Regulation of RhoA Activation and Actin Reorganization by Diacylglycerol Kinase ζ

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This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies as a partial fulfillment of the MSc program in Cellular & Molecular Medicine

December 2011

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ABSTRACT

Rho GTPases are critical regulators of actin cytoskeletal dynamics. The three most well characterized Rho GTPases, Rac1, RhoA and Cdc42 share a common inhibitor, RhoGDIα. It is only recently becoming clear how upstream signals cause the selective release of individual Rho GTPases from RhoGDI. For example, our laboratory showed that diacylglycerol kinase ζ (DGKζ), which converts diacylglycerol (DAG) to phosphatidic acid (PA), activates PAK1-mediated RhoGDI phosphorylation on Ser-101/174, causing selective Rac1 release and activation. Phosphorylation of RhoGDI on Ser-34 by PKCα has recently been demonstrated to selectively release RhoA, promoting RhoA activation. Here, I show DGKζ is required for optimal RhoA activation and RhoGDI Ser-34 phosphorylation. Both were substantially reduced in DGKζ-null fibroblasts and occurred independently of DGKζ activity, but required a function DGKζ PDZ-binding motif. In contrast, Rac1 activation required DGKζ-derived PA, but not PDZ-interactions, indicating DGKζ regulates these Rho GTPases by two distinct regulatory complexes. Interestingly, RhoA bound directly to the DGKζ C1A domain, the same region known to bind Rac1. By direct interactions with RhoA and PKCα, DGKζ was required for the efficient co-precipitation of these proteins, suggesting it is important to assemble a signalling complex that functions as a RhoA-specific RhoGDI dissociation complex. Consequently, cells lacking DGKζ exhibited decreased RhoA signalling downstream and disrupted stress fibers. Moreover, DGKζ loss resulted in decreased stress fiber formation following the expression of a constitutively active RhoA mutant, suggesting it is also important for RhoA function following activation. This is consistent

with the ability of DGKζ to bind both active and inactive RhoA conformations. Collectively, these findings suggest DGKζ is central to two distinct Rho GTPase activation complexes, each having different requirements for DGKζ activity and PDZ interactions, and might regulate the balance of Rac1 and RhoA activity during dynamic changes to the actin cytoskeleton.

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List of Abbreviations

A.A., amino acid;

Ad, adenovirus;

ATP, adenine tri-phosphate;

BSA, bovine serum albumin;

C, cysteine;

DAG, diacylglycerol;

DAPC, dystrophin-associated protein complex;

DGK, diacylglycerol kinase;

DMD, Duchenne muscular dystrophy;

DMEM, Dulbecco's modified Eagle medium;

EGF, epithelial growth factor;

ERM, ezrin-radixin-moesin;

F-actin, filamentous actin;

FBS, fetal bovine serum;

G-actin, globular actin;

GAP, GTPase-activating protein;

GDI, guanine-nucleotide dissociation inhibitor;

GDP, guanine di-phosphate

GEF, guanine-nucleotide exchange factor;

GST, glutathione S-transferase;

GTP, guanine tri-phosphate;

HA, hemagglutinin;

HIS-tag, hexa-histidine tag;

HRP, horseradish peroxidase;

IB, immunoblotting;

Ig, immunoglobulin;

IP, immunoprecipitation;

IP_3, inositol(1,4,5)-triphosphate;

KD, kinase dead;

kDa, kilo Dalton;

MARCKS, myristoylated alanice-rich C-kinase substrate;

mDia, mammalian homolog of *Drosophila* diaphanous;

MEF, mouse embryonic fibroblast;

MOI, multiplicity of infection;

MW, molecular weight;

N, asparagine;

NGF, nerve growth factor;

P, phosphate group;

PA, phosphatidic acid;

PAK, p21 activated kinase;

PBD, PAK1 binding domain;

PBS, phosphate buffered saline;

PDGF, platelet derived growth factor;

PDZ, Postsynaptic density protein-95/Disc-Large/Zona Occludens-1;

PFA, paraformaldehyde;

PH, pleckstrin homology;

PI(4,5)P_2, phosphatidylinositol (4,5) bisphosphate;

PIP5K, phosphatidylinositol-4-phosphate 5-kinase;

PKC, protein kinase C;

PLC, phospholipase C;

PMA, phorbol 12-myristrate 13-acetate;

pRB, retinoblastoma protein;

RasGRP, Ras guanyl-nucleotide releasing protein;

RBD, Rho-binding domain of Rhotekin;

RSB, reducing sample buffer;

ROCK, Rho-associated coiled-coil kinase;

SAM, sterile α motif;

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;

SEM, standard error of the mean;

Ser, Serine;

SNX, sorting nexin;

Syn, syntrophin;

TBST, Tris buffered saline-tween;

V, valine;

Wt, wild type;

Acknowledgments

I could not have completed this work without the help of numerous people. First, I thank my supervisor Dr. Stephen Gee for the opportunity to work in his laboratory and his continued support and encouragement. I greatly appreciate the independence and freedom he gave me to explore questions I found interesting. Our collaborators were instrumental to the completion of this thesis and include: Dr. Matthew Topham (University of Utah), who provided reagents and plasmids; Dr. Robin Parks (University of Ottawa), who created the adenoviral constructs; and finally, Dr. Atsuko Yoneda and Dr. John Couchman (University of Copenhagen) for sharing the pSer-34 RhoGDI antibody. Many thanks to members of my advisory committee, Dr. John Copeland, Dr. Jonathan Lee, and Dr. Chris Kennedy, for providing continued support, criticism, and interest in my project. It has been a pleasure to work with my fellow colleagues, past and present, in the Gee lab: Hanan Abramovici, Helga Agah, Tasfia Ahmed, Michèle Byham, Kun Cai, Marie-Josée Gandier, Alexander Steeves and Radi Zinoviev. Finally, I could not have asked for more support and encouragement from my close friends and family. I'll thank each of you in person.

Dedication

I dedicate the written component of this thesis and the last two years to the fictitious filmmaker, Sandy Bates, in the Woody Allen classic *Stardust Memories*. However, if your narcissism compels you to object to this dedication, I dedicate this thesis to you; but you can't have the two years – they're gone.

1. Introduction

INTRODUCTION

The cell is the basic unit of life. A key to our understanding of biology is appreciating the vast number of changes, both metabolically and structurally, that are essential for cells to perform their individual and collective roles within living organisms. Elucidating how specific molecules and signalling networks contribute to physiological and mechanical changes in the cell is critical to this endeavour.

The cytoskeleton

The eukaryotic cytoskeleton is an intracellular network of filaments essential for maintaining cell morphology. It is largely responsible for cell shape changes following the response to stimuli from the extracellular environment (Janmey, 1998). The cytoskeleton is comprised of three main types of filaments (Fuchs, 1996). Microfilaments, the smallest (6 nm in diameter), are primarily composed of actin polymers. Larger intermediate filaments (10 nm in diameter) are comprised of cell type-specific proteins, such as keratin found in skin cells, hair and nails. The largest filaments, microtubules (23 nm in diameter), are comprised mainly of α and β tubulin monomers. All three filament types undergo reorganization to meet cellular needs; however, the membrane associated-actin cytoskeleton responds most rapidly to external and internal cues and is the driving force behind initial shape changes (Hall, 1998; Heng and Koh, 2010; Janmey, 1998; Janmey and Lindberg, 2004; Pollard and Cooper, 2009; Schmidt and Hall, 1998).

Actin monomers (globular or G-actin) polymerize into filaments (filamentous or F-actin). Actin filaments adopt a variety of forms, including linear and branched. These filaments are organized into a diverse array of structures that cause local membrane

protrusions to allow cell migration, cytokinesis, endocytosis and vesicle movement (Pollard and Cooper, 2009). Conversely, changes in the lipid composition of the plasma membrane are closely tied to changes in actin dynamics. Many actin remodelling proteins are activated by specific signalling lipids that control the spatial and temporal regulation of actin dynamics (Dos Remedios et al., 2003; Uribe and Jay, 2009). It is this interplay between the plasma membrane and the underlying cortical actin cytoskeleton that causes mechanical changes to cell shape that are essential for the response to external and internal cues (Saarikangas et al., 2010).

Lipids regulate actin dynamics

Increasing evidence suggests plasma membrane lipids regulate actin dynamics (Saarikangas et al., 2010). For instance, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) controls the activity of several actin-associating proteins by increasing their plasma membrane association and subsequently promoting actin polymerization (Janmey, 1995; Janmey and Lindberg, 2004; Sechi and Wehland, 2000). The hydrolysis of $PI(4,5)P_2$ by phospholipase C (PLC) into diacylglycerol (DAG) and inositol 3-phosphate (IP_3) also contributes to changes in actin dynamics. IP_3 binding to the endoplasmic reticulum elicits Ca^{2+} release, which modulates the activity and function of many actin remodelling proteins (Janmey, 1994). DAG, the other product of $PI(4,5)P_2$ hydrolysis by PLC, is a crucial second messenger and binds/potently activates proteins with cysteine-rich C1 domains such as conventional protein kinase C (PKC) isoforms (Goni and Alonso, 1999; Griner and Kazanietz, 2007; Newton, 1997). Stimulating cells with DAG, or non-hydolyzable DAG analogs such as phorbol esters, causes dramatic reorganization

of the actin cytoskeleton (Keller et al., 1989). These compounds cause changes in actin dynamics by activating PKC and other DAG-activated enzymes.

DAG can be phosphorylated to yield phosphatidic acid (PA). PA is a lipid with distinct signalling properties (Cai et al., 2009). PA activates several proteins involved in actin reorganization such as phosphatidylinositol-4-phosphate 5-kinase (PIP5K), Raf-1, PKCζ and p21 activated kinase 1 (PAK1) (Bokoch et al., 1998; Limatola et al., 1994; Stace and Ktistakis, 2006; Yang et al., 2001). Moreover, PA can stimulate actin stress fiber assembly (Cross et al., 1996; Komati et al., 2005). It is now widely accepted that modulating these and other lipids in distinct plasma membrane regions can have significant effects on local and global changes in actin (Janmey and Lindberg, 2004). Thus, enzymes that alter the relative abundance of signalling lipids can significantly regulate the state of actin organization.

Many of these lipids regulate pathways that inevitably impinge on the activity of the Rho family of small GTPases (Rho GTPases) (Dermardirossian and Bokoch, 2005). Over 20 mammalian Rho GTPases are known to exist, the majority of which have been identified as critical regulators linking extracellular growth signals to actin cytoskeletal reorganization (Boureux et al., 2007; Hall, 1998). Therefore, many signalling lipids indirectly regulate actin dynamics by modulating the activity of specific Rho GTPase isoforms.

Rho GTPases are key actin regulators

Rho GTPases function as molecular switches, cycling between inactive GDP-bound and active GTP-bound conformations (Bourne et al., 1990; Bourne et al., 1991).

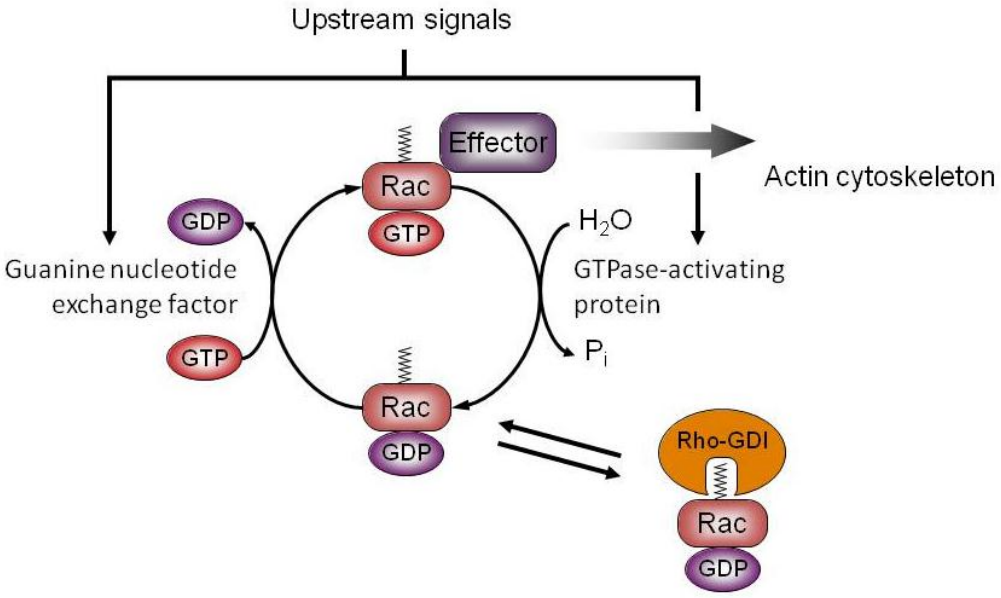
Early methods established direct roles for individual Rho GTPases in actin reorganization by making use of constitutively active and dominant-negative mutants of the three most studied Rho GTPases: Rac1, Cdc42 and RhoA. On the basis of these early studies, Rac1 is conventionally thought to regulate lamellipodia formation and membrane ruffling (Ridley et al., 1992). Cdc42 promotes filipodia extensions (Nobes and Hall 1995). Finally, RhoA is responsible for the assembly of actin stress fibers, focal adhesions, and contributes to actin-myosin contractility during migration and cell division (Charras, 2008; Paterson et al., 1990; Ridley and Hall, 1992).

New evidence has revealed an increasingly complicated picture for the spatial and temporal regulation of Rho GTPases. For example, while RhoA was traditionally thought to provide contractility at the tail-end of migrating cells, recent data has shown that RhoA is also important at the leading edge (Heasman et al., 2010; Pertz et al., 2006), a region of actin reorganization previously thought to be driven by Rac1 and Cdc42 activity (Ridley, 2004). Moreover, extensive crosstalk between Rho GTPases coordinates actin reorganization (Spiering and Hodgson, 2011). Collectively, spatial and temporal control of multi-protein signalling complexes involved in Rho GTPase regulation is required to organize specific signals into dynamic changes in the actin cytoskeleton (Pertz, 2010).

Regulation of Rho GTPase activity

GDP-GTP cycling of Rho GTPases is tightly controlled by numerous regulatory proteins (Figure 1.1). Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting the exchange of GDP for GTP. Conversely, GTPase-activating proteins (GAPs) inactivate Rho GTPases by enhancing their intrinsic GTPase activity (Spiering

Figure 1.1. Regulation of Rho GTPases. Rho GTPases (Rac, in this example) cycle between inactive GDP-bound and active GTP-bound conformations. The active conformations interact with specific downstream effectors, which propagate signal transduction and affect changes in the actin cytoskeleton. This cycle is tightly regulated by guanine nucleotide exchange factors (GEFs), which activate Rho GTPases by promoting the exchange of GDP for GTP, and by GTPase-activating proteins (GAPs), which inactivate Rho proteins by enhancing their intrinsic GTPase activity. Guanine nucleotide dissociation inhibitors (GDIs) sequester the Rho GTPases as soluble cytosolic complexes and prevent the C-terminal lipid group from inserting into the plasma membrane.


Upstream signals
Effector
Rac
GTP
Actin cytoskeleton
GDP
H_2O
Guanine nucleotide
exchange factor
GTPase-activating
protein
GTP
P_i
Rac
GDP
Rho-GDI
Rac
GDP

and Hodgson, 2011). Dozens of GEFs and GAPs exist, each with varying degrees of specificity and promiscuity with regard to individual Rho GTPase regulation (Spiering and Hodgson, 2011). This indicates that elaborate mechanisms must be in place to control specific Rho GTPase activity. Furthermore, many Rho GTPases localize to specific subcellular regions following extracellular cues (Ridley, 2006), implying they form multiple independent complexes in a spatially controlled manner with different GEFs, GAPs and other regulatory partners that control their activity and function.

Another important level of Rho GTPase regulation occurs by the Rho guanine nucleotide dissociation inhibitor (RhoGDI) (Olofsson, 1999). RhoGDI acts in four main ways to reduce Rho GTPase activity and inhibit their function: 1) RhoGDI associates with GDP-bound GTPases to prevent GDP to GTP exchange; 2) the hydrophobic binding cleft of RhoGDI masks the Rho GTPase C-terminal lipid moiety, thus preventing Rho GTPase association with GEFs and effectors at the plasma membrane; 3) RhoGDI can extract GTP-bound Rho GTPases from the plasma membrane, inhibiting GTP hydrolysis and disrupt association with effectors; and finally, 4) RhoGDI maintains Rho GTPase homeostasis as its loss causes cytosolic Rho GTPases to become misfolded and degraded (Boulter et al., 2010; Dermardirossian and Bokoch, 2005; Dovas and Couchman, 2005; Olofsson, 1999). Unlike GEFs and GAPs, only three RhoGDI isoforms exist in mammals: RhoGDI1 (RhoGDIα), RhoGDI2 (Ly/D4-GDI) and RhoGDI3 (RhoGDIγ) (Dovas and Couchman, 2005). Again unlike most GEFs and GAPs, RhoGDI interacts with multiple Rho GTPases, including Rac1, Cdc42 and RhoA (Garcia-Mata et al., 2011).

Structural studies of the most abundant isoform, RhoGDIα, have revealed two important functional domains: a roughly 70 amino acid flexible N-terminal domain that

includes transient helices between residues 9-20 and 36-58 that are stabilized by Rho GTPase binding, as well as a roughly 130 amino acid C-terminal immunoglobulin-like domain (Figure 1.2) (Keep et al., 1997; Golovanov et al., 2001; Grizot et al., 2001). The switch regions of Rho GTPases make contact with residues in the N-terminal domain. The isoprenyl group of Rho GTPases fits securely into the RhoGDI hydrophobic binding pocket in the C-terminal domain (Golovanov et al., 2001; Grizot et al., 2001). Thus, both the N- and C-terminus of RhoGDI are critical for Rho GTPase binding.

Recent studies suggest post-translational modifications of both regions can modulate Rho GTPase binding. For example, phosphorylation on RhoGDI Ser-34 by PKCα leads to the selective dissociation of RhoA from RhoGDI (Dovas et al., 2010). In contrast, PAK1-mediated phosphorylation of Ser-101/174, residues which lie adjacent to the isoprenyl-binding pocket, causes the selective release of Rac1 (Dermardirossian et al., 2004). These findings give rise to an interesting problem. Since both Rho GTPases interact with RhoGDI in essentially the same manner, why does phosphorylation affect the two Rho GTPases differently? One possible solution to this question lies with the ability of different Rho GTPases to form multi-protein complexes with unique sets of signalling proteins. Moreover, both of these selective release mechanisms require input from specific signalling lipids in the plasma membrane: $PI(4,5)P_2$ promotes non-canonical PKCα activation in the former example (Figure 1.3) (Corbalan-Garcia 2009; Dovas et al., 2010), while PA activates PAK1 in the latter (Abramovici et al, 2009).

Figure 1.2. RhoGDI structure when bound to Rho GTPases. The Rho GTPase, Rac1 in this case, is shown in blue and its C-terminal isoprenyl moiety in purple, while RhoGDI is shown in green. The yellow and orange regions represent the switch I and II regions of Rac1. GDP and Mg^{2+}, an important co-factor for binding, are also indicated. Amino acids 1-67 comprise the regulatory N-terminal domain of RhoGDIα in green. The switch regions (swI and swII) of Rho GTPases Rac1, Cdc42 and RhoA stabilize the RhoGDI helix–loop–helix motif between amino acids 34-58. The Rho GTPase isoprenyl moiety fits securely into the C-terminal immunoglobulin-like (Ig-like) motif. Image reproduced with permission from Grizot et al. 2001, ACS Publications, 1155 Sixteenth Street, NW, Washington, DC 20036, USA.

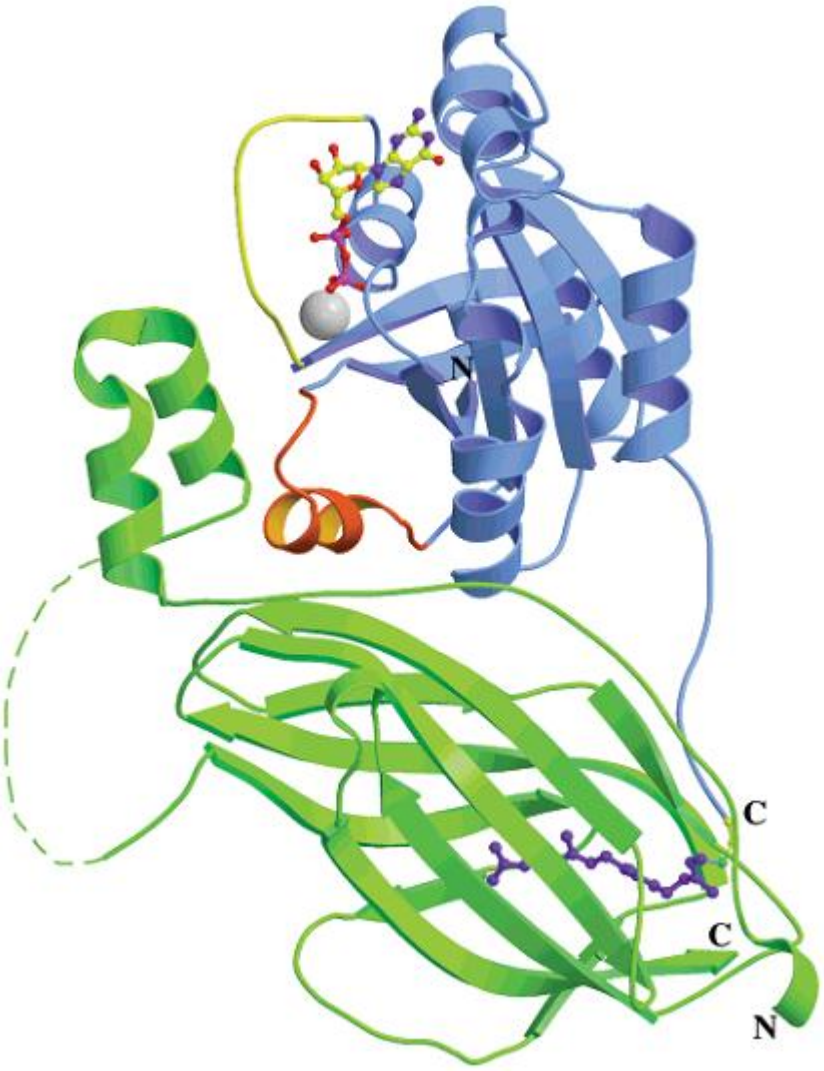
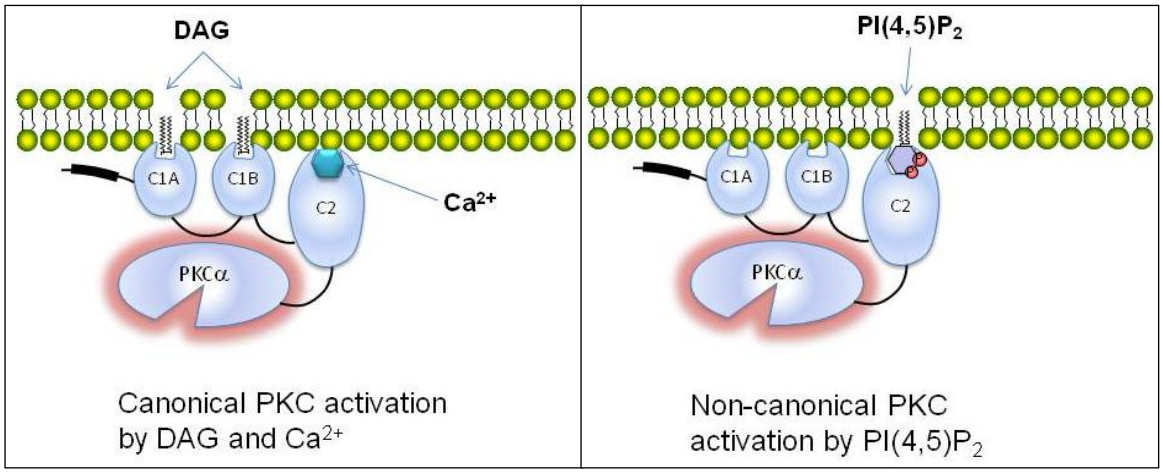

N
C
C
N

Figure 1.3. Schematic representations of the canonical (*left*) and non-canonical (*right*) pathways for conventional PKC activation. Canonical activation of conventional PKC isoforms such as PKCα includes the binding of DAG to C1 domains (left). PKCα-mediated phosphorylation of RhoGDI on Ser-34, however, uses the non-canonical pathway (right). This mechanism involves the binding of $PI(4,5)P_2$ to the C2 domain.


