NOTE TO USERS

uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Hanan Abramovici

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Neuroscience)

GRADE / DEGREE

Department of Neuroscience

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Diacylglycerol Kinase ζ Regulates Rac1-Mediated Remodeling of the Actin Cytoskeleton

TITRE DE LA THÈSE / TITLE OF THESIS

Stephen Gee

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Paul Albert

Christopher Kennedy

John Copeland

Nathalie Lamarche-Vane

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Diacylglycerol Kinase ζ Regulates Rac1-Mediated Remodeling of the Actin Cytoskeleton

Hanan Abramovici

This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies as a partial fulfillment of the Ph.D. program in Neuroscience

May 2008

Department of Neuroscience
Faculty of Medicine
University of Ottawa
Ottawa, Ontario
Canada

Your file Votre référence
ISBN: 978-0-494-48429-6
Our file Notre référence
ISBN: 978-0-494-48429-6

Permissions

March 25 2008

Our policy is below. #3 seems to apply in this case. Basically you're free to use it as long as you cite us properly.

Any author of an MBC article is permitted to reprint and/or archive according to the following guidelines:

1. To reprint the article-or any part of the article-in any book of which any of the article's authors is an author or an editor, provided that proper credit is attributed to the ASCB-published version of the article.

2. To post after first publication, a link to the online version of the article and/or the journal PDF version of the article and/or supplemental material on any of the author's web sites provided that proper copyright credit is made to the ASCB-published version of the article and that a link to the ASCB-published article is provided.

3. To revise, adapt, prepare derivative works, present, or distribute the article online or in print provided that all such use is for personal and noncommercial benefit of the authors as long as proper copyright credit is provided to the original published version of the article.

Eric Baker
Molecular Biology of the Cell
8120 Woodmont Ave., Suite 750
Bethesda, MD 20814-2762
301-347-9338
F: 301-347-9350

March 31, 2008.

Dear Hanan Abramovici:

Please be advised permission is granted to reuse Cell Motility and the Cytoskeleton: 64 (7) Pages: 549-567, ©2007 in your forthcoming publication/Thesis which will be published by University of Ottawa. Credit must appear on every copy using the material and must include the title; the author(s); and/or editor(s); Copyright (year and owner); and the statement "Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc." Please Note: No rights are granted to use content that appears in the work with credit to another source.

Good luck with thesis

Sincerely,

Brad Johnson, Permissions Assistant John Wiley & Sons, Inc.
111 River Street Hoboken, NJ 07030-5774 Permissions – Mail Stop 4-01
Tel: 201.748.6786 Fax: 201.748.6008 bjohns@wiley.com

Hello,

I would like permission to reprint in full the following article:

Regulation of Neurite Outgrowth in N1E-115 Cells through PDZ-Mediated Recruitment of Diacylglycerol Kinase ζ

Yury Yakubchyk, Hanan Abramovici, Jean-Christian Maillet, Elias Daher, Christopher Obagi, Robin J. Parks, Matthew K. Topham, and Stephen H. Gee

Molecular and Cellular Biology, August 2005, p. 7289-7302, Vol. 25, No. 16
0270-7306/05 doi:10.1128/MCB.25.16.7289-7302.2005

The article will be published in my doctoral dissertation at the University of Ottawa, Ottawa, Canada in partial fulfillment of the requirements for the doctoral degree. The publication is not for mass distribution, or for profit.

Thank you.

Best regards,
Hanan Abramovici

PERMISSION GRANTED
CONTINGENT ON AUTHOR PERMISSION (which you must obtain)
AND APPROPRIATE CREDIT
for Microbiology
artment
Date 3/27/08

Abstract

The lipid second messengers diacylglycerol (DAG) and phosphatidic acid (PA) regulate the activity and localization of actin reorganizing proteins that participate in cellular shape changes underlying important biological processes. DAG kinases (DGKs) phosphorylate DAG to yield PA, thereby attenuating the activity of DAG-activated proteins and stimulating proteins that respond to PA. Using cell biological and biochemical approaches, I explored the hypothesis that DGKζ regulates actin cytoskeleton remodeling in mammalian cells by associating with and promoting the activity of the Rho GTPase Rac1 and the Rac1-activated kinase PAK1. I found that DGKζ forms a complex with both proteins, and that PA from DGKζ promotes the release of Rac1 from its inhibitor RhoGDI, triggering Rac1 activation. DGKζ also regulates the localization and activity of PAK1. Furthermore, the ability of DGKζ to promote Rac1 activity appears to underlie important physiological processes requiring the restructuring of the actin cytoskeleton such as cell migration, neurite outgrowth, and myoblast fusion.

Localization of signaling complexes to specific cellular domains is required for complex changes in cell morphology. Because subcellular localization is critical for DGKζ function, I investigated the molecular mechanisms underlying its targeting to different cellular compartments. I found that both PKC-mediated phosphorylation of the MARCKS domain and binding of the C-terminus to syntrophins regulate DGKζ recruitment to the plasma membrane.

Collectively, my results define a molecular mechanism that regulates the recruitment of DGKζ to the membrane and that couples changes in DAG and PA to the activation of PAK1 and Rac1. These findings have uncovered a novel role for DGKζ in actin cytoskeleton organization.

Two different abbreviations for diacylglycerol kinase ζ are used in this thesis although they both refer to the same enzyme. "DGK-ζ" was used in earlier manuscripts. This was later changed to "DGKζ".

Table of Contents

List of Figures

List of Abbreviations

A, alanine;

A.A., amino acid;

AChRs, acetylcholine receptors;

Ad, adenovirus;

AI, autoinhibitory;

Ala, alanine;

Asn, asparagine;

Asp, aspartate;

BED, basic effector domain;

BSA, bovine serum albumin;

CRIB, Cdc42 and Rac interactive binding;

D, aspartate;

DAG, diacylglycerol;

DAP, dystrophin-associated protein;

DAPC, dystrophin-associated protein complex;

DGK, diacylglycerol kinase;

DAP, dystrophin-associated protein;

DMD, Duchenne muscular dystrophy;

DMEM, Dulbecco's modified Eagle medium;

ECM, extracellular matrix;

EGF, epidermal growth factor;

ERK, extracellular signal-regulated kinase;

ERM, ezrin-radixin-moesin;

F.A, focal adhesion;

F-actin, filamentous-actin;

FCS, fetal calf serum;

FGF, fibroblast growth factor;

Fil, filopodia;

FITC, fluorescein isothyocyanate;

FN, fibronectin;

Fx, focal contact;

G-actin, globular-actin;

GAP, GTPase-activating protein;

GEF, guanine-nucleotide-exchange factor;

GFP, green fluorescent protein;

Glu, glutamine;

Gly, glycine;

GST, glutathione S-transferase;

HA, hemagglutinin;

HRP, horseradish peroxidase;

IB, immunoblotting;

Ig, immunoglobulin;

IP, immunoprecipitation;

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

K, lysine;

KD, kinase-dead;

kDa, kiloDalton;

KLH, keyhole limpet haemocyanin;

Lam, lamellipodium;

LPA, lysophosphatidic acid;

mAb, monoclonal antibody;

MARCKS, myristoylated alanine-rich C-kinase substrate;

MEF, mouse embryonic fibroblast;

MOI, multiplicity of infection;

MW, molecular weight;

N, asparagine;

NF, neurofilament;

NGF, nerve growth factor;

NMJ, neuromuscular junction;

nNOS, neuronal nitric oxide synthase;

PA, phosphatidic acid;

PAK, p21-activated kinase;

PAX, paxillin;

PBD, p21-binding domain;

PBS, phosphate buffered saline;

PDGF, platelet-derived growth factor;

PDZ, Postsynaptic density protein-95/ Discs-large/ Zona Occludens-1;

PFA, paraformaldehyde;

PH, pleckstrin homology;

PI3K, phosphatidylinositol 3-kinase;

PI(4)P, phosphatidylinositol 4-phosphate;

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-myristate 13-acetate;

pRB, retinoblastoma protein;

Pro, proline;

pY, phosphorylated tyrosine;

R, arginine;

RA, Ras-association;

RasGRP, Ras guanyl-nucleotide-releasing protein;

RhoGDI, Rho GDP dissociation inhibitor;

RVH, recoverin homology;

SAM, sterile α motif;

SAPK, stress-activated protein kinase;

S.E.M, standard error of the mean;

Ser, serine;

S.F, stress fiber;

SNX27, sorting nexin 27;

SU, syntrophin unique;

Syn, syntrophin;

TA, tibialis anterior;

Thr, threonine;

TRPC, transient receptor potential channel;

TX-100, Triton X-100;

Tyr, tyrosine;

WT, wild-type;

YFP, yellow fluorescent protein;

α1-Syn, α1-syntrophin;

α-BgTx, α-bungarotoxin;

Acknowledgements

The work presented in this thesis would not have been possible without the support from many individuals over the years. First and foremost, I extend my sincere and deep gratitude to my supervisor Dr. Stephen Gee for giving me the opportunity to work in his laboratory, for giving me much scientific freedom, guidance and support, and for working together with me as a team on many interesting projects. Many thanks also go to our collaborator Dr. Matthew Topham who provided us with many reagents and advice over the years. I thank the members of my advisory committee: Drs. Bernard Jasmin, Rashmi Kothary, John Copeland, and Luc Sabourin for taking an active interest in my work and challenging me. I would also like to thank my fellow lab members over the years: Dr. Parmiss Mojtabaie, Jean-Christian Maillet, Helga Agah, Yury Yakubchyk, Andrea Ventimiglia, and Christopher Obagi. Thank also to Kim Wong and Rainy Tang.

I also extend my gratitude to the administrative staff of the CMM and NSC departments: Paul-Andre David, Donna Hooper, Sylvie Deblois, Blanche Dinelle, Gillian Lord, Denyse Blais, and Dr. Paul Albert. Thanks also to those institutions that provided me with financial support: The J. Armand Bombardier Foundation, the Ontario Graduate Scholarship Program, and the Canadian Institutes for Health Research.

Above all, I thank my family, loved ones and friends who supported me throughout all these years. You helped me make it!

Chapter 1

General Introduction

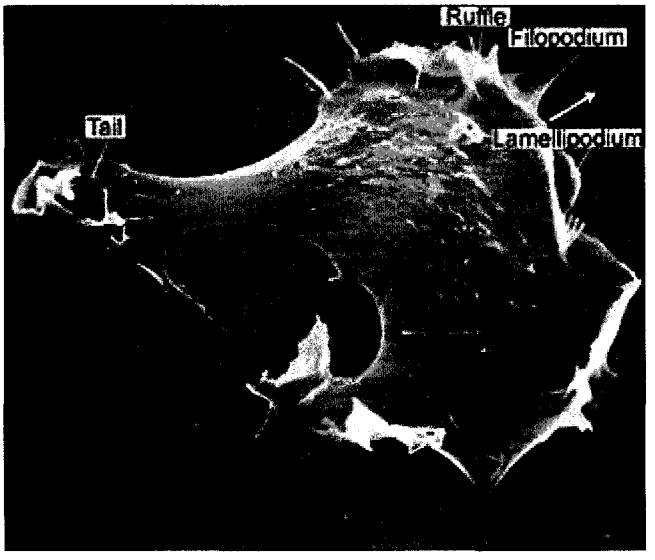
Eukaryotic cells undergo dynamic changes in cell shape that are carried out by an underlying cytoskeletal framework. The cytoskeleton is composed of an intricate network of three different sized protein filaments (Janmey, 1998). Microfilaments, the smallest, are composed primarily of actin (Janmey, 1995). Intermediate filaments are larger and vary in composition depending on cell type (Chou *et al.*, 2007; Godsel *et al.*, 2008). Microtubules, the largest diameter filaments, are composed mainly of tubulin (Janmey, 1995). Although all three filament systems undergo extensive reorganization, the actin cytoskeleton rapidly responds to cues and is generally the driving force behind the initial shape changes required for various cellular processes (Mitchison and Cramer, 1996; Hall, 1998; Machesky and Insall, 1999; Janmey and Lindberg, 2004).

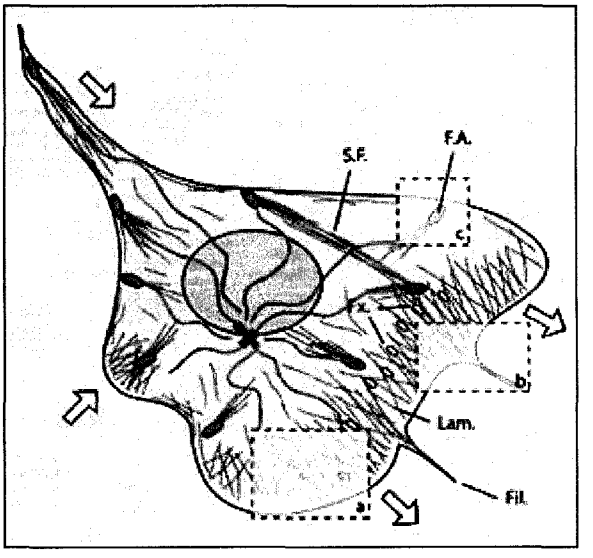
The Actin Cytoskeleton

Actin is a highly abundant protein found in all eukaryotic cells (Hall, 1994). Rapid remodeling of the actin cytoskeleton relies on polymerization of globular (G-actin) monomers to filamentous (F-actin) helical polymers or conversely, on filament depolymerization (Janmey, 1995). The non-muscle actin cytoskeleton, composed of β and γ actins, is organized into discreet structures designed to fulfill specialized functions. For example at the leading edge of motile cells, a highly compact canvas of β-actin filaments forms lamellipodia, veil-like cell surface protrusions (Zicha *et al.*, 2003) (Figure 1.1). On the other hand, tight bundling of actin filaments results in thin, finger-like extensions known as filopodia (Faix and Rottner, 2006) (Figure 1.1). Both structures are responsible for forward movement and for establishing new adhesive contacts at the front of the cell

Figure 1.1. Motile cells elaborate specialized membrane structures.

(*Left panel*): Scanning electron microscope photograph of a moving fibroblast with the leading edge on the right and the tail on the left. The cell displays a broad lamellipodium, and a number of filopodia and membrane ruffles. Together, these structures promote cell migration. (*Right panel*): Schematic diagram of a migrating cell. (*Dashed box a*): A meshwork of actin filaments (*red lines*) generates a protrusive force (*arrow*) resulting in outward bulging of the membrane and the formation of a lamellipodium (*Lam.*); (*Dashed box b*): Bundling of actin filaments produces thin, finger-like extensions called filopodia (*Fil.*). Focal complexes (*Fx.*) are transient agglomerations of actin and adhesion-associated proteins that form at the edges of lamellipodia and are responsible for traction. (*Dashed box c*): Focal adhesions (*F.A.*) result from the maturation of focal complexes and form a mechanical link between actin stress fibers (*S.F.*) and the extracellular matrix. Reproduced with permission from Encyclopedia of Biological Chemistry, Volume 1, J.V. Small and E. Vignal, Cell Migration, 356-361, Copyright Elsevier 2004.


Ruffle
Filopodium
Tail
Lamellipodium


S.F.
F.A.
Lam.
Fil.

(Borm *et al.*, 2005; Faix and Rottner, 2006; Misra *et al.*, 2007). De-adhesion of lamellipodia and their retrograde movement gives rise to membrane ruffles (Hoock *et al.*, 1991; Nobes and Hall, 1995) (Figure 1.1). Other structures such as actin stress fibers consist of long bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through specialized areas known as focal adhesion complexes (Figure 1.1) (Ridley and Hall, 1992; Hill and Gunning, 1993). Stress fibers are responsible for maintaining overall cell shape as well as regulating movement by controlling tension and contractility (Katoh *et al.*, 1998).

A growing body of evidence suggests that reorganization of the actin cytoskeleton requires tight spatial and temporal regulation of actin dynamics by a network of actin-remodeling proteins that work in concert with a variety of membrane phospholipids. Below I discuss some of the major protein and lipid players involved in coordinating the changes in actin underlying essential cell behaviors.

