a polybasic region within its carboxyl-terminal end, located just upstream from a covalently attached isoprenoid geranylgeranyl moiety. The polybasic region consists of a di-lysine motif and a di-arginine motif, separated by a single serine residue. Since the di-arginine motif directly precedes the geranylgeranyl moiety, we speculated that it might be in a better position to contact the membrane, compared to the di-lysine motif. Furthermore, the di-lysine motif has been shown to make important contacts with the γCOP subunit of the membrane-associated COPI complex (14). Given that the COPI complex is positioned such that its cargo recognition sites face the membrane, the di-lysine motif is likely directed away from the membrane surface in order to make contact with this complex. In fact, we showed that the di-arginine motif did not contribute to the interaction between Cdc42 and γCOP subunit, whereas the di-lysine motif is essential for this interaction. However, we demonstrated that the di-arginine motif within the carboxyl-terminal end of Cdc42 is necessary for binding to liposomes containing PIP_2, such that the affinity of Cdc42 for PIP_2 was effectively eliminated when the di-arginine motif was mutated to a pair of uncharged glutamine residues. These findings then raised the question of what might be the cellular consequences of the binding of Cdc42 to PIP_2.

3.4.2 The binding of Cdc42 to PIP_2 has important roles in its ability to impact cell growth- The anionic lipid $PI(4,5)P_2$ is predominantly located within the inner leaflet of the plasma membrane at concentrations that have been estimated to be as

high as 5% (16, 18, 22) and has been shown to bind to both regulators (i.e. guanine nucleotide exchange factors) and effectors of Cdc42 (19-21). Therefore, the recruitment of Cdc42 to PIP_2-enriched regions on the plasma membrane could facilitate its ability to become activated and/or to signal through its effector proteins. To see whether this might indeed be the case, we substituted two glutamine residues for the di-arginine motif within an activated Cdc42(F28L) background and examined the consequences of these substitutions for the signaling capabilities of Cdc42. Interestingly, we found that while mutating the di-arginine motif did not significantly affect the ability of activated Cdc42 to stimulate the formation of microspikes, which represents one of the best known cellular responses to Cdc42 (17), it prevented Cdc42 from inducing cellular transformation, as read-out by the growth of cells under serum-deprived conditions or the formation of colonies in soft-agar. Thus, substitutions for the di-arginine motif uncoupled the ability of Cdc42 to trigger actin cytoskeletal rearrangements necessary for generating microspikes/filopodia, from the stimulation of those signaling events that underlie the ability of Cdc42 to induce transformed phenotypes.

What do these findings imply for the role of PIP_2 in the cellular actions of Cdc42? An examination of the eptiope-tagged forms of Cdc42(F28L) versus the Cdc42(F28L,R193Q,R194Q) triple-mutant by immunfluorescence indicates that substituting for the carboxyl-terminal di-arginine motif does not lead to marked changes in the cellular localization of this GTPase. Apparently, Cdc42 is able to interact with the specific effector protein(s) necessary for mediating the actin cytoskeletal changes required for microspike formation from plasma membrane sites,

independent of whether or not they contain PIP_2. On the other hand, the ability of Cdc42 to engage the essential effector(s) for cellular transformation requires that Cdc42 binds to specific membrane locations that are rich in PIP_2. This could be the result of one or more Cdc42-effector proteins, which are necessary for sending transforming signals, having the capability to bind PIP_2 so as to be recruited to these specific membrane locations. The ability of PIP_2 to increase the overall affinity of Cdc42 for the membrane could provide an added advantage of enabling these membrane sites to better compete with RhoGDI for binding the geranylgeranyl moiety of Cdc42. However, another intriguing possibility is that the binding of PIP_2 to the carboxyl-terminal end of Cdc42 might help the GTPase to assume the proper activated conformational state to engage a specific effector protein that is essential for transformation and/or to induce a specific change in the activity of the effector. Previous work from our laboratory showed that the ability of RhoGDI to distinguish between the GDP- and GTP-bound forms of Cdc42 was dependent upon Cdc42 being associated with membranes (9). Specifically, whereas the binding of RhoGDI to the GDP- versus GTP-bound forms of Cdc42 in solution was essentially indistinguishable, clear differences were observed when monitoring the interactions of these nucleotide-bound forms of Cdc42 with liposomes. This then implies that the lipid bilayer interacts with Cdc42 in a manner that significantly influences its ability to assume an activated conformational state for engaging specific target/effector proteins. Moreover, the presence of PIP_2 at specific membrane sites might further tune the conformation of activated Cdc42, enabling it to engage specific targets, through the interactions of this lipid with the carboxyl-terminal di-arginine motif of the GTPase.

Future studies will be further directed toward examining how the lipid bilayer might help to influence the ability of activated Cdc42 to form signaling complexes that are important for its transforming capability, and whether a specific Cdc42-effector protein(s) is recruited to membrane sites where PIP_2 has accumulated.

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CHAPTER 4

CONCLUSIONS

A number of regulatory processes in the cell are mediated by GTPases. Perhaps no other group of GTPases has exerted as broad of an influence as the Ras superfamily, which has made them the central focus of many basic and biomedical research-oriented laboratories. Over the past three decades, substantial progress has been made in characterizing their cellular functions and their roles in disease. Equally substantial milestones have been reached *in vitro*, through biochemical reconstitution experiments and 3-D structure determinations. However, in the vast majority of *in vitro* studies, an important component has been omitted. This has to do with the fact that four out of the five subgroups within the Ras superfamily, namely the Rho, Rab, Arf and Ras family members, are modified by the posttranslational addition(s) of a lipid chain, that enables them to bind to membranes in the cell. In our work, we set out to explore the influence the lipid bilayer has on Rho GTPase signaling and how their membrane interactions are regulated. To this end, we developed *in vitro* techniques that enabled us to make real-time measurements of the interaction of the Rho family member, Cdc42, with synthetic membranes. We were able to demonstrate important contributions made by key regulators, RhoGDI, RhoGAPs and RhoGEFs, and specific phospholipids in modulating these interactions. In the end, we gained a newfound appreciation for the role the lipid bilayer plays in Cdc42 signaling and a better understanding of how Cdc42 cycles on and off the membrane and, in essence, moves about in the cell.