DAG
C1A
C1B
C2
Ca2+
PKCα
Canonical PKC activation
by DAG and Ca2+
PI(4,5)P2
C1A
C1B
C2
PKCα
Non-canonical PKC
activation by PI(4,5)P2

Rho GTPase/RhoGDI dissociation mechanisms

Several different mechanisms have emerged as possible RhoGDI dissociation factors. One includes the displacement of Rho GTPases by factors including integrins, ERM proteins and the p75 NGF receptor (Kim et al., 2002; Maeda et al., 1999; Takahashi et al., 1997; Yamashita and Tohyama, 2003). Another involves direct phosphorylation of RhoGDI by protein kinases (see Table 1.1) (Dermardirossian et al., 2004; Dovas et al., 2010; Mehta et al., 2001). Conversely, phosphorylation of Rho GTPases themselves appears to generally increase their association with RhoGDI, thereby inhibiting their activity (Garcia-Mata et al., 2011). Collectively, this indicates that different kinases modulate individual Rho GTPase/RhoGDI complexes by phosphorylating either Rho GTPases themselves or RhoGDI.

Recent findings suggest signalling lipids that regulate Rho GTPase activation are generated by lipid modifying enzymes residing within specific signalling complexes (Garcia-Mata et al., 2011). These lipids act locally on proteins, such as PKCα or PAK1, to activate specific downstream signalling pathways that effect changes in the actin cytoskeleton (Almena and Mérida, 2011; Cocco et al., 2004). For example, the assembly of the Rac1/RhoGDI dissociation complex requires the lipid kinase diacylglycerol kinase-ζ (DGKζ) (Figure 1.4). DGKζ stably associates with Rac1, PAK1 and RhoGDI (Abramovici et al., 2009; Yakubchyk et al., 2005). Following stimulation by platelet derived growth factor (PDGF), PA produced by DGKζ activates PAK1 (Abramovici et al., 2009). Once activated, PAK1 phosphorylates RhoGDI and releases Rac1, which interacts with GEFs and effectors at the plasma membrane (Abramovici et al., 2009; Dermardirossian et al., 2004). Consistent with these findings, cells lacking DGKζ have

Table 1.1. RhoGDI post-translational modifications that modulate Rho GTPases binding. Phosphorylation of RhoGDI generally serves to decrease affinity for Rho GTPases. Several specific sites identified as important for Rho GTPase binding are a listed, along with the kinase responsible. Table adapted from Garcia-Mata et al., 2011.

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Table 1.1. RhoGDI post-translational modifications and Rho GTPase binding			
Phosphorylation Site	**Kinase**	**Effect**	**Sources**
Ser-34 on RhoGDIα	PKCα	RhoA dissociation	Dovas et al., 2010
Ser-96 on RhoGDIα	PKCα	RhoA and RhoG dissociation	Elfenbein et al., 2009
Ser-101/174 on RhoGDIα	PAK1	Rac1 dissociation	Dermardirossian et al., 2004
Ser-174 on RhoGDIα	PKA	Inhibits binding to RhoA	Qiao et al., 2008
Tyr? on RhoGDIα	PKCζ	RhoA, Rac1 and Cdc42 dissociation	Chianale et al. 2010
Tyr-27/156 on RhoGDIα and RhoGDI2	Src	RhoA, Rac1 and Cdc42 dissociation	Dermardirossian et al., 2006; Wu et al., 2009
Tyr-156? on RhoGDIα	Fer	Rac1 dissociation	Fei et al., 2010

Figure 1.4. Release of Rac1 from RhoGDI. 1) DGKζ conversion of DAG to PA causes PAK1 to undergo auto-phosphorylation, thereby activating it. The C-terminal lipid moiety of Rac1-GDP is sequestered in a hydrophobic pocket of RhoGDI. 2) Once activated, PAK1 phosphorylates RhoGDI at two serine residues in the C-terminal immunoglobulin-like domain (Ser-101/174) causing the selective release of Rac1. DGKζ binding to Rac1 may also facilitate insertion of the lipid group into the plasma membrane. 3) Membrane translocation allows Rac1 to interact with a GEF and become activated. For all known post-translational modifications to RhoGDI, and their effect on RhoGTPase binding, please see Table 1.1. Adapted from Abramovici et al., 2009.

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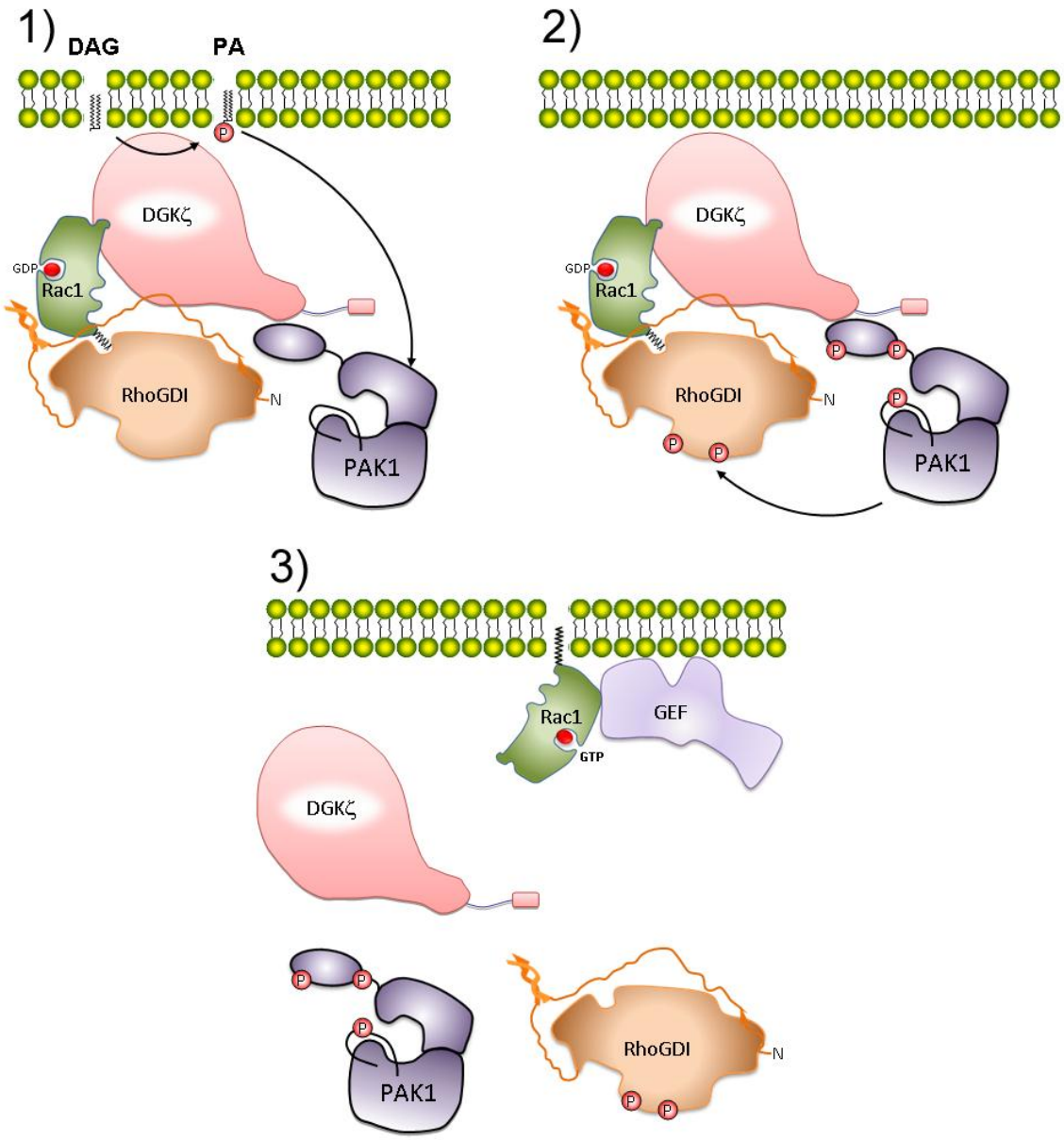

1)
DAG
PA
P
DGKζ
GDP
Rac1
RhoGDI
N
PAK1
2)
DGKζ
GDP
Rac1
P
P
RhoGDI
N
P
P
P
PAK1
3)
Rac1
GEF
GTP
DGKζ
P
P
P
PAK1
RhoGDI
N
P
P

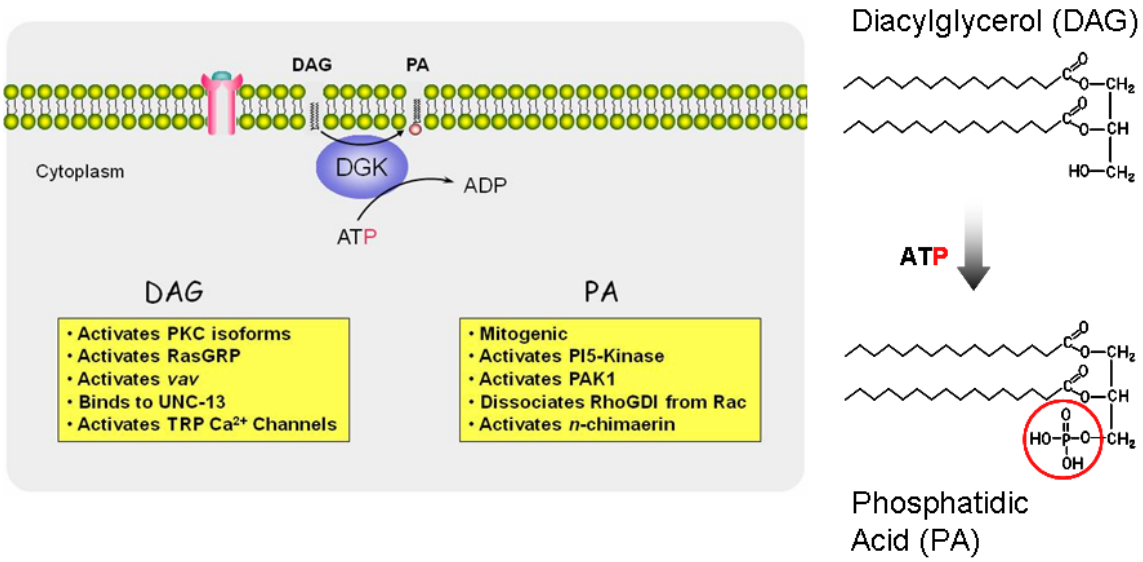
mislocalized PAK1, significantly reduced PAK1 and Rac1 activity levels, decreased Rac1-dependent actin structures and attenuated cell migration (Abramovici et al., 2009).

PA derived from another isozyme, DGKα, can also stimulate Rac1/RhoGDI dissociation. In this case, following hepatocyte growth factor (HGF) stimulation of endothelial cells, DGKα-derived PA activates an atypical PKC isoform, PKCζ, to promote Rac1 release from RhoGDI (Chianale et al., 2007; Chianale et al., 2010). It is currently unclear how PKCζ specifically performs this task, although it is suspected that it phosphorylates a yet unidentified tyrosine residue on RhoGDI (Chianale et al., 2010; Garcia-Mata et al., 2011). Collectively, these studies indicate the importance of forming distinct multi-protein complexes, together with lipid modifying enzymes such as DGKs, in a spatially controlled manner to allow selective Rho GTPase/RhoGDI dissociation. Given the previous examples, regulation of Rho GTPases by DGKs is likely an important intermediary for the dynamic relationship between changes to the lipid composition of the plasma membrane and the actin cytoskeleton.

DGKs are a large family of lipid modifying enzymes

DGKs sit at a pivotal position in lipid signalling by effectively terminating DAG while simultaneously producing PA (Figure 1.5) (Cai et al., 2009). DGKs play critical roles in diverse signalling pathways regulated by these two lipids (Topham, 2006). Ten mammalian DGK isozymes have been identified to date (Topham and Epand, 2009). The DGK family becomes even larger when taking into account the fact that six of the isozymes are known to be differentially spliced (Luo et al., 2004b; Shulga et al., 2011).

Figure 1.5. DGKs terminate DAG signalling and stimulate PA signalling. DGKs utilize ATP to phosphorylate DAG, yielding PA. By metabolizing DAG, DGKs attenuate the function of DAG-activated signalling proteins including conventional PKC isozymes, RasGRP, and others. The product of the reaction, PA, positively regulates several important signalling molecules including PAK1, PI5K and atypical PKC isozyme PKCζ. Thus, DGKs regulate the balance of functions controlled by these two important signalling lipids. Source: Cai et al., 2009.


DAG
PA
Cytoplasm
DGK
ADP
ATP
DAG
• Activates PKC isoforms
• Activates RasGRP
• Activates vav
• Binds to UNC-13
• Activates TRP Ca2+ Channels
PA
• Mitogenic
• Activates PI5-Kinase
• Activates PAK1
• Dissociates RhoGDI from Rac
• Activates n-chimaerin
Diacylglycerol (DAG)
ATP
Phosphatidic
Acid (PA)

Individual isozymes vary in expression patterns, subcellular localization, structural domains and modes of regulation (Tables 1.2 and 1.3). Two structural elements common among all DGKs are a catalytic domain and at least two cysteine-rich C1 domains (Topham and Epand, 2009). DGK catalytic domains are composed of two subunits: a catalytic domain and an accessory domain (or, extended catalytic domain). ATP binding to the catalytic domains can be interrupted by mutating a glycine in this region to alanine, rendering the enzyme catalytically inactive (Shulga et al., 2011). These mutants have proved useful in confirming the catalytic function of DGKs in lipid metabolism and signalling.

DGK C1 domains are homologous to PKC diacylglycerol/phorbol ester-binding motifs. Despite this homology, only two DGK isozymes tested, β and γ, could bind DAG analogues (Shindo et al., 2003). Consistent with this finding, sequence alignments previously suggested the C1 domains of other DGK isozymes were too different from those in PKCs to bind DAG (Hurley et al., 1997). Rather, DGK C1 domains have been proposed to serve as sites for important protein-protein interactions (Mérida et al., 2008; Shulga et al., 2011).

In addition to the catalytic and C1 domains, individual DGK isozymes contain unique structural domains. DGKs are divided into five different subtypes based on commonality with respect to shared structural elements (Figure 1.6). For example, Type 1 DGKs (DGKα, DGKβ, and DGKγ) have calcium-binding motifs. Type 1 DGK activity is increased considerably in the presence of Ca^{2+}. Type 2 DGKs (DGKδ, DGKη and DGKκ) have pleckstrin homology (PH) motifs and sterile alpha motifs (SAM); PH domains mediate protein-protein interactions and associations with phosphatidylinositol

Table 1.2. Mammalian DGK tissue distribution and subcellular localization. The expression profiles and subcellular localization of all known mammalian DGK isozymes. Sources include: Goto et al., 2008; Ohanian and Ohanian, 2001; Raben and Tu-Sekine, 2008; Topham, 2006; Shulga et al., 2011; and van Blitterswijk and Houssa, 2000.

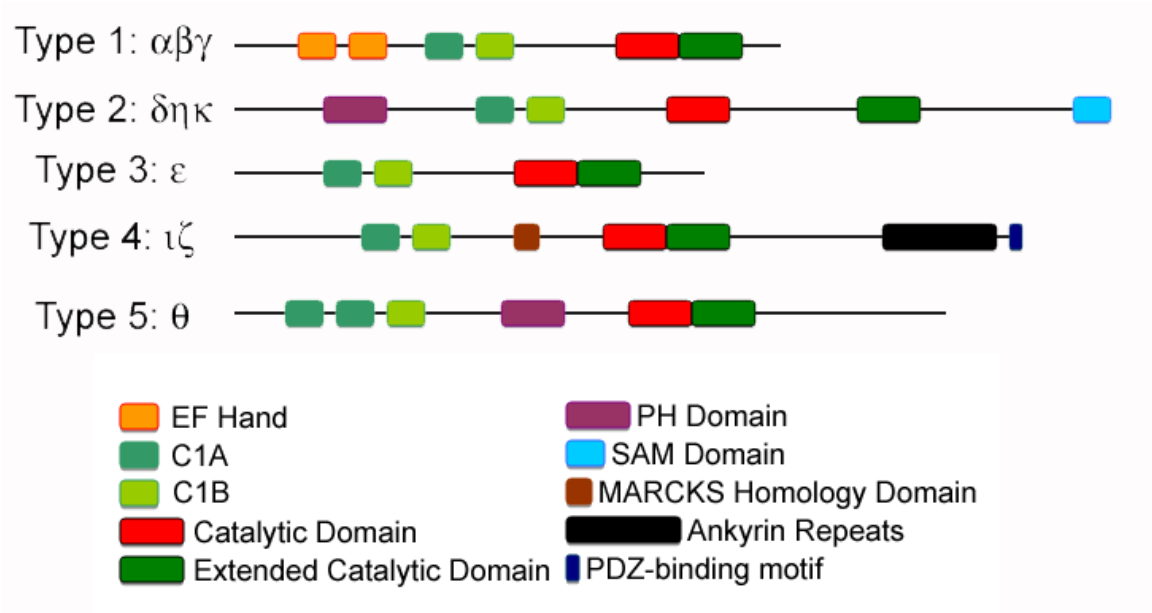
Table 1.2. Mammalian DGK tissue distribution and subcellular localization

DGK isozyme (size in kDa)	Confirmed Tissue Expression	Subcellular Localization
DGKα (80 kDa)	Thymus, spleen, kidney, brain, lung and endothelial cells	Plasma membrane, cytosol and nucleus
DGKβ (90 kDa)	Brain, adrenal gland, intestine and small arteries	Actin cytoskeleton
DGKγ (90 kDa)	Retina and brain	Cytoskeleton, golgi apparatus and nucleus
DGKδ (130 kDa)	Skeletal muscle, testis, liver and leukocytes	Endoplasmic reticulum, endosomes and golgi apparatus
DGKη (134 kDa)	Testis, lung, spleen, brain, heart, muscle, kidney and liver	Cytosol and endosomes
DGKκ (142 kDa)	Testis, brain and ovary	Plasma membrane and cytosol
DGKε (64 kDa)	Testis, skeletal muscle, lung, pancreas, retina and brain	Endoplasmic reticulum and actin cytoskeleton
DGKζ (104 kDa)	Thymus, brain, intestine, eye, endothelial cells, heart, lung, small arteries and muscle	Plasma membrane, cytosol, nucleus and actin cytoskeleton
DGKι (117 kDa)	Retina and brain	Plasma membrane, cytosol and nucleus
DGKθ (110 kDa)	Brain, intestine, duodenum, liver and small arteries	Plasma membrane and nucleus

Table 1.3. Known regulators of DGKs. DGK regulators. Sources: Sakane et al., 2007; Topham et al., 1998 ; Yakubchyk et al., 2005; and Wattenberg et al., 2006.

Table 1.3. Known regulators of DGKs		
DGK isozyme	**Known Upstream Regulator**	**Effect on DGK**
DGKα	Src	Activation by Tyr 334 phosphorylation
	Ca^{2+}	Activation
	$PI(4,5)P_2$ and PIP_3	Activation; translocation to plasma membrane
DGKβ	Ca^{2+}	Activation
	$PI(4,5)P_2$	Activation
DGKγ	Ca^{2+}	Activation
	PKCγ	Activation by Ser 776/779 phosphorylation
	Phorbol ester	Translocation to plasma membrane
DGKδ	PKCα	Inhibition of plasma membrane localization by phosphorylation at Ser 22/26
DGKκ	Src	Tyr 78 phosphorylation
DGKζ	Src	Activation by phosphorylation
	PKCα	MARCKS domain phosphorylation by PKCα causes: decreased catalytic activity; nuclear exit; DGKζ dissociation from PKCα and pRB and active Rac1; plasma membrane localization
	ERK	Inhibition of actin cytoskeletal localization by phosphorylation
	Leptin Receptor	Not determined
	Syntrophin	Recruitment to actin cytoskeleton
	pRB	Activation in nucleus
DGKθ	RhoA	Inactivation
	PCKε	Phosphorylation causes increase plasma membrane localization

Figure 1.6. Structural differences of mammalian DGK isozymes. The ten members of the mammalian DGK family classified under five different subtypes on the basis of shared structural homology. A catalytic domain and at least two C1 domains are common to all DGKs. Type 1 DGKs have two EF hand motifs, responsible for binding Ca^{2+}. Type 2 DGKs are unique in that they have PH and SAM domains. Type 3 DGK is the most simple in structure. Type 4 DGKs have a domain homologous to the PKCα phosphorylation site of the MARCKS protein, as well as four ankyrin repeats and a C-terminal PDZ-binding motif. Type 5 DGK has a PH domain and is the only member to have three C1 domains. Adapted from Topham and Epand, 2009.