Molecular Regulators of Actin Remodeling

The Rho GTPases RhoA, Rac1 and Cdc42 are pivotal regulators of signaling pathways linking extracellular growth signals to actin restructuring (Ridley *et al.*, 1992; Ridley and Hall, 1992; Nobes and Hall, 1995; Van Aelst and D'Souza-Schorey, 1997). The idea that Rho GTPases could remodel the actin cytoskeleton originated from studies using C3 transferase; an exoenzyme produced by the bacterium *Clostridium botulinum* that inactivates Rho proteins (Sekine *et al.*, 1989; Aktories and Hall, 1989; Aktories, 1994). Treatment of cells with C3 transferase causes rapid actin stress fiber disassembly (Chardin *et al.*, 1989; Paterson *et al.*, 1990). Ectopic expression of a constitutively active RhoA mutant or addition of serum or lysophosphatidic acid (LPA), a major serum component, to serum-

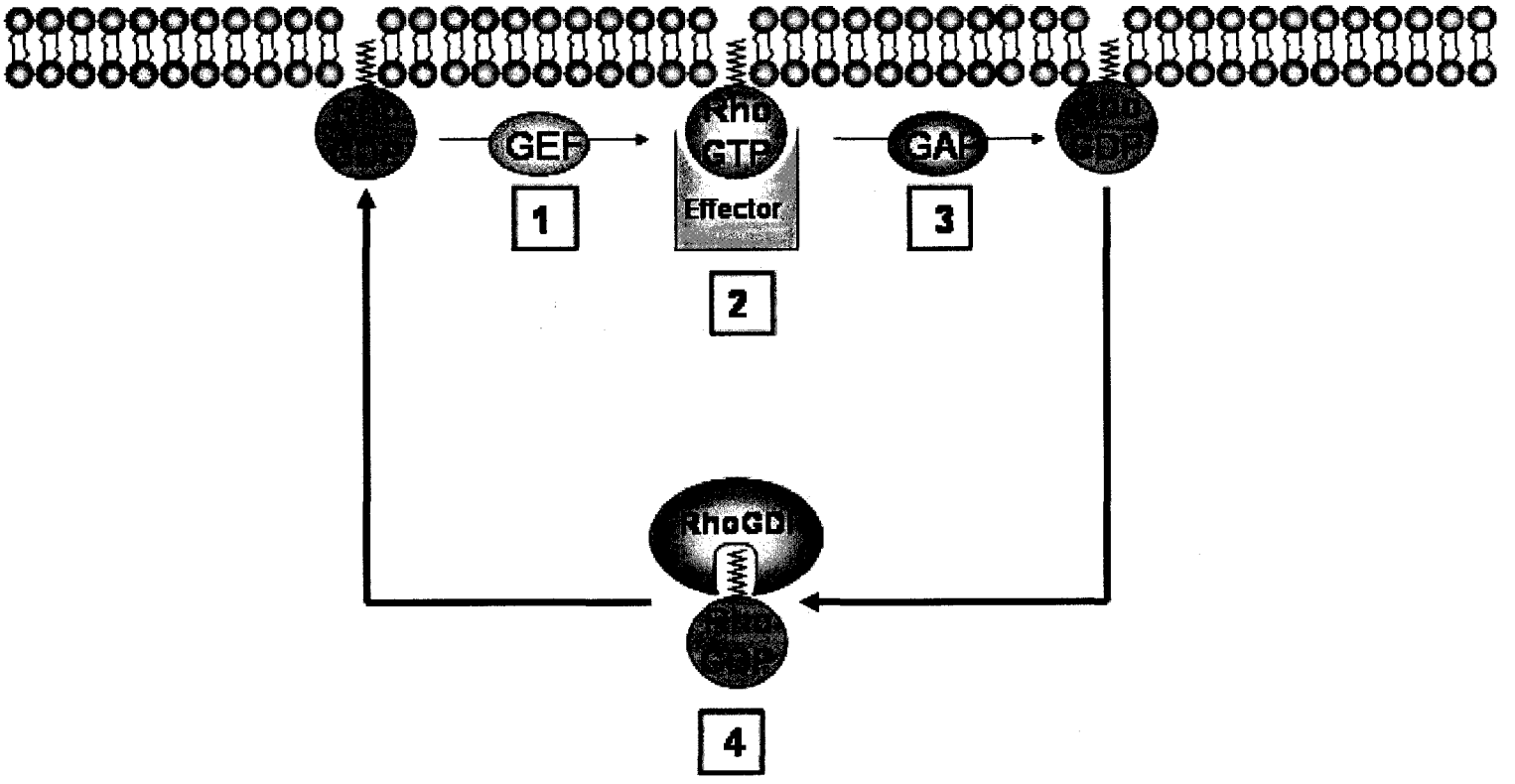
starved cells induces actin stress fiber assembly and the formation of focal adhesions (Paterson *et al.*, 1990; Ridley and Hall, 1992). On the other hand, inactivation of RhoA blocks LPA-induced stress fiber assembly (Ridley and Hall, 1992). Rac1 regulates actin polymerization and the formation of lamellipodia and membrane ruffles, structures observed following addition of growth factors or engagement of integrins (Ridley *et al.*, 1992; Kozma *et al.*, 1995; Price *et al.*, 1998). Cdc42 regulates the formation of peripheral-actin microspikes, including filopodia, in response to bradykinin or integrin activation (Nobes and Hall, 1995; Kozma *et al.*, 1995; Price *et al.*, 1998). Collectively, these findings firmly establish Rho GTPases as the proteins chiefly responsible for regulating actin-dependent structures triggered by growth factors or adhesion.

The Rho GTPase Cycle

Rho GTPases cycle between an inactive, primarily cytosolic GDP-bound state, and an active membrane-associated, GTP-bound state (Figure 1.2). Guanine-nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the exchange of GDP for GTP, thus allowing GTPases to bind effector proteins (Olofsson, 1999). GTPase-activating proteins (GAPs) return GTPases to their inactive GDP-bound state by accelerating GTP hydrolysis. The activity of the Rho GTPases is further modulated by the Rho GDP dissociation inhibitors (RhoGDIs). RhoGDIs bind GDP-bound GTPases preventing GDP to GTP exchange, and maintain GTPases in an inactive form by blocking their activation by GEFs (Olofsson, 1999; Dermardirossian and Bokoch, 2005). GDIs also

Figure 1.2. The Rho GTPase Cycle.

(1) Binding of GDP-bound Rho GTPases to GEFs promotes GDP-GTP exchange. (2) GTP-bound Rho GTPases can associate with downstream effectors. (3) GAPs accelerate GTP-hydrolysis bringing GTPases back to their inactive GDP-bound state. (4) RhoGDI associates with GDP-bound Rho GTPases in the cytosol and prevents their association with GEFs, GAPs, and the plasma membrane. Adapted from Dransart et al. (2005).


GEF
Rho
GTP
Effector
GAP
1
2
3
RhoGDI
4

interact with GTP-bound GTPases inhibiting GTP hydrolysis, blocking both intrinsic and GAP-catalyzed GTPase activity, and preventing interactions with effector targets (Olofsson, 1999; Dermardirossian and Bokoch, 2005). Furthermore, GDIs modulate the translocation of Rho GTPases from the cytosol to the plasma membrane by keeping the GTPases in a soluble cytosolic complex and preventing the GTPase C-terminal geranylgeranyl motif from inserting into the plasma membrane (Olofsson, 1999; Dermardirossian and Bokoch, 2005). Once released from GDIs, Rho proteins interact with membrane-associated effector proteins (Olofsson, 1999; Robbe *et al.*, 2003; Dermardirossian and Bokoch, 2005). GDIs can also extract membrane-associated GTPases, bringing them back to the cytosol (Bokoch *et al.*, 1994; Van Aelst and D'Souza-Schorey, 1997; Sasaki and Takai, 1998; Olofsson, 1999). Since GDIs play a major role in controlling GTPase activity, the GDI-GTPase complex must be tightly regulated. In addition, individual GTPases are activated in response to different stimuli leading to GTPase-specific responses. Therefore, particular mechanisms must exist to dissociate different GTPases from GDI complexes (Dermardirossian and Bokoch, 2005).

Regulation of RhoGDI-GTPase Dissociation

Several distinct GDI-GTPase dissociation mechanisms have so far been described. One involves displacement or sequestration of RhoGDI from RhoA by members of the Ezrin-Radixin-Moesin (ERM) family of membrane-cytoskeleton linkers (Takahashi *et al.*, 1997), the tyrosine kinase Etk (Kim *et al.*, 2002), and the p75 neurotrophin receptor (Yamashita and Tohyama, 2003). Another mechanism involves phosphorylation of GDI by protein kinases. Phosphorylation of RhoGDI by protein kinase C α (PKCα) has been linked to the release of GDI from RhoA (Mehta *et al.*, 2001), Src-mediated phosphorylation of

RhoGDI to the release of RhoA, Cdc42 and Rac1 (Dermardirossian *et al.*, 2006), and phosphorylation of RhoGDI by p21-activated kinase 1 (PAK1) to the selective release of Rac1 (Dermardirossian *et al.*, 2004). PAK1 plays an important role in actin reorganization. In the following sections I discuss the regulation of PAK1 by both GTPases and lipids, and expand on the roles of lipids in actin remodeling.

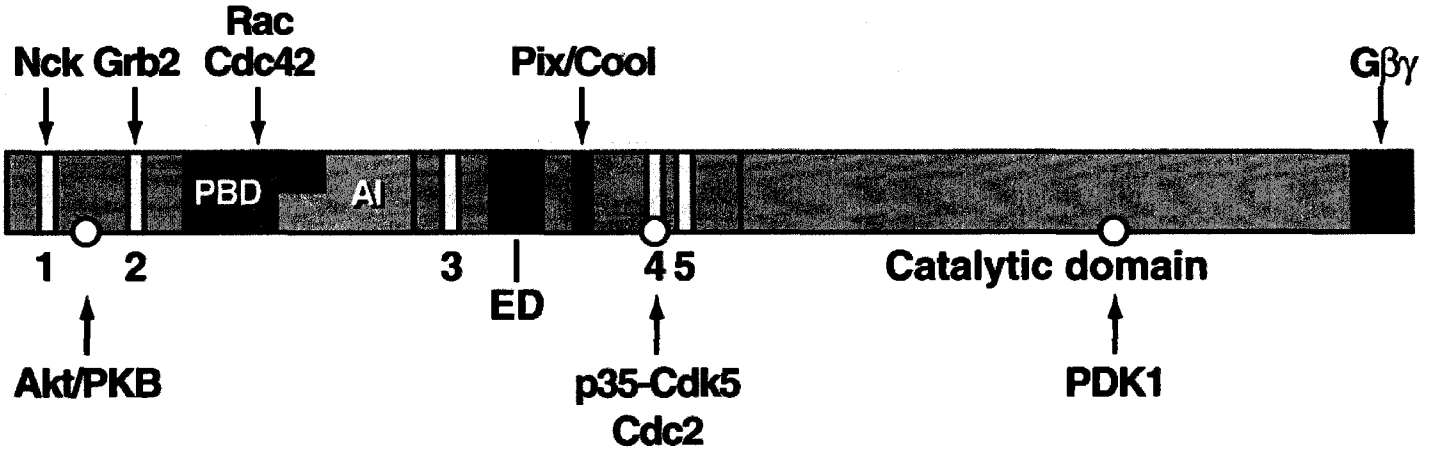
PAK

Six mammalian PAK isoforms have been identified and all play significant roles in actin cytoskeleton reorganization (Bokoch, 2003; Kumar *et al.*, 2006). Both GTPase-dependent and -independent mechanisms regulate PAK1 activity. The GTP-dependent mechanism involves binding of Cdc42- or Rac-GTP to the so-called Cdc42 and Rac Interactive Binding (CRIB) domain of PAK1. The slightly larger p21-binding domain (PBD) contributes to overall GTPase binding affinity (Manser *et al.*, 1994; Sells and Chernoff, 1997; Knaus and Bokoch, 1998; Lei *et al.*, 2000). The PBD overlaps, but is distinct from the autoinhibitory segment that forms part of the inhibitory switch that controls the basal kinase activity of PAK1 (Figure 1.3) (Lei *et al.*, 2000). GTPase binding leads to a series of conformational changes that destabilizes the folded structure of the inhibitory switch domain and therefore activates the enzyme (Bokoch, 2003).

PAK1 can also be activated by GTPase-independent mechanisms, some of which involve the action of negatively charged lipids such as phosphatidic acid (PA). PA also promotes the dissociation of Rac1 from RhoGDI (Chuang *et al.*, 1993a; Bokoch *et al.*, 1998). It is believed that these lipids induce a partial disruption of the GTPase-GDI complex, enabling interaction with membrane-associated GEFs and subsequent nucleotide exchange

Figure 1.3. Schematic diagram indicating features of PAK1 structure.

The PAK1 backbone is shown in orange and the catalytic domain (aa 255–529) in bright blue. The p21 (Rac/Cdc42)-binding domain or PBD is shown in purple overlapping with the pale green autoinhibitory (AI) domain. Yellow boxes are the five PXXP putative SH3-binding motifs, the green box is the noncanonical proline-rich Pix/Cool SH-3 binding motif, and the red box is the ED-rich region of unknown significance. The white circles represent identified sites of phosphorylation by Akt (Ser21), Cdc2/Cdk5 (Thr212), and PDK1 (Thr423). Reproduced with permission from Bokoch (2003).


Rac
Nck Grb2 Cdc42
Pix/Cool
Gβγ
PBD
AI
1
2
3
ED
4 5
Catalytic domain
Akt/PKB
p35-Cdk5
Cdc2
PDK1

(Faure *et al.*, 1999). Since PAK1-mediated phosphorylation of RhoGDI appears to be responsible for the selective release of Rac1, these findings raise the possibility that PA may in fact dissociate Rac from RhoGDI by activating PAK1. Furthermore, the existence of GTPase-dependent and independent mechanisms of PAK1 activation could help explain how PAK1 can paradoxically act both upstream as well as downstream of Rac1.

GTPase-independent mechanisms of PAK1 activation, particularly by lipids, suggest PAK1 must associate with the plasma membrane in order to be active. Furthermore, agonist binding to receptors not only stimulates the formation and metabolism of lipid species that activate PAK1 but also triggers actin cytoskeleton remodeling (Bokoch *et al.*, 1998). Therefore, plasma membrane lipids appear to play an important role in regulating actin organization. Below, I have summarized the role of lipids in actin dynamics.

Lipids Regulate Actin Reorganization

Plasma membrane lipids have been traditionally regarded as constituents of a permeability barrier and at most as a source of second messengers for signal transduction (Janmey and Lindberg, 2004). However, there is now compelling evidence to suggest a central role for a number of lipids in actin dynamics. For example, phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) promotes actin polymerization by controlling the activity of many actin-associated proteins as well as enhancing actin association with the plasma membrane (Janmey, 1995; Sechi and Wehland, 2000; Janmey and Lindberg, 2004).

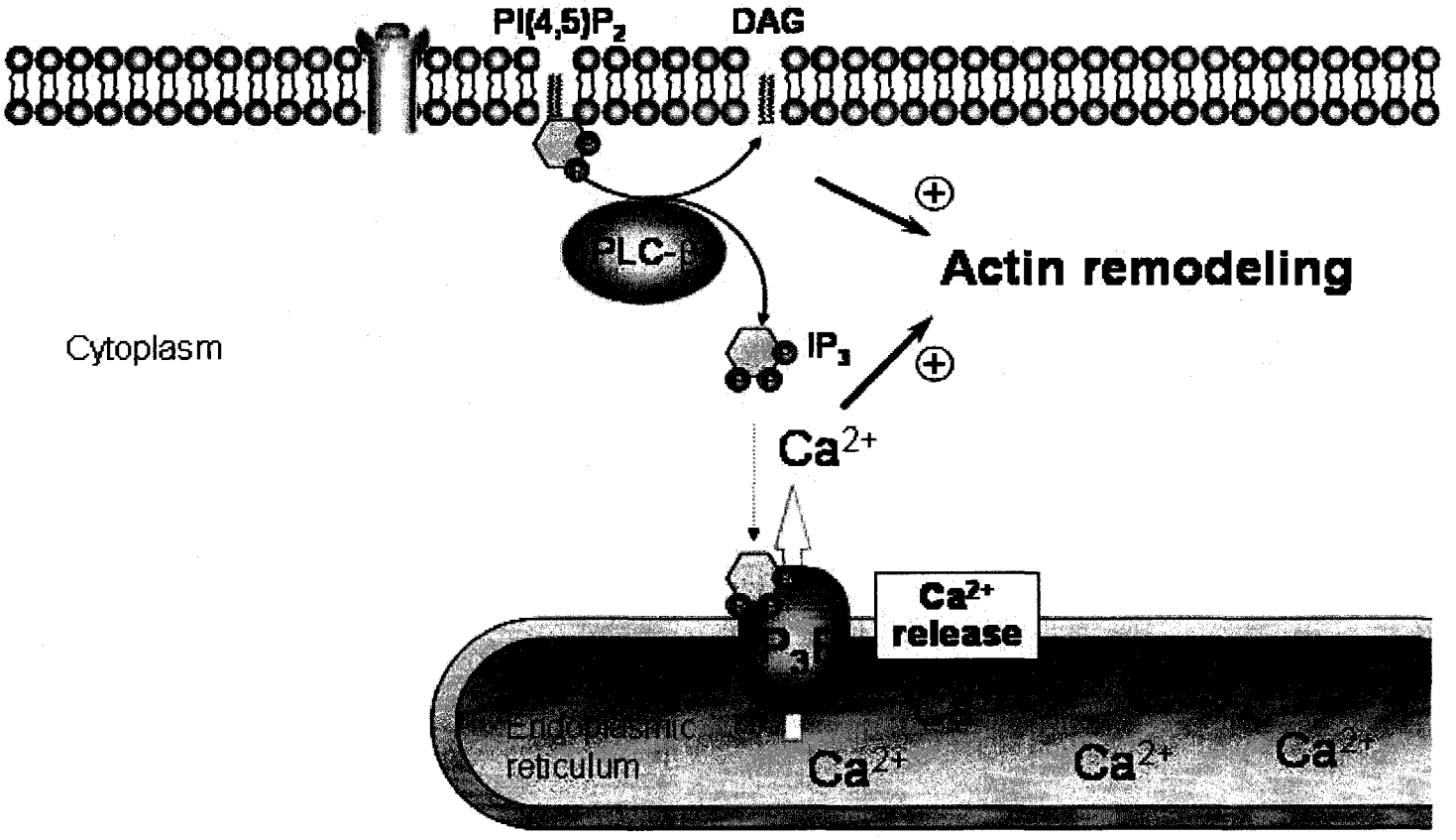
Actin reorganization is also associated with phospholipase C-mediated hydrolysis of $PI(4,5)P_2$, resulting in a transient rise in the levels of diacylglycerol (DAG) and inositol

(1,4,5)-trisphosphate (IP_3) (Figure 1.4) (Topham and Prescott, 1999). DAG is an intermediate in the biosynthesis and degradation of triglycerides, glycerophospholipids, and glyceroglycolipids as well as an important second messenger molecule (Goni and Alonso, 1999). Stimulation of cells with DAG or non-hydrolyzable DAG analogs such as phorbol esters induces dramatic changes in the cytoskeleton including disassembly of actin stress fibers and an accompanying increase in membrane ruffling (Schliwa *et al.*, 1984; Meigs and Wang, 1986; Kitano *et al.*, 1986; Ridley *et al.*, 1992). Binding of IP_3 to endoplasmic reticulum receptors triggers the release of Ca^{2+} (Figure 1.3), which itself regulates a variety of actin-binding proteins (Janmey, 1994).