An emerging theme, throughout the Ras superfamily, is the need to engage effectors at multiple locations in the cell. With the vast majority of these interactions occurring on the surfaces of membranes, optimizing their membrane residence time becomes an extremely important objective. How does a given GTPase remain bound to the membrane for a sufficient period of time to engage its downstream targets, while maintaining the capability to localize to multiple cellular locations? How this is achieved turns out to be case-specific, as each subfamily has its own needs regarding signaling locations and functional time frames. Two prominent members of the Ras subfamily, H- and N-Ras, need to signal at both the plasma membrane and the endomembranes. Since they do not have as effective a membrane anchor as the Rho subfamily's geranylgeranyl moiety, they must rely on the labile attachment of palmitoyl groups to remain bound to the membrane surface. Dissociating from the membrane then becomes the function of the de-palmitoylation machinery. Once this occurs, the GTPases are free to reach virtually any location within the cytosol. In these cases, the cellular distribution of the respective palmitoyltransferases and de-palmitoylases for the Ras subfamily members largely determines their spatial range of influence.

The Rho GTPases are perhaps even more widely known for regulating events at a variety of different locations in the cell. Cdc42 is a remarkable example, as it plays an active role in many key spatial processes, including cell polarity, cell migration, and vesicle trafficking. In Chapter 2, we reconstituted the interaction of geranylgeranylated Cdc42 with synthetic membranes and uncovered interesting and unexpected characteristics. If one were to take a snapshot of the partitioning of Cdc42

between lipid bilayers and aqueous solution, as monitored through *in vitro* assays and corroborated by *in vivo* cellular imaging, it would likely be concluded that the residence time for Cdc42at the membrane is all but permanent. However, in taking advantage of the time-resolution of our *in vitro* approaches, we determined that Cdc42 has a residence time of only 10 seconds at the membrane, making a case for a much more dynamic interaction than expected. Thus, in contrast to being constrained at a given membrane surface, Cdc42 has the ability to dissociate from its lipid bilayer within seconds and explore the cellular milieu.

In the hectic environment of the cell, where the Rho GTPases need to reach multiple locations, they also need a safeguard to prevent them from indiscriminantly associating with any given membrane surface, as this could jeopardize their ability to engage the appropriate membrane sites that contain their signaling targets. Like the Rho GTPases, similar regulatory mechanisms are required to ensure that Rab GTPases traffic between the proper membrane sites. Thus the Rab GTPases utilize a regulatory protein (RabGDI) that helps these GTPases to 'exit' from one membrane site and to ultimately associate with an appropriate 'acceptor' membrane site. While in complex with the RabGDI, the Rab GTPases become susceptible to a release mechanism as catalyzed by what are referred to as RabGDI releasing factors. One might assume that a strict analogy exists between the actions of RabGDI and those of RhoGDI, despite the fact that these two regulatory proteins fail to exhibit structural homology. The family of proteins collectively referred to as RhoGDI (often designated RhoGDI-1, RhoGDI-2, and RhoGDI-3) were discovered and named for their strong inhibition of GDP dissociation which, at face value, would suggest that RhoGDI functions

primarily as a negative regulator. However, we now realize that is an oversimplification, as they also play a number of other critically important roles, including their ability to stabilize their respective GTPase in the cytosol. Depletion of RabGDI in yeast is lethal, resulting in the inhibition of vesicle transport at multiple stages of the secretory pathway. Studies from mammalian cells indicate that RhoGDI is essential for the ability of Cdc42 to transform NIH3T3 fibroblasts.

In Chapter 2, we took mechanistic approaches to better understand how RhoGDI influences the interactions of Cdc42 with membranes. Despite its potent ability to influence how Cdc42 partitions between membranes and the cytosol, we found, quite unexpectedly, that RhoGDI has no direct influence over the rate at which Cdc42 dissociates from the membranes. Instead of directly driving the dissociation of Cdc42 from membranes, RhoGDI takes advantage of the intrinsic ability of Cdc42 to dissociate from the lipid bilayer. Following the dissociation of Cdc42 from the membrane surface, the hydrophobic binding pocket provided by the C-terminal half of RhoGDI then encapsulates the geranylgeranyl moiety of Cdc42, thereby significantly slowing the re-binding of Cdc42 to membranes. This regulatory action of RhoGDI helps to ensure that Cdc42 binds to specific membrane-signaling sites. Indeed, previous studies from our laboratory showed that the depletion of RhoGDI from mammalian cell lines caused the widespread delocalization of Cdc42 throughout the endomembranes of the cell. This can explain the beneficial role played by RhoGDI in Cdc42's signaling activities and in the ability of oncogenic mutants of Cdc42 to transform cells.

A common characteristic of many small GTPases is the dependence of their membrane association on the state of their bound guanine nucleotide. This has been demonstrated most dramatically within the Arf subfamily, where nucleotide exchange from GDP to GTP induces conformational changes that strengthen their association with membranes. Consequently, the spatial distribution of Arf GEFs and Arf GAPs dictates the subcellular localization of the Arf GTPases. Given that the majority of small GTPase signaling activities occur at the membrane, it seems logical that their activation is coupled to their membrane binding capability and, once their signaling activities are no longer needed, they are re-localized to the cytosol. In our work, we have demonstrated a similar case for the Rho GTPases. Binding measurements made previously in our laboratory showed that RhoGDI was apparently indifferent to the nucleotide state of Cdc42 in solution, as it could bind both the GDP- and GTP-bound forms of Cdc42 with equal affinity. However, we then made the surprising finding that when Cdc42-RhoGDI interactions were assayed in the presence of liposomes, the affinity of RhoGDI for the GTP-bound state of Cdc42 is reduced tenfold. Kinetic modeling confirmed that it was the initial recognition of Cdc42 at the membranes by GDI, and not their release, that depended on the nucleotide-bound state of Cdc42. Importantly, beyond the role that the lipid bilayer plays as a binding platform for Cdc42 interactions with its signaling partners, the membrane surface also influences the conformation of Cdc42. This novel finding had particular relevance to a puzzling structural attribute of Cdc42, found earlier in our laboratory. The x-ray crystal structure of activated Cdc42 was found to adopt an almost identical conformation to that of GDP-bound Cdc42 and, only upon the binding of a signaling effector was

Cdc42 able to assume an activated conformation. In this work, we show that the lipid bilayer is capable of shaping the activated conformation of Cdc42.

In Appendix 1, we elaborated upon the regulatory actions of RhoGDI on our membrane association of Cdc42 by examining the additional effects of RhoGAPs and RhoGEFs. By enhancing GTP hydrolysis, the RhoGAP (Cdc42GAP) greatly accelerated the rate of dissociation of Cdc42 from membranes that occurs in the presence of RhoGDI. Interestingly, RhoGEFs are still capable of stimulating nucleotide exchange on Rho GTPases, while in complex with GDI. Indeed, the the catalytic domain of Dock180 was able to achieve this on RhoGDI-bound Rac1at low nanomolar concentrations. Based on the relatively low affinity that RhoGDI exhibits for GTP-bound Rho GTPases at the membrane, we can propose a model for how the Rho GTPases are preferentially recruited to a location in the cell (Figure A.4). Following the upstream activation of a RhoGEF, the cytosolic RhoGDI-bound Cdc42 undergoes (GDP-GTP) nucleotide exchange. By virtue of the GTPase inhibitory activity of RhoGDI, the GTP-bound activated state of Cdc42 is preserved until it reaches a membrane location where it exhibits a significantly weakened affinity for RhoGDI, causing the GDI to dissociate and paving the way for Cdc42 to engage specific signaling effectors. Signaling is ultimately terminated through the actions of a RhoGAP. This results in the re-binding of RhoGDI to Cdc42 and the release of the RhoGDI-Cdc42 complex into the cytosol where it awaits another activation event.