Type 1: αβγ
Type 2: δηκ
Type 3: ε
Type 4: ιζ
Type 5: θ
EF Hand
C1A
C1B
Catalytic Domain
Extended Catalytic Domain
PH Domain
SAM Domain
MARCKS Homology Domain
Ankyrin Repeats
PDZ-binding motif

lipids while SAM domains support homo- and hetero-dimerization. DGKε, the only Type 3 DGK, is the simplest structurally. Unlike other DGKs, DGKε has an affinity for arachidonoyl-DAG. Type 4 DGKs (DGKζ and DGKι) have domains structurally homologous to the PKCα phosphorylation site on the MARCKS protein. Type 4 DGKs are also unique in that they have four ankyrin repeats and a C-terminal PDZ (postsynaptic density 95, PSD-95; disc large, Dlg; zonula occludens-1, ZO-1)-binding motif. The only Type 5 DGK, DGKθ, has a PH domain and three C1 domains. These different structural motifs allow individual DGK isozymes to form unique multi-protein complexes in distinct subcellular compartments, suggesting diverse functions for each subtype.

Like many other lipid modifying enzymes, DGK activity is spatially restricted (Kobayashi 2007; Sakane et al., 2007; Wattenberg et al., 2006). Because DGKs localize to different subcellular regions, it is thought that individual isozymes metabolize distinct DAG pools in cells (Almena and Mérida, 2011; Topham and Epand, 2009). Moreover, DGKs are expressed in various tissue types (see Table 1.2). More than one DGK can be present at a time in a given cell type; when this is the case, they are often from different subtypes (Topham and Ephand 2009). This suggests different subtypes have unique cell-type specific functions. Strikingly, members of the same subtypes can have drastically different functions when present in a given cell-type, such as the opposing effects of Type 4 DGKs, DGKζ and DGKι, on Ras signalling (Regier et al., 2005).

Biological roles of DGKζ

DGKζ is a ubiquitously expressed isoform and a component of several distinct protein complexes in many cell types and tissues (Rincón et al., 2011). Studies of this

isoform have provided important clues to our understanding of how this enzyme family regulates diverse cellular processes. In the brain, dendritic spine maintenance requires the removal of DAG at synapses by DGKζ in complex with the postsynaptic density protein PSD-95 (Kim et al., 2009). In the heart, DGKζ attenuates cardiac hypertrophy, left ventricular remodelling, and improves survival following myocardial infarction by interacting with G protein coupled receptors (Harada et al., 2007; Niizeki et al., 2008). DGKζ loss promotes Ras activation in T-cells, resulting in a greater immune response in knockout mice (Zhong et al., 2003; Zhong et al., 2007). DGKζ attenuates Ras signalling by binding to and negatively regulating RasGRP1, a Ras GEF activated by DAG (Topham and Prescott, 2001). mTOR – a critical regulator of protein synthesis, cell growth and survival – is also negatively regulated by DGKζ (Gorentla et al., 2011). How DGKζ achieves selectivity for such diverse pathways is still not entirely clear.

Subcellular localization of DGKζ is a central determinant of its function (Rincón et al., 2011). This is important for all DGKs as they must be targeted to compartments of the cell where DAG is generated (Topham, 2006). In the cytoplasm, DGKζ regulates actin cytoskeletal reorganization and changes to cell morphology (Abramovici and Gee, 2007; Abramovici et al., 2009; Luo et al., 2004a; Yakubchyk et al., 2005). Consistent with this function, DGKζ has also been found in the cytoskeletal fraction of cells (Yakubchyk et al., 2005). In the nucleus, DGKζ dynamically associates with heterochromatin (Hasegawa et al., 2008). It also negatively regulates cell cycle progression as a partner and downstream target of the retinoblastoma (pRB) tumour suppressor in the nucleus (Los et al., 2006). Nuclear DAG levels increase to allow for G1/S transition (Banfic et al., 1993). pRB binds and activates DGKζ, which reduces

nuclear DAG levels causing G1/S cell cycle arrest (Evangelisti et al., 2007; Evangelisti et al., 2009; Los et al., 2006). Together, accumulating evidence suggests DGKζ forms distinct multi-protein complexes in specific subcellular regions to allow for diverse roles in cell signalling.

Regulation of DGKζ localization and activity

Our laboratory originally isolated DGKζ from a yeast-two hybrid scene using the PDZ-domain of γ1-syntrophin as bait (Hogan et al., 2001). The five known syntrophin isoforms (α1, β1, β2, γ1 and γ2) act as adaptor proteins linking ion channels and signalling proteins to the dystrophin-associated protein complex (DAPC) by a direct interaction with the C-terminus of dystrophin, the product of the gene mutated or missing in Duchene Muscular Dystrophy (DMD) (Froehner et al., 1997). The C-terminal PDZ-binding motif of DGKζ was sufficient to associate with syntrophin PDZ domains; this interaction was interrupted with the attachment of a C-terminal FLAG epitope tag (Hogan et al., 2001). Disrupting the DGKζ/syntrophin interaction resulted in DGKζ mislocalization in cells and tissues (Abramovici et al., 2003). This data suggests syntrophin isoforms provide localization cues for targeting DGKζ to correct subcellular regions, which is likely critical for regulating DGKζ activity.

In addition to syntrophins, another important factor responsible for correct DGKζ subcellular targeting is PKCα (Figure 1.7). PKCα phosphorylates the MARCKS homology domain of DGKζ, causing nuclear exit and increased plasma membrane localization of DGKζ (Abramovici et al., 2003; Topham et al., 1998). Consistent with

this, a DGKζ mutant that mimics phosphorylation of this domain is constitutively membrane bound (Abramovici et al., 2003).

An important step for conventional PKC regulation occurs at the level of DAG depletion. DGKζ directly interacts with and negatively regulates PKCα by metabolizing DAG (Luo et al., 2003a; Luo et al., 2003b). DAG levels accumulate considerably following growth factor stimulation, and overcome the ability of DGKζ to deplete DAG; this results in PKCα activation. Once activated, PKCα phosphorylates the MARCKS homology domain of DGKζ (Topham et al., 1998). Phosphorylation at this site serves to dissociate the PKCα/DGKζ complex and also to reduce DGKζ activity (Luo et al., 2003a; Luo et al., 2003b); this increases the available pool of DAG and sustains PKCα activity (Figure 1.8). These studies indicate the balance of DGKζ and PKCα activity depends largely on the local availability of specific signalling lipids.

PKCα is an important kinase linking extracellular growth signals to actin cytoskeleton reorganization (Larsson, 2006). Dynamic changes in actin taking place following PKCα activation, for example by $PI(4,5)P_2$ or DAG (or phorbol esters) stimulation, are principally attributed to the regulation of RhoA activity downstream (Dovas et al., 2006; Dovas et al., 2010; Holinstat et al., 2003; Mehta et al., 2001). Due to the established mutual regulation of DGKζ and PKCα, it is plausible that DGKζ contributes to actin remodelling by regulating RhoA, in addition to the previously established role for DGKζ in Rac1 regulation.

Figure 1.7. Regulation of DGKζ subcellular targeting by syntrophins and PKCα. As a lipid kinase, DGKζ needs to be targeted to membrane compartments in the cell that generate DAG. The C-terminal PDZ-binding motif of DGKζ has a high affinity for syntrophin PDZ domains. As scaffold/adaptors, syntrophin regulates the correct subcellular targeting of DGKζ. Phosphorylation of DGKζ by PKCα on the MARCKS homology domain is also responsible for regulating DGKζ localization. Phosphorylated DGKζ leaves the nucleus and has increased plasma membrane association.

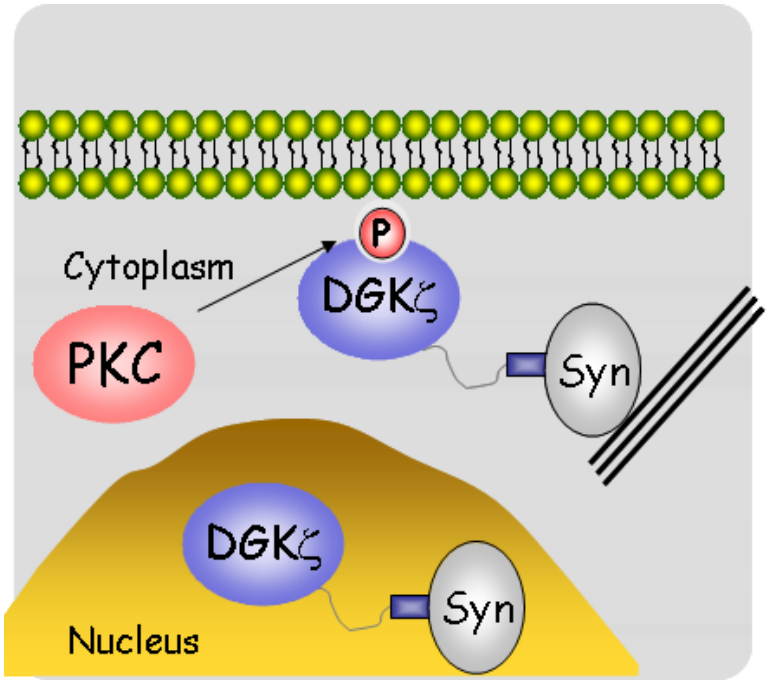
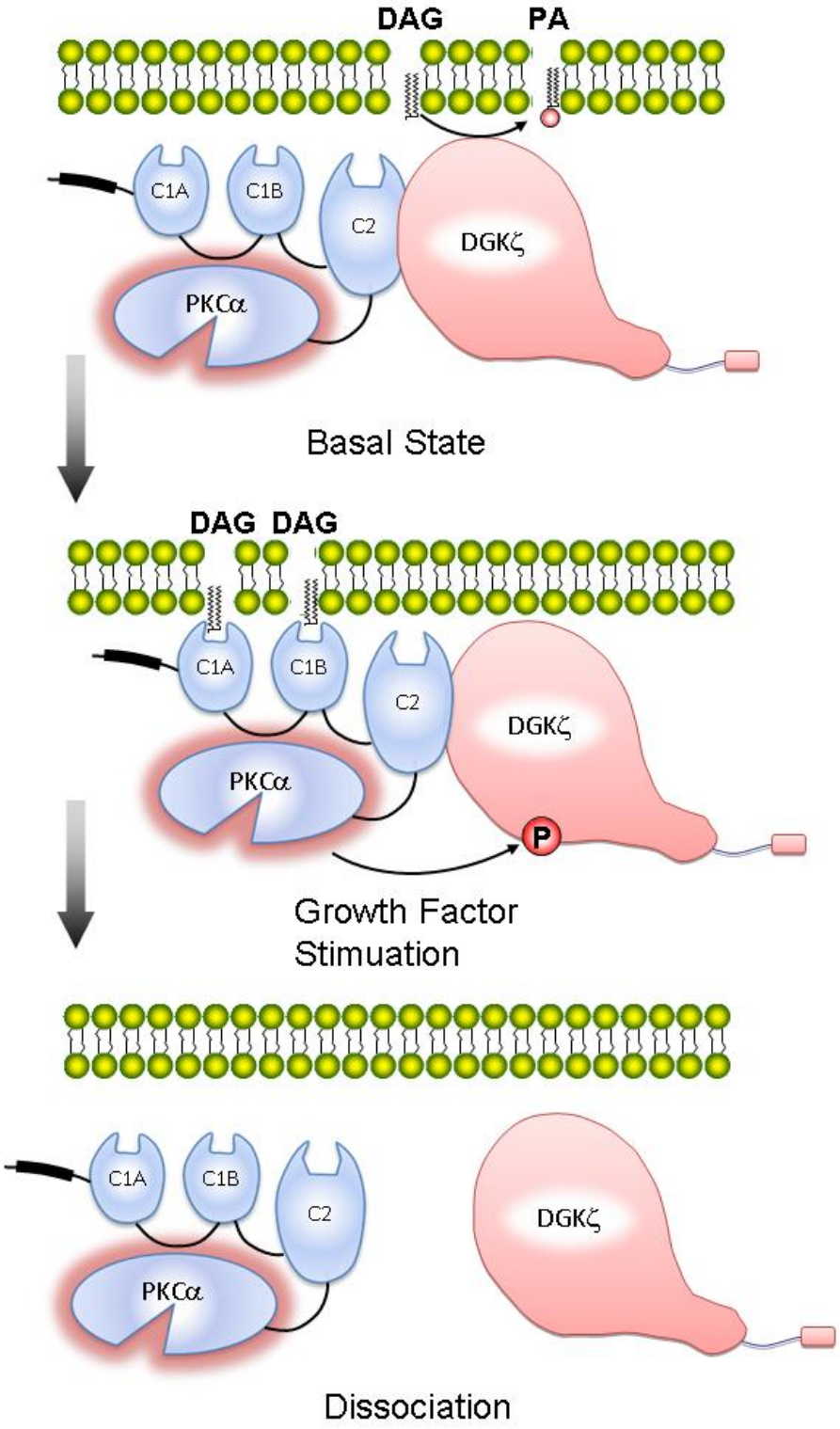

Cytoplasm
P
DGKζ
PKC
Syn
DGKζ
Syn
Nucleus

Figure 1.8. Dual DGKζ and PKCα regulation. DGKζ phosphorylates DAG into PA, thereby decreasing DAG levels required to activate PKCα (basal state). Following growth factor stimulation, however, DAG levels dramatically increase, activating PKCα to phosphorylate DGKζ on the MARCKS homology domain. Phosphorylation of DGKζ decreases DGKζ activity and causes the DGKζ/PKCα complex to dissociate, sustaining PKCα activation. Adapted from Luo et al., 2003a.


DAG
PA
C1A
C1B
C2
DGKζ
PKCα
Basal State
DAG DAG
C1A
C1B
C2
DGKζ
PKCα
P
Growth Factor
Stimuation
C1A
C1B
C2
PKCα
DGKζ
Dissociation

The roles of DGKζ in actin dynamics

DGKζ has been shown to associate with several important actin regulatory proteins, including Rac1, PAK1, RhoGDI, PKCα, RhoA and PIP5K (Abramovici et al., 2009; Luo et al., 2004a; Topham et al., 1998; Yakubchyk et al., 2005). The latter example reveals an addition role for DGKζ in lipid modulation. PIP5K, an enzyme responsible for synthesizing $PI(4,5)P_2$, is potently activated by DGKζ-derived PA (Luo et al., 2004a). In cells, these two lipid modulating enzymes associate and co-localize to actin filaments. Furthermore, DGKζ and PIP5K cooperatively promote actin polymerization and lamellipodia formation.

To date, the primary role of DGKζ in actin dynamics is attributed to a direct interaction with Rac1 (Yakubchyk et al., 2005). Acting in concert with Rac1, DGKζ plays a significant role in numerous actin-driven processes, including: neurite outgrowth in cultured N1E-511 cortical neurons (Yakubchyk et al., 2005); the fusion of mono-nucleated C2 myoblasts into differentiated poly-nuclear myotubes (Abramovici and Gee, 2007); and cell migration and spreading in mouse embryonic fibroblasts (MEFs) (Abramovici et al., 2009). As introduced earlier, DGKζ is also critical for Rac1/RhoGDI dissociation and Rac1 activation (Abramovici et al., 2009). In addition to Rac1, DGKζ has been shown to associate with another Rho GTPase, RhoA (Yakubchyk et al., 2005). The significance, however, of this interaction has not yet been explored.

Research Objectives

Preliminary data from our laboratory showed, in addition to reduced Rac1 activity, DGKζ-null MEFs also have reduced RhoA activity. Additional studies from our lab showed DGKζ co-immunoprecipitated with RhoA when the two proteins were co-expressed in Cos-7 cells. We therefore hypothesized DGKζ exists in a signalling complex with RhoA and regulates its activity. The goals of my research were to:

1. Establish if DGKζ formed separate signalling complexes with Rac1 and RhoA or, alternatively, whether they were part of the same complex.
2. Determine how DGKζ regulates RhoA activity.

In the course of my studies, a new mechanism for RhoA/RhoGDI dissociation by PKCα-mediated RhoGDI phosphorylation was identified (Dovas et al., 2010). Given this new information, I expanded these studies to determine whether DGKζ regulates RhoA release from RhoGDI in a manner analogous to the role played by DGKζ in PAK1-mediated Rac1/RhoGDI dissociation (Abramovici et al., 2009).

3. Determine the biological consequences of DGKζ loss on Rho-dependent actin structures and the activity of downstream Rho-targets.

2. Materials & Methods

MATERIALS & METHODS

Antibodies and reagents

An affinity-purified rabbit polyclonal antibody raised against the N-terminus of DGKζ was described previously (Topham *et al.*, 1998). Mouse monoclonal and rabbit polyclonal hemagglutinin (HA) and mouse monoclonal tubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal RhoA, monoclonal and polyclonal RhoGDI were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal Rac1 was from BD Transductions Laboratory. Mouse monoclonal His6 antibody was from Abcam (Cambridge, MA). Rabbit polyclonal pSer34 RhoGDI antibody was a generous gift from Dr. Atsuko Yoneda and Dr. John Couchman (Copenhagen, Denmark). Rabbit polyclonal antibodies against phospho-Cofilin (Ser 3) and phospho-Myosin Light Chain (Ser 19) were from Cell Signalling (Boston, MA). Mouse monoclonal c-Myc was from Roche Applied Science (Indianapolis, IN). AlexaFluor 350-, 488- and 594-conjugated secondary antibodies and phalloidin were from Invitrogen (Carlsbad, CA). Horseradish peroxidise (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies were from Sigma-Aldrich (St. Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich (St. Louis, MO). The PKC inhibitor Gö6976 was from Calbiochem (San Diego, CA).

Plasmids

All constructs used in this thesis are listed in Table 2.1. Plasmids encoding wild type DGKζ, a DGKζ mutant with a glycine to aspartate substitution in the catalytic domain completely eliminates DGKζ activity (DGKζ^{kd}), the DGKζ construct lacking

Table 2.1. Constructs used to study the role of DGKζ in actin dynamics. The following table is a list of constructs used to investigate DGKζ regulation of RhoA and actin dynamics in this thesis. See Materials & Methods for cloning details. Sources include: Abramovici et al., 2003; Froehner et al., 1997; Garcia-Mata et al., 2011; Hogan et al., 2001; Kim et al., 2009; Luo et al., 2003a; Luo et al., 2003b; Rincón et al., 2007; Ridley and Hall, 1992; Topham et al., 1998; Yakubchyk et al., 2005.

Table 2.1. Constructs used to study the role of DGKζ in actin dynamics	
Construct Name	**Details**
DGKζ^{wt}	Wild type DGKζ
DGKζ^{kd}	Kinase dead DGKζ; Glycing to Aspartate mutation in the catalytic domain renders DGKζ catalytically inactive
DGKζ^{FLAG}	DGKζ C-terminal FLAG-tag; disrupts PDZ-binding motif; inhibits binding to syntrophins; mislocalized
DGKζ$^{\Delta 97\text{-}233}$	DGKζ mutant lacking C1A-C1B domains; does not bind Rac1 or RhoA
DGKζ$^{1\text{-}99}$	DGKζ fragment containing the N-terminal region
DGKζ$^{97\text{-}152}$	DGKζ fragment containing the C1A domain exclusively
DGKζ$^{172\text{-}233}$	DGKζ fragment containing the C1B domain exclusively
DGKζ$^{97\text{-}233}$	DGKζ fragment containing the C1A-C1B domains; binds directly to Rac1
DGKζ$^{227\text{-}332}$	DGKζ fragment containing the MARCKS homology domain; regulatory site of PKCα phosphorylation
DGKζ$^{238\text{-}467}$	DGKζ fragment containing the catalytic domain
DGKζ$^{446\text{-}652}$	DGKζ fragment containing the extended catalytic domain; binds directly to PKCα
DGKζ$^{783\text{-}928}$	DGKζ fragment containing the C-terminal region; includes ankyrin repeats and PDZ-binding motif; binds directly to the PDZ domain of syntrophin, SXN27 and PSD-95
RhoA	Wild type RhoA
RhoA^{V14}	Glycine 14 substitution to Valine; constitutively GTP bound; over-expression causes actin stress fiber formation in MEFs
RhoA^{N19}	Threonine 19 substitution to Asparagine; dominant negative; GDP bound
RhoGDI	Wild type RhoGDIα; universal Rho GTPase inhibitor
RhoGDI 1-67	N-terminal regulatory domain of RhoGDIα
RhoGDI 68-204	C-terminal immunoglobulin-like domain of RhoGDIα
RhoGDI 34-204	Extended C-terminal domain of RhoGDIα
RhoGDI Δ34-58	Mutant lacking helix-loop-helix structure in the N-terminal regulatory domain of RhoGDIα
α1-syntrophin	Wild type α1-syntrophin; scaffolds signalling proteins to the actin cytoskeleton through a direct interaction with dystrophin; associates with the DGKζ PDZ-binding motif
GST	Glutathione S-transferase; negative control for GST-fusion protein pull-down assays

both C1 domains (DGK$\zeta^{\Delta 97\text{-}233}$), as well as DGKζ with a C-terminal FLAG epitope tag (DGKζ^{FLAG}), all with three tandem, N-terminal HA epitope tags, have all been previously described (Topham et al., 1998; Hogan et al., 2001).

The pGEX-4T-1, pGEX-4T-2 and pGEX-4T-3 cloning vectors containing glutathione S-transferase (GST) were purchased from Amersham Parmacia Biotech. GST-fusion proteins of DGKζ fragments, including DGK$\zeta^{1\text{-}99}$, DGK$\zeta^{97\text{-}233}$, DGK$\zeta^{227\text{-}332}$, DGK$\zeta^{238\text{-}467}$, DGK$\zeta^{446\text{-}652}$ and DGK$\zeta^{783\text{-}928}$ have been previously described (Yakubchyk et al., 2005). GST-fusion proteins of the DGKζ C1A (DGK$\zeta^{97\text{-}152}$) and C1B domains (DGK$\zeta^{172\text{-}233}$) were created by amplifying desired regions using PCR and ligating them into EcoRI and XhoI sites in the pGEX4T-1 vector.

The Rhotekin-RBD construct was previously described (Ren et al., 1999). Rhotekin RBD in pGEX-2T was obtained from Dr. Rashmi Kothary. The PAK1-PBD construct was previously described (Sander et al., 1998); however, the construct does not encode amino acids 56-272 of PAK1 as stated in Sander et al., 1998. Rather, it encodes amino acids 56-141. This PAK1 PBD construct in pGEX-2TK was obtained from Dr. John Collard. Wild type RhoA GST-fusion protein was previously described (Yakubchyk et al., 2005). For GST-fusion proteins of Rho GTPase mutants, RhoA^{V14}, RhoA^{N19}, Rac1^{V12} and Rac1^{N17} were previously subcloned into pGEX-4T-1 between BamHI and EcoRI sites. RhoGDI and mutants thereof were cloned using wild type RhoGDI in the sites BamHI and XhoI of pGEX-4T-1. RhoGDI 1-67, 68-204, 1-33, 34-67, 34-204, delta 34-58 constructs were created by amplifying desired regions by PCR and ligating them into pGEX-4T-1 BamHI and XhoI sites. Wild type RhoA, RhoGDI and Rac1 fusion

proteins were subcloned in pET-32a between EcoRI and XhoI restriction sites immediately following HIS and S-protein epitope tags.