Tight control of DAG levels is critical because DAG binds and activates a number of actin remodeling proteins such as PKCs, chimaerins, and Vav and because high DAG levels are associated with aberrant cellular function and malignant transformation (Ahmed *et al.*, 1990; Gulbins *et al.*, 1994; Caloca *et al.*, 1997; Topham and Prescott, 1999; Caloca *et al.*, 1999; Johnson *et al.*, 2007). Furthermore, there is evidence to suggest that DAG is produced and acts locally in a spatially regulated manner at sites of receptor activation and not stored in a common pool (van der Bend *et al.*, 1994; Shirai *et al.*, 2000; Payrastre *et al.*, 2001; Luo *et al.*, 2003a; Gonzales and Anderson, 2006). Such an arrangement allows the precise regulation of intended DAG targets without simultaneous activation of unintended targets, an important consideration in biological processes requiring spatially restricted remodeling of the actin cytoskeleton.

Figure 1.4. Hydrolysis of $PI(4,5)P_2$ yields DAG and IP_3.

DAG binds and activates a number of proteins involved in actin remodeling. IP_3 binds to receptors on the endoplasmic reticulum, resulting in the release of Ca^{2+} from intracellular stores. Ca^{2+} also plays a role in actin reorganization.


PI(4,5)P2
DAG
PLC-β
Actin remodeling
Cytoplasm
IP3
Ca2+
Ca2+ release
IP3R
Endoplasmic reticulum
Ca2+
Ca2+
Ca2+

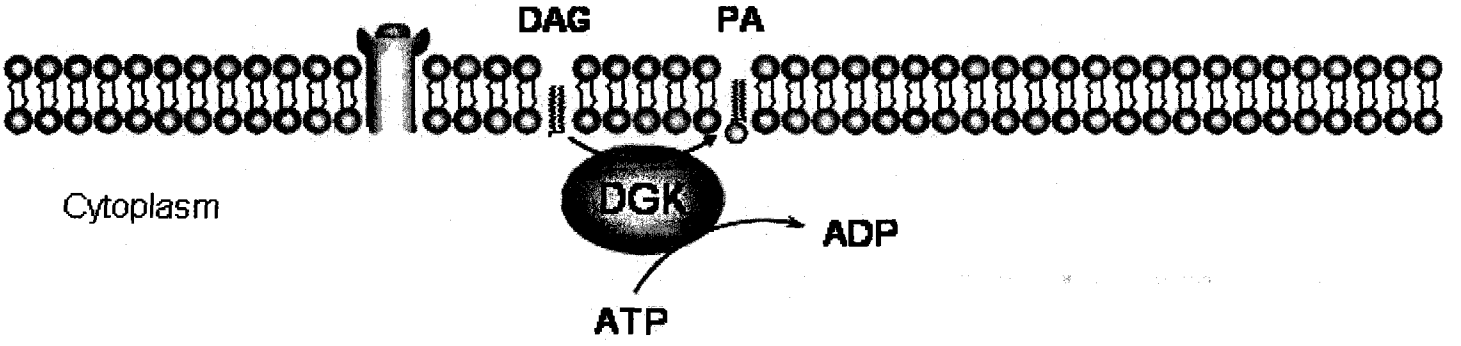
Diacylglycerol kinases (DGKs) Regulate the Levels of DAG and PA

The DGKs are a family of enzymes that phosphorylate DAG to yield PA (Figure 1.5). PA stimulates actin stress fiber assembly (Cross *et al.*, 1996; Siddiqui and English, 1997; Komati *et al.*, 2005) and activates a number of proteins with significant roles in actin reorganization such as phosphatidylinositol-4-phosphate 5-kinase (PIP5K) and PAK1 (Figure 1.4) (Bokoch *et al.*, 1998; Ishihara *et al.*, 1998). Thus, DGKs could regulate actin remodeling by attenuating signaling pathways stimulated by DAG or by activating pathways controlled by PA.

My dissertation focused primarily on discovering biological roles for DGKζ, one of 10 mammalian DGK isoforms. Our laboratory identified this isozyme in a yeast-two hybrid screen as a binding partner for syntrophin, a scaffold protein of the dystrophin-associated protein complex (DAPC) (Hogan *et al.*, 2001). Initial experiments and clues from the literature suggested a possible role for DGKζ and syntrophin in actin restructuring. I have explored this theme using three well-studied experimental paradigms in which actin remodeling underlies morphological changes critical for function: myoblast fusion, neurite outgrowth and cell migration. In each case, the relationship of lipid signaling to actin reorganization was not well studied, but there was evidence to suggest a role for DGKζ. In the following sections I provide relevant background information that will help the reader appreciate the biological diversity of DGKs and their potential roles in cellular signaling, with a focus on cytoskeletal dynamics.

Figure 1.5. DGK regulates the levels of DAG and PA at the plasma membrane.

Stimulation of growth factor receptors (*pink symbols*) results in transient accumulation of the lipid second messenger DAG. DAG binds and activates a number of targets (*left yellow box*). DGK attenuates DAG-mediated signaling by phosphorylating DAG to yield PA. PA also binds and activates its own set of targets (*right yellow box*). Adapted from Topham et al. (1999).



DAG

- Activates PKC isoforms
- Activates RasGRP
- Elevated in transformed cells

PA

- Activates PIP-5-Kinase
- Activates PAK1
- Dissociates RhoGDI from Rac
- Promotes actin polymerization

A Mammalian Family of DGK Isoforms

DGKs are evolutionarily conserved from bacteria to mammals, indicating an important role in fundamental cellular processes. Ten mammalian DGK isoforms have been identified and are classified into five subtypes based on structural homology (Figure 1.6 and Figure 1.7) (Sakane *et al.*, 2007). In general, DGKs have a common catalytic region and two to three cysteine-rich C1 domains homologous to the diacylglycerol/phorbol ester-binding motifs found in PKCs (Merida *et al.*, 2008). Otherwise, the various subtypes have unique protein domains, indicating they are each regulated in distinct fashion. Several DGK isoforms are also alternatively spliced (Figure 1.6), suggesting additional levels of control of DGK expression and activity (Sakane *et al.*, 2007).

Mammalian DGK activities have been identified in a broad range of tissues and cell types. Because more than one DGK isoform may be expressed in a particular cell type, the different isozymes likely perform specialized roles, either by recognizing distinct DAG pools generated in response to different stimuli or by accumulating in specific membrane compartments (Topham and Prescott, 1999). There is also growing evidence that individual DGK isozymes exert their specific roles through interactions with distinct target proteins (Sakane *et al.*, 2007).

DGKs and Actin Reorganization

Early studies suggested a link between DGKs and the actin cytoskeleton. For example, DGK activity was detected in purified cytoskeletal fractions (Payrastre *et al.*, 1991; Grondin *et al.*, 1991) and stimulation of cells with growth factors was associated with increased DGK activity and significant reorganization of the actin cytoskeleton

Figure 1.6. Structural organization of the mammalian diacylglycerol kinase family.

Mammalian DGKs are divided into five subtypes based on the indicated structural motifs. All DGKs have in common a catalytic domain and two or three C1 motifs. Some DGKs (β, γ, δ, η, ζ, ι) are also alternatively spliced. DGKζ and a closely related isoform, DGKι, belong to the type IV subgroup of DGKs. They are distinguished from other DGK isoforms by a region homologous to the phosphorylation site domain (PSD) of the MARCKS protein, four ankyrin repeats near the C-terminus and a C-terminal PDZ-binding motif (Hogan *et al.*, 2001). RVH, recoverin domain; Pro, proline; Glu, glutamine; PH, pleckstrin homology; SAM, sterile α motif; PDZ, PSD-95, DLG, ZO-1; MARCKS, myristoylated alanine-rich C kinase substrate; Gly, glycine; RA, Ras-associating. Reproduced from Biochimica et Biophysica Acta, 2007, 1771: 793-806. Copyright 2007, Elsevier.

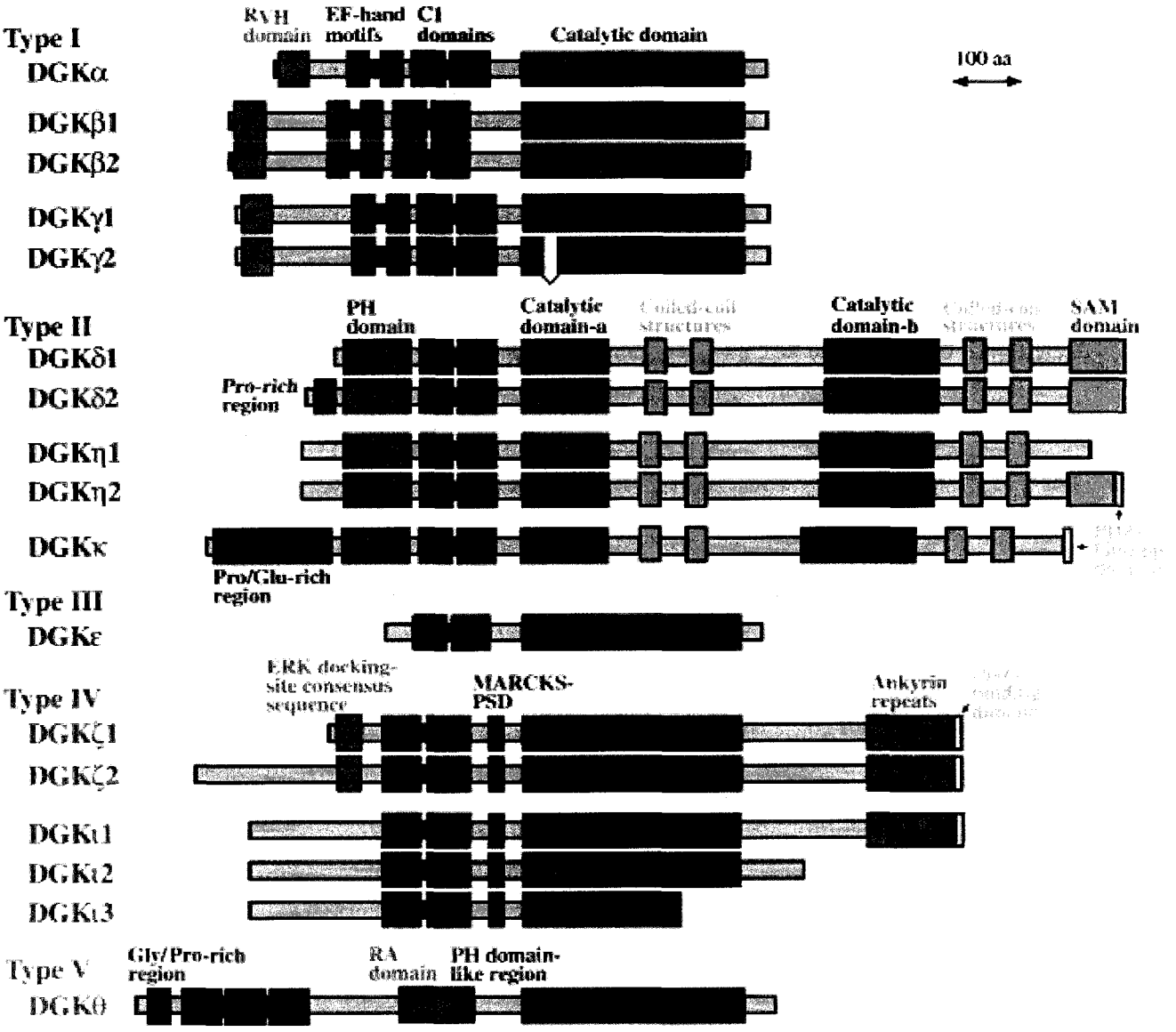
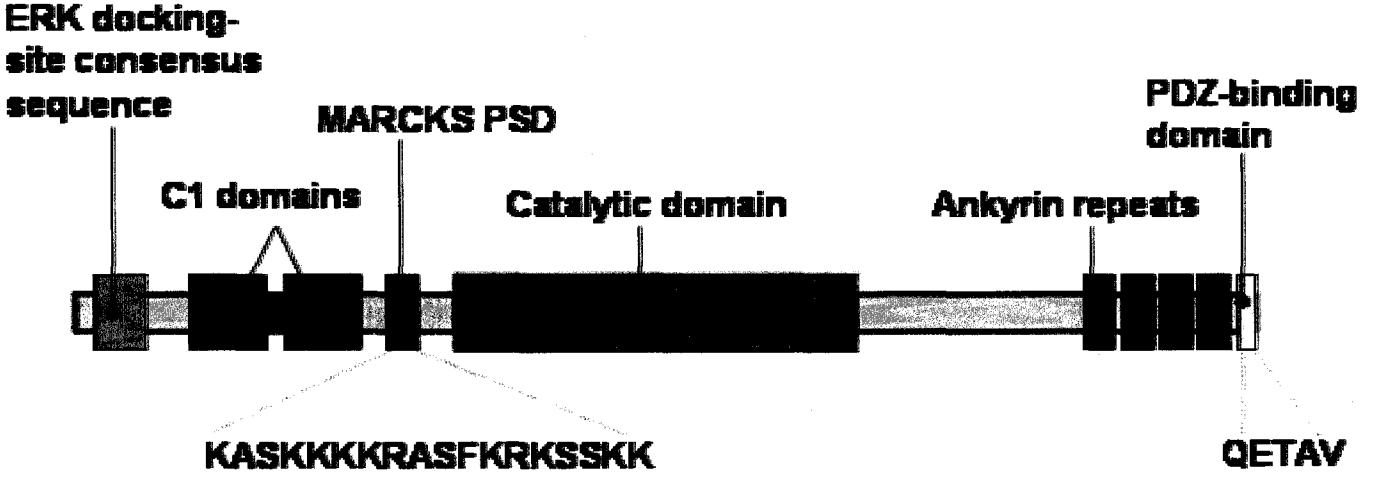

Type I
RVH domain
EF-hand motifs
C1 domains
Catalytic domain
100 aa
DGKα
DGKβ1
DGKβ2
DGKγ1
DGKγ2
Type II
PH domain
Catalytic domain-a
Coiled-coil structures
Catalytic domain-b
SAM domain
DGKδ1
DGKδ2
Pro-rich region
DGKη1
DGKη2
DGKκ
Pro/Glu-rich region
Type III
DGKε
Type IV
ERK docking-site consensus sequence
MARCKS-PSD
Ankyrin repeats
DGKζ1
DGKζ2
DGKι1
DGKι2
DGKι3
Type V
Gly/Pro-rich region
RA domain
PH domain-like region
DGKθ

Figure 1.7. Domain structure of DGKζ. The N-terminus contains an ERK docking-site consensus sequence (*blue-green box*) and two C1 domains (*dark blue boxes*). The MARCKS phosphorylation site domain (*brown box*) is rich in basic amino acid residues such as lysine (*K*) and arginine (*R*) and this motif is similar to some nuclear localization signals. The serines (*S*) are targets of PKC-dependent phosphorylation (Topham *et al.*, 1998). The catalytic domain (*green box*) is responsible for DAG phosphorylation. The C-terminus contains four ankyrin repeats (*light blue boxes*) and a PDZ-binding motif (*yellow box*) which corresponds to a class I PDZ-binding consensus sequence (*QETAV*) (Hogan *et al.*, 2001).