In contrast to the common view that the membrane simply serves as a signaling site where Cdc42 meets its signaling targets, we have obtained evidence suggesting that the lipid bilayer influences the conformation state of Cdc42. In Chapter 3, we

explored the effects that specific phospholipids might have on the ability of Cdc42 to bind to membranes. Located immediately proximal to the geranylgeranyl moiety of Cdc42 are two highly-conserved pairs of basic residues, which could conceivably confer upon Cdc42 a binding preference for negatively charged membranes. Our *in vitro* reconstitution system enables us to examine how different anionic lipids contribute to the binding of Cdc42 to lipid bilayers. These studies showed that only PIP_2 is capable of targeting Cdc42 to the membranes. Indeed, the intrinsic rate of dissociation of Cdc42 from liposomes was reduced by more than tenfold when relatively low percentages of PIP_2 were included. With PIP_2 exclusively localized to the plasma membrane, and shown in many cases to be sequestered into microdomains, this anionic phospholipid may provide a mechanism for recruiting Cdc42 to specific regions in the cell that contain its key signaling partners. We went on to show that the interaction of Cdc42 with PIP_2 is facilitated by a pair of arginine residues directly neighboring its geranylgeranyl moiety. Perhaps most interesting was our finding that disrupting the interactions of oncogenic Cdc42 mutants with PIP_2 led to a significant reduction in the ability of Cdc42 to transform mammalian cells, while leaving its ability to influence cell morphology intact. Thus, by blocking the ability of Cdc42 to bind PIP_2, we are able to uncouple the roles of this GTPase in actin remodeling from its ability to regulate cell growth.

In conclusion, this thesis highlights the important area of G-protein signaling that concerns their interactions with the membrane. Given that the vast majority of GTPases engage their regulators and targets at the membrane surface, it is perhaps not surprising that membranes might contribute more to their cellular functions than

providing docking platforms. Moreover, it seems reasonable to expect that many of the cellular and biological functions attributed to the various GTPases that associate with membranes will be highly dependent on their interactions with specific lipids.

APPENDIX 1

THE EFFECTS OF REGULATORY PROTEINS ON THE MEMBRANE ASSOCIATION/DISSOCIATION CYCLE OF THE RHO GTPASES

Introduction

Previous work from Chapters 2 and 3 have treated the association of Cdc42 with GDI and membranes in terms of a relatively simple partitioning between the membrane and the cytosol. However, it would be more realistic to view this as a cycling process, where Cdc42 is continuously recruited and then released from membranes. Given their extensive involvement in a number of spatially-important processes, Cdc42, and the Rho GTPases in general, need a timely mechanism to reach multiple locations in the cell (1). By taking advantage of the tightly regulated membrane association-dissociation cycles, the cell can utilize Rho GTPase-signaling activities at multiple cellular locations. In the study described below, we explore important steps of the membrane association-dissociation cycles of the Rho GTPases in our *in vitro* reconstituted system, with the objectives of seeing how different regulatory proteins influence this process. From the work described in Chapters 2 and 3, we now have a more detailed picture of how Cdc42 is released from membranes. However, our understanding of other important points in this process, namely how Cdc42 is released from RhoGDI and its concurrent recruitment to membranes, as well as the events that lead up to its next engagement with GDI, remains incomplete.

Considering the preference of GDI for the inactive GDP-bound state of Cdc42 at the membrane, over its GTP-bound state, it seems reasonable to suspect that the fundamental Rho regulators, RhoGEFs and RhoGAPs, might be capable of modulating the association of Cdc42 with GDI and, as a result, impact its membrane-to-cytosol partitioning. Indeed, *in vivo* work has shown that the RhoGEFs and RhoGAPs make important contributions to the subcellular localization of the Rho GTPases (2,3). Should this be the case, it would suggest the membrane association-dissociation cycle of Cdc42 is coordinated with its GTP-binding/GTPase cycle. As it is, much work from our laboratory and others have demonstrated that a rapid GTP-binding/GTPase cycle for Cdc42, as occurs with the fast-cycling (Cdc42(F28L)) mutant, potentiates its signaling activities and is capable of transforming mammalian cells(4). In contrast, the GTPase-defective mutants of Cdc42, which are locked in their GTP-bound state, are not only transformation-defective, but often are toxic to cells. Thus, simply maintaining Cdc42 in an activated state is not sufficient for stimulating the signaling pathways necessary for transformation. Rather, Cdc42 may need to cycle between its GDP-bound and GTP-bound states, possibly take advantage of the regulatory actions of RhoGDI. To explore the degree of RhoGEF and RhoGAP involvement, we utilized our *in vitro* reconstitution system, consisting of geranylgeranylated Cdc42, GDI, and liposomes of defined lipid composition, to examine how the RhoGAP, Cdc42GAP, or the Rho GEF, Dock180, influence Rho GTPase partitioning between GDI and membranes.

The prototypic signaling circuit of all small G-proteins consists of a GEF for activation and a GAP for deactivation (5). Rho-specific GAPs operate by engaging

the GTP-bound form of the Rho GTPase and accelerating GTP hydrolysis by inserting a highly conserved arginine residue into position to stabilize the developing negative charge of the GTP+Pi transition state, while additionally stabilizing the switch domains (6,7). Cdc42GAP is a relatively potent Rho-specific GAP, exhibiting a k_{cat} that was reported to be 35 s^{-1}, which is approximately 350 times faster than the rate of membrane release by Cdc42 in presence of GDI (8).

Rho-GEF activity is carried out by two distinct families, i.e. the Dbl and the DOCK families of proteins. The Dbl (Diffuse B-cell Lymphoma) family originated in the unicellular eukaryotes and has remained a constant evolutionary companion to the Rho GTPases (9). Comprising 69 members, the Dbl family regulates a large fraction of the Rho family GTPases, including the Cdc42, Rac, and Rho proteins, by engaging and remodeling their switch domains in a manner that indirectly disrupts their binding to the magnesium ion that coordinates the guanine nucleotide (10). With the affinity for magnesium reduced, the bound nucleotide dissociates along with the departing magnesium.