The method for cloning and production of adenoviral constructs encoding HA-tagged wild type DGKζ (DGKζ^{wt}), mutant with C-terminal FLAG tag DGKζ^{FLAG}, kinase-dead mutant (DGKζ^{kd}), and myc-tagged RhoA^{V14}, RhoA^{N19} and α1-syntrophin have been previously described (Yakubchyk et al., 2005).

Cell culture, transfection and adenoviral infection

Primary MEFs were isolated from wild type (wt or +/+) and DGKζ-deficient (null or –/–) embryos (13.5 d after coitus) as previously described (Robertson, 1987). Immortalized cell lines were created using SV40 (Abramovici et al., 2009). MEFs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin and grown at 37°C, 5% CO_2. For serum starving conditions, MEFs were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin at 37°C, 5% CO_2 overnight.

MEFs were transfected using FuGENE 6 (Roche, Indianapolis, IN) according to manufacturer's instructions. For adenoviral over-expression experiments, unless otherwise indicated, MEFs were infected with adenoviruses at a multiplicity of infection (MOI) of 100 for 1 hour at 37°C. Cells were further incubated for 24-36 hours under standard growth conditions.

SDS-PAGE and immunoblotting

Equivalent amounts of protein from samples were boiled in reducing sample buffer (RSB) for 10 minutes, spun down and separated by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Proteins were detected by staining gels with Coomassie Brilliant Blue unless required for immunoblotting. For immunoblotting, proteins were transferred to PVDF membranes and stained with Ponceau-S to confirm protein transfer and then blocked in blocking buffer (5% skim milk in TBST, pH 7.5) for 30 minutes. For phospho-status experiments membranes were blocked in filter sterilized 3% BSA in TBST, pH 7.5. Unless otherwise indicated, primary antibodies were diluted in blocking buffer and incubated with membranes for 1 hour. Membranes were then washed for 15 minutes in TBST 3-4 times. Secondary HRP-conjugated antibodies were diluted 1:2000 in blocking buffer and incubated with membranes for 1 hour, then washed for 15 minutes in TBST 3-4 times. Membranes were then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 2 minutes and exposed on Kodak Imaging Center.

RhoA and Rac1 activity assays

Active RhoA levels were measured using a GST-RBD pull-down assay (Ren et al., 1999; Ren and Schwartz, 2000). The cells were serum starved overnight and then stimulated with 20% fetal bovine serum (FBS) containing media for 3 minutes. Following stimulation, media was quickly removed, and cells were immediately harvested in chilled lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM $MgCl_2$, and protease inhibitors). Lysates were centrifuged at 18,000 x *g* for 5

minutes at 4°C. Equivalent amounts of protein were incubated with GST-RBD beads for 30 minutes at 4°C. The beads were collected and washed with lysis buffer, boiled in RSB, and eluted proteins assayed for bound RhoA by immunoblotting. For rescue experiments, DGKζ-null MEFs were infected with adenovirus constructs harbouring wild type DGKζ ($DGK\zeta^{wt}$), kinase-dead ($DGK\zeta^{kd}$) and a mutant with a C-terminal FLAG tag ($DGK\zeta^{FLAG}$). 24 hours after infection, cells, including wild type and null controls, were serum starved overnight; the following day RhoA activity was measured as above.

Active Rac1 levels were measured using a GST-PBD pull-down assay (Sander et al., 1998) and were performed as previously described (Abramovici et al., 2009). Briefly, the cells were serum starved overnight and then stimulated with PDGF for 5 minutes. The cells were immediately harvested in chilled lysis buffer. Lysates were cleared by centrifugation and equivalent amounts of protein were incubated with GST-PBD beads for 30 minutes at 4°C. The beads were washed with lysis buffer, boiled in RSB, and eluted proteins assayed for bound Rac1 by immunoblotting. Rescue experiments were carried out essentially as described above for RhoA with the exception that cells were stimulated with PDGF as opposed to serum.

Phosphorylated Ser-34 RhoGDI experiments

The phosphorylated RhoGDI Ser-34 experiment was carried out similarly to those conducted by Dovas et al. (Dovas et al., 2010). Briefly, cells were serum starved overnight and stimulated with 800 nM PMA for 10 or 20 minutes in the presence or absence of PKCαβ specific inhibitor, Gö6976, at a concentration of 1 μM. DMSO was used as a control. For serum stimulation experiments, serum starved cells were stimulated

for 5 or 10 minutes with DMEM containing 20% FBS. The PKCαβ specific inhibitor, Gö6976, was used similarly to PMA stimulation experiments to confirm RhoGDI phosphorylation was due to PKCα. For rescue experiments, null cells were infected as described above. Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN)) and cleared by centrifugation at 4ºC. Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted using an antibody that recognizes the phosphorylated Ser-34 residue of RhoGDI (a generous gift from the laboratory of Drs. Atsuko Yoneda and John Couchman, Copenhagen, DK). Total RhoGDI-, Tubulin- and, in the case of rescue experiments, DGKζ-specific antibodies were used as loading controls.

Immunoprecipitations

Cells for immunoprecipitation were used 24-36 hours following transfection/infection. All steps were carried out on ice or at 4°C. Cells were washed with ice-cold PBS, pH 7.4 and lysed in 0.5 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% Triton X-100, and the following protease inhibitors: 10 mg/mL of leupeptin, anti-pain, benzamidine HCl, 4-(2-aminoethyl)-benzenesulfonyl fluoride HCl and pepstatin A). The cells were incubated on ice for 15 minutes and then scraped from the dish and centrifuged at 10,000 x *g* for 10 minutes at 4°C. Supernatant was removed and an aliquot of this starting material was boiled in RSB (Input). One to five μg of antibody (depending on the antibody) was added to 1000 μg of protein and incubated at 4°C for 2 hours. Immuno-complexes were purified using 50 μL of 50%

slurry of washed protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for one hour. The immune complexes were collected by centrifugation, washed three times for 5 minutes each with lysis buffer lacking detergent and then eluted by boiling in RSB. Samples were then subjected to SDS-PAGE, transferred to PVDF membranes and probed with relevant antibodies to detect co-precipitation of proteins.

Recombinant protein expression and purification in bacteria

BL21 E.coli cells were transformed with desired constructs and plated on LB with antibiotics Chloramphenicol and Ampicillin. To express protein in bacteria, a 50 mL culture of LB with Chloramphenicol and Ampicillin picked from a single colony was grown in a shaker at 37ºC overnight. The following day, cells were collected and resuspended in 10 mL of fresh LB without antibiotics and transferred to 250 mL flasks of LB with Chloramphenicol and Ampicillin. Bacterial cultures were grown at 37ºC. Protein expression was induced with 1mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) once cells reached an optical density of 0.6-0.8 (595λ). Cultures were collected by centrifugation after protein was allowed to express at 37ºC for three hours. Some constructs expressed best at 25ºC or 18ºC for 16 hours overnight.

For purification, pellets were resuspended in ice-cold lysis buffer (50mM Tris HCl, pH 7.4, 0.5M NaCl, 0.25M $MgCl_2$ and protease inhibitors). Once resuspended, samples were sonicated on ice. Triton X-100 was added to a final proportion of 10%, mixed gently, and samples were centrifuged at 4°C. Supernatant was incubated with pre-washed Glutathione Sepharose 4B slurry for one hour. GST-fusion proteins were collected from supernatant by centrifugation and washing in lysis buffer containing

Triton detergent three times and again washed three times in lysis buffer without Triton. GST-fusion proteins were run on SDS-PAGE and stained with Coomassie Brilliant Blue to confirm expression and purification was successful. Fusion protein concentration was determined by comparing the relative amount of GST-fusion proteins to a BSA standard also run on SDS-PAGE.

Glutathione S-transferase (GST) pull-downs and overlays

MEFs over-expressing HA-tagged wild type DGKζ were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitors) and equal amounts of protein were incubated with immobilized GST-fusion proteins for 1 hour, washed 4 times with lysis buffer to reduce the likelihood of non-specific interactions, and eluted proteins were assayed for bound HA-DGKζ by immunoblotting. GST-alone was consistently used as a negative control for binding.

For binding of GST-RhoA to HA-DGKζ in the presence and absence of PMA, MEFs were exposed to three conditions: serum starved or stimulated with 1μM PMA for 20 minutes in the presence or absence of the PKCαβ specific inhibitor Gö6976 at a concentration of 1μM. Cells were treated with Gö6976 for 15 minutes before PMA stimulation. GST-pull-downs were carried out as described above.

For overlay experiments, recombinant RhoA harbouring S-protein and HIS-tag was purified from bacterial extracts using HIS-column chromatography. Purified RhoA was analyzed by Coomasie Blue staining following SDS-PAGE and concentration was determined by spectrophotometery (A_{280}). 150μg of purified protein was diluted in 4 mL of blocking buffer and incubated overnight with membranes containing GST-fusion

proteins for various mutants spanning the length of DGKζ. Membranes were washed in TBST for 15 minutes 4 times and probed with HRP-conjugated S-protein for 2 hours. Membranes were washes in TBST for 15 minutes 4 times before incubating with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 2 minutes and exposed with Kodak Imagining Centre.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as previously described (Abramovici and Gee, 2007). Briefly, the cells were fixed in 4% paraformaldehyde for 15 minutes and rinsed with PBS 3 times. After permeabilization with 0.5% Triton X-100 in PBS for 10 minutes, the cells were incubated with blocking buffer (1% BSA in PBS; filter sterilized) for 30 minutes at room temperature. Unless otherwise indicated, primary antibodies were diluted in blocking buffer at a ratio of 1:100, unless otherwise indicated. Secondary fluorescent conjugated antibodies were diluted 1:300. Alexa-Fluor 350 phalloidin was used to visualize actin, and diluted in blocking buffer at a ratio of 1:1000. The cover-slips were mounted onto glass slides using Fluoromount-G (Southern Biotech) and sealed with nail polish. Carl-Zeiss fluorescent microscope with AxioVision software was used to obtain images.

Cell spreading assays

Serum starved cells were washed in PBS and then harvested with 2 mL Trypsin-EDTA. Trypsin was inactivated with 2 mL of soybean Trypsin inhibitor (1mg/mL). Cells were collected by centrifugation and washed three times with 1% BSA in DMEM (filter

sterilized). Finally, cells were resuspended in 5 mL of 1% BSA in DMEM (filter sterilized) and held in suspension at 37ºC for 1 hour. Cell were re-plated at a density of $2x10^4$ on fibronectin coated cover-slips for indicated times, fixed in 4% paraformaldehyde (PFA), washed twice in PBS and prepared for immunofluorescence microscopy.

Co-localization studies

Wild type MEFs seeded on collagen coated coverslips were infected with adenoviruses harbouring HA-DGKζ and either myc-$RhoA^{V14}$ or myc-$RhoA^{N19}$. 24 hours following infection, cells were fixed in 4% PFA, washed twice in PBS and prepared for immunofluorescence microscopy.

Statistical analyses

Microsoft Office Excel and SigmaPlot software were used to perform graphs and statistical calculations. Data were expressed as means ± standard error of the mean (SEM). Differences were considered statistically significant if $P < 0.05$ (*) following Student's *t*-test.

3. Results

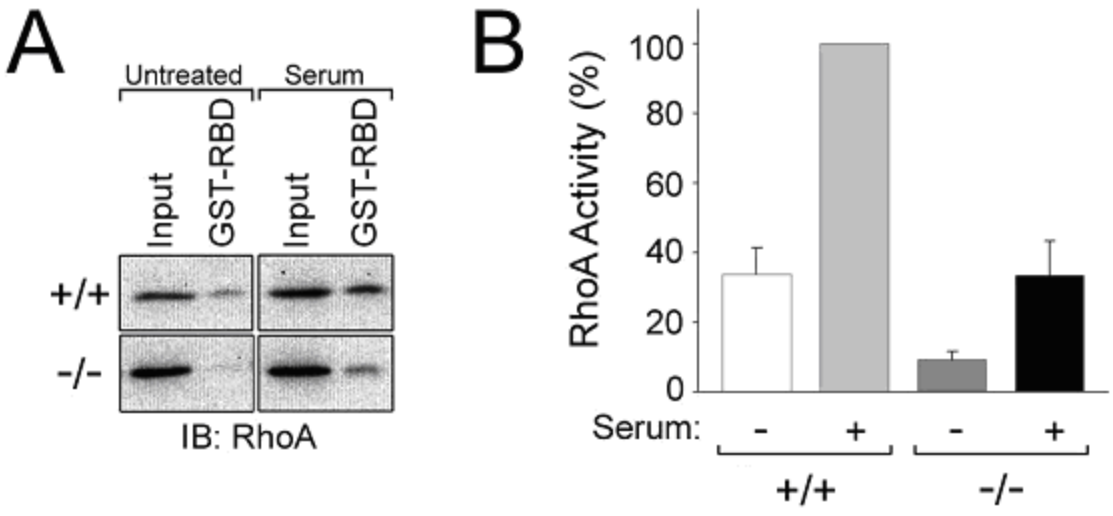
Results

Decreased RhoA activity in the absence of DGKζ

Previously, work from our laboratory established the role of DGKζ in Rac1 activation using DGKζ-null MEFs. Compared with wild type MEFs, MEFs lacking DGKζ had reduced Rac1 activity levels, but normal Cdc42 activity (Abramovici et al., 2009). Here, I sought to investigate RhoA activity in the absence of DGKζ using these same cell lines. The level of GTP-bound (active) RhoA was assayed in lysates from wild type and DGKζ-null MEFs using a Rho-effector pull-down assay (Ren et al., 1999; Ren and Schwartz, 2000). A GST-fusion protein of the Rho-binding domain (RBD) from the Rho-effector Rhotekin exclusively captures the (active) GTP-bound conformation of RhoA from cell lysates. Immunoblotting for RhoA following the RBD pull-down revealed a substantial decrease in (active) GTP-bound RhoA in resting DGKζ-null cell lysates compared to wild type lysates (Figure 3.1A).

Lysophosphatidic acid (LPA) is an abundant component of serum and potent activator of RhoA signalling (Chrzanowska-Wodnicka and Burridge, 1996). Consequently, stimulating starved MEFs with serum induced a substantial increase in RhoA-GTP levels in wild type cell lysates. The relative increase for serum-stimulated null cell lysates was roughly similar (~3-fold) but the absolute levels were still significantly lower than in wild type cells (Figure 3.1B). This dramatic overall decrease in active RhoA-GTP levels in the null cells suggests DGKζ is critical for efficient RhoA activation.

Figure 3.1. Reduced RhoA activity in DGKζ-null MEFs. (**A**) GST-fusion protein of the Rho binding domain of Rhotekin (GST-RBD) was used to capture GTP-bound RhoA from untreated or serum-stimulated wild type (+/+) and DGKζ-null (-/-) MEFs. (A) Bound GTP-RhoA was immunoblotted with a monoclonal anti-RhoA antibody. Input represents 10% of the extract used for the pull-down assay. (**B**) Graph showing the quantification of RhoA activity in A. The values were normalized to the amount of active RhoA in serum stimulated wild type cells and are the average of three-independent experiments.

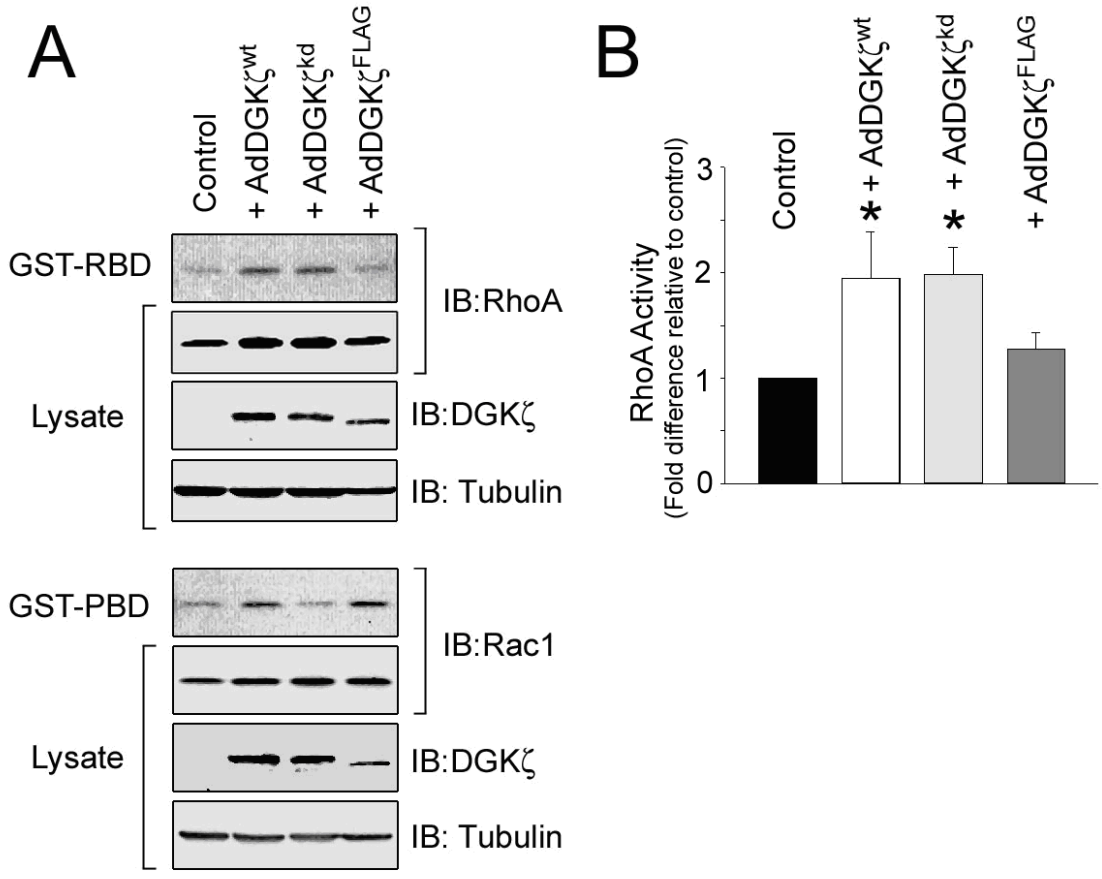

A
Untreated
Serum
Input
GST-RBD
Input
GST-RBD
+/+
-/-
IB: RhoA
B
RhoA Activity (%)
100
80
60
40
20
0
Serum:
-
+
-
+
+/+
-/-

DGKζ catalytic activity is dispensable for RhoA activation

To verify that decreased RhoA activity in DGKζ-null MEFs is due directly to the DGKζ loss, the HA-tagged wild type protein was reintroduced into null cells by adenoviral infection and its expression was verified by immunoblotting cell lysates with an anti-DGKζ antibody. Equal loading was confirmed using an antibody against α-tubulin. Active GTP-bound levels of RhoA in the lysate of null cells expressing HA-DGKζ (AdDGKζ^{wt}) was noticeably increased compared with uninfected control cell lysates (Figure 3.2A). A kinase-dead DGKζ mutant (AdDGKζ^{kd}), which harbours a glycine to aspartate substitution in the catalytic domain to completely eliminate catalytic activity, also rescued RhoA activity. Quantification of multiple independent rescue experiments revealed a near equal rescue of RhoA activity by DGKζ^{kd} and the wild type protein (Figure 3.2B). This result suggests DGKζ catalytic activity is not necessary to rescue RhoA activation in these cells.

To identify mechanistic differences or similarities between RhoA and Rac1 activation by DGKζ, rescue of Rac1 activity was assayed in lysates from DGKζ-null MEFs and cells over-expressing AdDGKζ^{wt}, AdDGKζ^{kd} and AdDGKζ^{FLAG} using a Rac1-effector pull-down assay (Sander et al., 1998). In contrast to RhoA activity, Rac1 activity rescue in null cells required DGKζ catalytic activity (Figure 3.2B), consistent with our previously published findings (Abramovici et al., 2009). Interestingly, a mutant with a C-terminal FLAG epitope tag (AdDGKζ^{FLAG}), which disrupts the interaction of DGKζ with the PDZ domain of syntrophins (Hogan et al., 2001), was insufficient to rescue RhoA activity (Figure 3.2A), but efficiently rescued Rac1 activity (Figure 3.2B). These results indicate the RhoA activation defect in null cells is due primarily to the

Figure 3.2. RhoA activity rescue independent of DGKζ kinase activity, but requires functional PDZ-binding motif. (**A**) DGKζ-null MEFs were infected with adenoviruses encoding HA-tagged wild type DGKζ ($DGK\zeta^{wt}$), a kinase dead DGKζ construct ($DGK\zeta^{kd}$), and a DGKζ construct with a C-terminal FLAG-tag that blocks binding to syntrophins ($DGK\zeta^{FLAG}$) (Hogan et al., 2001). RhoA activity was assayed by serum stimulated condition as in Figure 3.1A. GST-fusion protein of the p21-binding domain of PAK1 (GST-PBD) was used to capture GTP-bound Rac1 from MEFs stimulated with PDGF. Membranes were probed with anti-DGKζ to verify expression and anti-tubulin as a control for equal loading. (**B**) Graph represents quantification of RhoA rescue. The values were normalized to the amount of active RhoA in uninfected serum stimulated DGKζ-null cells (Control) and are the average of three-independent experiments. Asterisks (*) represent a statistically significant different from control ($p<0.05$).