ERK docking-site consensus sequence
MARCKS PSD
PDZ-binding domain
C1 domains
Catalytic domain
Ankyrin repeats
KASKKKKRASFKRKSSKK
QETAV

(Landreth *et al.*, 1985; Wiegant *et al.*, 1986; van Bergen en Henegouwen PM *et al.*, 1989; Roy *et al.*, 1989; Payrastre *et al.*, 1991)

Additional evidence came from a report showing that Rac1 immunoprecipitates from brain contained DGK activity, suggesting DGKs function in conjunction with Rac1 to regulate actin dynamics (Tolias *et al.*, 1998). Consistent with this idea, the results presented in Chapter 3 revealed a direct interaction between the ζ isoform and Rac1 as well as a mechanism for the regulation of Rac1 activity by DGKζ (Chapter 5). These findings together with other studies suggest DGKζ forms regulated signaling complexes with proteins having key roles in actin organization (Topham and Prescott, 2001; Luo *et al.*, 2004a)

Signaling complexes direct localized changes in the state of actin assembly and disassembly underlying the diverse responses of eukaryotic cells to discrete stimuli. Since proteins may be used in varying combinations to achieve specific biological responses, it is essential that the correct repertoire of such molecules come together to form distinct signaling modules (Pawson and Scott, 1997). Membrane lipids play an important role in this regard by restricting the recruitment of proteins to sites of receptor activation and by coordinating their spatial arrangement, conformation and activity (Pawson and Scott, 1997; Ile *et al.*, 2006; Fernandis and Wenk, 2007). Finally, signaling at the membrane slows the diffusion of the reactants, concentrates them in a small volume, and enhances the extent of complex formation, thereby increasing the intensity of the signal being transduced (Kholodenko *et al.*, 2000). Signaling at the membrane is thus an important feature of signal transduction.

A number of studies propose that DGK activity is topologically restricted and tightly associated with receptor stimulation, suggesting DAG (and PA) signaling is spatially

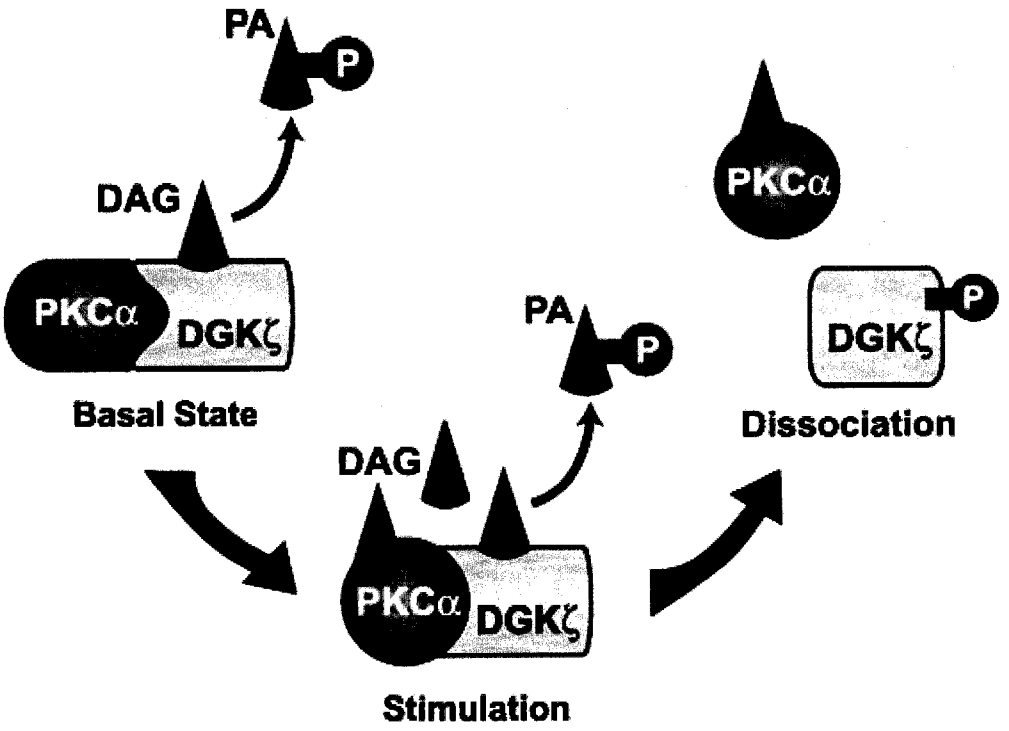
regulated (van der Bend *et al.*, 1994; Topham and Prescott, 1999; Topham and Prescott, 2001; Luo *et al.*, 2003a; Luo *et al.*, 2004a). In other words, rather than entering a non-specific pool, DAG (or its metabolite PA) acts locally to regulate a single target protein present in a complex with a DGK in response to specific signals. Three such DGKζ-containing complexes have been described in detail (see below).

DGKζ Negatively Regulates PKCα and RasGRP

PKCs regulate a broad array of cell functions, such as growth, differentiation, apoptosis, and cytoskeletal reorganization (Nishizuka, 1995; Black, 2000; Dempsey *et al.*, 2000). The binding of DAG to the C1 domains of PKC results in PKC activation (Newton, 2001). Because PKC phosphorylates a large number of protein targets, DAG levels must be precisely regulated both spatially and temporally. A study by our collaborators showed DGKζ directly interacts with and negatively regulates PKCα by metabolizing DAG (Luo *et al.*, 2003a). However, increased DAG production following growth factor stimulation overcomes the ability of DGKζ to metabolize DAG and results in PKCα activation (Figure 1.8). Once activated, PKCα phosphorylates the DGKζ MARCKS domain. This attenuates DGKζ activity and dissociates the two proteins, resulting in increased DAG levels and prolonged PKCα activation. In this way, PKCα regulates its own activity by attenuating the activity of its suppressor DGKζ.

Figure 1.8. Model for the spatial regulation between DGKζ and PKCα.

In the basal condition, DGKζ phosphorylates DAG preventing it from activating PKCα. Following stimulation, local DAG levels increase and overcome the ability of DGKζ to phosphorylate DAG. High DAG levels activate PKCα which then phosphorylates DGKζ causing the two proteins to dissociate. This favors transient or prolonged activation of PKCα. Reproduced from The Journal of Cell Biology, 2003, 160: 929-937. Copyright 2003, The Rockefeller University Press.


PA
P
DAG
PKCα
DGKζ
Basal State
PA
P
DAG
PKCα
DGKζ
Stimulation
PKCα
DGKζ
P
Dissociation

The role of PKCs in remodeling of the actin cytoskeleton has been extensively documented (Larsson, 2006). Activation of some PKC isoforms (such as PKCα) is associated with dissolution of stress fibers, formation of membrane ruffles and migration (Rifkin *et al.*, 1979; Schliwa *et al.*, 1984; Meigs and Wang, 1986; Kitano *et al.*, 1986; Miyata *et al.*, 1989; Ridley *et al.*, 1992; Lewis *et al.*, 1996; Myat *et al.*, 1997; Uberall *et al.*, 1999; Larsson, 2006). PKCs (including PKCα) regulate Rho GTPases by modulating the activity of both Rho GEFs and GAPs and by phosphorylating and repressing RhoGDI (Fleming *et al.*, 1997; Brandt *et al.*, 2002; Mehta *et al.*, 2002; Holinstat *et al.*, 2003; Tatin *et al.*, 2006; Knezevic *et al.*, 2007). DGKζ could therefore regulate PKCα-mediated reorganization of the actin cytoskeleton by attenuating the activity of PKCα.

Epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) elevate DAG levels, activate Ras and produce dramatic changes in the actin cytoskeleton (Hasegawa-Sasaki, 1985; Mulcahy *et al.*, 1985; Gibbs *et al.*, 1990; Satoh *et al.*, 1990; Satoh *et al.*, 1993; Montgomery *et al.*, 1997; Wang *et al.*, 1998). In addition, activated mutants of Ras induce substantial morphological changes in a number of cell types, suggesting Ras activation is a key signal for growth factor-induced actin reorganization (Wahrman *et al.*, 1985; Bar-Sagi and Feramisco, 1986; Sistonen *et al.*, 1987; Bhattacharya *et al.*, 1988; Higgins and Ryan, 1989; Lombardi *et al.*, 1990; Dartsch *et al.*, 1994).

The finding that DAG binds and activates RasGRP (a GEF for Ras), which results in increased Ras activity, provided an additional route for lipid-mediated modification of actin organization (Ebinu *et al.*, 1998; Kawasaki *et al.*, 1998; Tognon *et al.*, 1998). A later study showed RasGRP directly associates with DGKζ in a signaling complex (Topham and

Prescott, 2001). By metabolizing DAG that would otherwise activate RasGRP, DGKζ negatively regulates RasGRP, and hence Ras activity. Furthermore, DGKζ and RasGRP were colocalized at the leading edge of migrating cells, an area of intense actin remodeling. These findings raise the possibility that DGKζ regulates actin dynamics by controlling the activity of RasGRP and Ras.

Taken together, the published reports on DGKζ interactions with PKCα and RasGRP suggest a general mechanism for regulation of DGKζ-associated proteins: DGKζ interacts with a DAG-activated protein and inhibits its activity by locally metabolizing signaling DAG. As described below, DGKζ may also positively regulate proteins activated by PA.

DGKζ Positively Regulates PIP5K

$PI(4,5)P_2$ is catabolized by hydrolysis to DAG and IP_3 or by dephosphorylation to phosphatidylinositol 4-phosphate (PI(4)P) and is synthesized by PIP5K-mediated phosphorylation of PI(4)P (Nasuhoglu *et al.*, 2002; Wong *et al.*, 2005). The PIP5K family of isozymes is subdivided into two types (I and II), each comprising α, β, and γ isoforms (Weernink *et al.*, 2004). The finding that type I enzymes accumulate in structures undergoing extensive actin reorganization and are activated *in vitro* by acidic phospholipids such as PA suggest they could be regulated by DGKs (Moritz *et al.*, 1992; Jenkins *et al.*, 1994; Ishihara *et al.*, 1996; Ishihara *et al.*, 1998; Honda *et al.*, 1999; Luo *et al.*, 2004a; Kisseleva *et al.*, 2005). Indeed, DGKζ and PIP5KIα colocalize (together with actin) in lamellipodia and at the leading edge of migrating cells (Luo *et al.*, 2004a). They also associate in a signaling complex and DGKζ-derived PA activates PIP5KIα. These results suggest DGKζ positively

regulates PIP5KIα activity and promotes local actin polymerization by raising $PI(4,5)P_2$ levels.

Further support for a role for PIP5Kα in actin remodeling came from studies which showed that Rac1 and RhoA colocalize with and activate PIP5KIα, leading to increased $PI(4,5)P_2$ levels (Ren *et al.*, 1996; Tolias *et al.*, 1998; Weernink *et al.*, 2004). Moreover, Rac1 associates with a complex that includes a RhoGDI, a type I PIP5K and a DGK (Tolias *et al.*, 1998). Although the identity of the DGK isoform was not determined with certainty, the possibilities included the zeta (ζ), theta (θ), or gamma (γ) isoforms (Tolias *et al.*, 1998; Tsushima *et al.*, 2004). Based on initial experiments and clues from the literature, we hypothesized that DGKζ was the unidentified DGK. As described in Chapters 3 and 5, I have pursued the theme that DGKζ regulates proteins involved in actin organization by studying the interaction of DGKζ with Rac1. My results suggest DGKζ positively regulates Rac1 by relieving RhoGDI inhibition.

Regulation of DGKζ Activity by Subcellular Localization

Subcellular localization is undoubtedly a key determinant of DGK function because the enzyme must gain access to a membrane compartment where DAG is generated. Although translocation from the cytosol to the membrane seems to be a general mechanism governing the activation of all DGKs, different isoforms likely rely on distinct regulatory regions as a means for redistributing to a particular membrane domain (Santos *et al.*, 2002; Sakane *et al.*, 2002; Nagaya *et al.*, 2002; Hozumi *et al.*, 2003; Imai *et al.*, 2005; Matsubara *et al.*, 2006; Kobayashi *et al.*, 2007).

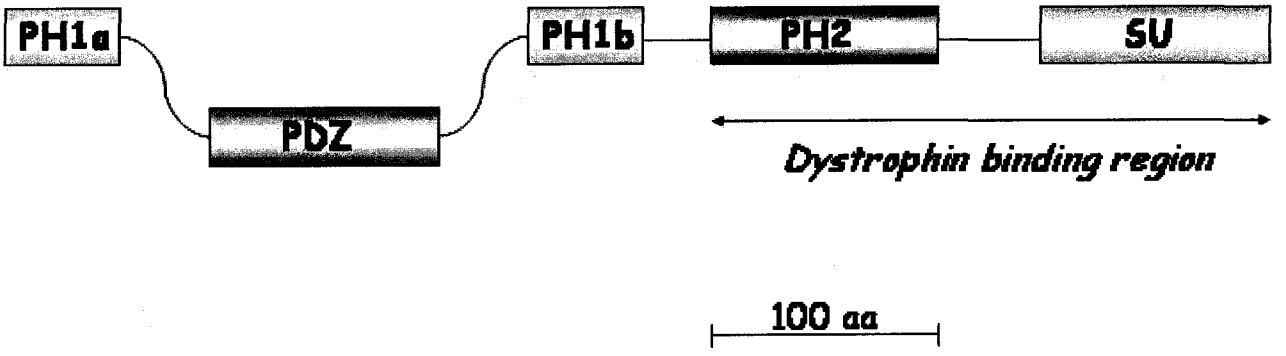
Previous findings from our laboratory revealed that the C-terminal PDZ (Post-synaptic density protein of 95 kDa, *Drosophila* Discs large protein, and the Zona occludens protein 1) -binding motif in DGKζ binds to the PDZ domain of syntrophins, a family of scaffolding proteins that associate with the cytoskeletal protein dystrophin (Hogan *et al.*, 2001). Disrupting the DGKζ/syntrophin interaction resulted in DGKζ accumulation in the nucleus of HeLa cells, suggesting syntrophins regulate the partitioning of DGKζ between the nucleus and the cytoplasm. This finding was consistent with the known role of syntrophins in regulating the subcellular localization of partner proteins. I pursued the idea that syntrophins provide localization cues that target DGKζ to the correct subcellular domain, and that this is likely critical for regulated DGKζ activity. The results of these studies are presented in Chapters 2 and 3.

Syntrophins

Syntrophins are a family of scaffolding proteins that anchor a variety of signaling molecules at the plasma membrane (Hogan *et al.*, 2001). There are five known syntrophin isoforms (α1, β1, β2, γ1, γ2), each the product of a separate gene (Adams *et al.*, 1993; Ahn *et al.*, 1994; Ahn *et al.*, 1996; Piluso *et al.*, 2000). Although syntrophins are ubiquitously expressed, each isoform has a unique tissue distribution (Alessi *et al.*, 2006). All syntrophins share the same basic structure: an N-terminal bipartite pleckstrin-homology (PH1) domain, a PDZ domain, a second pleckstrin homology domain (PH2) and a C-terminal syntrophin unique (SU) domain (Figure 1.9) (Ahn *et al.*, 1996; Piluso *et al.*, 2000).

Figure 1.9. Domain structure of syntrophins.

Syntrophins have an N-terminal bipartite pleckstrin homology (PH1) domain; a PDZ domain; a second PH domain (PH2), and a C-terminal syntrophin unique (SU) domain. Adapted from Ahn et al. (1996).


PH1a
PDZ
PH1b
PH2
SU
Dystrophin binding region
100 aa

PDZ Domains

PDZ domains are modules of 80 to 100 conserved amino acids found in most organisms and are common protein-protein interaction domains (Jemth and Gianni, 2007). They are often arranged in tandem arrays and are associated with other interaction domains to form multi-domain scaffolding proteins which assemble macromolecular structures at specialized membrane areas (Li and Montell, 2000; Kim and Sheng, 2004).

PDZ-mediated clustering of molecular signaling complexes increases signaling efficiency, specificity, and sensitivity (Zhang and Wang, 2003). For example, a reaction product from an upstream signaling protein can be quickly and directly channeled to a downstream protein without relying on diffusion. The end result of such spatially coupled reactions is enhanced signaling speed, which is often essential for proper cell homeostasis. Furthermore, since many signaling pathways share common substrates, signaling specificity can only be achieved by the selective recruitment of a subset of designated proteins into spatially isolated signaling complexes. The alternative would be random cross-talk between proteins without a necessarily productive outcome. Lastly, the assembly and clustering of numerous signaling molecules into larger molecular weight complexes allows a biological system to respond to a relatively weak initial signal by reducing the activation threshold necessary for a response (Bray *et al.*, 1998; Levchenko *et al.*, 2000). Collectively, these features allow cells to rapidly, efficiently and discreetly direct the flow of information that controls cellular responses.

PDZ domains are classified on the basis of the sequence of their preferred C-terminal ligands (Kim and Sheng, 2004). The PDZ domain of syntrophins belongs to the class I type of PDZ domains (Sheng and Sala, 2001). Type I PDZ domains typically recognize a (Q/E)-(S/T)-X-V-COOH tetrapeptide ligand motif where X can be any amino acid (Songyang *et*

al., 1997). In addition to binding short peptide motifs (~10 a.a.) at the extreme C-termini of partner proteins (Zhang and Wang, 2003), PDZ domains can also bind internal peptide sequences that adopt a β-hairpin structure (Hillier *et al.*, 1999), and they can also heterodimerize with other PDZ domains (Brenman *et al.*, 1996a; Brenman *et al.*, 1996b).