The DOCK (Dedicator of Cytokinesis) family emerged during early metazoa. Consisting of 11 members, the DOCK family's regulatory activities focus specifically on the Rac and Cdc42 proteins (11). Similar to the Dbl family, the DOCK proteins engage the switch regions of their respective GTPase, but in this case, the coordinated magnesium ion is directly perturbed through the insertion of a key valine, facilitating its dissociation along with the bound guanine nucleotide (12). While the Dbl and DOCK families operate through distinct catalytic mechanisms, they both achieve the

common endpoint of enhancing nucleotide dissociation through their association with the switch domains of the Rho GTPases.

In this work, we were interested in seeing if Rho GTPases are accessible to GEF-engagement, and hence vulnerable to nucleotide exchange, while in complex with GDI. RhoGDI is often depicted as a kinetic trap for the Rho GTPases, stably sequestering the GTPases in the cytosol, only to release them through specific regulatory events, like phosphorylation or conformational changes induced by a so-called 'GDI-displacement factor (GDF) (13).' Our kinetic characterization of the interaction of Cdc42 with RhoGDI showed it to be more dynamic than expected, with the complex exhibiting a half-life of roughly 40 seconds (14). Thus, although Cdc42 forms a tight complex with GDI, it still could remain accessible for RhoGEF-mediated nucleotide exchange, in the 30 second to 1 minute range, well within the approximate time frame of many of its cellular activities. Once GTP loading is achieved, GDI could then provide a service for Cdc42 by preserving its activated GTP-bound state, through its GIP activity (15), until the release of Cdc42 at the membrane. Here, we examine how RhoGEFs affect the dynamics of the interaction of GDI with Rho GTPases, in our model system, utilizing the limit catalytic domain of Dock180. Due to the preference of DOCK180 for Rac, relative to Cdc42, we used insect cell-expressed, purified Rac1 in these studies. Regarding its association with GDI, Rac1 behaves similarly to Cdc42, as both GTPases preferentially bind to GDI in membranes in their GDP-bound state (Figures 2.1 and 2.9).

Methods

Protein purification

Cdc42 and Rac1 were purified as His_6-tagged protein from baculovirus infected Sf21 insect cells, as described in Chapter 2 Methods.

RhoGDI and the limit GAP domain of Cdc42GAP (residues 234-462) were purified from *E. coli* cells harboring plasmids encoding N-terminal GST of each construct, as described in Chapter 2 Methods. The limit guanine nucleotide exchange domain of Dock180 (specifically its DHR2 domain) was purified from *E. coli* cells harboring plasmids encoding it as an N-terminal His_6-tagged construct.

Fluorescence

For fluorescence based assays of Cdc42-liposome association, a Varian Cary Eclipse fluorimeter in the counting mode was used. Excitation and emission wavelengths were 365 and 440 nm, respectively. Samples were stirred continuously at 25°C in TBSM. To prepare HAF (Hexdecanoylaminofluorescein) labeled lipids for FRET assays, 1.25 nmol HAF (Molecular Probes) were vortexed with 50 μL of lipids (1 mg/ml). In order to monitor the release of Cdc42 from liposomes, Cdc42 was preloaded with Mant-nucleotide (GTP, GDP or GMP-PNP) and incubated with 30 μL of HAF-containing liposomes at RT for 5 min. The mixture was added to the cuvette, bringing Cdc42 concentration to 40 nM. At indicated time-points 50 nM RhoGDI and 10 nM Cdc42GAP were added and fluorescence was recorded for 20 min. Traces monitored the changes in Mant fluorescence due to changes in FRET between Mant-nucleotide-bound Cdc42 and liposomes containing HAF.

Nucleotide exchange assay

Competition between GDI and the guanine nucleotide exchange factor Dock180 was measured with prenylated Rac1. Rac1 was preloaded with Mant-GDP in a 25 μl volume. After transferring the mixture to the cuvette (final concentration Rac1 60 nM, Mant GDP 200 nM), 10 μM unlabeled GTP and 80 nM GDI were added for 10 min. At the indicated time point, different concentrations of the DHR2 domain of Dock180 were added. Traces monitored the loss of Mant fluorescence due to exchange.

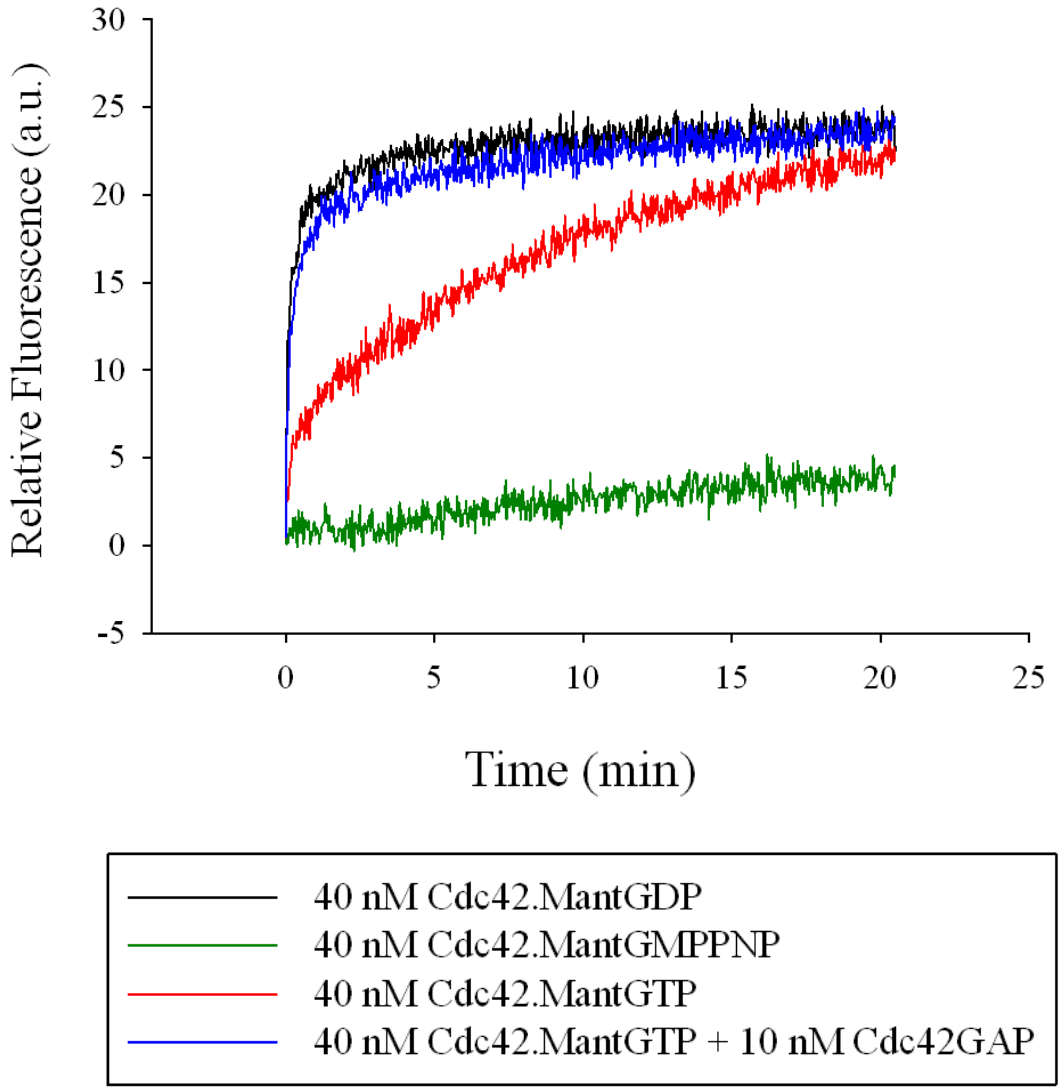
For radioactive assays of Rac1-liposome association, Rac1 40 nM was preloaded with $\alpha[^{32}P]$GTP (2300 cpm/pmol, 10 μM) by 8 mM EDTA-stimulated nucleotide exchange in the presence of 0.1 mg/ml RSE-prepared liposomes. Rac1 was then allowed to hydrolyze its bound nucleotide to $\alpha[^{32}P]$GDP by 20 min incubation at room temperature in the presence of excess magnesium (14 mM). This also prevented further EDTA-stimulated nucleotide exchange. The protein was then incubated with 45 nM GDI for 10 min, followed by a 10 min treatment with 100 nM of the DHR2 domain of Dock180, in the presence of 100 μM $\alpha[^{32}P]$GTP. The mixture was pelleted by centrifugation for 10 min at 16,000 $\times$ *g*. Radioactivity counts were separately analyzed in the supernatant and pellet fractions.