A
Control
+ AdDGKζwt
+ AdDGKζkd
+ AdDGKζFLAG
GST-RBD
IB:RhoA
Lysate
IB:DGKζ
IB: Tubulin
GST-PBD
IB:Rac1
Lysate
IB:DGKζ
IB: Tubulin
B
RhoA Activity
(Fold difference relative to control)
0
1
2
3
Control
* + AdDGKζwt
* + AdDGKζkd
+ AdDGKζFLAG

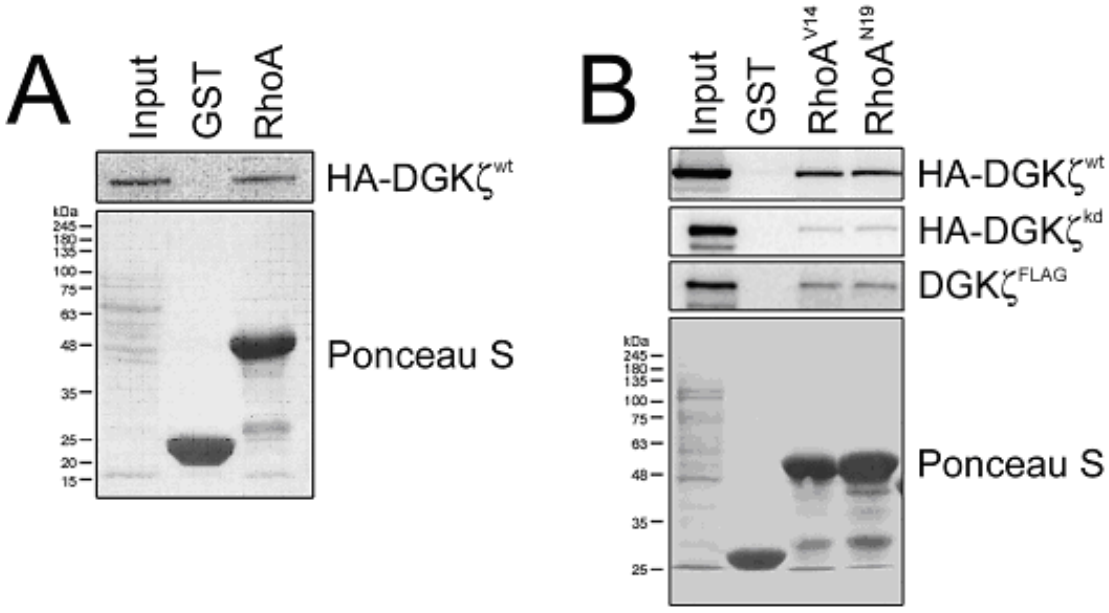
loss of DGKζ. Moreover, the functional requirements for DGKζ-dependent RhoA activation, namely catalytic activity and PDZ interaction(s), appear to be distinct from those of Rac1.

DGKζ stably associates with RhoA

Previous published findings from our laboratory have shown that DGKζ and RhoA stably associate in GST-pull-down experiments (Yakubchyk et al., 2005). To assess the interaction between DGKζ and RhoA in MEFs, a GST-fusion protein of RhoA was incubated with lysates from MEFs over-expressing HA-tagged wild type DGKζ. Consistent with previous findings (Yakubchyk et al., 2005), GST-RhoA captured over-expressed HA-DGKζ from MEFs, while GST-alone did not (Figure 3.3A). GST-fusion proteins of constitutively active and dominant negative RhoA mutants, GST-RhoA^{V14} and -RhoA^{N19} respectively, were tested to identify whether DGKζ binds preferentially to the active or inactive conformations of RhoA. Interestingly, both GST-RhoA^{V14} and GST-RhoA^{N19} captured HA-DGKζ from adenoviral infected MEF lysates (Figure 3.3B). GST-alone, again, was used as a negative control for binding. Collectively these data suggest DGKζ stably associates with both active and inactive RhoA conformations *in vitro*.

DGKζ^{kd} rescued RhoA activity in null MEFs, while DGKζ^{FLAG} was inefficient to rescue RhoA activity, suggesting the DGKζ^{FLAG} mutant might not be able to interact with RhoA. Following pull-down experiments similar to those described above, GST-fusion proteins of RhoA^{V14} and RhoA^{N19} efficiently captured both over-expressed HA-tagged DGKζ^{kd} and FLAG-tagged DGKζ^{FLAG} from transfected MEF lysates (Figure 3.3B). This

Figure 3.3. RhoA and DGKζ form a stable complex *in vitro*. (**A**) Detergent extracts of MEFs infected with an adenovirus bearing HA-tagged wild type DGKζ (HA-DGKζ^{wt}) constructs were incubated with immobilized GST or a GST-RhoA fusion protein. The amount of each fusion protein is shown in the Ponceau S blot (bottom). Bound proteins were analyzed by immunoblotting with an anti-HA antibody. Input represents 10% of the total extract. (**B**) Similar to A, detergent extracts of MEFs transfected with HA-DGKζ^{wt}, HA-tagged kinase dead DGKζ (DGKζ^{kd}), or a C-terminally FLAG tagged DGKζ (DGKζ^{FLAG}), were incubated with immobilized GST or a GST-RhoA fusion protein using GST, a constitutively active RhoA mutant (GST-RhoA^{V14}) or a dominant negative RhoA mutant (RhoA^{N19}). Near equal amounts of fusion protein is shown in the Ponceau S blot (bottom). Bound proteins were analyzed by immunoblotting with an anti-HA antibody for DGKζ^{wt} and DGKζ^{kd}, and anti-DGKζ for DGKζ^{FLAG}. Input represents 10% of the total extract.


A
Input
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RhoA
HA-DGKζwt
kDa
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180
135
100
75
63
48
35
25
20
15
Ponceau S
B
Input
GST
RhoAV14
RhoAN19
HA-DGKζwt
HA-DGKζkd
DGKζFLAG
kDa
245
180
135
100
75
63
48
35
25
Ponceau S

data indicates that neither of these mutants interfere with the ability of DGKζ to associate with RhoA.

DGKζ forms a signalling complex with RhoA and PKCα in cells

To confirm the interaction of DGKζ with active and inactive RhoA in cells, epitope-tagged versions of each protein (HA and myc, respectively) were co-expressed in COS-7 cells and were subjected to reciprocal immunoprecipitation and immunoblotting with epitope-tag specific antibodies. HA-DGKζ was efficiently co-precipitated by anti-myc antibodies when co-expressed with constitutively active myc-RhoA^{V14} or dominant-negative myc-RhoA^{N19}, but not when expressed alone (Figure 3.4A). In the reciprocal experiment, myc-tagged RhoA^{V14} and RhoA^{N19} were effectively co-precipitated by anti-HA antibodies from extracts of co-transfected cells, but not from cells expressing HA-DGKζ alone (Figure 3.4B). No proteins were precipitated by control mouse IgG. In other control experiments, no bands were detected in immunoprecipitates from untransfected cell lysates using either antibody.

MEFs co-expressing HA-DGKζ with either myc-RhoA^{V14} or RhoA^{N19} were subjected to immunofluorescence. The distribution of HA-DGKζ and myc-RhoA^{V14} showed substantial overlap (Figure 3.5). Cells expressing RhoA^{V14} had prominent stress fibers and both DGKζ and RhoA^{V14} were concentrated at the tips of pseudopodia where stress fibers terminated. In cells co-expressing HA-DGKζ and RhoA^{N19}, the two proteins were diffusely distributed and were concentrated in the same cellular regions. Collectively, this data is consistent with biochemical evidence suggesting the two proteins interact in cells. Moreover, in contrast to effectors which exclusively bind the

Figure 3.4. DGKζ co-immunoprecipitates (IP) with both constitutively active RhoA^{V14} and dominant negative RhoA^{N19}. (**A**) Cos-7 cells infected with an adenoviral construct encoding HA-tagged wild type DGKζ were left untreated or were transfected with myc-RhoA^{V14} or -RhoA^{N19} and allowed to recover for 24 hours. Cell extracts were immunoprecipitated with control IgG or with an anti-myc antibody. The immune complexes were analyzed by immunoblotting (IB) with an anti-HA antibody (*upper panels*). The expression and IP efficiency of the RhoA^{V14} and RhoA^{N19} mutants were monitored by IB of the cell lysates and the IPs, respectively, with anti-myc (*lower panels*). (**B**) In the reverse experiment, RhoA was detected in HA, but not control IgG IPs. No myc-reactive bands were detected in untransfected cells. IP experiments conducted by Jean-Christian Maillet.

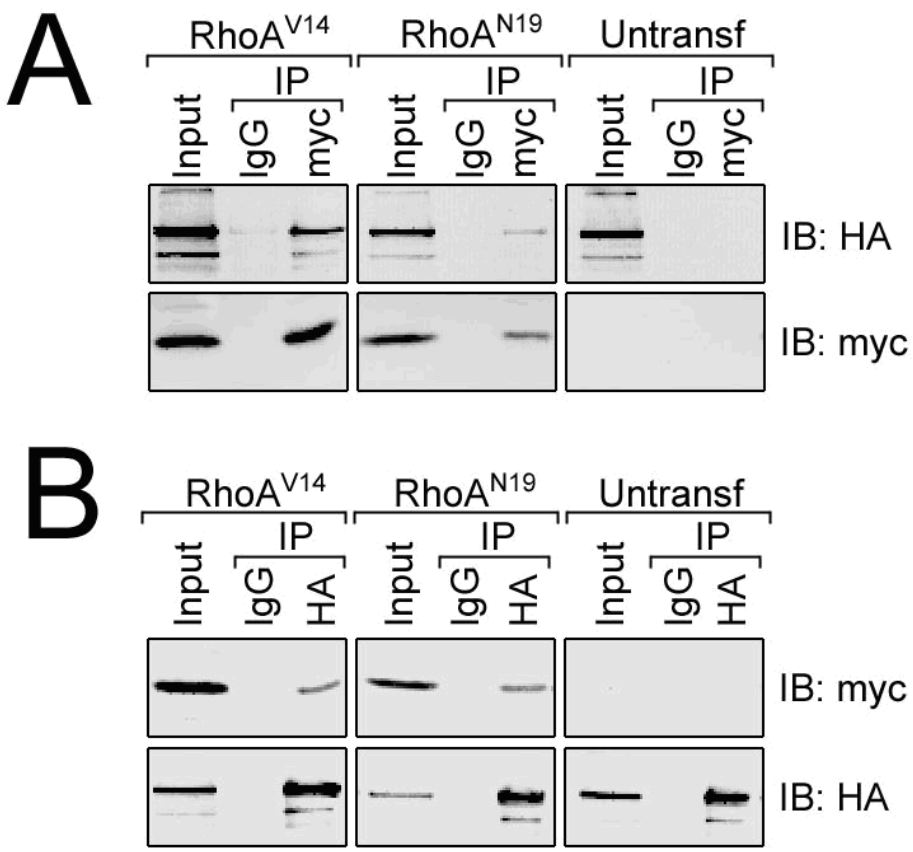
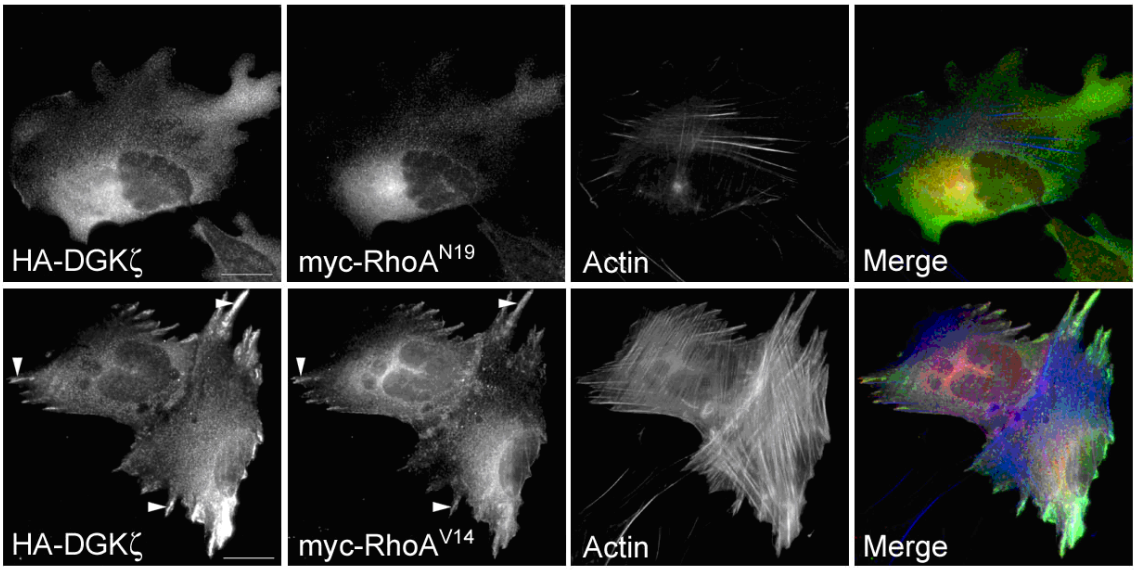

A
RhoA^{V14}
RhoA^{N19}
Untransf
IP
Input
IgG
myc
IB: HA
IB: myc
B
RhoA^{V14}
RhoA^{N19}
Untransf
IP
Input
IgG
HA
IB: myc
IB: HA

Figure 3.5. DGKζ expression overlaps with active and inactive RhoA in MEFs. MEFs co-expressing HA-DGKζ (green) with myc-RhoA^{V14} or -RhoA^{N19} (red) were fixed in PFA and immuno-stained with antibodies against HA-DGKζ and myc-RhoA constructs and secondary antibodies conjugated to AlexaFluor-488 and -594, respectively. Actin (blue) was visualized using AlexaFluor-350 conjugated-phalloidin. Arrowheads in the lower panels represent distinct regions of co-localization between DGKζ and RhoA^{V14}. Scale bars = 20 μm.

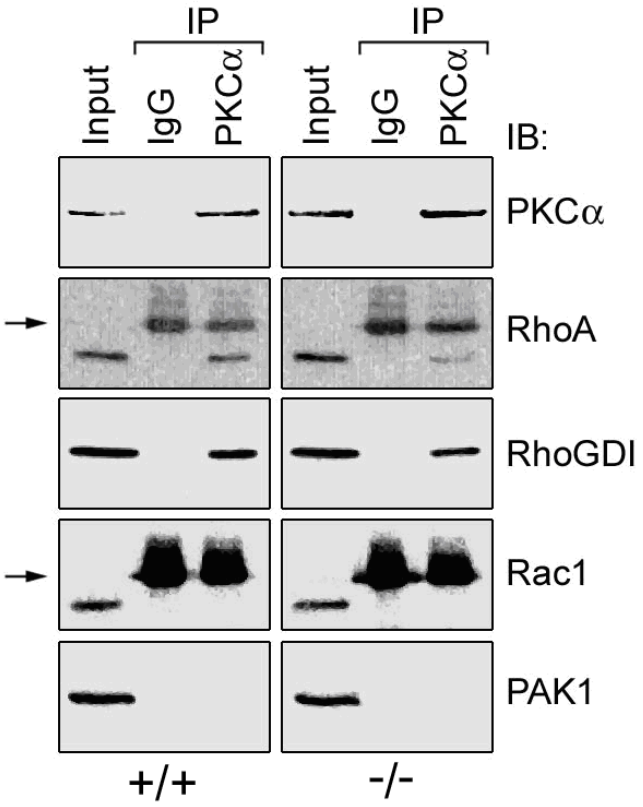

HA-DGKζ
myc-RhoA^{N19}
Actin
Merge
HA-DGKζ
myc-RhoA^{V14}
Actin
Merge

active form of Rho GTPases, data here indicates DGKζ stably associates with both active and inactive RhoA conformations.

A major point of regulation for the Cdc42, Rac1 and RhoA GTPases is their sequestration in an inactive, cytosolic complex by their common inhibitor, RhoGDI (Garcia-Mata et al., 2011). Our laboratory has previously demonstrated that Rac1 is part of a multi-protein signalling complex with DGKζ, PAK1 and RhoGDI (Abramovici et al., 2009). DGKζ-derived PA activates PAK1, which subsequently phosphorylates RhoGDI, triggering Rac1 release. More recently, it was shown that PKCα phosphorylates RhoGDI on Ser-34 to selectively release and activate RhoA (Dovas et al., 2010). Since DGKζ interacts with PKCα (Luo et al., 2003) and RhoA, we hypothesized that it might be important for the assembly of the RhoA/RhoGDI dissociation complex.

Consistent with the idea that DGKζ stabilizes the PKCα/RhoA interaction, RhoA efficiently co-precipitated with PKCα from wild type MEF extracts, but not from extracts of DGKζ-null cells (Figure 3.6). Quantification of replicate blots showed a ~2.5-fold decrease in the amount of co-precipitated RhoA from DGKζ-null cells. RhoGDI was present in PKCα immunoprecipitates from both cell lines, suggesting its interaction with PKCα does not require DGKζ. In contrast, neither Rac1 nor PAK1 co-immunoprecipitated with PKCα, indicating the RhoA/RhoGDI dissociation complex exists independently from the Rac1/RhoGDI complex. Collectively, these data suggest DGKζ is important in the assembly of PKCα and RhoA into a RhoA-specific RhoGDI dissociation signalling complex.

Figure 3.6. DGKζ is required for efficient RhoA-PKCα co-IP. Wild type (+/+) and DGKζ-null (-/-) cell extracts were immunoprecipitated with control IgG or anti-PKCα. The immune complexes were analyzed by immunoblotting with an anti-PKCα antibody to confirm successful immunoprecipitation and with antibodies raised against RhoA, RhoGDI, Rac1 and PAK1. Arrow indicates 25kDa IgG from immunoprecipitation.


IP
IP
Input
IgG
PKCα
Input
IgG
PKCα
IB:
PKCα
RhoA
RhoGDI
Rac1
PAK1
+/+
-/-

Direct interaction of DGKζ with RhoA

To determine if DGKζ binds directly to RhoA and to identify the interacting regions, a soluble His_6 fusion protein of RhoA (containing an S-tag epitope) was used to overlay various GST fusion proteins of DGKζ, shown schematically (Figure 3.7A). RhoA consistently bound to a DGKζ fusion protein containing amino acids 97 to 233, which includes two cysteine-rich regions homologous to the C1A and C1B motifs of PKCs (Figure 3.7B) (Hurley et al., 1997). RhoA did not bind to GST or other GST-DGKζ domains, demonstrating the specificity of this interaction. In supporting experiments, GST-$RhoA^{V14}$ and GST-$RhoA^{N19}$ fusion proteins efficiently captured HA-tagged wild type DGKζ from lysates of transfected MEFs (Figure 3.3B), but failed to capture a DGKζ mutant missing amino acids 97-233 (HA-DGKζ$^{\Delta 97\text{-}233}$) (Figure 3.7C), suggesting these residues are required for RhoA binding. Interestingly, this is the same region previously defined as the binding site for Rac1 (Yakubchyk et al., 2005). As a control for binding, HA-DGKζ$^{\Delta 97\text{-}233}$ was efficiently captured by the PDZ domain of α1-syntrophin (Figure 3.7C), indicating that the deletion does not affect DGKζ function in this regard. Additional overlay experiments further refined the RhoA binding site to the C1A domain (a.a. 97-152) of DGKζ (Figure 3.7D). In reciprocal pull-down experiments, the C-terminal half of RhoA (a.a. 101-193), but not the N-terminal half (a.a. 1-100) nor GST alone, was sufficient to capture HA-DGKζ from MEF cell lysates (Figure 3.8). Collectively, these data indicate the C1A domain of DGKζ, and the C-terminus of RhoA, are important for the interaction between these two proteins.

Figure 3.7. RhoA binds directly to the C1 domains of DGKζ. (**A**) The indicated regions of DGKζ were fused to the C-terminus of GST. DGKz structure adapted from Topham and Epand, 2009. (**B**) The constructs shown in A were expressed in bacteria. Bacterial lysates were analyzed by SDS-PAGE and transferred to a PVDF membrane. The lower panel represents Ponceau S staining of bacterial lysates following expression of DGKζ constructs. Asterisks (*) indicated bands that correspond to GST-fusion proteins. The same membrane was overlayed with HIS-tag purified, recombinant RhoA containing an S-tag epitope, at 4°C overnight and then probed with S-protein conjugated to HRP (top panel). (**C**) Wild type MEFs were transfected with HA-tagged DGKζ mutant missing amino acids 97-233 (HA- DGKζ$^{\Delta 97\text{-}233}$). After 48 hours, the cells were lysed and extracts were incubated with beads charged with GST-alone, RhoA^{V14}, RhoA^{N19} or GST fused to the PDZ domain of α1-syntrophin (α-Syn). Near equal levels of fusion protein are shown in the Ponceau S blot (bottom). Bound proteins were analyzed by immunoblotting with anti-HA. Input represents 10% of the total extract. (**D**) Further delineation of the RhoA binding site on DGKζ was conducted using deletion constructs of the C1 domains (C1) and carried out as described in Figure 3.7B with constructs including just the C1A domain (97-152) and the C1B domain (172-233) of DGKζ. Overlay was conducted as described in 8B.