The PDZ domain of syntrophins binds to the C-terminus of numerous signaling proteins (Gee *et al.*, 1998; Schultz *et al.*, 1998; Iwata *et al.*, 1998; Hasegawa *et al.*, 1999; Lumeng *et al.*, 1999; Ort *et al.*, 2000; Garcia *et al.*, 2000; Adams *et al.*, 2001; Buechler *et al.*, 2002; Connors *et al.*, 2004; Luo *et al.*, 2005; Chen *et al.*, 2006). In addition to their accepted role as protein-protein recognition domains, there is evidence that syntrophin PDZ domains can also function as lipid binding motifs (Zimmermann *et al.*, 2002). For example, Yan et al. (2005) showed that the PDZ domain of α1-syntrophin could bind brain lipid extracts. Furthermore, the lipid binding site and the PDZ ligand-binding site on the α1-syntrophin PDZ domain did not appear to overlap with one another (Yan *et al.*, 2005). Such an arrangement could conceivably allow simultaneous binding of lipid and protein ligands, giving syntrophins the ability to target proteins to membrane domains enriched in particular lipids thereby contributing to the spatial regulation of signaling.

Pleckstrin Homology (PH) Domains

PH domains are abundant protein modules that play important roles in cellular signaling and cytoskeletal organization because they have an affinity for a number of inositol phospholipids (Lemmon and Ferguson, 2000). PH domains target the proteins that contain them to the cell membrane in response to lipid signals generated by receptor activation.

The PH domain organization of syntrophins is unique because the N-terminal PH domain (PH1) is split into two halves (a and b) by insertion of the PDZ domain (Yan *et al.*, 2005). Although the PH1 domain of syntrophins has been shown to specifically bind $PI(4,5)P_2$ (Chockalingam *et al.*, 1999), the PH1a-PDZ-PH1b supra-module binds a variety of phospholipids, including PA, with higher affinity than either the PH1 or PDZ modules alone (Yan *et al.*, 2005). Furthermore, the two halves of the split PH1 domain associate with each other to form a canonical PH domain, with the inserted PDZ module having a negligible impact on the conformation of the PH1 domain (Yan *et al.*, 2005). The finding that syntrophins bind certain phospholipids suggests they are intimately linked to the plasma membrane and that they could be recruited to lipid microdomains formed as a consequence of receptor activation. Many of the phospholipids that bind syntrophins play important roles in remodeling of the actin cytoskeleton, raising the possibility that syntrophins are involved in this process. Consistent with this notion, the PH2 and SU domains of syntrophins, which mediate binding to dystrophin and its homologues, also contain high-affinity binding sites for F-actin (Iwata *et al.*, 1998).

The Dystrophin-associated Protein Complex

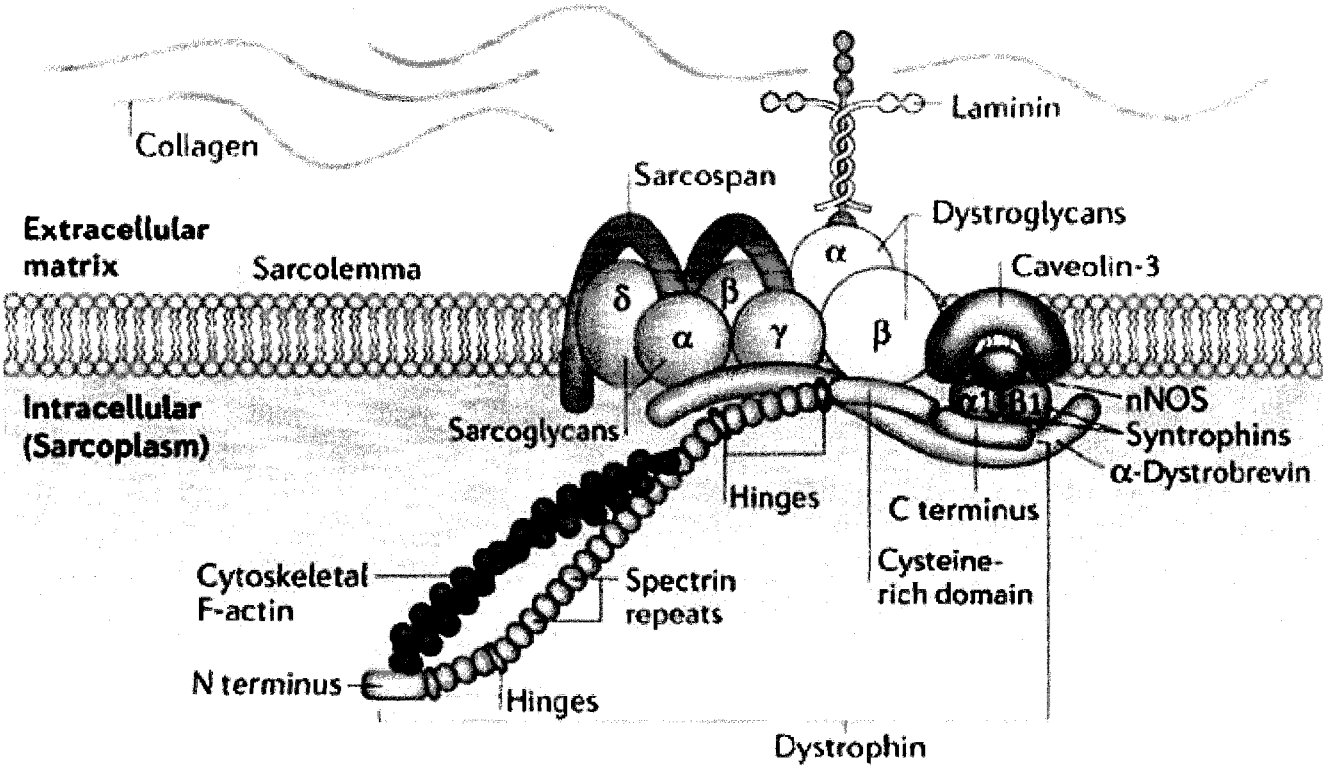
Dystrophin is a member of the dystrophin-associated protein complex (DAPC) that includes α- and β-dystroglycan, α-, β-, γ-, and δ-sarcoglycans, sarcospan, α-dystrobrevin, and one or more syntrophins (Davies and Nowak, 2006). The N-terminal region of dystrophin binds F-actin. The central portion is composed of spectrin repeats, giving dystrophin its characteristic elastic properties. The C-terminal region of dystrophin binds dystrobrevins, syntrophins and the intracellular portion of β-dystroglycan, which anchors

dystrophin to the plasma membrane (Figure 1.10) (Dalkilic and Kunkel, 2003; Lapidos *et al.*, 2004).

The role of the DAPC has been best characterized in muscle because humans missing or expressing mutated forms of dystrophin suffer from an X chromosome-linked disorder known as Duchenne muscular dystrophy (DMD). DMD is characterized by progressive wasting of the skeletal musculature, development of profound weakness, joint contractures, and kyphoscoliosis (Blake *et al.*, 2002). The prognosis is poor, and patients often die in their late teens or mid-twenties from respiratory or cardiac dysfunction (Anderson *et al.*, 2002).

Two primary hypotheses have developed regarding the functions of the DAPC. The first is that the DAPC physically links the intracellular F-actin cytoskeleton to the extracellular matrix, enabling force transduction from the contractile apparatus in the muscle myofiber to the basement membrane (Lynch *et al.*, 2000; Bloch and Gonzalez-Serratos, 2003; Ervasti, 2003; Judge *et al.*, 2006). In addition, the elastic properties of dystrophin protect muscle cells from the stress caused by the force of muscle contraction (Davies and Nowak, 2006).

Figure 1.10. The dystrophin-associated protein complex (DAPC). The DAPC is comprised of sarcoplasmic proteins (α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)), transmembrane proteins (β-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (α-dystroglycan and laminin). Dystrophin is localized at the sarcolemma and has a long central rod domain made up of spectrin repeats interspersed with hinge regions. The N-terminus of dystrophin binds to the cytoskeleton through filamentous (F)-actin. The C-terminus of dystrophin binds to the DAPC. In this way, the DAPC links the intracellular cytoskeleton to the membrane and the extracellular matrix. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews: Molecular Cell Biology 7(10): 762-73, Copyright 2006.


Collagen
Laminin
Sarcospan
Dystroglycans
Extracellular matrix
Sarcolemma
Caveolin-3
δ
α
β
γ
α
β
Intracellular (Sarcoplasm)
Sarcoglycans
α1 β1
nNOS
Syntrophins
α-Dystrobrevin
Hinges
C terminus
Cysteine-rich domain
Cytoskeletal F-actin
Spectrin repeats
N terminus
Hinges
Dystrophin

However, dystrophin and dystrophin-associated proteins (DAPs) are also expressed in nervous tissue (Lidov *et al.*, 1990; Lidov *et al.*, 1993; Gorecki *et al.*, 1994; Blake *et al.*, 1999; Piluso *et al.*, 2000; Moukhles and Carbonetto, 2001). Moreover, a subset of DMD patients exhibit cognitive deficits, suggesting a different role for the DAPC in neurons (Anderson *et al.*, 2002). Consistent with this idea, mice with mutations in dystrophin or dystrobrevin exhibit defects in the maturation and functioning of a subset of inhibitory synapses leading to behavioral abnormalities (Grady *et al.*, 2006). Brain-selective deletion of dystroglycan in mice results in defects in cerebral cortical layering and aberrant migration of granule cells (Moore *et al.*, 2002). In *Drosophila*, dystrophin and dystroglycan regulate photoreceptor axonal pathfinding, a process that requires extensive remodeling of the actin cytoskeleton (Shcherbata *et al.*, 2007). DAPs are also highly enriched at the neuromuscular junction and have been implicated in agrin-stimulated clustering of nicotinic acetylcholine receptors (AChR), a process that depends on reorganization of the actin cytoskeleton by Rac1, RhoA and Cdc42 (Gee *et al.*, 1994; Campanelli *et al.*, 1994; Weston *et al.*, 2000; Dai *et al.*, 2000; Banks *et al.*, 2003; Weston *et al.*, 2007). Collectively, these studies suggest an important role for the DAPC in both the nervous system and the neuromuscular system. Furthermore, they suggest the DAPC plays a role in signal transduction and remodeling of the actin cytoskeleton. These functions extend beyond the traditional role of the DAPC as a mechanical support during muscular contraction.

Rationale for Selection of Thesis Topic

Reorganization of the actin cytoskeleton underlies a number of critical biological processes and there is considerable evidence suggesting a role for specific membrane lipids in this regard. Many studies have focused on the role of $PI(4,5)P_2$ in actin remodeling, but

DAG and PA also have a significant impact on actin dynamics. Consistent with this, the available evidence supports a role for DGKζ in the regulation of actin restructuring that underlies changes in morphology associated with several fundamental cellular processes. However, further studies are needed to understand exactly how DGKζ functions in this regard and in particular, how its enzymatic activity is regulated. Translocation of DGK from the cytosol to the plasma membrane is a general mechanism for activation of the enzyme but how this is controlled remains unclear.

The results presented in Chapter 2 showed that syntrophins and PKC-mediated phosphorylation of the MARCKS domain regulate the association of DGKζ with the plasma membrane of muscle cells. Furthermore, a DGKζ mutant with a blocked syntrophin-binding motif resulted in dramatic changes in the actin cytoskeleton of myoblasts and myotubes (Chapter 2). These experiments provided evidence that the DGKζ-syntrophin interaction plays a role in regulating the localization of DGKζ as well as modulating actin reorganization and justified a more detailed examination of DGKζ's role in this process. In subsequent studies, we employed three different cell culture paradigms to explore possible biological roles for DGKζ:

1. Neurite outgrowth in N1E-115 neuroblastoma cells.
2. Fusion of mononucleated C2C12 myoblasts into myotubes.
3. Migration of mouse embryonic fibroblasts.

These systems were chosen because DGKζ and syntrophins are abundantly expressed in each cell type and because actin reorganization plays an essential role in each process. Furthermore, both DAG and PA have been implicated in all three processes but the exact relationship of lipid signaling to actin dynamics in these model systems remained unclear.

Lastly, all three paradigms are useful models of processes encountered during development, in neuromuscular disorders, wound healing, and cancer. In the following sections I provide relevant background information and highlight the unresolved questions for each of the three experimental systems.

Myoblast Fusion

The musculature plays a primordial role in the functioning of many organisms because it is responsible for mobility. Organisms from fruit flies to mammals possess an elaborate musculature comprised of arrays of muscle fibers generated by the fusion of committed muscle precursors known as myoblasts (Abmayr *et al.*, 2003). If the resulting musculature is to be properly patterned, be of the appropriate size and be functional, specific regulatory systems must exist to control the fusion of myoblasts during development or in response to traumatic injury or exercise (Blau, 1993; Doberstein *et al.*, 1997). Skeletal muscle may also constitute a target organ for gene therapy, as engineered myoblasts can be induced to fuse with mature muscle, forming a hybrid organ in the adult (Blau, 1993; Miller and Boyce, 1995). This last point is of particular importance in diseases where repopulation of the damaged muscle by healthy myoblasts constitutes a possible therapeutic approach (Doberstein *et al.*, 1997; Chen *et al.*, 2003). Therefore, unraveling the molecular mechanisms regulating myoblast fusion would constitute an important step toward understanding muscle development and developing therapeutic strategies to treat muscular degeneration.

The fruit fly *Drosophila melanogaster* has become a staple of modern myoblast fusion research because the musculature is relatively simple and well characterized (Bate, 1990). *Drosophila* is also a genetically simpler organism and amenable to genetic

manipulation, allowing the function of individual genes to be easily dissected. Furthermore, the entire process of muscle formation is accomplished rapidly and many fusion events in various stages of completion can be observed in single thin sections of developing muscle (Bate, 1990; Paululat *et al.*, 1999). Lastly, many of the gross morphological changes that occur between fusing myoblasts in *Drosophila* appear to be conserved in mammals (Doberstein *et al.*, 1997).

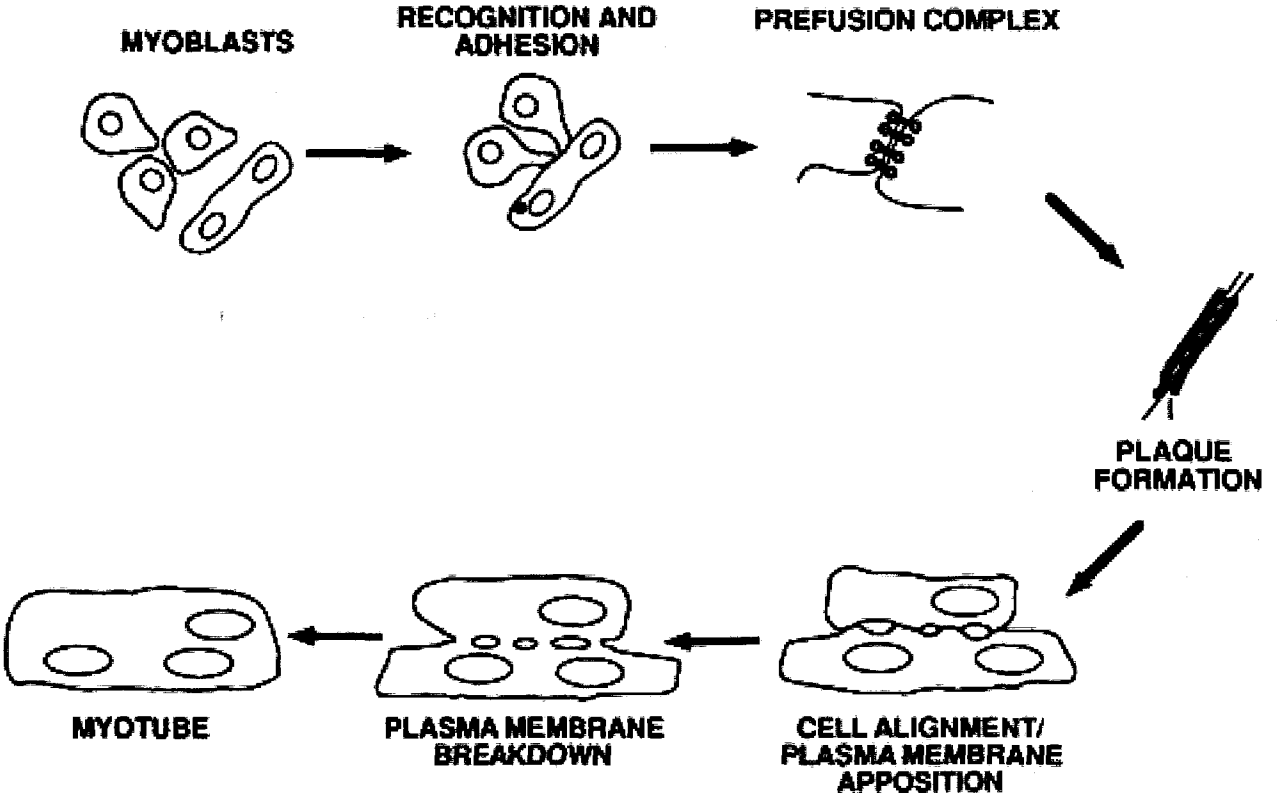
In *Drosophila*, differential gene expression in the embryonic myoblast population defines two groups of myoblasts: founder cells and fusion-competent cells (Chen and Olson, 2004). Myoblast fusion occurs between founder cells which act as "seeds", whereas the fusion-competent cells are attracted to the founders and migrate towards them (Menon and Chia, 2001). Once they are in close proximity to founders, fusion-competent cells adopt a tear-shaped morphology and extend filopodia (or pseudopodia) with the tips of the protrusions attaching to the founder cell membrane (Ruiz-Gomez *et al.*, 2000).