A.3 Results

A.3.1 Cdc42GAP activity greatly accelerates the release of GTP-bound Cdc42from model membranes- We selected conditions for our experiments, such that the GDI was able to exclusively bind to the GDP-bound form of Cdc42 in membranes and not the GTP-bound form. Figure A.1 shows the release of Mant-nucleotide-bound Cdc42 (40 nM) from fluorescein-labeled membranes as occurs in the presence of GDI (50 nM). Under these conditions, most of the Mant-GDP-bound Cdc42 was released from the membranes within the first minute, while the Mant-GMPPNP-bound Cdc42 remained associated with the membranes. Mant-GTP-bound Cdc42 exhibited a slow release that occurred over the course of 20 minutes, which essentially matched the intrinsic rate of GTP hydrolysis by Cdc42. Adding a catalytic amount of Cdc42GAP significantly accelerated the release of Mant-GTP-bound Cdc42 from membranes, with a rate that was indistinguishable from that for Mant-GDP-bound Cdc42.

A.3.2 GDI-bound Rac1 remains accessible to nucleotide exchange catalyzed by the GEF Dock180- To assess whether Rho GTPases are able to undergo GEF-stimulated nucleotide exchange while in complex with GDI, geranylgeranylated Rac1 was loaded with MantGDP and incubated with RhoGDI, allowing them to form a stable complex. The complex was then exposed to a ten-fold excess of unlabeled GTP, as illustrated in Figure A.2A. At the 2-minute time-point, the limit-functional domain of the Rac1- specific GEF Dock180 was introduced (Figure A.2B). At

Figure A.1 Cdc42GAP accelerates the release of GTP-bound Cdc42 from membranes in the presence of GDI. Insect cell recombinant Cdc42 (40 nM), pre-loaded with MantGDP (black), MantGMPPNP (green), or MantGTP (red and blue), was added to liposomes prepared from 30 μg/mL of labeled lipids. At t = 0, 50 nM RhoGDI was added. Cdc42GAP (10 nM) was added to one of the samples containing MantGTP-bound Cdc42 (blue), at t = 0. The release of Cdc42 from the liposomes was monitored by using the de-quenching of Mant-fluorescence (λ_{ex} = 365 nm, λ_{em} = 440 nm).

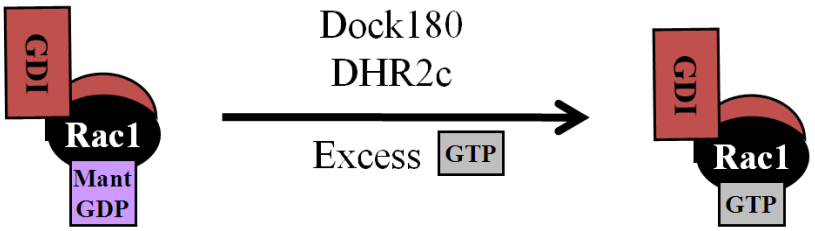

Relative Fluorescence (a.u.)
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
Time (min)
40 nM Cdc42.MantGDP
40 nM Cdc42.MantGMPPNP
40 nM Cdc42.MantGTP
40 nM Cdc42.MantGTP + 10 nM Cdc42GAP

concentrations as low as 30 nM, DHR2c efficiently catalyzes nucleotide exchange within the GDI-bound Rac1, as indicated by the decrease in Mant fluorescence where MantGDP is replaced by unlabeled GTP. Upon further increasing the levels of DHR2c to 150 nM, an upper limit is reached, where nucleotide exchange is no longer further accelerated and fails to achieve the rate of nucleotide exchange that is reached in the absence of GDI (purple curve). Thus, while GDI allows its bound GTPases to undergo guanine nucleotide exchange, it imposes an upper limit on the rate.

Based on the ability of DHR2c to catalyze nucleotide exchange in the presence of GDI, we next wanted to confirm that this level of GDP-GTP exchange was sufficient for the translocation of Rac1 from GDI to membranes. Rac1 was loaded with $\alpha[^{32}P]$GTP in the presence of rapid solvent exchange-prepared liposomes, as illustrated in Figure A.3A. Excess magnesium was then added, locking in the bound GTP and allowing it to hydrolyze to $\alpha[^{32}P]$GDP. RhoGDI was added and the GDP-bound Rac1 was released from the membranes. 100 nM DHR2c was then added and the membranes were pelleted and analyzed separately from the supernatant for radioactivity counts. The results are shown in Figure A.3B. In the sequence of events, Rac1, alone, was completely recovered in the lipid pellet. Adding GDI led to its partitioning into the supernatant, while DHR2c reversed this effect, enabling Rac1 to partition back to the membranes. Thus, by loading Rac1 with GTP, in the presence of

Figure A.2 DOCK180 can catalyze nucleotide exchange upon GDI-associated Rac1. **A)** Schematic of DOCK180-mediated nucleotide exchange of GDI-bound Rac.MantGDP for unlabeled GTP. **B)** Insect cell recombinant Rac1 (60 nM) was preloaded with MantGDP (200 nM) and incubated with 80 nM GDI for 5 minutes to allow the formation of the GDI-Rac.MantGDP complex. Subsequently, 20 μM unlabeled GTP was added. At t = 2 minutes, varying concentrations of the DHR2c domain of DOCK180 were added. DOCK180-catalyzed exchange of Rac1 was followed in real time by monitoring the decrease in Mant-fluoresence (λ_{ex} = 365 nm, λ_{em} = 440 nm).