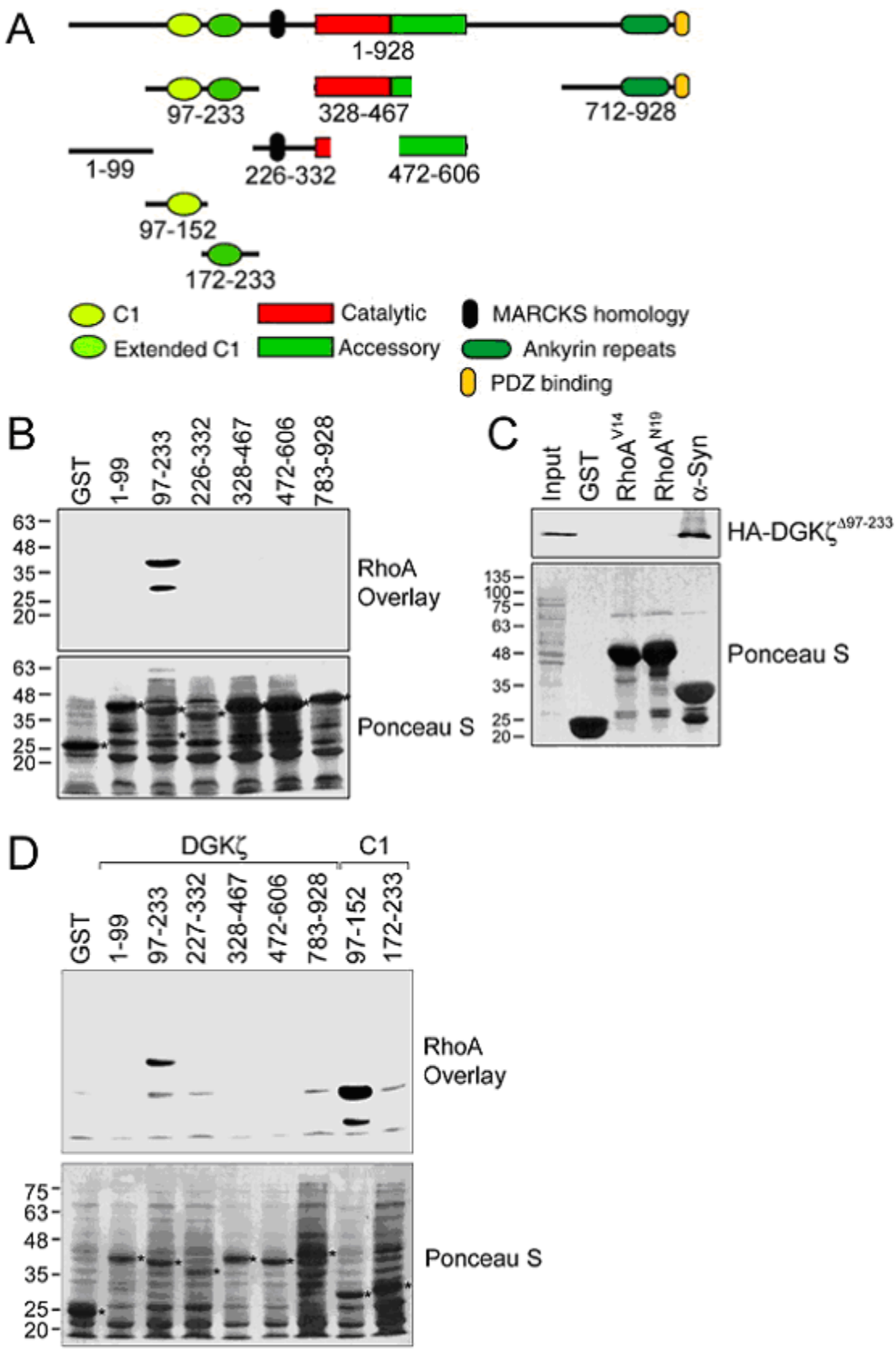
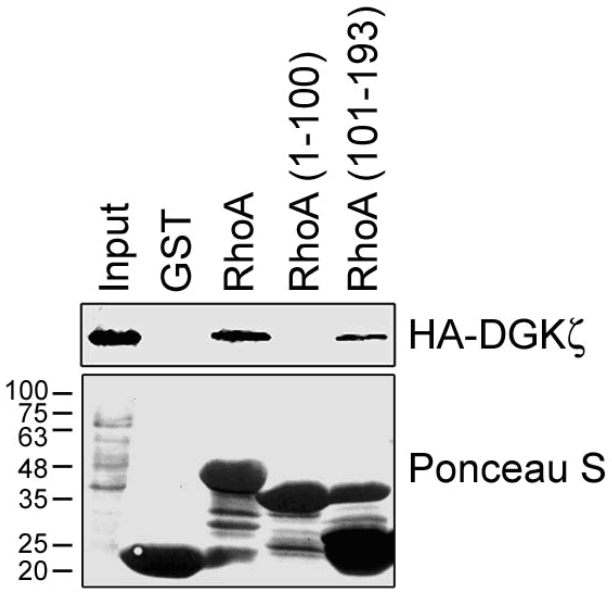

A
1-928
97-233
328-467
712-928
1-99
226-332
472-606
97-152
172-233
C1
Extended C1
Catalytic
Accessory
MARCKS homology
Ankyrin repeats
PDZ binding
B
GST
1-99
97-233
226-332
328-467
472-606
783-928
63
48
35
25
20
RhoA
Overlay
Ponceau S
C
Input
GST
RhoA^V14
RhoA^N19
α-Syn
HA-DGKζ^Δ97-233
135
100
75
63
48
35
25
20
Ponceau S
D
DGKζ
C1
GST
1-99
97-233
227-332
328-467
472-606
783-928
97-152
172-233
RhoA
Overlay
75
63
48
35
25
20
Ponceau S

Figure 3.8. DGKζ associates with the C-terminus of RhoA. The region of RhoA responsible for binding DGKζ was narrowed down by incubating extracts from cells over-expressing HA-tagged wild type DGKζ with GST-alone, GST-RhoA, the N-terminus of RhoA (1-100) or the C-terminus of RhoA (101-193). The amount of each fusion protein is shown in the Ponceau S blot (bottom). Bound proteins were analyzed by immunoblotting with anti-HA. Input represents 10% of the total extract.


Input
GST
RhoA
RhoA (1-100)
RhoA (101-193)
HA-DGKζ
100
75
63
48
35
25
20
Ponceau S

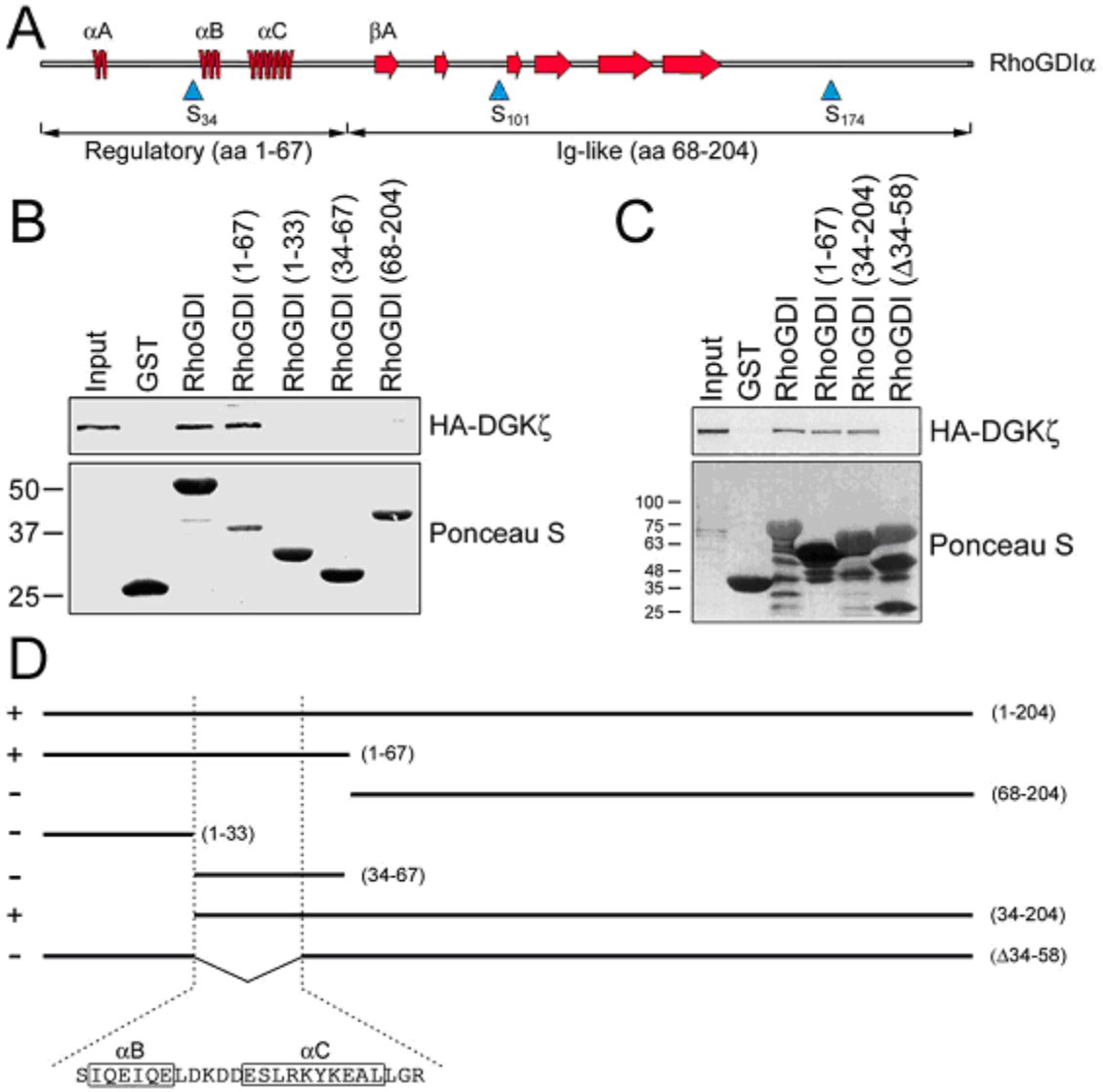
DGKζ binds to the regulatory region of RhoGDI

RhoGDIα is composed of two structurally distinct regions: a flexible N-terminal regulatory domain (residues 1–67) that forms three alpha helices when in complex with Rho GTPases and a C-terminal immunoglobulin-like domain (residues 68–204) (Figure 3.9A). The serine residue (Ser-34) phosphorylated by PKCα, which regulates RhoA/RhoGDI association, lies adjacent to the αB helix within the regulatory arm of RhoGDI. Using pull-down assays, the DGKζ-binding region of RhoGDI was narrowed down to the N-terminal regulatory domain. A GST fusion protein comprising amino acids 1-67 was sufficient to capture HA-tagged DGKζ from transfected cell lysates, while a fusion protein containing amino acids 68-204 failed to capture DGKζ (Figure 3.9B). Smaller GST fusion proteins (residues 1-33 and 34-67) were not sufficient to bind DGKζ. However, in the context of the full length protein, residues 1-33 were dispensable for binding, while 34-58 were essential (Figure 3.9C). Together, these results suggest DGKζ binds to the region of RhoGDI immediately C-terminal to Ser-34 and raise the intriguing possibility that DGKζ regulates phosphorylation of this key residue.

Reduced Ser-34 phosphorylation of RhoGDI by PKCα in the absence of DGKζ

To determine if DGKζ is required for PKCα-mediated phosphorylation of RhoGDI on Ser-34, wild type and DGKζ-null cells were stimulated with PMA, a potent activator of PKC. Despite the requirement for $PI(4,5)P_2$ and not DAG for PKCα-mediated RhoGDI phosphorylation, Dovas et al. used PMA, a DAG analog, to activate this process as stimulating cells with PMA can activate also PKCα indirectly through

Figure 3.9. The N-terminal regulatory region of RhoGDI binds DGKζ. (**A**) Schematic showing the secondary structure of RhoGDI and selected phosphorylation sites. PAK1 phosphorylation of Ser-101/174 causes Rac1 dissociation while PKCα phosphorylation of Ser-34 promotes RhoA release. (**B and C**) Pull-down of HA-tagged wild type DGKζ with full length, N-terminal (residues 1-67), two N-terminal fragments (residues 1-33 and 34-67), a deletion of the N-terminal region between amino acids 34-58 and C terminal (residues 68-204) RhoGDI. MEFs were infected with adenoviral vector encoding HA-DGKζ and cell extracts were incubated with beads charged with GST-alone or with GST-fusion proteins of the various RhoGDI described above. Near equal levels of each fusion protein is shown in the Ponceau S blot (bottom). Bound proteins were immunoblotted with anti-HA. Input represents 20% of the extract used for the pull-down. (**D**) Schematic representation of RhoGDI deletion constructs used to test binding to DGKζ through GST-pull-down experiments. A plus sign (+) indicates binding to DGKζ, whereas a minus sign (-) indicates no detected interaction. The amino acid sequence of the RhoGDI region responsible for binding DGKζ is also included. Figure 3.9B by Alexandria Fottinger.


A
αA
αB
αC
βA
RhoGDIα
S34
S101
S174
Regulatory (aa 1-67)
Ig-like (aa 68-204)
B
Input
GST
RhoGDI
RhoGDI (1-67)
RhoGDI (1-33)
RhoGDI (34-67)
RhoGDI (68-204)
HA-DGKζ
Ponceau S
50
37
25
C
Input
GST
RhoGDI
RhoGDI (1-67)
RhoGDI (34-204)
RhoGDI (Δ34-58)
HA-DGKζ
Ponceau S
100
75
63
48
35
25
D
(1-204)
(1-67)
(68-204)
(1-33)
(34-67)
(34-204)
(Δ34-58)
αB
αC
SIQEIQELDKDDESLRKYKEALLGR

integrin and cell adhesion signalling pathways upstream (Dovas et al., 2010; Lub et al., 1997). Immunoblot analysis of the cell lysates was carried out using an affinity purified antibody that specifically recognizes Ser-34-phosphorylated RhoGDI (Dovas et al., 2010). A faint band corresponding to the size of RhoGDI was detected in lysates of untreated wild type cells (Figure 3.10A). There was a dramatic increase in intensity of the band after 10 min of PMA stimulation and a further increase at 20 min. The increase in phosphorylated RhoGDI at Ser-34 was blocked by a PKCαβ-specific inhibitor, Gö6976, consistent with previous findings in other cell lines confirming PKCα is the kinase responsible for phosphorylation at this residue (Dovas et al., 2010). In contrast to wild type MEFs, PMA stimulation of DGKζ-null cells did not elicit the same increase in phosphorylated Ser-34 at either time point. Quantification of signal intensities at the 20 min time point between three independent experiments showed a significant decrease ($p<0.05$) in pSer-34 levels in DGKζ-null cells compared to wild type (Figure 3.10B). Physiologically consistent with the RhoA activation defect in DGKζ-null cells following serum stimulation, similar results were obtained for pSer-34 levels using serum-stimulated cells (Figure 3.11A and 3.11B). Together, these data suggest PKCα-mediated phosphorylation of Ser-34 is defective in DGKζ-null cells.

Exogenous expression of HA-tagged wild type DGKζ (AdDGKζ) in PMA-stimulated null cells was sufficient to restore pSer-34 RhoGDI levels close to those seen in control wild type MEFs (Figure 3.12). The kinase dead mutant (AdDGKζ^{kd}) also rescued the phosphorylation defect, indicating catalytic activity is dispensable for its role in this complex. Consistent with the results of the rescue of RhoA activation,

Figure 3.10. PKCα phosphorylation of RhoGDI on Ser-34 attenuated in DGKζ-null MEFs following PMA stimulation. (**A**) Starved wild type (+/+) and DGKζ-null (-/-_ cells were incubated with DMSO, or stimulated with PMA for 10 or 20 minutes in the presence or absence of the PKC-specific inhibitor, Gö6976. Phosphorylation of RhoGDI on serine 34 (pSer-34) was analyzed using a phospho-specific rabbit polyclonal antibody (Dovas et al., 2010). Antibodies against RhoGDI and tubulin were used as controls for equal loading. (**B**) Graph represents quantification of phosphorylated RhoGDI following 20 minutes of stimulation in wild type and DGKζ-null MEFs from three independent experiments. Asterisk (*) designates statistical significance ($p<0.05$).

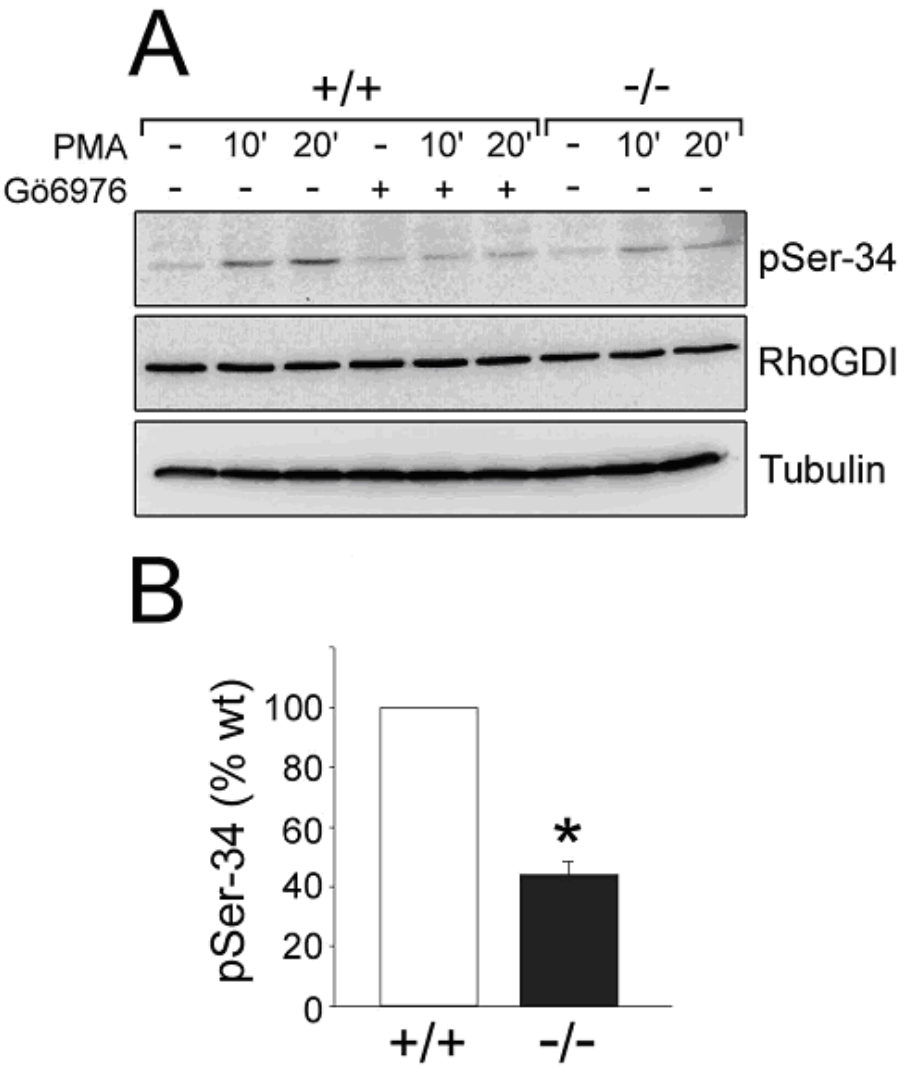

A
+/+
-/-
PMA
-
10'
20'
-
10'
20'
-
10'
20'
Gö6976
-
-
-
+
+
+
-
-
-
pSer-34
RhoGDI
Tubulin
B
pSer-34 (% wt)
100
80
60
40
20
0
*
+/+
-/-

Figure 3.11. PKCα phosphorylation of RhoGDI on Ser-34 attenuated in DGKζ-null MEFs following serum stimulation. (**A**) Starved wild type (+/+) and DGKζ-null (-/-) were left untreated or stimulated with 20% fetal bovine serum (FBS) containing media (Serum) for 5 or 10 minutes in the presence or absence of the PKC-specific inhibitor, Gö6976. Phosphorylation of RhoGDI on Ser-34 (pSer-34) was analyzed using a phospho-specific rabbit polyclonal antibody (Dovas et al., 2010). Antibodies against RhoGDI and tubulin were used as controls for equal loading. (**B**) Graph represents quantification of phosphorylated RhoGDI following 10 minutes of stimulation with serum in wild type and DGKζ-null MEFs from three independent experiments. Asterisk (*) designates statistical significance ($p<0.05$).

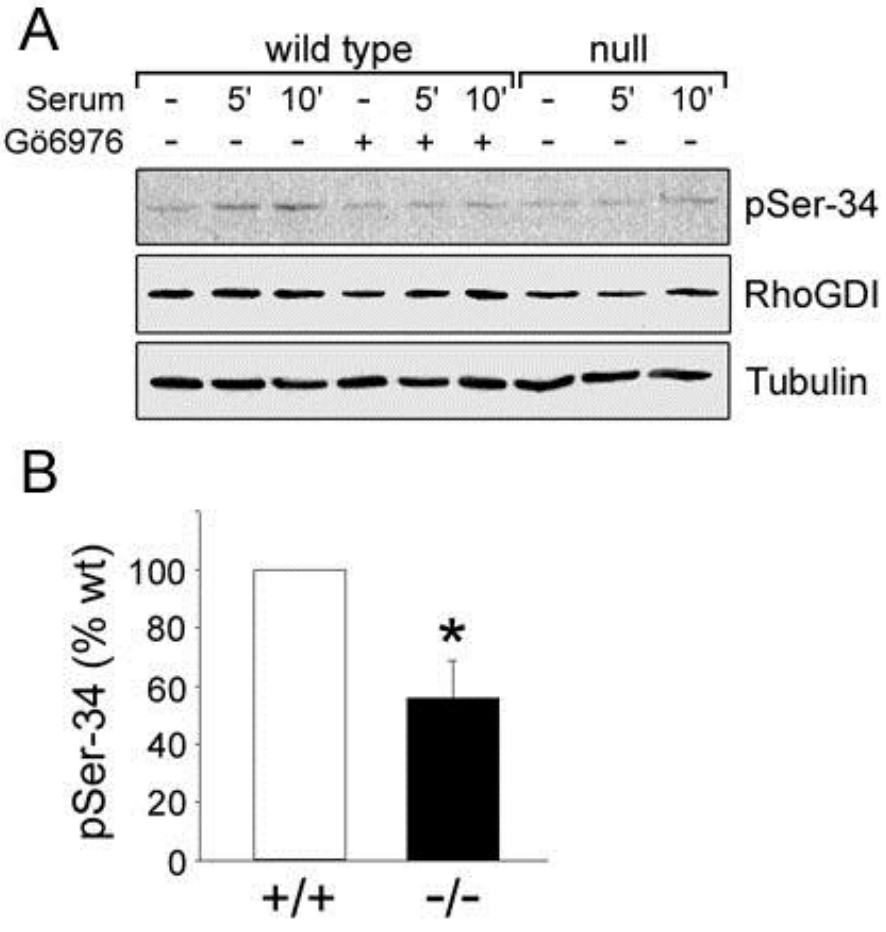
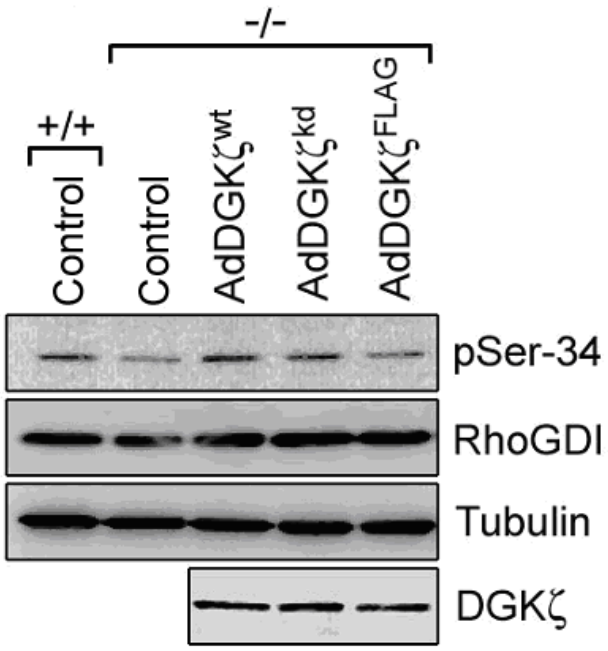

A
wild type
null
Serum - 5' 10' - 5' 10' - 5' 10'
Gö6976 - - - + + + - - -
pSer-34
RhoGDI
Tubulin
B
pSer-34 (% wt)
100
80
60
40
20
0
*
+/+
-/-

Figure 3.12. RhoGDI pSer-34 rescue consistent with RhoA activity rescue experiments. Adenoviral constructs of wild type DGKζ (DGKζ^{wt}), a kinase dead mutant (DGKζ^{kd}) and a C-terminally FLAG-tagged DGKζ that disrupts association with syntrophins (DGKζ^{FLAG}) were expressed in DGKζ-null MEFs. Untreated wild type (+/+) and DGKζ-null (-/-) cells were used as controls (Control). The cells were starved overnight, and then stimulated with PMA for 20 minutes. Cell extracts were run on SDS-PAGE, transferred to a PVDF membrane, and were probed for phosphorylated RhoGDI on Ser-34. Antibodies against RhoGDI and tubulin were used as loading controls. An antibody against DGKζ was used to confirm expression of DGKζ constructs.