Each group of myoblasts expresses distinct trans-membrane receptors that mediate myoblast attraction and adhesion, and genetic deletion of these receptors results in randomly oriented filopodia and a lack of myoblast fusion (Ruiz-Gomez *et al.*, 2000; Bour *et al.*, 2000; Strunkelnberg *et al.*, 2001; Chen and Olson, 2004). Once contact has been established, fusion-competent cells move towards the founders and the cells align along their long axes, bringing the cell membranes into close proximity (Chen and Olson, 2004). Detailed electron microscopy studies of myoblast fusion in *Drosophila* show that pairs of myoblasts establish a "prefusion complex" consisting of groups of paired vesicles and associated electron-dense

material both inside and outside the cells (Figure 1.11) (Doberstein *et al.*, 1997). The complex then resolves into electron-dense plaques along the plasma membranes of the apposed cells, most likely by fusion of the paired vesicles with their respective plasma membranes. Formation of pores between the apposed cells is then followed by vesiculation of the plasma membranes along their shared lengths, and the residual plasma membranes are presumably disposed of and/or recycled. These findings reveal the complexity of myoblast fusion and suggest that intricate mechanisms must exist to regulate the dramatic changes taking place in the plasma membrane and to coordinate these changes with other cellular processes.

Cell migration, filopodial extension, cell-cell contact, alignment and fusion all require changes in cell shape driven by remodeling of the actin cytoskeleton. Pharmacological inhibition of actin polymerization blocks myoblast fusion (Sanger *et al.*, 1971; Sanger and Holtzer, 1972; Blau and Epstein, 1979; Constantin *et al.*, 1995). In *Drosophila*, perturbation of Rac expression and function promotes precocious fusion or blocks fusion altogether by inducing aberrant plasma membrane morphology and inhibiting a late step in membrane pore formation (Luo *et al.*, 1994; Doberstein *et al.*, 1997; Hakeda-Suzuki *et al.*, 2002). More recently another actin remodeling protein D-WIP, a *Drosophila* homolog of the Verprolin/WASp Interacting protein, has been implicated in localized actin polymerization required to enlarge nascent pores, break down membranes via endocytosis or push vesiculated membrane particles formed during the late stages of fusion away from areas of cell-cell contact (Massarwa *et al.*, 2007). The actin cytoskeleton also plays a role in targeted exocytosis of pre-fusion vesicles containing uncharacterized fusigenic proteins or chemicals (Kim *et al.*, 2007). Collectively, these findings suggest an intimate association between the

Figure 1.11. Schematic outlining the steps in myoblast fusion in *Drosophila* at the ultrastructural level. First, myoblasts identify and adhere to fusion targets, either muscle pioneer cells or existing myotubes. This step may involve multiple separate stages, including chemoattraction of myoblasts to fusion targets, cell–cell communication for identification of target cells, and cell adhesion. Pairs of cells that have correctly identified appropriate fusion targets then set up prefusion complexes at contact points where fusion will eventually begin. These complexes include paired vesicles and their associated electron dense material. The myoblasts become elongated, and align themselves along their long axes. After an unknown signal, the prefusion complex resolves into a short-lived electron-dense plaque. Next, fusion pores form, making the cytoplasm of the fusing cells continuous. The pores expand and the plasma membrane breaks down into smooth sacs of membrane. These sacs become rounder in profile through time and eventually accumulate in groups of clear, irregularly shaped vesicles before recycling or disposal. Reproduced from The Journal of Cell Biology, 1997, 136: 1249-1261. Copyright 1997, The Rockefeller University Press.


MYOBLASTS
RECOGNITION AND ADHESION
PREFUSION COMPLEX
PLAQUE FORMATION
CELL ALIGNMENT/ PLASMA MEMBRANE APPOSITION
PLASMA MEMBRANE BREAKDOWN
MYOTUBE

membrane and the actin cytoskeleton and an important role for the actin cytoskeleton in regulating membrane dynamics during myoblast fusion.

In mammals, myoblast-myoblast adhesion is mediated by a distinct set of calcium-dependent trans-membrane adhesion molecules known as cadherins (Abmayr *et al.*, 2003). Myoblasts express N- and M-cadherins, and both proteins associate with the actin cytoskeleton (Abmayr *et al.*, 2003). N-cadherin is thought to play an important role in myoblast fusion because treatment of myoblasts with anti-N-cadherin antibodies blocks myoblast aggregation and slows or blocks myotube formation altogether (Knudsen, 1990; Pouliot *et al.*, 1990; Mege *et al.*, 1992). Meanwhile, stimulation of N-cadherin receptors increases $PI(4,5)P_2$ levels through RhoA and Rac1-mediated recruitment and activation of PIP5KIγ exclusively to sites of N-cadherin adhesion (El Sayegh *et al.*, 2007). This localized accumulation of $PI(4,5)P_2$ promotes actin polymerization and increases the strength of cell-cell contacts. Together, these studies suggest stimulation of adhesion receptors, such as cadherins, drives localized changes in the actin cytoskeleton through the recruitment of lipid kinases and Rho GTPases to sites of cadherin activation.

There is also evidence to suggest a role for other lipids in myoblast fusion besides $PI(4,5)P_2$. For example, rapid increases in the levels of both DAG and PA are detected shortly after myoblast adhesion (Wakelam, 1983). Paradoxically, global generation of DAG by addition of exogenous phospholipase C or by DAG analogs such as TPA inhibit myoblast fusion (Nameroff *et al.*, 1973; Cohen *et al.*, 1977; Kent, 1979). Together, these findings suggest that proper spatial regulation of DAG and PA-mediated lipid signaling is important for myoblast fusion.

DAG could regulate myoblast fusion through PKC-dependent signaling pathways (David *et al.*, 1990; Vaidya *et al.*, 1991; Dulong *et al.*, 2004) whereas PA could regulate fusion by promoting the dissociation of Rac1 from RhoGDI (Chuang *et al.*, 1993a), allowing Rac1 to bind downstream effectors required for fusion. We explored the possibility that DGKζ plays a role in myoblast fusion because DGKζ is highly expressed in muscle, modulates the levels of DAG and PA, associates with Rac1 and regulates actin remodeling (Chapter 4).

Neurite Outgrowth

Nervous system function depends on the ability of neurons to form precise and highly complex connections. Terminally differentiated neurons are highly polarized with distinct subcellular compartments, including one or a multitude of dendritic processes arising from the cell body, as well as a single extended axon (Gao *et al.*, 1999). This polarized structure, with dendrites responsible for receiving and processing information and axons responsible for signal propagation, is critical for establishing the brain circuitry that allows information to be handled appropriately (Newey *et al.*, 2005).

During early embryogenesis neurons begin as simple spheres, but in postmitotic neurons the spherical shape is broken as a localized bud forms and neurites begin to sprout (Figure 1.12) (da Silva and Dotti, 2002). Neuritogenesis is guided by the growth cone, a highly motile structure that responds to molecular cues in the environment and is located at the tip of the growing neurite (da Silva and Dotti, 2002; Kalil and Dent, 2005). The central region of the growth cone contains bundled microtubules, while the periphery is populated by actin filaments that form a meshwork within lamellipodia or bundles within filopodia (Figure 1.13) (Luo, 2002; Kalil and Dent, 2005).

Figure 1.12. Schematic of morphological changes in neurons during development.

(A) Neurons begin as spheres and signals in the extracellular milieu promote the protrusion of one or more processes depicted in (B) and (C). The extension of these processes is driven by specialized organelles known as growth cones which are located at the distal tips of neurites. (D) The processes mature into multiple dendrites and a single axon (*drawn as the long process located directly below the cell body and turning to the right*). Dendrites travel short distances and branch extensively while axons travel longer distances and form synapses with dendrites of other neurons (E). Inset in (E) shows dendritic spines, small protrusions on dendritic trees that play important roles in cognition. Reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 18 ©2002 by Annual Reviews www.annualreviews.org.

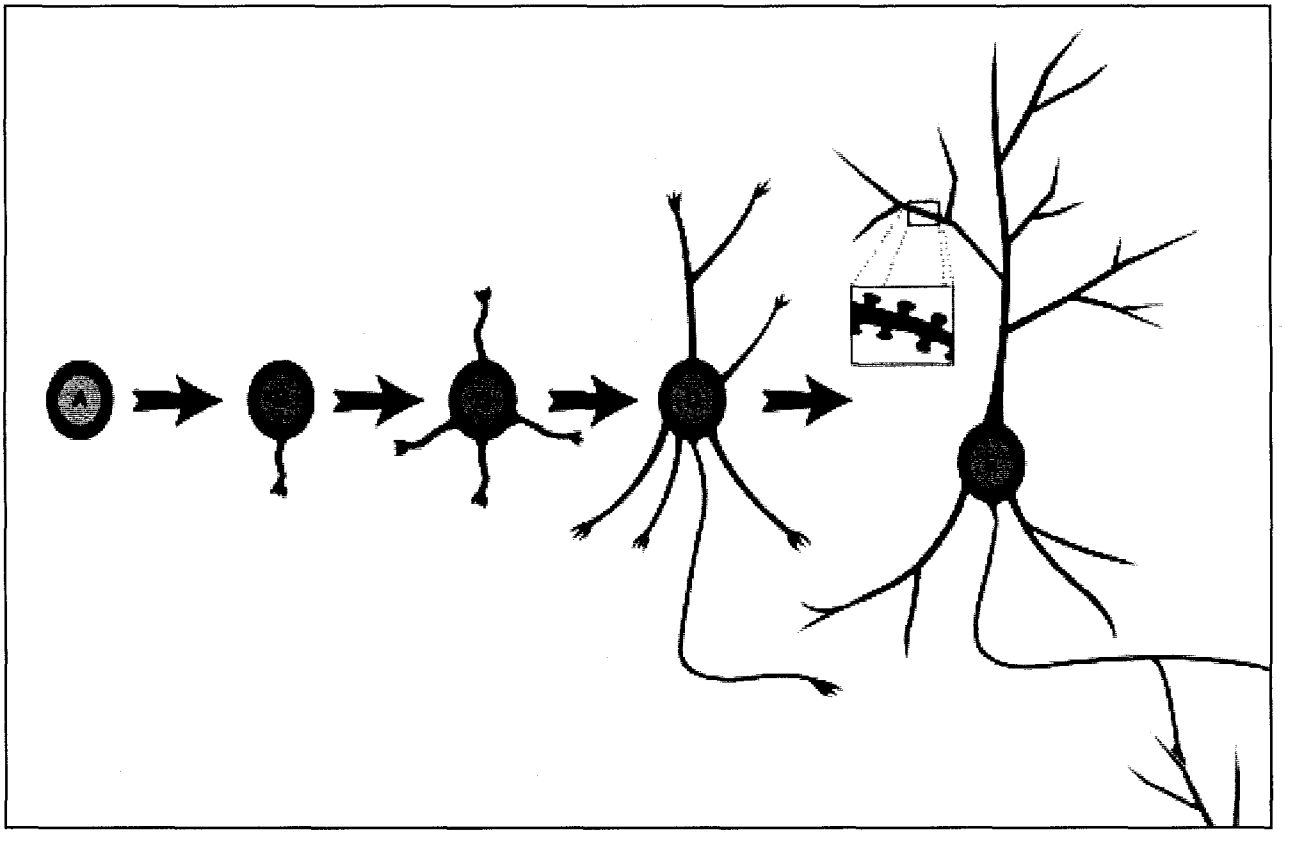
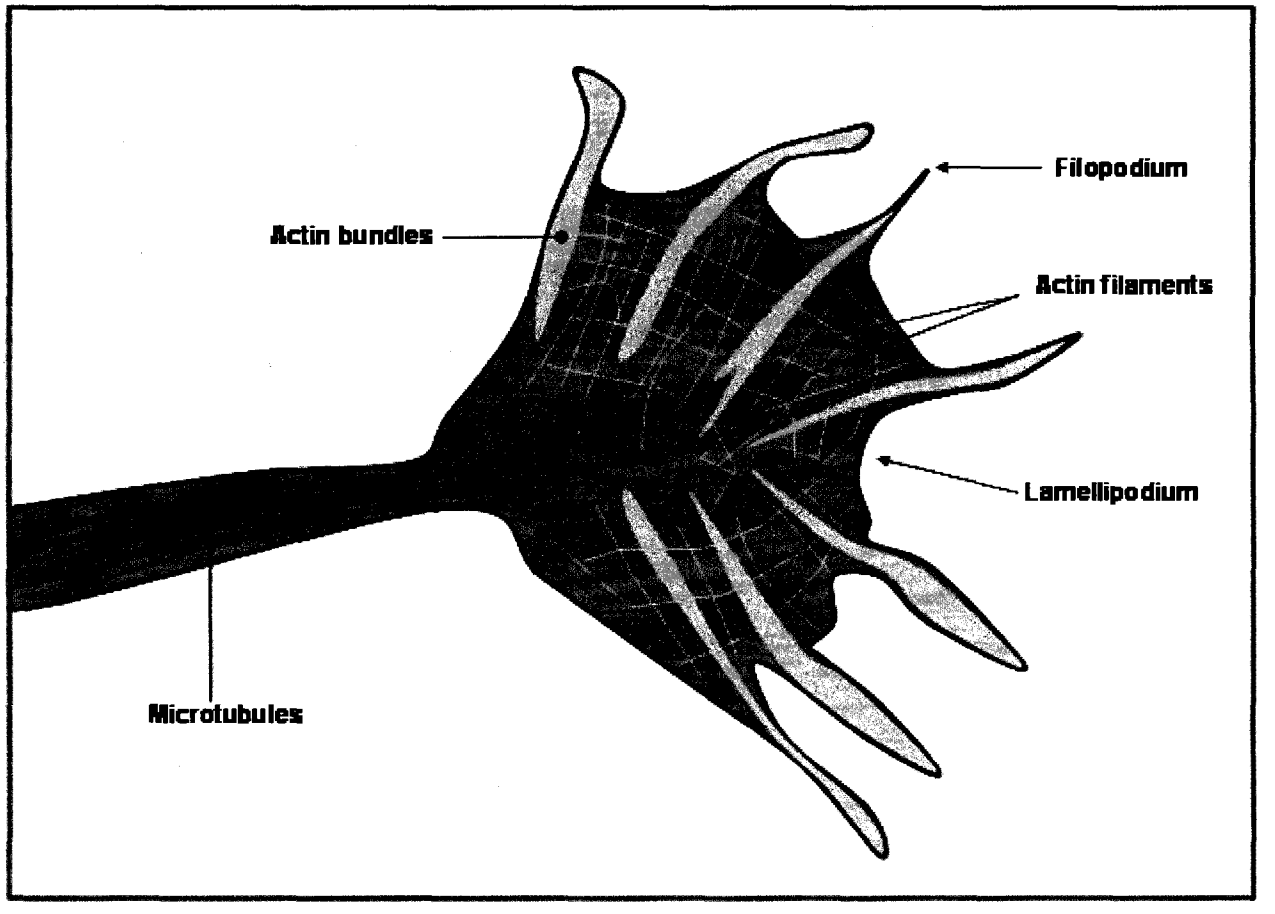


Figure 1.13. Schematic representation of a neuronal growth cone.

Neurite outgrowth is led by a specialized organelle at the tip of the extending neurite known as the growth cone. Growth cones are composed of finger-like filopodia and veil-like lamellipodia that drive the movement of the growth cone. Filopodia contain tightly cross-linked bundles of F-actin (*thick light gray lines*) whereas lamellipodia are formed by a cross-linked meshwork of F-actin filaments (*thin light gray lines*). Microtubules (*black lines*) project from the axon to the central region of the growth cone. Adapted from Luo et al.(2002).


Filopodium
Actin bundles
Actin filaments
Lamellipodium
Microtubules

Neuritogenesis is triggered by the activation of membrane receptors in response to a variety of extracellular cues (da Silva and Dotti, 2002). Nerve growth factor (NGF) or fibroblast growth factor (FGF)-induced neuritogenesis in neuronal cell lines is accompanied by phosphoinositide hydrolysis, an increase in DAG and PA levels, and actin cytoskeleton remodeling in growth cones and axonal shafts (Traynor, 1984; Williams *et al.*, 1994; Li and Wurtman, 1998; Watanabe *et al.*, 2004b; Lykissas *et al.*, 2007). This signaling pathway also appears to be used in neurite outgrowth stimulated by NCAM-, N-cadherin-, L1- and netrin (Cybulsky *et al.*, 1993; Williams *et al.*, 1994; Kennedy *et al.*, 1994; Serafini *et al.*, 1994; Wrenn *et al.*, 1996; Xie *et al.*, 2006; Choi *et al.*, 2007).