A.


GDI
Rac1
Mant
GDP
Dock180
DHR2c
Excess
GTP
GDI
Rac1
GTP

B.

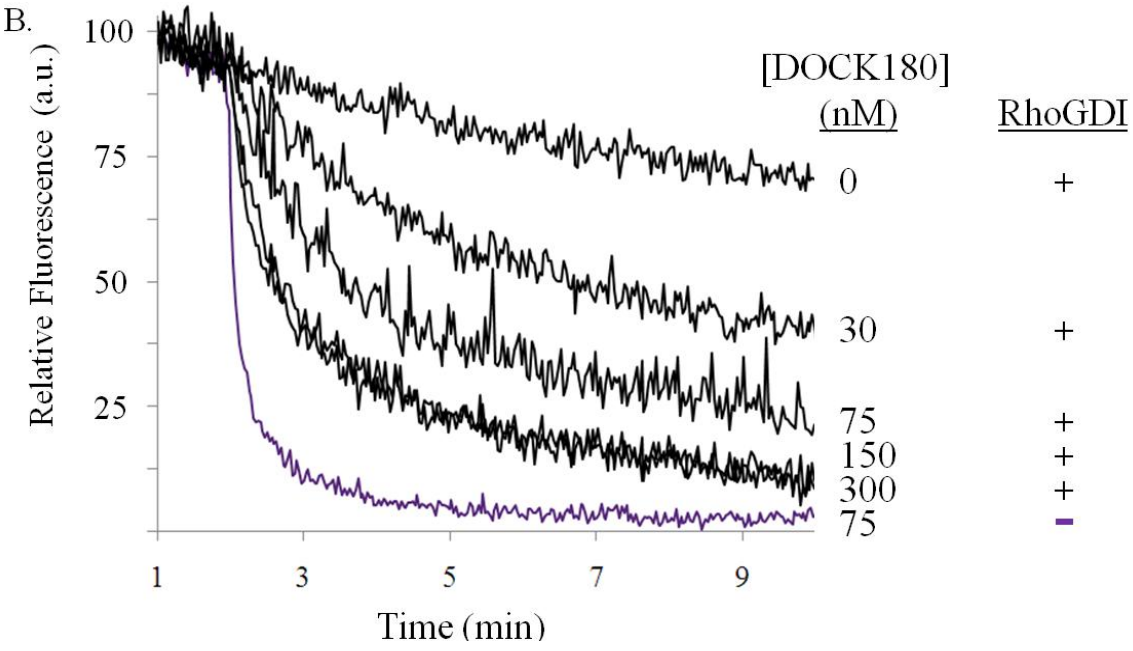
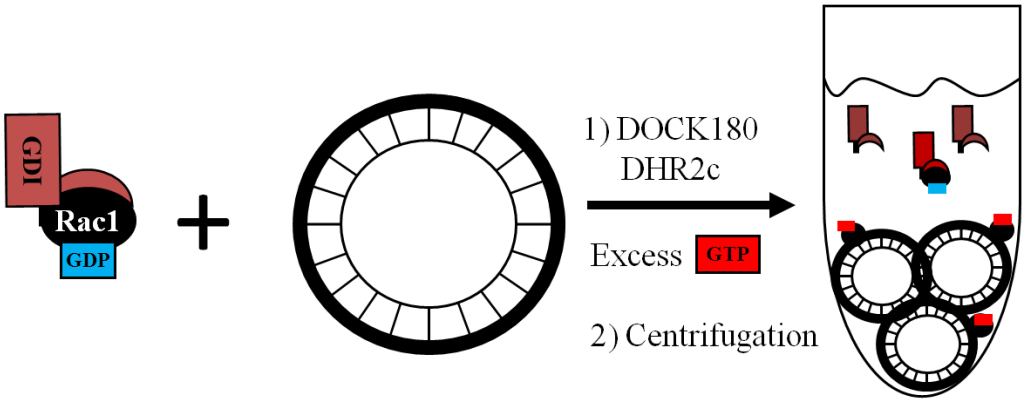
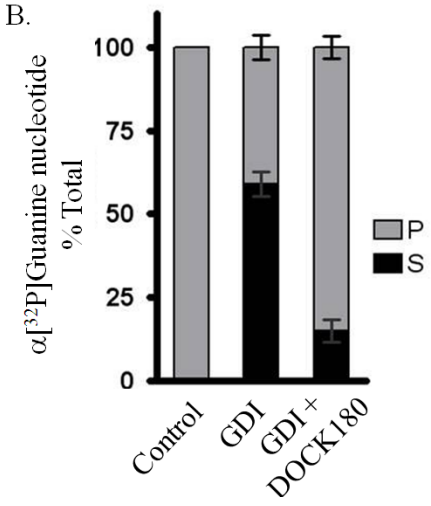

Relative Fluorescence (a.u.)
100
75
50
25
1
3
5
7
9
Time (min)
[DOCK180]
(nM)
RhoGDI
0
+
30
+
75
+
150
+
300
+
75
-

Figure A.3 DOCK180-catalyzed partitioning of Rac1 from GDI to liposomes. **A)** Schematic of DOCK180-mediated GDP(blue)-to-GTP(red) exchange of GDI-associated Rac1 and its partitioning into liposomes. **B)** Insect cell recombinant Rac1 (40 nM) was added to 100 μg/mL liposomes, prepared by rapid solvent exchange, and EDTA-loaded with 10 μM $\alpha[^{32}P]$GTP. Subsequently, the Rac1-bound $\alpha[^{32}P]$GTP was hydrolyzed to $\alpha[^{32}P]$GDP by the addition excess magnesium in a 20 minute incubation at room temperature (*1). 45 nM GDI was added to allow the formation of the GDI-Rac1.$\alpha[^{32}P]$GDP complex (*2). Still in the presence of excess unbound $\alpha[^{32}P]$GTP, 100 nM DOCK180 DHR2c domain was added to catalyze the $\alpha[^{32}P]$GDP-$\alpha[^{32}P]$GTP exchange of GDI-bound Rac1 (*3). At different endpoints throughout the procedure, as indicated by the asterisks, the liposomes were pelleted by centrifugation and the radioactivity was measured in both the supernatant (**S**) and the lipid pellet (**P**) to assess the extent of Rac1's liposome-to-solution partitioning. (*1 = control, *2 = GDI, *3 = GDI + DOCK180) All measurements were performed in triplicate.