-/-
+/+
Control
Control
AdDGKζwt
AdDGKζkd
AdDGKζFLAG
pSer-34
RhoGDI
Tubulin
DGKζ

AdDGKζ^{FLAG} failed to rescue Ser-34 phosphorylation. Collectively, these results indicate DGKζ is required for optimal Ser-34 phosphorylation by PKCα. Furthermore, these findings suggest decreased RhoA activity in DGKζ-null cells is due, at least in part, to attenuated RhoA release from RhoGDI.

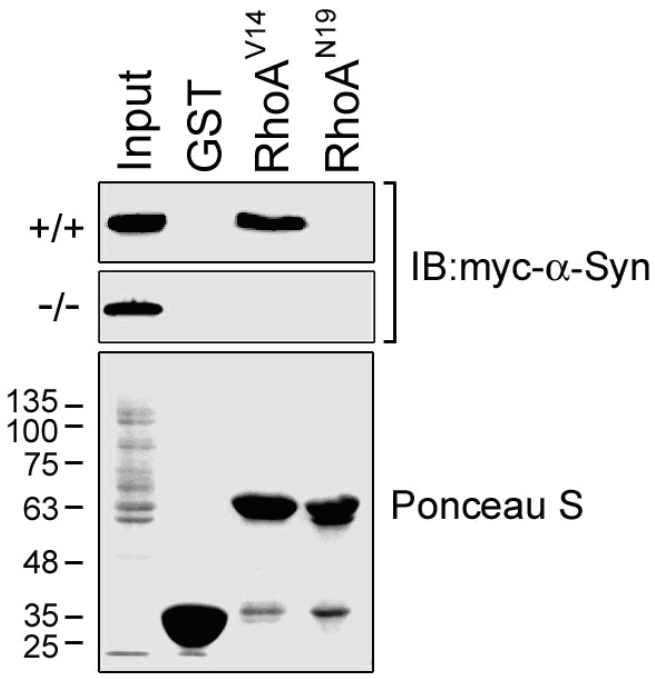
Syntrophin interacts with active RhoA in the presence of DGKζ

The failure of DGKζ^{FLAG} to sufficiently rescue RhoA activation and Ser-34 phosphorylation suggests the interaction of DGKζ with syntrophins might be important for RhoA activation, as the C-terminal FLAG-tag disrupts DGKζ/syntrophin interactions (Hogan et al., 2001). To evaluate potential interactions between syntrophin and RhoA, GST-fusion proteins of RhoA^{V14} and RhoA^{N19} were used to capture myc-tagged α-syntrophin (α-syn) from lysates of transfected MEFs. Myc-α-syn was captured by GST-RhoA^{V14} but not by RhoA^{N19} or GST alone (Figure 3.13). When the same experiment was carried out in DGKζ-null cells, syntrophin failed to bind to RhoA^{V14}. These pull-downs indicate syntrophin interacts exclusively with active RhoA and does so only in the presence of DGKζ, suggesting DGKζ is a component of distinct multi-protein complexes with active and inactive RhoA.

Phosphorylation of DGKζ increases binding to RhoA

Dual regulation of Rac1 and RhoA activity by DGKζ implies different upstream signals might control the selectivity of DGKζ for these Rho GTPases. RhoA activity and

Figure 3.13. RhoA^{V14} associates with α-syntrophin exclusively in the presence of DGKζ. Wild type (+/+) and DGKζ-null (-/-) MEFs were transfected with myc-tagged α1-syntrophin. After 48 hours, the cells were lysed and extracts were incubated with beads charged with GST-alone, GST-RhoA^{V14}, or GST-RhoA^{N19}. Ponceau S (bottom panel) reveals near equal levels of fusion proteins. Bound protein was detected by immunoblotting with anti-myc. Input represents 10% of the starting material.

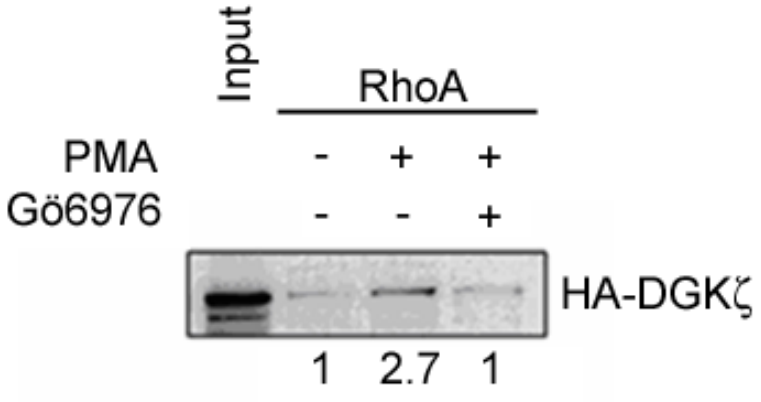

Input
GST
RhoA^{V14}
RhoA^{N19}
+/+
-/-
IB:myc-α-Syn
135
100
75
63
48
35
25
Ponceau S

PKCα-mediated RhoGDI phosphorylation on Ser-34 were attenuated in DGKζ-null MEFs, thus it is plausible that DGKζ selectively regulates RhoA downstream of PKCα signalling. Interestingly, PKCα phosphorylation of DGKζ is known to both reduce binding to active Rac1 and decrease DGKζ catalytic activity (Luo et al., 2003b; Yakubchyk et al., 2005), which is dispensable for RhoA activation but required for Rac1. To test the effect of PKCα phosphorylation on DGKζ and RhoA binding, MEFs expressing HA-tagged wild type DGKζ were left untreated or stimulated with PMA in the presence or absence of a PKCαβ specific inhibitor, Gö6976. In cell stimulated with PMA, DGKζ bound 2.7 times more to RhoA compared with non-stimulated cells (Figure 3.14). The PKC inhibitor reversed this increase as RhoA bound DGKζ at levels similar to untreated cells. These data suggest phosphorylation of the DGKζ MARCKS domain by PKCα increases DGKζ binding to RhoA. Collectively, these findings suggest PKCα might sustain RhoA activity by promoting DGKζ to preferentially associate with RhoA over Rac1.

Impaired actin stress fiber assembly during cell spreading in the absence of DGKζ

Assessing cell behaviour following re-plating on extra-cellular matrix factors such as fibronectin is an invaluable tool to gauge the roles of individual proteins and signalling networks in cell spreading (Pankov and Yamada, 2002). Moreover, *in vitro* adhesion assays are directly relevant to understanding how signalling pathways control tissue development *in vivo* (Dobereiner et al., 2005). When cells spread on adhesive substrates such as fibronectin, the balance of activity of two Rho effectors, ROCK and mDia1,

Figure 3.14. DGKζ phosphorylation by PKCα increases DGKζ binding to RhoA. (**A**) MEFs over-expressing wild type HA-tagged DGKζ were stimulated with PMA in the presence and absence of Gö6976, a PKCαβ-specific inhibitor. Equal levels of protein from these lysates were incubated with immobilized GST-RhoA^{V14}. Bound proteins were analyzed by immunoblotting with an anti-HA antibody. Input represents 10% of total extract. The numbers below reflect fold change in binding under the three conditions.

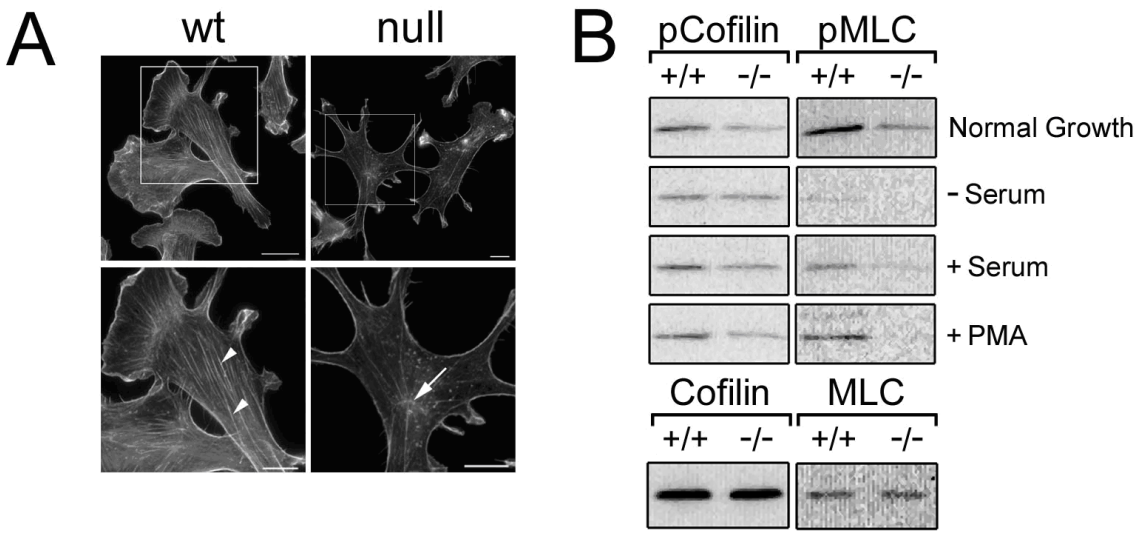

Input
RhoA
PMA
- + +
Gö6976
- - +
HA-DGKζ
1 2.7 1

determines the thickness and proper organization of actin stress fibers (Defilippi, 1999; Watanabe et al., 1999). To determine if DGKζ absence alters stress fiber formation, wild type and DGKζ-null MEFs spreading on fibronectin were fixed following 2 hour of re-plating and stained with phalloidin to visualize F-actin. The majority of wild type MEFs adopted a broad morphology and had stress fibers that spanned the diameter of the cell and were regularly distributed (Figure 3.15A). Conversely, DGKζ-null MEFs did not have well defined actin stress fibers. Cells lacking DGKζ occasionally exhibited radial F-actin structures phenotypically identical to those observed by Watanabe et al. following a disruption in the ratio of ROCK to mDia activity (Watanabe et al., 1999). Because of mutual inhibition between Rac1 and ROCK (Huveneers and Danen, 2009), and decreased Rac1 activity in the DGKζ-null cells (Abramovici et al., 2009), decreased ROCK inhibition by Rac1, relative to mDia, might cause such an imbalance. Taken together, our results suggest DGKζ loss perturbs Rho-mediated stress fiber formation in MEFs.

Decreased phosphorylation of Rho/ROCK targets in DGKζ-null MEFs

Downstream of RhoA, mDia and ROCK cooperate to both promote polymerization of actin filaments and obstruct the severing of actin filaments, respectively. An important downstream target of ROCK includes LIM Kinase, which phosphorylates Cofilin on Ser-3 to prevent the severing of F-actin to G-actin (Maekawa et al., 1999). In addition, ROCK is responsible for myosin light chain (MLC) phosphorylation on Ser-19 (Kosako et al., 2000). Phosphorylation at this site is important for actin-myosin contractility during cell migration and furrow ingression during cytokenesis. Conversely, Rac1 has been shown to negatively regulate actin-myosin

Figure 3.15. Impaired actin stress fiber formation in spreading DGKζ-null MEFs. (**A**) Representative images of wild type (+/+) and DGKζ-null (-/-) MEFs taken at 120 minutes after plating on fibronectin coated coverslips. The cells were fixed and labelled with AlexaFluor 488-conjugated phalloidin to visualize F-actin. The bottom panels are magnified images of the boxed regions at 120 minutes. The arrowheads indicate long, regularly distributed stress fibers. The arrow indicates a condensed F-actin structure in -/- cells. Scale bars = 20 μm. (**B**) Lysates of +/+ and -/- MEFs from four different conditions (normal growth, serum starved, serum stimulated for 5 minutes, and PMA stimulated for 5 minutes) were immunoblotted (IB) with phospho-specific antibodies against two proteins downstream of Rho/ROCK signalling: phosphorylated Cofilin at Ser-3 and phosphorylated myosin light chain (MLC) at Ser-19. Equal loading was confirmed by IB with anti-Cofilin and anti-myosin light chain (MLC).


A
wt
null
B
pCofilin
pMLC
+/+
-/-
+/+
-/-
Normal Growth
- Serum
+ Serum
+ PMA
Cofilin
MLC
+/+
-/-
+/+
-/-

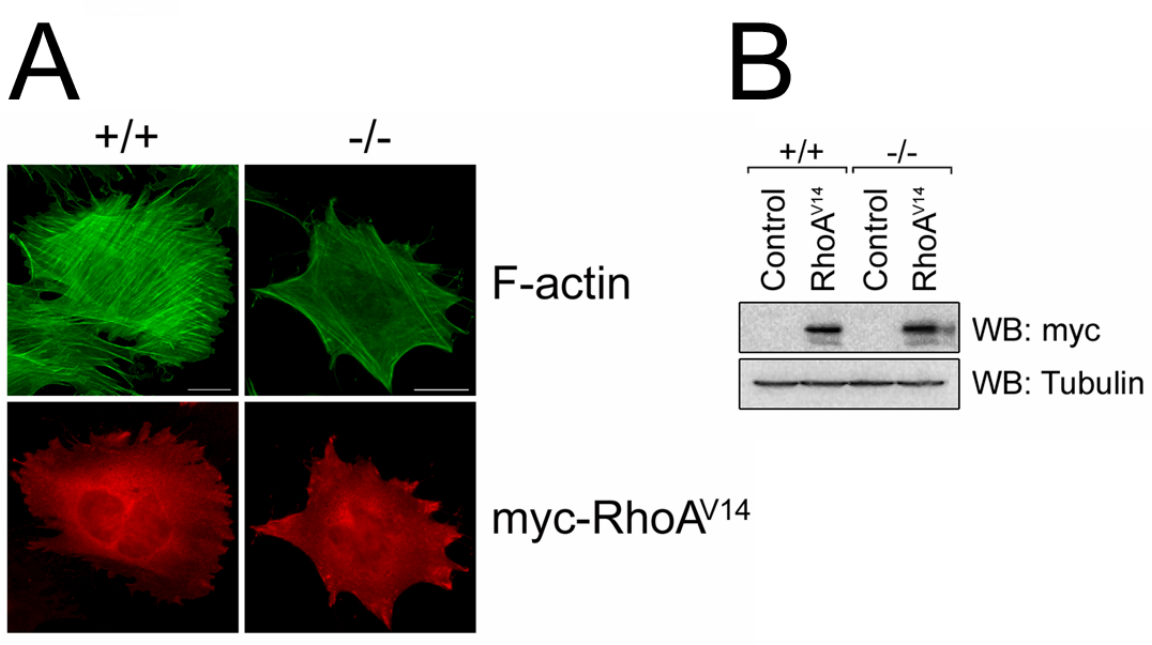
contractility by inhibiting MLC phosphorylation at Ser-19 (Sanz-Moreno et al., 2008). ROCK promotes pMLC levels two fold: 1) by inhibiting myosin phosphatase, and 2) by direct MLC phosphorylation (Totsukawa et al., 2003; Zhao and Manser, 2005). As ROCK establishes actin-myosin contractility and inhibits F-actin severing, mDia concurrently acts through profilin to polymerize G-actin into F-actin (Kovar, 2006). Loss of adequate actin stress fiber assembly and decreased RhoA activity in DGKζ-null cells implies reduced activity of these two effectors.

To investigate the actin defect in DGKζ-null cells biochemically, phospho-specific antibodies that recognize downstream Rho/ROCK targets, pCofilin (Ser-3) and pMLC (Ser-19), were used to immunoblot lysates from wild type and null cells under four different conditions: normal growth, serum starved, serum stimulated and PMA stimulated. Phosphorylation levels for Cofilin and MLC were reduced in DGKζ-null cells compared to wild type in almost all conditions (Figure 3.15B). In addition to ROCK, however, LIMK activation and Cofilin phosphorylation have been reported by PAK downstream of Rac1 and Cdc42 activation (Edwards et al., 1999; Zhao and Manser, 2005). Since DGKζ-null cells have normal Cdc42 activity but decreased RhoA and Rac1 activity (Abramovici et al., 2009; Figure 3.1), the relative contribution of Rac1 and RhoA for pCofilin is unclear for some of these conditions. Regardless, serum is a potent RhoA activator (Chrzanowska-Wodnicka and Burridge, 1996). pCofilin levels increased substantially in wild type cells following RhoA activation by serum stimulation. Conversely, pCofilin levels did not increase cells lacking DGKζ under this condition. This suggests Rho/ROCK activity is indeed perturbed in the absence of DGKζ, consistent with disrupted actin stress fiber formation in DGKζ-null cells during spreading.

Reduced RhoA^{V14} induced actin stress fibers in the absence of DGKζ

Exogenous over-expression of a constitutively active RhoA mutant (RhoA^{V14}) in fibroblasts is sufficient to induce actin stress fiber formation (Paterson et al., 1990; Ridley et al., 1992). DGKζ forms a stable complex and co-localizes with both inactive and active RhoA, raising the possibility that DGKζ might remain associated with RhoA following GTP-loading and therefore could contribute to RhoA function downstream. To test this idea, we compared actin stress fiber formation in wild type and DGKζ-null cells infected with the constitutively active RhoA^{V14} mutant. As expected, myc-tagged RhoA^{V14} expression induced abundant actin stress fibers in wild type MEFs (Figure 3.16A). In contrast, RhoA^{V14}-induced stress fiber formation was noticeably attenuated in MEFs lacking DGKζ. We verified that RhoA^{V14} was expressed at equivalent levels in wild type and null lysates (Figure 3.16B). These results suggest DGKζ is necessary for RhoA function following activation, consistent with the ability of DGKζ to associate with both active and inactive RhoA conformations. Therefore, DGKζ appears to be critical for RhoA activation and downstream functions following activation.

Figure 3.16. Reduced RhoA^{V14}–induced stress fibers in DGKζ-null MEFs. (**A**) Wild type (+/+) and DGKζ-null (-/-) MEFs were infected with an adenoviral construct expressing constitutively active myc-RhoA^{V14} (red) and visualized with anti-myc, followed by AlexaFluor 594-conjugated secondary antibody. Actin filaments were stained using AlexaFluor 488-conjugated Phalloidin (green). Images were obtained with equal exposure time. (**B**) To confirm equal expression, +/+ and -/- extracts from cells expressing myc-RhoA^{V14} were analyzed by immunoblotting with anti-myc. Tubulin was used as a loading control. WB: western blot.


A
+/+
-/-
F-actin
myc-RhoA^{V14}
B
+/+
-/-
Control
RhoA^{V14}
Control
RhoA^{V14}
WB: myc
WB: Tubulin

4. Discussion

DISCUSSION

Major findings

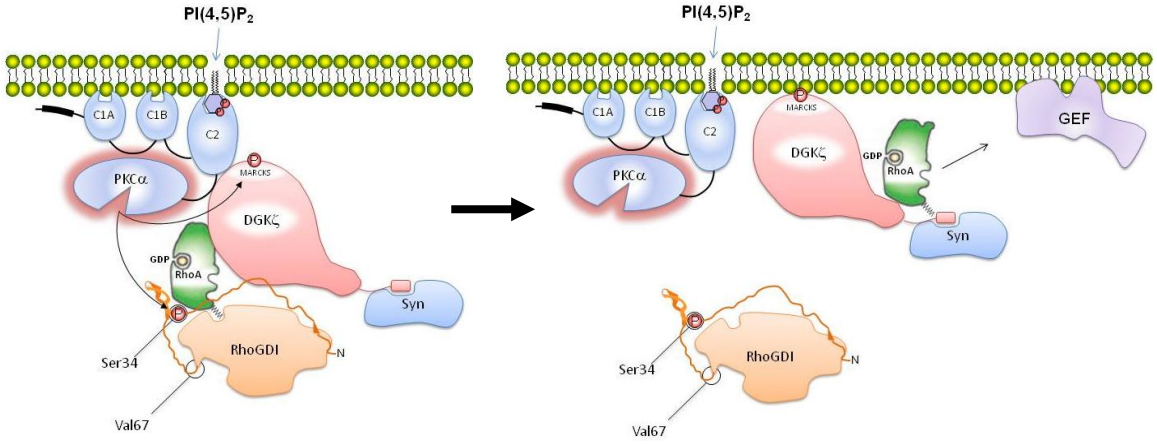
MEFs lacking DGKζ have reduced RhoA activity and impaired Rho-dependent actin stress fiber formation. This defect in RhoA activity is due, at least in part, to the inability of PKCα to efficiently phosphorylate RhoGDI on Ser-34 in the absence of DGKζ. Phosphorylation at this key residue is required for the selective release of RhoA from RhoGDI (Dovas et al., 2010). Data presented here suggests DGKζ binds directly to RhoA and is a core component of a RhoA/RhoGDI dissociation complex (Figure 4.1). Moreover, DGKζ activity was dispensable for this role, indicating that it acts as a scaffold for RhoA release and activation.

DGKζ is a component of the RhoA/RhoGDI dissociation complex

Rac1, Cdc42 and RhoA share a common inhibitor, RhoGDIα. Accumulating evidence suggests phosphorylation of RhoGDI selectively releases individual Rho GTPases (Dermardirossian et al., 2004; Dermardirossian et al., 2006; Dovas et al., 2010). For example, the kinases PAK1, PKCα and Src have been shown to phosphorylate RhoGDI on different residues and modify its binding to specific Rho GTPases (see Table 1.1). However, Rac1, Cdc42 and RhoA share highly conserved regions that stabilize binding to RhoGDI (Garcia-Mata et al., 2011). Therefore, how phosphorylation on a specific RhoGDI site releases only one Rho GTPase and not others is still unclear.

Accumulating evidence suggests specificity for selective Rho GTPase/RhoGDI dissociation mechanisms is achieved by the formation of distinct signalling complexes

Figure 4.1. Model for RhoA/RhoGDI dissociation. Left, DGKζ assembles a signalling complex with PKCα, RhoA/RhoGDI, and possibly a syntrophin isoform (Syn) (or other PDZ-containing protein in place of Syn). PI(4,5)P_2 binds to the C2 domain of PKCα and activates it. Activated PKCα phosphorylates RhoGDI on Ser-34, releasing RhoA. Active PKCα can phosphorylate DGKζ and decrease its activity, which is dispensable for this mechanism. Right, membrane translocation allows RhoA to interact with a GEF at the plasma membrane and become activated.


PI(4,5)P2
C1A
C1B
C2
PKCα
MARCKS
DGKζ
GDP
RhoA
Syn
Ser34
RhoGDI
N
Val67
PI(4,5)P2
C1A
C1B
C2
PKCα
MARCKS
DGKζ
GDP
RhoA
GEF
Syn
Ser34
RhoGDI
N
Val67

with RhoGDI (Garcia-Mata et al., 2011). Our laboratory has previously shown that Rac1 exists in a multi-protein complex with DGKζ, PAK1 and RhoGDI in MEFs (Abramovici et al., 2009). An independent group has reported a similar mechanism involving the assembly of a Rac1/RhoGDI dissociation complex by DGKα in endothelial cells (Chianale et al., 2010). Loss of DGKζ or DGKα significantly reduced Rac1 release from RhoGDI in MEFs and endothelial cells, respectively. Together, these reports demonstrate specificity for Rho GTPase/RhoGDI dissociation mechanisms following different upstream signals requires the formation of unique multi-protein complexes.