DAG-binding proteins such as PKCs and chimaerins have been implicated in neuritogenesis (Morrison *et al.*, 1988; Burry, 1998; Bonsall and Rehder, 1999; Rosner and Vacun, 1999; Weeks *et al.*, 1999; Zeidman *et al.*, 1999; Kabir *et al.*, 2001; Benitez-King *et al.*, 2001; Hall *et al.*, 2001; Choe *et al.*, 2002; Troller *et al.*, 2004; Kolkova *et al.*, 2005; Gatlin *et al.*, 2006; Beg *et al.*, 2007; Bellon *et al.*, 2007; Wegmeyer *et al.*, 2007; Shi *et al.*, 2007). The role of PKC is complex because it is linked to both neurite outgrowth and retraction. On the other hand, Rac-specific GAPs such as chimaerins appear to antagonize neurite outgrowth by promoting growth cone collapse (Beg *et al.*, 2007; Wegmeyer *et al.*, 2007; Shi *et al.*, 2007). Although there is evidence to suggest a role for PA in promoting neuritogenesis (Watanabe *et al.*, 2004a), the exact mechanism involved remains unresolved.

Rac1, Cdc42 and RhoA play specific roles in neuritogenesis (Luo *et al.*, 1994; Luo *et al.*, 1996; Luo, 2000; Hakeda-Suzuki *et al.*, 2002; Ng and Graham, 2002; Luo, 2002; Govek *et al.*, 2005). Rac1 and Cdc42 drive the formation of lamellipodia and filopodia, structures which promote growth cone motility and neurite extension, while RhoA generally inhibits neurite outgrowth by directing growth cone collapse and neurite retraction (Kozma *et al.*,

1997; Yamaguchi *et al.*, 2001; Dan *et al.*, 2002; Miyashita *et al.*, 2004; Hajdo-Milasinovic *et al.*, 2007). While the function of these GTPases in neuritogenesis is well established, the molecular mechanisms governing their activity remain unclear. However, it is known that lipids such as PA promote the release of Rac1 from RhoGDI, leading to Rac1 activation (Chuang *et al.*, 1993a) as well as triggering neuritogenesis (Watanabe *et al.*, 2004a). By synthesizing PA, DGKs would be well suited to regulate Rac1-mediated neurite outgrowth. Indeed, DGK-derived PA drives neuritogenesis in N1E-115 neuroblastoma cells (Clejan *et al.*, 1996).

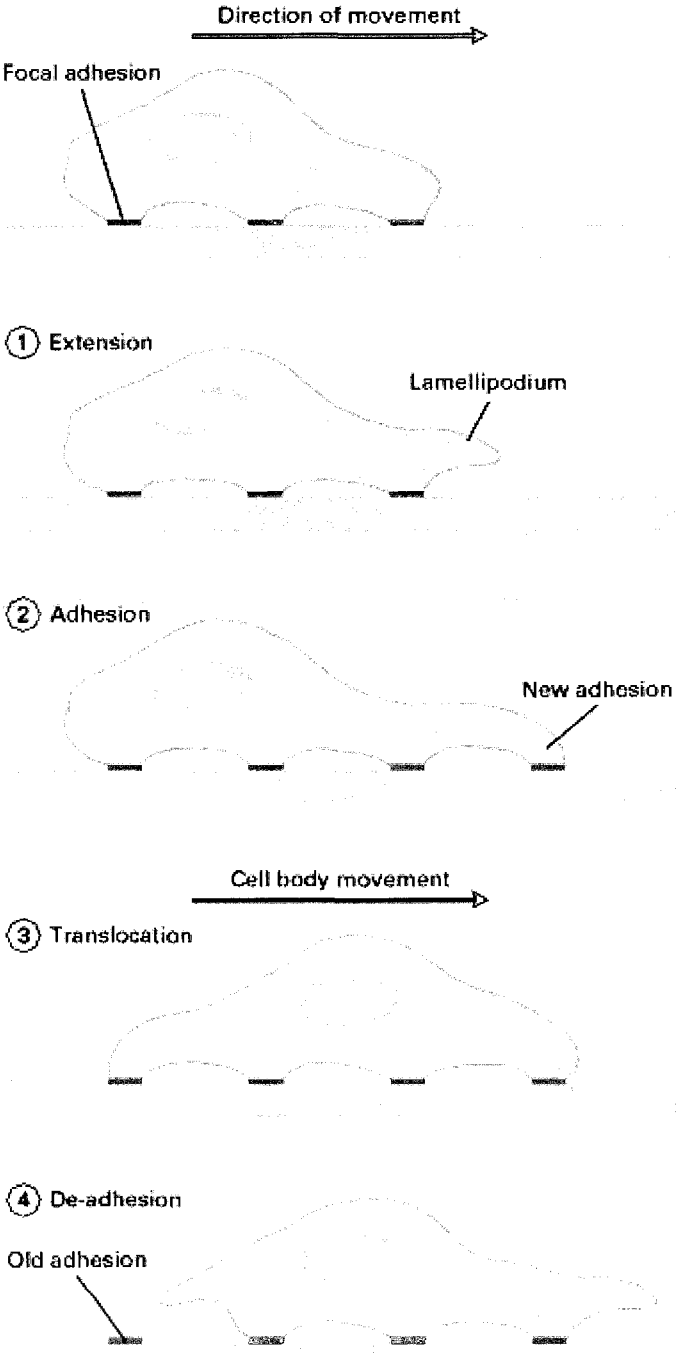
DGKζ is highly expressed in the nervous system (Hogan *et al.*, 2001; Hozumi *et al.*, 2003) and accumulates at the periphery of F-actin rich cell extensions in A172 neuronal cells (Topham and Prescott, 2001) raising the possibility that it plays a role in neuritogenesis. The binding of DGKζ to syntrophins may also be important for neurite outgrowth because syntrophins regulate the intracellular localization of DGKζ and because PDZ proteins regulate cell polarity and organize signaling complexes at synapses (Kim and Sheng, 2004; Chen and Olson, 2005; Nishimura *et al.*, 2005; Straight *et al.*, 2006; Wells *et al.*, 2006; Wu *et al.*, 2007). Collectively, these findings justified our interest in exploring the role of DGKζ and syntrophins in neurite outgrowth (Chapter 3).

Cell Migration

In eukaryotes cell migration underlies embryogenesis, wound closure, neutrophil chemotaxis in response to infection, and tumor invasion and metastasis (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Ridley, 2001). Migration involves four distinct steps: protrusion, adhesion, contraction and de-adhesion/tail retraction (Figure 1.14),

Figure 1.14. Schematic of the steps in cell migration.

(1) Movement begins with the protrusion of one or more lamellipodia from the leading edge of the cell. (2) Some lamellipodia adhere to the extracellular matrix via focal adhesions. (3) The bulk of the cytoplasm then moves forward. (4) Adhesions at the trailing edge of the cell are dissolved allowing the cell to move forward. Reproduced from Molecular Cell Biology, 4th Edition, W.H. Freeman and Company, Copyright 2000.


Direction of movement
Focal adhesion
1 Extension
Lamellipodium
2 Adhesion
New adhesion
Cell body movement
3 Translocation
4 De-adhesion
Old adhesion

processes driven in large part by reorganization of the actin cytoskeleton (Mitchison and Cramer, 1996).

Protrusive structures such as filopodia and lamellipodia form at the leading edge of motile cells, are highly dynamic, and contain dense arrays of actin filaments whose barbed ends are pointed in the direction of protrusion (Small, 1988; Mitchison and Cramer, 1996). In addition to being responsible for forward movement, these protrusive structures also establish nascent adhesions at the leading edge (Mitchison and Cramer, 1996; Price *et al.*, 1998; Partridge and Marcantonio, 2006; Guillou *et al.*, 2008). Failure of lamellipodia to adhere to the extracellular substrate leaves the membrane hovering above the surface and typically leads to rearward curling, resulting in a ruffled appearance (Figure 1.1) (Borm *et al.*, 2005). While these so-called "peripheral" ruffles are generally found at the leading edge of motile cells, a distinct, circular shaped ruffle can also appear on the dorsal surface of cells stimulated by growth factors (Mellstrom *et al.*, 1988; Eriksson *et al.*, 1992; Buccione *et al.*, 2004). The precise function of circular ruffles is unclear but they have traditionally been associated with bulk fluid-phase uptake, also known as macropinocytosis (Dowrick *et al.*, 1993; Swanson and Watts, 1995; Dharmawardhane *et al.*, 2000; Lanzetti *et al.*, 2004; Araki *et al.*, 2007). More recently, macropinocytosis has been suggested as a mechanism for recycling plasma membrane and for bulk internalization of growth factor receptors as well as endocytosis of E and N-cadherins (Falcone *et al.*, 2006; Orth and McNiven, 2006; Sharma and Henderson, 2007; Bryant *et al.*, 2007). Circular ruffles have also been proposed to function in three-dimensional invasion through extracellular matrix (Suetsugu *et al.*, 2003) and in the breakdown of stress fibers into a fine cortical actin meshwork that can be reused during lamellipodial extension (Buccione *et al.*, 2004).

The formation of protrusive structures is triggered by growth factors, phorbol esters, or adhesion to the extracellular matrix (Schliwa *et al.*, 1984; Meigs and Wang, 1986; Kitano *et al.*, 1986; Ridley *et al.*, 1992; Price *et al.*, 1998). These stimuli activate Rac1 and its downstream target PAK1 (Manser *et al.*, 1995; Dharmawardhane *et al.*, 1997; Bokoch *et al.*, 1998; Knaus and Bokoch, 1998; Benard *et al.*, 1999; Tu and Wigler, 1999). Rac1-deficient fibroblasts are defective in lamellipodia and ruffle formation, cell adhesion and cell spreading and migrate more slowly than their wild-type counterparts (Guo *et al.*, 2006; Vidali *et al.*, 2006). Fibroblasts stably expressing constitutively active PAK1 have large leading edge lamellipodia and are more motile than wild-type controls (Sells *et al.*, 1999). Conversely, cells expressing a kinase dead PAK1 mutant display multiple, randomly oriented lamellipodia which impede directional motility (Sells *et al.*, 1999). Rac1 and PAK1 therefore play important roles in actin remodeling underlying cell motility, and their activities must be well regulated for the cell to respond appropriately to stimuli.

Since cell migration is associated with the breakdown of $PI(4,5)P_2$ to DAG, PA synthesis, and increased Rac1 and PAK1 activity (Wright *et al.*, 1988; Boonen *et al.*, 1993; Zhou *et al.*, 1995; Siddiqui and English, 1997; Sells *et al.*, 1999; Evers *et al.*, 2000; Knight *et al.*, 2000; Pankov *et al.*, 2005; Choi *et al.*, 2007; ten Klooster and Hordijk, 2007; van Rheenen *et al.*, 2007) and because our findings indicated that DGKζ associates with Rac1, we hypothesized that DGKζ had a role to play in regulating cell migration. We explored this hypothesis in Chapter 5.

Statement of Problem and Hypothesis

Communication between the plasma membrane and the actin cytoskeleton drives complex cell behaviors such as myoblast fusion, neurite outgrowth, and cell motility. Fluctuations in the lipid composition of the membrane often accompany changes in the activity of Rho GTPases but it remains to be understood precisely how the two processes are linked.

The **central hypothesis** of this thesis is that DGKζ regulates Rac1-mediated remodeling of the actin cytoskeleton underlying myoblast fusion, neuritogenesis and cell migration by controlling the activity of DAG- and PA-responsive proteins that affect Rac1 activity. Furthermore, the regulation of DGKζ activity is critically dependent upon its correct subcellular localization, which is controlled by the interaction of the DGKζ C-terminus with syntrophins. Improper subcellular targeting of DGKζ leads to aberrant changes in the actin cytoskeleton, which may have consequences for normal cellular function.

Chapter 2

Diacylglycerol kinase-ζ localization in skeletal muscle is regulated by phosphorylation and interaction with syntrophins

Hanan Abramovici[†¶], Angela B. Hogan[†¶], Christopher Obagi[†], Matthew K. Topham[‡], and Stephen H. Gee*[†]

*Corresponding author

[†]Department of Cellular and Molecular Medicine
Center for Neuromuscular Disease
University of Ottawa
451 Smyth Rd
Ottawa, ON K1H 8M5
Canada

[‡]Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112 USA

Running Title: DGK-ζ Subcellular Localization in Muscle

Characters: 52,340

Key Words: Actin cytoskeleton, dystrophin, muscular dystrophy, protein kinase C, MARCKS

Published in Molecular Biology of the Cell 14: 4499-4511.

Contribution from authors

Hanan Abramovici and Stephen Gee co-wrote the manuscript. Hanan Abramovici performed experiments depicted in Figures 2.5-2.7, Figure 2.8A and Figure 2.9.

Acknowledgements

This work was supported by the Neuromuscular Research Partnership Program, an alliance of the Amyotrophic Lateral Sclerosis Society of Canada, the Muscular Dystrophy Association of Canada and the Canadian Institutes of Health Research (CIHR). S.G. is supported by a CIHR New Investigator Award. We thank Drs. Bernard Jasmin and Stephen Prescott for helpful discussions. ¶These authors contributed equally.

Abstract

Syntrophins are scaffolding proteins that link signaling molecules to dystrophin and the cytoskeleton. We previously reported that syntrophins interact with diacylglycerol kinase-ζ (DGK-ζ), which phosphorylates diacylglycerol (DAG) to yield phosphatidic acid (PA). Here, we show syntrophins and DGK-ζ form a complex in skeletal muscle whose translocation from the cytosol to the plasma membrane is regulated by protein kinase C (PKC)-dependent phosphorylation of the DGK-ζ MARCKS domain. DGK-ζ mutants that do not bind syntrophins were mislocalized and an activated mutant of this sort induced atypical changes in the actin cytoskeleton, indicating syntrophins are important for localizing DGK-ζ and regulating its activity. Consistent with a role in actin organization, DGK-ζ and syntrophins were colocalized with filamentous (F)-actin and Rac in lamellipodia and ruffles. Moreover, extracellular signal-related kinase (ERK)-dependent phosphorylation of DGK-ζ regulated its association with the cytoskeleton. In adult muscle, DGK-ζ was colocalized with syntrophins on the sarcolemma and was concentrated at neuromuscular junctions (NMJs), whereas in type IIB fibers it was found exclusively at NMJs. DGK-ζ was reduced at the sarcolemma of dystrophin-deficient *mdx* mouse myofibers, but was specifically retained at NMJs indicating that dystrophin is important for the sarcolemmal, but not synaptic localization of DGK-ζ. Together, our findings suggest syntrophins localize DGK-ζ signaling complexes at specialized domains of muscle cells, which may be critical for the proper control of lipid signaling pathways regulating actin organization. In dystrophic muscle, mislocalized DGK-ζ may cause abnormal cytoskeletal changes that contribute to disease pathogenesis.

Introduction

Scaffold proteins are thought to enhance the efficiency and specificity of signal transduction by organizing proteins involved in the same signaling pathway into macromolecular assemblies and localizing them to specific intracellular domains (Burack and Shaw, 2000). Syntrophins are scaffold proteins that link signaling molecules to the dystrophin family of cytoskeletal proteins (Albrecht and Froehner, 2002). Dystrophin is the product of the gene mutated or missing in patients with Duchenne muscular dystrophy (DMD), a fatal, inherited disorder causing progressive weakness and wasting of skeletal muscles (Rando, 2001). Dystrophin lies on the inner face of the plasma membrane of skeletal muscle fibers, where it links cortical actin with a multi-subunit complex (the dystrophin-glycoprotein complex) that spans the plasma membrane and connects to the surrounding extracellular matrix (Durbeej and Campbell, 2002). Mutations in dystrophin are thought to disrupt this linkage, causing sarcolemmal instability and contraction-induced lesions in the membrane (Petrof *et al.*, 1993). In the absence of dystrophin, syntrophins fail to localize at the sarcolemma (Adams *et al.*, 2001).

The syntrophin family comprises five isoforms (α1, β1, β2, γ1 and γ2) encoded by separate genes, but with the same modular domain organization. Each isoform has two tandem pleckstrin homology (PH) domains, a PSD-95, discs-large, ZO-1 (PDZ) domain, and a C-terminal syntrophin-unique region (SU) (Adams *et al.*, 1995; Ahn *et al.*, 1996; Piluso *et al.*, 2000). The latter half of syntrophin, which includes the PH2 and SU domains, mediates the interaction with members of the dystrophin family of proteins, including dystrophin, utrophin, and dystrobrevin (Albrecht and Froehner, 2002). This leaves the N-terminal half,

which includes the PDZ domain, available to interact with other proteins. Thus, syntrophins provide a link between signaling proteins and the actin cytoskeleton via dystrophin.