A.


GDI
Rac1
GDP
1) DOCK180
DHR2c
Excess
GTP
2) Centrifugation

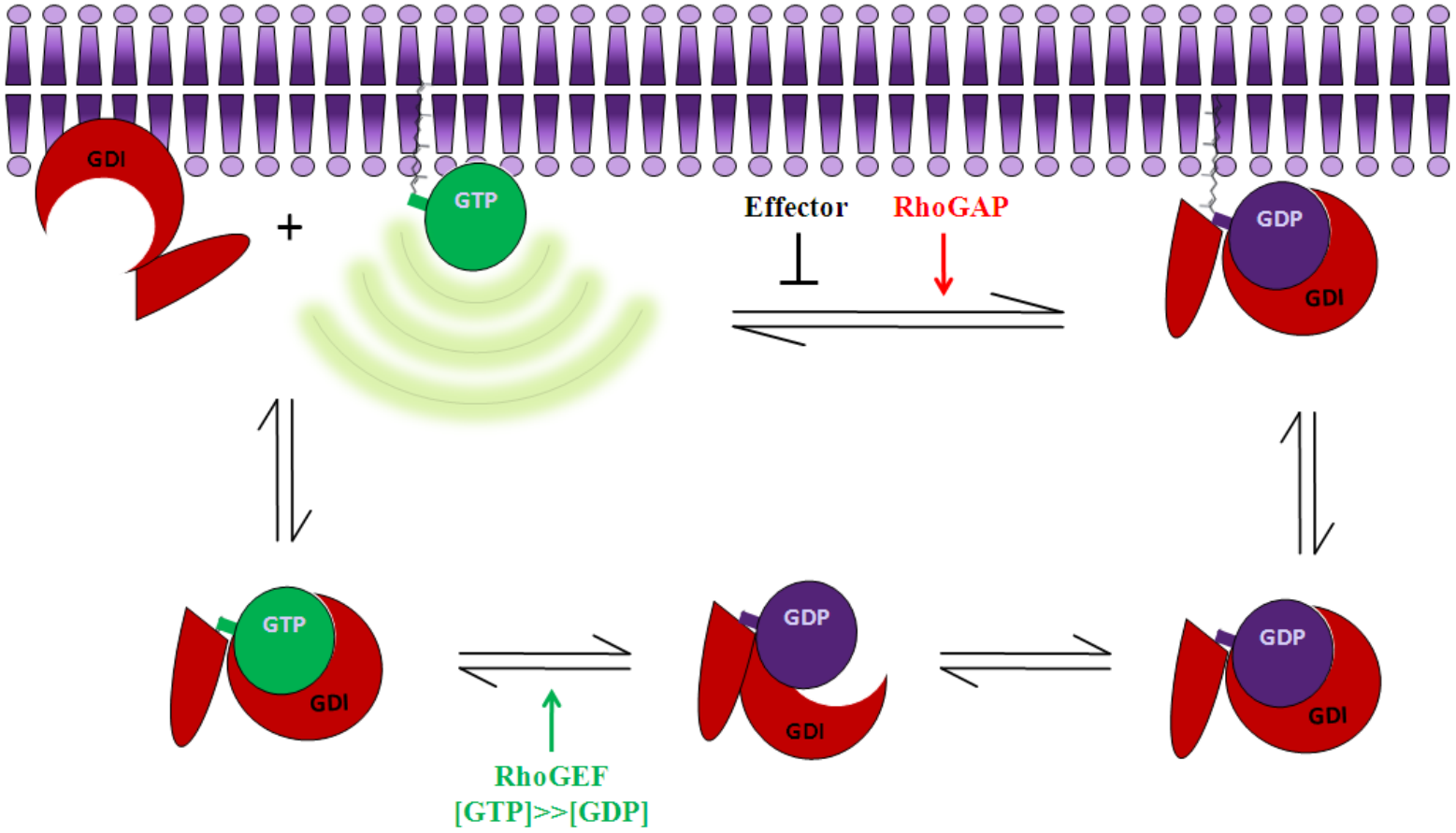
B.


α[32P]Guanine nucleotide
% Total
100
75
50
25
0
P
S
Control
GDI
GDI+
DOCK180

membranes, the Rac-GEF DHR2c was in effect able to catalyze the re-partitioning of the active GTPase to the lipid surface.

A.3.3 The Rho GTPase membrane association/dissociation cycle- Taken together, our findings described here complement the model presented in Figure A.7, and provide a more developed picture regarding how Rho GTPases are recruited to and released from the membrane, with the assistance of their three major classes of regulatory proteins (Figure A.4). In the scheme, we start with Cdc42 in its activated GTP-bound state, where it associates with its effectors at the membrane. This signaling persists until Cdc42 undergoes GTP hydrolysis. Once in the GDP-bound state, the GDI can then engage Cdc42 and the GDI-Cdc42 complex is released from the membrane as described in Chapter 2. In the cytosol, GDI exerts a bottleneck of sorts, by slowing the rate at which Cdc42 can undergo GEF-mediated nucleotide exchange, corresponding to a half-life of approximately 30 seconds. However, once a threshold of RhoGEF activation is achieved, GTP exchange ensues and the GTP-bound Cdc42 is then able to associate with membranes and is effectively released from the GDI.

Figure A.4 Model depicting the Rho GTPase membrane association/dissociation cycle. Initially, the Rho GTPase is in its activated GTP-bound state (shown in green) at the membrane, where its rate of GTP-hydrolysis is inhibited by effectors and accelerated by RhoGAPs. Upon GTP hydrolysis, the GDP-bound Rho GTPase (shown in purple) is engaged by RhoGDI (red). The Rho GTPase dissociates from the membranes as a complex with RhoGDI and the geranylgeranyl moiety of the GTPase is encapsulated by the hydrophobic binding pocket of RhoGDI, slowing their rate of membrane re-association. The cytosolic complex of RhoGDI-Rho GTPase exists in a dynamic equilibrium, where the Rho GTPase remains accessible to guanine nucleotide exchange by RhoGEFs. Following RhoGEF-mediated GDP-GTP exchange, the affinity of the GTP-bound Rho GTPase for GDI becomes significantly weakened when they reach the membrane, resulting in the dissociation of the Rho GTPase-GDI complex.