My results indicate DGKζ exists in a RhoA/RhoGDI dissociation complex independent from Rac1. RhoA-release requires PKCα phosphorylation of RhoGDI on Ser-34 (Dovas et al., 2010). DGKζ-null MEFs showed significantly decreased levels of RhoGDI Ser-34 phosphorylation by PKCα, indicating DGKζ is important for optimal RhoA/RhoGDI dissociation. DGKζ binds the RhoGDI helix-loop-helix region directly C-terminal to Ser-34, suggesting it might stabilize the surrounding structure and present this residue for phosphorylation by PKCα.

PKCα and RhoA failed to efficiently co-immunoprecipitate in the absence of DGKζ, suggesting it is critical for the assembly of these two proteins in a complex. A previous study showed that PKCα and RhoA functionally associate *in vivo* (Chang et al., 1998). Chang et al. were unable to detect a direct physical interaction between the two proteins, suggesting additional factors likely mediate their binding. A later study showed PKCα can interact directly with RhoA *in vitro* (Slater et al., 2001). Collectively these findings, together with data showing DGKζ binds directly to both proteins (Luo et al.,

2003; Figure 3.7.), suggests PKCα and RhoA form a weak interaction in cells that is enhanced and/or stabilized by the presence of DGKζ.

DGKζ regulates RhoA activation independent of catalytic activity

Canonical activation of conventional PKC isoforms involves DAG binding to their C1 domains (Figure 1.3). PKCα and DGKζ have previously been shown to associate (Luo et al., 2003a). In this complex, DAG metabolism by DGKζ attenuates PKCα activity. If PKCα required DAG to phosphorylate RhoGDI on Ser-34, DGKζ should inhibit this process. However, PKCα utilizes non-canonical activation by PI(4,5)P_2 binding to the C2 domain in order to phosphorylate RhoGDI (Dovas et al., 2010; Figure 1.3.). Therefore, the conversion of DAG to PA by DGKζ is likely irrelevant for this process. This is consistent with my finding that a kinase-dead DGKζ mutant was sufficient to rescue RhoA activity and RhoGDI Ser-34 phosphorylation and supports the idea that DGKζ functions as a scaffold.

DGKζ-derived PA is known interact with and activate PIP5K, an enzyme that produces PI(4,5)P_2 (Luo et al., 2004). Therefore, an interesting possibility is that PIP5K resides in the same signalling complex as PKCα and RhoA. DGKζ would stimulate the local production of PI(4,5)P_2 for PKCα activation, leading to RhoA/RhoGDI dissociation. Because the kinase-dead DGKζ mutant rescues RhoGDI phosphorylation, this indicates DGKζ activity might not be essential for PI(4,5)P_2 accumulation. PA derived from phospholipase D (PLD) is also known to activate PIP5K (van den Bout and Divecha, 2009), and might compensate for PI(4,5)P2 production in the absence of DGKζ activity.

This type of arrangement would provide an additional level of signalling specificity by limiting activation by lipids to the immediate RhoA/RhoGDI dissociation complex.

Kinase independent roles for DGK isozymes

There is evidence to suggest DGKs have functions independent of their catalytic activity. A previous study showed that two members of the Type 2 subfamily, DGKδ and DGKη, modulate the same Ras/B-Raf/C-Raf/MEK/ERK signalling cascade, albeit via different mechanisms and targets (Yasuda et al., 2009). DGKδ functioned conventionally by metabolizing DAG to PA, while DGKη activity was dispensable. Yasuda et al. suggested DGKη functions as a scaffolding protein for signalling in this process. My results showing DGKζ activity is dispensable for RhoA activation provide an additional example of DGK function not being limited to lipid metabolism/signalling. I suspect future studies making use of kinase-dead mutants will reveal additional catalytically independent roles for DGK isozymes and expand the scope of their significance in signal transduction.

PDZ requirements for Rac1 and RhoA activation

Rescue experiments utilizing a mutant with a C-terminal FLAG-tag, DGKζ^{FLAG}, offered additional insight into the mechanistic differences between Rac1 and RhoA activation by DGKζ. The C-terminal FLAG-tag disrupts the PDZ-binding motif of DGKζ, rendering DGKζ mislocalized (Hogan et al., 2001; Abramovici et al., 2003). Interestingly, exogenously over-expressed DGKζ^{FLAG} construct rescued Rac1 activity, but not RhoA. Thus, Rac1 and RhoA activation have differing requirements for DGKζ PDZ interactions.

My data do not currently reveal how DGKζ^{FLAG} interferes with RhoA activation yet has no effect on Rac1 activation. Because of the well established role for syntrophins in controlling correct DGKζ targeting in the cell (Abramovici et al., 2003; Abramovici et al., 2007; Hogan et al., 2001; Yakubchyk et al., 2005), abrogating this interaction might interrupt the ability of DGKζ to localize and/or scaffold a RhoA-specific activation complex. α1-syntrophin did not associate with inactive RhoA in the presence or absence of DGKζ, making it unlikely that this isoform is part of a RhoA-specific activation complex. It is therefore also possible that only a subset of these isoforms, each with distinct expression patterns (Froehner et al., 1997), might be involved in RhoA activation. Therefore, a role for individual syntrophins in RhoA signalling might be cell type specific and deserves further study.

Despite high specificity for many PDZ domains and their targets (Gee et al., 2000), another possibility is that DGKζ binds one or more PDZ domain containing proteins important for RhoA activation. One such protein could be the PDZ containing Rho-specific GEF, PDZ-RhoGEF (Fukuhara et al., 1999). If DGKζ associates with this GEF, the inability of DGKζ^{FLAG} to rescue RhoA activation could be due to the failure of DGKζ to couple PDZ-RhoGEF to RhoA and allow GDP/GTP exchange. The DGKζ C-terminal PDZ-binding motif is known to associate with numerous PDZ domain proteins, including different syntrophin isoforms, sorting nexin 27 (SNX27) and PSD-95 (Hogan et al., 2001; Kim et al., 2009; Rincón et al., 2007); therefore, a possible DGKζ/PDZ-RhoGEF interaction should be explored. Nevertheless, such an interaction would not fully explain the failure of DGKζ^{FLAG} to rescue RhoGDI Ser-34 phosphorylation, unless this complex requires the presence of such a GEF. Therefore, additional binding and

activity studies are required to dissect the specific mechanistic differences between Rac1 and RhoA activation with regard to DGKζ PDZ interactions. Apart from this uncertainty, the differing requirement of DGKζ PDZ-interactions for Rac1 and RhoA support the hypothesis that DGKζ forms independent signalling complexes with these Rho GTPases.

Rac1 and RhoA share the same binding site on DGKζ

DGKζ interacted with both active and inactive RhoA conformations. In contrast, another mammalian isozyme, DGKθ, interacts exclusively with the active RhoA conformation (Houssa et al., 1999). Active RhoA binds directly to the DGKθ catalytic domain. While the extended catalytic domain of DGKζ shares homology with the Rho-binding regions of DGKθ and another protein, $p27^{kip1}$ (Besson et al., 2004), RhoA bound directly to the DGKζ C1A domain and not the extended catalytic domain. Rac1 also binds directly to the DGKζ C1A domain (Yakbuchyk et al., 2005; Gee, unpublished observation), suggesting these Rho GTPases share the same or proximal binding sites on DGKζ. Further binding delineation will reveal whether RhoA and Rac1 interact with the same site or distinct regions of the C1A domain.

Since RhoA binds DGKθ and inhibits its activity (Houssa et al., 1999), this raises the interesting possibility that Rho GTPase binding might regulate DGKζ. While Rac1 and RhoA do not associate with the DGKζ catalytic domain directly, the ability of Rac1 and/or RhoA to modulate DGKζ activity, targeting, and/or function upon binding should be explored. Seeing as these Rho GTPases bind the same region of DGKζ, and have

different requirements for DGKζ catalytic activity and PDZ interactions, it is possible that crosstalk between these Rho GTPases might occur at the level of DGKζ regulation.

Our results do not presently differentiate between whether Rac1 and RhoA compete for binding to DGKζ or exist in different spatially controlled complexes in the cell. However, PKCα immunoprecipitations suggest Rac1 and RhoA exist in distinct RhoGDI dissociation complexes with DGKζ, favouring the hypothesis that DGKζ does not bind both Rho GTPases at the same time. Therefore, spatio-temporal regulation of DGKζ might control its availability for either Rho GTPase and therefore modulate the balance of Rac1/RhoA signalling during actin reorganization.

How does DGKζ achieve selectivity for Rac1 and RhoA?

Rac1 and RhoA activity is tightly controlled in space and time to allow for the sequential series of events required to reorganize the actin cytoskeleton (Spiering and Hodgson, 2011). This is especially evident during cell spreading, a critical process for the development of tissues (Gumbiner, 1996; Parsons et al., 2010). Rac1 and Cdc42 are turned on early in spreading to promote cell protrusion through the formation of lamellipodia and filopodia, respectively, and are later turned off. The converse is true of RhoA. RhoA activity is initially suppressed to allow spreading but gradually increases to initiate the organization of focal adhesions and strong adherence by stress fibers (Clark et al., 1998; Price et al., 1998; Rottner et al., 1999). Since DGKζ loss results in decreased Rac1 and RhoA activity and disrupts actin reorganization in spreading cells, it might be involved in regulating the activity of both Rho GTPases at different times and/or in different regions of the cell during spreading and adhesion.

Rac1/RhoA crosstalk during spreading largely manifests itself as mutual inhibition (Huveneers and Danen, 2009). Rac1/RhoA antagonism implies there must be mechanisms to both decrease Rac1 activation while promoting RhoA activation during later events in cell spreading. As RhoA activity increases during spreading, ROCK phosphorylates the Rac-specific GAP, FilGAP, to suppress Rac1 activity (Ohta et al., 2006). Decreasing Rac1 activity is imperative during spreading as events downstream of Rac1 are equally capable of suppressing RhoA and ROCK (Nimnual et al., 2003). Eventual RhoA activation is attributed to many factors, including decreased inhibition from Rac1 signalling, inhibition of a Rho-specific GAP, p190 RhoGAP, and PKCα activation (Arthur and Burridge, 2001; Dovas et al., 2006; Dovas et al., 2010; Mehta et al., 2001; Ohta et al., 2006; Wildenberg et al., 2006). Therefore, PKCα might regulate DGKζ specificity for RhoA over Rac1 during later events in cell spreading.

PKCα-mediated phosphorylation of DGKζ decreases its affinity for active Rac1 (Yakubchyk et al., 2005). Conversely, DGKζ binding to RhoA was increased following phosphorylation by PKCα. Moreover, phosphorylation reduces DGKζ catalytic activity (Luo et al., 2003b), which is dispensable for RhoA activation but required for Rac1 activation. It follows that PKCα might sustain RhoA activity at later events during spreading by phosphorylating DGKζ, causing it to preferentially bind and activate RhoA. Furthermore, decreased DGKζ activity following phosphorylation would help reduce Rac1 activation as spreading progresses. Taken together, my data suggests that controlling the assembly of DGKζ in different signalling complexes, and modulating DGKζ activity, might regulate Rac1/RhoA selectivity and regulation during dynamic changes to actin.

DGKζ regulates RhoA function following activation

Three independent lines of evidence support the hypothesis that DGKζ is important for RhoA function following activation. The first is that DGKζ binds active RhoA, suggesting it is important for targeting active RhoA. Second, MEFs lacking DGKζ show reduced $RhoA^{V14}$-induced actin stress fibers. If DGKζ was only involved in RhoA activation and not downstream functions, the introduction of a constitutively active mutant in cells lacking DGKζ should have behaved similarly in both wild type and null cells. Finally, α1-syntrophin associates with active RhoA exclusively in the presence of DGKζ, suggesting DGKζ is important for the assembly of protein complexes with RhoA after activation. Active RhoA can bind and activate PKCα (Slater et al., 2011), therefore the decreased ability of these two proteins to co-precipitate from null cells also supports the hypothesis that DGKζ might help target active RhoA to effectors and/or other regulatory partners, such as PKCα.

Does DGKζ form independent signalling complexes with active and inactive RhoA?

The inability of α1-syntrophin to associate with GDP-bound RhoA suggests DGKζ might form distinct multi-protein complexes with RhoA, one important for RhoA activation and the other for downstream function(s). Results here indicate a RhoA "activation complex" contains DGKζ, RhoA, RhoGDI and PKCα. A possible "targeting complex" might include DGKζ and RhoA in complex with α1-syntrophin and/or other currently unknown regulatory factors such as GEFs, GAPs and/or effectors.

Future studies should focus on immunoprecipitating these possible DGKζ/RhoA complexes. Purified DGKζ/RhoA complexes from starved and stimulated MEFs could be subjected to mass spectroscopy. The rationale behind this approach would be to confirm the existence of distinct DGKζ/RhoA complexes under different cellular conditions, and identify additional regulatory proteins involved in such complexes. Moreover, it will be important to further elucidate the minimal binding regions responsible for such interactions, as replacing single protein components with point or deletion mutants that interfere with binding will help dissect important mechanistic details for the role of DGKζ in Rho GTPase regulation and actin reorganization.

DGKs regulate the actin cytoskeleton

Accumulating evidence in the last two decades suggest many DGKs are involved in cytoskeletal regulation. Early studies showed that stimulating cells with growth factors caused significant changes to actin and DGK activity (Landreth et al., 1985; Roy et al., 1989; Payrastre et al., 1991). Furthermore, DGK activity was identified in purified cytoskeletal fractions from cells (Payrastre et al., 1991; Grondin et al., 1991; Tan and Boss, 1992). The initial characterization of the Rac1/RhoGDI complex identified an unknown DGK member (Tolias et al., 1998), later confirmed to be DGKζ (Abramovici et al., 2009). This raises the possibility that DGKs modulate changes to the cytoskeleton by regulating Rho GTPases. Intervening years of study have confirmed that several mammalian DGK isozymes function in cytoskeletal dynamics via Rho GTPase regulation.

A role for individual DGK isozymes in cytoskeletal regulation appears to be conserved in the evolution of higher eukaryotes (See Table 4.1 for a comprehensive list

Table 4.1. DGKs involved in cytoskeletal reorganization. An accumulating body of evidence suggests cytoskeletal regulation constitutes an important evolutionarily conserved function for DGKs. At least six of the ten mammalian isozymes have already been implicated in the regulation of actin dynamics; the majority link lipid signals in the plasma membrane to changes in actin by regulating Rho GTPases. The following table represents a comprehensive list of DGKs associated with cytoskeletal reorganization. Sources: Abramovici et al., 2003; Abramovici and Gee, 2007; Abramovici et al., 2009; Baldanzi et al., 2010; Chianale et al., 2010; de la Roche et al., 2002; Gee, unpublished observation; Houssa et al., 1999; Hozumi et al., 2009; McMullan et al., 2006; Nakano et al., 2009; Tan and Boss, 1992; Tsushima et al., 2004; Yakubchyk et al., 2005; Yasuda et al., 2007 ; and Yamada et al., 2003.

Table 4.1. DGKs involved in cytoskeletal reorganization			
DGK isozyme	**Role in Cytoskeletal Dynamics**	**Rho GTPase Regulation**	**Sources**
DGK (*D. carota*)	DGK activity found in cytoskeletal fractions	Not determined	Tan and Boss, 1992
DGKA (*D. discoideum*)	Organizes myosin-II assembly; regulates actin-myosin contractility	Not determined	de la Roche et al., 2002
DGK-1 (*C. elegans*)	Not determined	Inhibited by binding RHO-1	McMullan et al., 2006
DGKα	Promotes lamellipodia/membrane ruffling	Rac1 release from RhoGDI; Rac1 activation	Chianale et al., 2010
DGKβ	Promotes dendrite outgrowth and spine maturation; localizes with actin stress fibers	Not determined	Hozumi et al., 2009; Tsushima et al., 2004
DGKγ	Suppresses lamellipodia/membrane ruffling; localizes to cytoskeleton	Activation of Rac GAP, β_2-chimaerin; Rac1 inhibition	Yamada et al., 2003; Yasuda et al., 2007
DGKθ	Not determined	Inhibited by binding active RhoA; effect on RhoA not fully explored	Baldanzi et al., 2010
DGKε	Localizes to actin stress fibers	Not determined	Nakano et al., 2009
DGKζ	Promotes neurite outgrowth, myoblast fusion, actin stress fiber assembly, lamellipodia/membrane ruffling, and cell migration; localizes to actin cytoskeleton	Rac1 and RhoA release from RhoGDI; Rac1 and RhoA activation	Abramovici et al., 2003 ; Abramovici et al., 2007; Abramovici et al., 2009; Yakubchyk et al., 2005
DGKι?	Suppress membrane ruffling	Rac1 inhibition?	Gee, unpublished observation

For example, DGK activity was found in the cytoskeletal fraction of plants (Arisz et al., 2009; Tan and Boss, 1992). In *D. discoideum*, the balance of DAG and PA levels by DGK controls the cytoskeletal dynamics (de la Roche et al., 2002). Deleting the single DGK gene (dgkA) in *D. discoideum* disrupts myosin II assembly, important for actin based cell motility (Senju and Miyata, 2009). In *C. elegans*, the Rho orthologue RHO-1 binds to and inactivates DGK-1, resulting in elevated DAG levels at neurotransmitter-release sites (McMullan et al., 2006). Likewise in mammals, RhoA, but not Rac1 or Cdc42, binds the DGKθ catalytic domain, negatively regulating its activity (Houssa et al., 1999).

Accumulating evidence suggests many mammalian DGKs directly regulate the activity of specific Rho GTPases. For example, Type 1 DGKs, DGKα and DGKγ, regulate Rac1 in opposing ways. This is consistent with the notion that even closely related isozymes can have opposing functions. DGKγ activates a Rac-specific GAP, β_2-chimaerin, in response to epidermal growth factor (EGF) (Goto et al., 1994; Tsushima et al., 2004; Yasuda et al., 2007). In contrast to Rac1/RhoGDI dissociation and Rac1 activation by DGKα (Chianale et al., 2010), DGKγ promotes β_2-chimaerin and thereby suppresses Rac1 activity. The only other Type 1 member, DGKβ, localizes with actin stress fibers and has been reported to promote dendrite outgrowth and spine maturation in neurons (Kobayashi et al., 2007; Hozumi et al., 2009). The link between DGKβ and Rho GTPases has not yet been investigated. In addition to DGKβ, DGKε has also been reported to localize with actin stress fibers (Nakano et al., 2009). Unfortunately, the physiological significance of this localization was not explored. Strikingly, unpublished data from our laboratory suggests DGKι loss causes a significant increase in Rac1-

induced membrane ruffling in MEFs (Gee, unpublished observation). In contrast, loss of a closely related Type 4 isozyme, DGKζ, causes a significant decrease in Rac1-induced membrane ruffling (Abramovici et al., 2009). Therefore, Type 4 DGKs ζ and ι, much like Type 1 DGKs α and γ, appear to have opposing functions with regard to Rac1 regulation.

In addition to Rac1 regulation, data here suggest DGKζ regulates RhoA. Cells lacking DGKζ exhibited attenuated Rho/ROCK signalling downstream and disrupted Rho-dependent actin stress fiber assembly. While RhoA has conventionally been thought to regulate actin-myosin contractility important for actin stress fiber assembly (Wheeler and Ridley, 2004), a recent study has challenged this idea. Surprisingly, the genetic deletion of RhoA from MEFs did not affect stress fiber formation (Melendez et al., 2011). Instead, it was only the concurrent knockdown of RhoB or RhoC, in the absence of RhoA, which disrupted this function. RhoA, RhoB and RhoC therefore appear redundant for actin stress fiber formation. Since these three isoforms share roughly 85% sequence homology (Wheeler and Ridley, 2004), the severe actin stress fiber defect in spreading DGKζ-null cells indicates DGKζ might regulate RhoB and/or RhoC activity also.

Binding assays similar to those performed here to confirm the DGKζ/RhoA interaction should be used to explore possible interactions of DGKζ with RhoB and RhoC. Also, since the Rhotekin RBD domain recognizes GTP-bound RhoA, RhoB and RhoC (Ren and Schwartz, 2000), future studies should immunoblot for RhoB and RhoC following Rho-activity assays to identify whether the activity of these isoforms is compromised in the absence of DGKζ. If DGKζ regulates these additional isoforms, it will suggest an even broader role for DGK regulation of Rho GTPases.

Conclusions

DGKs comprise a large family of lipid kinases important for the correct functioning of various biological systems, including the immune, nervous and cardiovascular systems (Shulga et al., 2011). Consistent with their significance in many different tissues, DGK dysregulation has been implicated in numerous human diseases, including cancer, epilepsy, heart disease, and type II diabetes; as a result, many individual isozymes have been suggested as potential drug targets (Sakane et al. 2008). Given the emerging importance of individual DGKs in human health, more understanding of DGK regulation and function might help to advance diagnostic tools and treatments for a number of diseases.

Accumulating evidence suggest many DGKs function to regulate cell morphology via Rho GTPases. Changes to the actin cytoskeleton by Rho GTPases help drive cell migration, cytokinesis and vesicle trafficking (Ridley, 2006). Therefore, Rho GTPase regulation by DGKs indicates many additional functions for this family of lipids kinases at the cellular level. Furthermore, DGK/Rho GTPase regulation has broader implications for human health. Aberrations in signalling pathways regulated by Rho GTPases have been implicated in numerous human diseases, including many cancers and developmental disorders (Gomex del Pulgar et al., 2005; Vega and Ridley, 2008; Visvikis, O. et al., 2011). Therefore, some of the pathologies associated with dysregulated DGK activity might be due to a loss in adequate DGK/Rho GTPase regulation.

Evidence presented here provides an additional example of DGK regulating changes to cell morphology by the modulation of Rho GTPases. My results indicate that DGKζ is required for optimal RhoA/RhoGDI dissociation and RhoA activity in MEFs.

This complements previous findings from our laboratory showing Rac1 release from RhoGDI requires DGKζ (Abramovici et al., 2009). Together these studies indicate DGKζ modulates the activity of two Rho GTPases through distinct signalling complexes. Our findings are consistent with the idea that tight spatio-temporal control of distinct signalling complexes and plasma membrane lipids account for selective Rho GTPase regulation (Dermardirossian and Bokoch, 2005; Pertz, 2010). Consequently, factors that regulate DGKζ activity and localization in space and time might contribute to selectivity in the balance of Rac1/RhoA activation. Collectively, these exciting findings provide evidence that DGKζ sits at a pivotal signalling position for changes to cell morphology, selectively activating Rac1 and/or RhoA in response to specific internal and external cues.

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