GDI
+
GTP
Effector
RhoGAP
GDP
GDI
GTP
GDI
GDP
GDI
GDP
GDI
RhoGEF
[GTP]>>[GDP]

A.4 Discussion

A.4.1 In the presence of RhoGDI, RhoGEF- and RhoGAP-activity modulates the partitioning of the Rho GTPases on and off the membrane, respectively- Based on the preferential binding by GDI to GDP-bound Cdc42, relative to the GTP-bound Cdc42, in the membranes (described in Chapter 2), we hypothesized that this selectivity may be exploited to modulate the rate at which Cdc42 associates the and dissociates from membranes. An activated RhoGAP, in catalyzing GTP hydrolysis, would help to accelerate the release of Cdc42 from membranes in the presence of GDI. Conversely, an activated RhoGEF, in catalyzing the exchange of GDP for GTP, would accelerate the apparent rate of recruitment of Cdc42 to the membranes.

We first showed that Cdc42GAP is able to markedly accelerate the release of GTP-bound Cdc42 from membranes in the presence of GDI. This suggests that RhoGAPs impact Cdc42-signaling in a critical way, as they would help to ensure the timely release of the GTPase from the membrane surface.

Somewhat unexpectedly, we found that geranylgeranylated Rho GTPases, when in their GDP-bound state, complexed to GDI, are still accessible to GEF-mediated nucleotide exchange. Based on our reconstitution experiments described in Chapter 2, Cdc42 is released from GDI within the time-frame of 40 seconds. Here, we show that DHR2c catalyzes nucleotide exchange on the close relative of Cdc42, Rac1, in complex with GDI, at a rate corresponding to a half-life of 30 seconds, which is reasonably similar to the expected rate of release of the GTPase from GDI. We

further went on to show that these conditions are sufficient to translocate Rac1 to the membranes.

GDI displacement factors (GDF's) were conceived of shortly after RhoGDI was found to stimulate the membrane-to-cytosol partitioning of Rho GTPases. What followed were reports of GDF activity for a diverse set of proteins, including integrins, the oncogenic membrane scaffold protein Ezrin, the neurotrophin receptor p75, and the Cdc42/Rac effector p21-activated kinase (PAK) (16-19). While many of these cases are intriguing, further work is needed before any of these candidates can be established as having genuine GDF activity.

Here, we demonstrate that the displacement of Rho GTPases from GDI, in some circumstances, may not require GDI to undergo posttranslational modification or allosteric regulation, but might be achieved by virtue of their accessibility to RhoGEFs. Our measurements suggests that the Rac1-GDI complex exists in a more dynamic equilibrium than expected, where the dissociation of GDI from Rac1's switch domains occurs at an apparent rate of 0.1 s^{-1}, providing ample opportunity for guanine nucleotide exchange by a RhoGEF and the subsequent release of Rac1 from GDI to the membrane (Figures 2.7B, A.2-4). There are many reasons why the RhoGEFs might make suitable candidates as GDI displacement factors. Given that most of the Cdc42 effectors are membrane-localized, coupling the membrane-recruitment of Cdc42 to GDP-GTP exchange would provide the benefit both of properly positioning and activating the GTPase. Furthermore, the release of Cdc42 from GDI would be under the regulatory modes of the RhoGEFs, reducing the risks of unintentional recruitment to the membrane. Given the vast number of upstream pathways that

stimulate the activation of the RhoGEFs, the recruitment of Cdc42 to the membrane would be subject to many different signaling cues.

A.4.2 The Rho GTPase membrane association/dissociation cycle- In this work, we propose a general model depicting the cycling of Cdc42 on and off the membrane, given our observations to date (Figure A.4). Since most of the signaling effectors are localized to the membrane, this would additionally ensure that Cdc42 will remain at the membrane throughout the duration of its GTP-bound state. The rate of GTP hydrolysis is known to be inhibited by its interaction with effector binding (20), whereas it is accelerated by its interaction with RhoGAPs. Thus, it is likely that effectors and RhoGAPs are in direct competition at this point. Once Cdc42 has been converted to its GDP-bound state, it becomes more "receptive" to GDI. With Cdc42 no longer bound to effectors, it is released into the cytosol as a complex with GDI, where its lipid tail is bound by the hydrophobic pocket of GDI, significantly reducing its rate of re-association with membranes.

Once in a stable cytosolic complex, GDI provides three potentially important advantages for Cdc42's signaling activities: 1) membrane-binding selectivity, 2) an activation barrier, and 3) GIP activity following GDP/GTP exchange. When faced with the challenge of translocating significant distances within the cell, as might occur during epithelial polarization, cell migration, or bud-site selection, lipidated Cdc42 needs a safeguard against indiscriminant association within the endomembranes that lack its signaling partners. Even with a lower than expected membrane residence time

of 5-10 seconds, the millimolar concentration of membranes in the cell could present an obstacle to Cdc42 in terms of its ability to localize to specific membrane signaling sites. In fact, this was observed in NIH 3T3 stable cell lines generated in our laboratory expressing the GDI-binding defective mutant of Cdc42, Cdc42(R66A). Cdc42(R66A) appeared to be exclusively localized at the endomembranes, whereas a significant fraction of wild-type Cdc42 was observed, as expected, at the plasma membrane (21). Other laboratories have shown that knocking down GDI in budding yeast resulted in a slowed recruitment of Cdc42 to the bud site during the budding process (22). By having Cdc42 in complex with GDI, its geranylgeranyl is effectively shielded, thus preventing it from nonspecifically associating with cellular membranes. The partial inhibition by GDI of guanine nucleotide exchange further protects against nonspecific membrane association. By limiting RhoGEF access to Cdc42, GDI provides what could be an activation threshold which reduces the risk of spurious, or premature, activation and membrane recruitment. Once a threshold of RhoGEF-catalyzed activation is achieved, GDI-bound Cdc42 can undergo guanine nucleotide exchange where its GTP-bound state becomes stabilized by GDI and ready for delivery to the membranes to resume another round of signaling.

In reconstituting each of the steps in the membrane association-dissociation cycle of Cdc42 with its three fundamental regulators, GDI, RhoGEFs, and RhoGAPs, two important implications emerge. First, the binding and release of Cdc42 from membranes is coupled to its signaling activation and de-activation, respectively. Just as they work in opposing fashion to regulate the nucleotide-bound state of Cdc42, the RhoGEFs and RhoGAPs also in effect compete for influence over the membrane-to-

cytosol partitioning of Cdc42. Second, the complex regulatory modes of the many members of the RhoGEF- and RhoGAP families add countless dimensions to the ways in which Cdc42 can be activated/recruited to the membrane and de-activated/released thereafter, contributing to the multifunctional roles of this GTPase in different tissues and organisms.

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