We previously identified an interaction between the PDZ domain of γ1-syntrophin and the C-terminus of DGK-ζ (Hogan *et al.*, 2001), an enzyme that phosphorylates DAG to yield PA. DAG is a lipid second messenger that transiently accumulates in cells stimulated by growth factors and other agonists (Hodgkin *et al.*, 1998). It exerts its effects primarily by activating proteins that contain DAG-responsive C1 domains such as PKC isoforms, Ras-guanyl nucleotide-releasing protein, Unc-13 and chimaerins (Topham and Prescott, 1999). These proteins are key regulators of a variety of cellular functions; therefore DAG signaling must be strictly controlled for an appropriate response. By metabolizing DAG, DGKs attenuate the activity of DAG-activated proteins. Moreover, because PA is mitogenic and modulates the activity of enzymes like phosphatidylinositol-4-phosphate 5-kinase, Raf-1 kinase, atypical PKCs and others (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000), DGKs also have positive regulatory roles in signal transduction.

DGK-ζ is one of nine mammalian isozymes that differ remarkably in their structure, modes of tissue expression and enzymatic properties (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000). Their structural diversity and different cellular localizations suggest different isoforms modify distinct DAG signaling events and are regulated by separate molecular mechanisms. However, translocation from the cytosol to various membrane compartments appears to be a general mechanism for activation of DGK isoforms.

Recently, Santos et al. (2002) showed that DGK-ζ translocates to the plasma membrane of T-cells in response to activation of an exogenously expressed muscarinic type I

receptor. DGK-ζ also translocates to the nucleus, where it regulates the amount of nuclear DAG (Topham *et al.*, 1998). Both events appear to be dynamically regulated by PKC-mediated phosphorylation of the MARCKS domain, a cluster of basic and serine (Ser) residues homologous to the phosphorylation site domain of the MARCKS protein (McLaughlin and Aderem, 1995). Additionally, we have shown that the nuclear accumulation of DGK-ζ is regulated by its interaction with γ1-syntrophin (Hogan *et al.*, 2001). DGK-ζ mutants with a non-functional C-terminal PDZ-binding motif accumulated in the nucleus, suggesting syntrophins are important for the stable retention of DGK-ζ in the cytoplasm. Because both syntrophins and DGK-ζ are expressed in multiple tissues and cell types, we hypothesized that their interaction may be a common mechanism for targeting the enzyme to specialized subcellular domains.

Here, we investigated the molecular mechanisms regulating the localization of DGK-ζ in skeletal muscle. We show that syntrophins are important for the stable association of DGK-ζ with the sarcolemma, that the two proteins form a complex whose subcellular localization is dynamically regulated by phosphorylation, and that they are involved in the regulation of the actin cytoskeleton.

Materials and Methods

Reagents

To make polyclonal antisera to human DGK-ζ, a peptide (CSERDAGPEPDKAPRRLNK) was synthesized, conjugated to KLH, and injected into rabbits. Antibodies were affinity purified from serum on immobilized peptide. The specificity of this antibody was verified as in (Bunting *et al.*, 1996) and by preabsorption of antibodies with the immunizing peptide. The BF-F3 mAb against type IIB myosin heavy chain isoform was from the German Collection of Microorganisms and Cell cultures (Braunschweig, Germany). A mAb to Rac1 was from BD Biosciences (San José, CA) and rabbit polyclonal anti-HA was from Zymed Laboratories (San Francisco, CA). Texas Red-conjugated α-BgTx was from Molecular Probes (Eugene, OR), FITC-conjugated phalloidin was from Sigma-Aldrich (St. Louis, MO) and Texas Red-, FITC-, and peroxidase-conjugated secondary antibodies were from Jackson Immuno-Research Laboratories (West Grove, PA).

Plasmids

To visualize DGK-ζ in transfected cells, we used a construct with three tandem N-terminal hemagglutinin (HA) epitope tags as described in (Topham *et al.*, 1998). A DGK-ζ construct with a C-terminal FLAG epitope tag and a mutant with all four Ser residues in the MARCKS domain changed to aspartate (Asp) (DGK-ζ^{M1}), have been described previously (Topham *et al.*, 1998; Hogan *et al.*, 2001). To construct DGK-$\zeta^{M1\text{-}FLAG}$, the mutant MARCKS domain was cut from DGK-ζ^{M1} with Sma I and Hind III enzymes and shuttled into DGK-ζ^{FLAG} in pcDNA3.1 digested with the same enzymes.

To visualize α1-syn in transfected cells, we made a construct encoding the full-length protein with two tandem N-terminal myc epitope tags. This was done by isolating a 1.3 kb restriction fragment by digestion of the α1-syn clone BC1012 (in pBluescript SK (-)) with EcoR I and BamH I restriction enzymes. A fragment encoding the N-terminus of α1-syn was amplified by the polymerase chain reaction using specific primers then digested with EcoR I and Apa I. Complementary oligonucleotides containing 5' Nhe I and 3' Apa I sites and encoding two myc epitope tags were synthesized, annealed and ligated along with the other fragments into pQBI25-fc1 (Quantum Biotechnologies) cut with Nhe I and BamH I.

Mutations near the ankyrin repeats of DGK-ζ were introduced using site directed mutagenesis: An Mxa I restriction site (2429T-->C) was engineered into the DGK-ζ cDNA then oligonucleotides encoding the desired mutations were ligated into the Bsa BI + Xma I restriction sites as described in (Topham *et al.*, 1998). The Raf-ER and MKP3 expression plasmids were a gift from Dr. Andrew Thorburn (Wake Forest University, Winston-Salem, NC).

Muscle Homogenates

Skeletal muscles were harvested from adult male C57BL/6 mice, flash-frozen in liquid nitrogen and stored at –80°C. 1-2 grams of frozen muscle was added to 10 volumes of homogenization buffer (25 mM Tris, pH 7.4, 1mM EDTA, 1mM EGTA, 100mM NaCl) plus protease inhibitors (10 μg/mL each of leupeptin, antipain, 4-(2-aminoethyl)-benzenesulfonyl-fluoride HCl (AEBSF), pepstatin A, and benzamidine HCl). The mixture was homogenized in a Waring blender then with a PT-3100 Polytron (Kinematica, Luzern, Switzerland). Nuclei were pelleted for 10 min at 1,000 x g. The supernatant was centrifuged at 48,000 x g

for 20 min, removed and assayed for protein content using the Bio-Rad Protein Assay kit (Hercules, CA). A fraction of the sample was mixed with 5X SDS-PAGE sample buffer and heated for 5 min at 95°C.

Cell Culture and Transfection

C2C12 myoblasts were grown on dishes coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in DMEM high-glucose with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine to 80-100% confluency. To induce differentiation, cells were switched to fusion medium containing 5% horse serum.

C2 myoblasts were transfected 18-24 h after plating at 70-80% confluency using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. For transfections of myoblasts plated on glass coverslips, 1 μg of purified DNA was added to 3 μl of FuGENE 6 (3:1 ratio) diluted in serum-free DMEM to a final volume of 100 μl. The mixture was incubated for 20 min at RT and added to 3 ml of growth medium in 35 mm dishes containing the coverslips. For large-scale transfections, 5.3 μg of DNA was added to 8 μl of FuGENE 6 (3:2 ratio) diluted in serum-free DMEM to a final volume of 100 μl. Transfections proceeded for 18-24 h.

For Raf activation experiments, plasmids were transfected into COS-7 cells as described in (Topham *et al.*, 1998). 24 h later, cells were treated with 2 μM estrogen for 30 min and then harvested. Equivalent amounts of protein were used for SDS-PAGE followed by western blotting using anti-DGK-ζ antibodies.

Subcellular Fractionation

Cultures of C2 myoblasts in 100 mm dishes were washed 3 X with cold PBS, then scraped into 1 ml of PBS and centrifuged. All subsequent steps were performed on ice or at 4°C. The cells were resuspended in 200 µl of lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1mM $MgCl_2$, 1 mM $CaCl_2$ and protease inhibitors) and lysed by 10 s sonication with a Branson Sonifier equipped with a 5 mm tip (power output 1, at 50% duty cycle). An aliquot (*Total* fraction) was removed and boiled in SDS-PAGE loading buffer. The lysates were centrifuged at 14,000 x *g* for 10 min to remove nuclei and unbroken cells. Then the supernatant was collected and centrifuged at 100,000 x g for 1 h at 4°C. This supernatant (*Cytosol* fraction) was removed and the pellet was resuspended by vortexing in buffer containing 1% Triton X-100 (TX-100) then centrifuged at 100,000 x g for 1 h at 4°C. The final supernatant (*Membrane* fraction) was collected and the pellet (*Cytoskeleton* fraction) was resuspended and boiled in SDS-PAGE loading buffer.

Immunofluorescence

C57BL6 and C57BL/10ScSn-Dmd^{MDX}/J (*mdx*) mice were sacrificed by CO_2 overdose. The tibialis anterior, soleus, and plantaris muscles were immediately removed, placed in Histo Prep (Fisher Scientific, Fair Lawn, NJ), frozen in liquid nitrogen-cooled isopentane, and stored at –80°C. 8 µm thick transverse sections were cut on a cryostat and stored at -80 °C. The sections were thawed, ringed with a hydrophobic boundary and rinsed with buffer A (0.5% BSA and 0.15% glycine in PBS, pH 7.4). Then they were fixed with 4% paraformaldehyde, washed, and blocked in buffer A + 5% normal goat serum. They were then incubated with affinity-purified antibody diluted in buffer A + 0.3% TX-100 in a closed,

damp chamber for 1 h at RT or overnight at 4 °C. The sections were then rinsed in buffer A and incubated with a 1:300 dilution of FITC- or Texas Red dye-conjugated secondary antibody in buffer A + 0.3% TX-100 for 1 h at RT. The sections were rinsed in PBS for 3 x 15 min and cover-slipped with Fluoromount G (EMS, Fort Washington, PA). Images were captured on a Zeiss Axioskop microscope equipped with an Axiocam digital camera using Axiovision 3.0 software. Images were processed using Adobe Photoshop 5.5.

Quantification of Membrane Localization

C2 myoblasts were fixed and stained as in (Hogan *et al.*, 2001). Transfected cells were scored as having cytoplasmic, partially membrane-associated, or completely membrane-associated protein according to the following criteria: A cytoplasmic localization was defined as protein diffusely and equally distributed throughout the cell. Complete membrane localization was defined as protein highly concentrated at the plasma membrane around the entire perimeter of the cell. Cells with partial membrane localization had protein concentrated at the plasma membrane in at least one part of the cell. In double-labeled cells, each fluorophore was scored separately. The results are the average of three separate experiments with a minimum of 200 cells per condition.

Immunofluorescence intensity profile plots were made using Scion Image software (Scion Corp., Frederick, MD) and data exported and graphed using SigmaPlot 8.0 (SPSS Inc., Chicago, IL). Scanned regions were selected by drawing a 50 μm reference line, 8 pixels wide, near the middle of the cell so as to include the plasma membrane, cytoplasm and nucleus. Values for the profile plots were derived by averaging the pixel intensity values across the width of the line.

Immunoprecipitation

All steps were carried out at 4°C or on ice. 5 day-old C2 myotube cultures were washed twice with 10 ml of ice-cold PBS pH 7.4 then lysed with 0.5 ml lysis buffer (50mM Tris, pH 7.5, 150mM NaCl, 1% TX-100 and protease inhibitors) per 100 mm dish. Cells were lysed on ice for 30 min, then scraped and centrifuged for 10 min at 4°C. The supernatant was collected and an aliquot of this starting material (*Input*) was boiled in reducing SDS-PAGE sample buffer. Samples were pre-cleared for 1-2 h using 50 µl of a 50% slurry of washed Protein A/G Plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). One to five µg of antibody was added to 0.8 ml of pre-cleared supernatant and incubated at 4°C with mixing for 2 h. Then 50 µl of Protein A/G Plus agarose beads were added and incubated an additional 2-3 h at 4°C with mixing. The immune complexes were collected by centrifugation and washed 3 x 10 min with lysis buffer. Proteins were eluted from the beads by boiling in SDS-PAGE sample buffer. The samples were then centrifuged at 21,000 x g for 1-2 min and subjected to SDS-PAGE and western blot analysis.

Pull-down Assays

Bacterial fusion proteins were expressed and purified as in (Gee *et al.*, 2000). 2.5 mg of GST or a fusion protein consisting of GST and amino acids 787-928 of human DGK-ζ (GST-DGK-ζ) (Hogan *et al.*, 2001) were incubated with 500 µl of a 50% slurry of Glutathione-Sepharose 4B beads (Amersham-Pharmacia Biotech Inc., Piscataway, NJ) for 20 min at 4°C, followed by washing in PBS, pH 7.2. Then an equal volume of beads (100 µl) was incubated with the soluble fraction of mouse skeletal muscle homogenates for 2 h at 4°C with constant mixing. A fraction of the starting material (*Input*) was boiled in SDS-PAGE

loading buffer. The beads were washed twice with ice-cold PBS pH 7.2, then once with PBS + 1% TX-100. The bound proteins were eluted in an equal volume of loading buffer and boiled for 5 min.

Results

DGK-ζ and Syntrophins Associate in Skeletal Muscle

Western blotting indicated that both DGK-ζ and syntrophins are detectable in extracts of adult skeletal muscle (Figure 2.1A and B). To verify the biochemical association of DGK-ζ and syntrophins, we performed pull-down experiments from muscle extracts using a GST fusion protein of DGK-ζ. The bound proteins were analyzed by immunoblotting for syntrophins. GST-DGK-ζ, but not GST alone captured a significant fraction of syntrophins in the extract (Figure 2.1B). For coimmunoprecipitations, C2 myotubes were used since more DGK-ζ could be solubilized from cells than from muscle tissue. DGK-ζ was efficiently precipitated by its specific antibody, but not by control rabbit IgG (Figure 2.1C). Although syntrophins were specifically coimmunoprecipitated by the DGK-ζ antibody, only a fraction of the total bound DGK-ζ. This result may be explained by the fact that syntrophins have additional binding partners in skeletal muscle (Brenman *et al.*, 1996a; Gee *et al.*, 1998). Nevertheless, these results suggest DGK-ζ and syntrophins form a stable complex in muscle cells.

DGK-ζ and Syntrophins Colocalize in Skeletal Muscle

Indirect immunofluorescence labeling of transverse sections of mouse tibialis anterior (TA) muscle revealed a fiber-type specific pattern of DGK-ζ expression, which was blocked by preincubation of the antibody with its immunogenic peptide (Figure 2.2A and B). Higher magnification revealed strong staining of the sarcolemma and punctate intracellular staining

Figure 2.1. DGK-ζ and syntrophins associate in skeletal muscle. (A) DGK-ζ migrates as a doublet of ~116 kDa in immunoblots of mouse skeletal muscle homogenates. (B) Beads charged with GST alone or with a GST-DGK-ζ fusion protein were incubated with skeletal muscle extracts. GST-DGK-ζ, but not GST, captured a significant portion of syntrophin in the offered extract. Input = 5% of starting material. (C) DGK-ζ and syntrophins are coimmunoprecipitated from muscle cell extracts. Lysates of C2 myotubes were immunoprecipitated with an antibody to DGK-ζ or with control IgG. The immunoprecipitates were analyzed by immunoblotting for DGK-ζ and syntrophins. With longer exposures, DGK-ζ was detectable in the Input lane. Input = 5% of starting material.

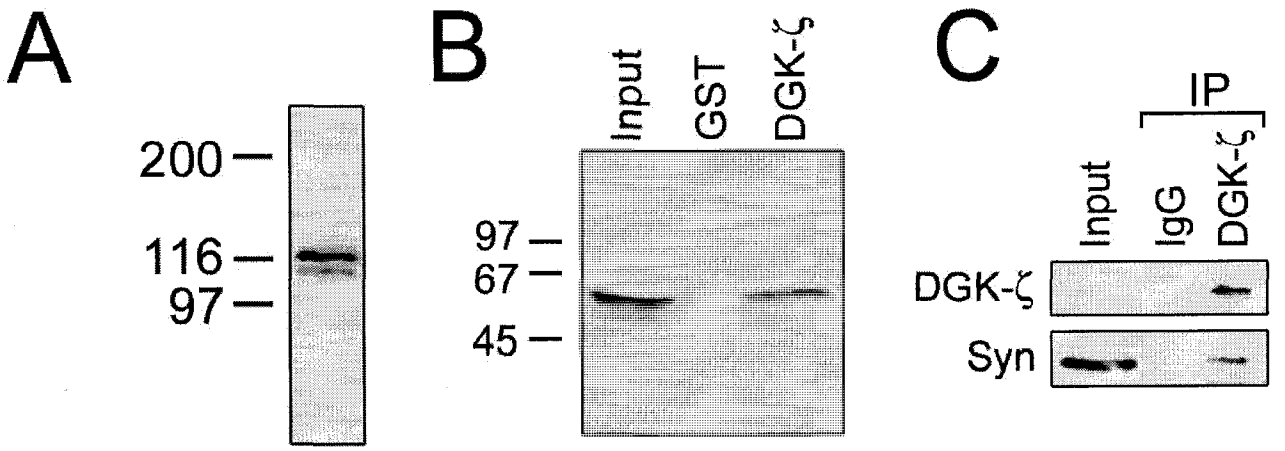
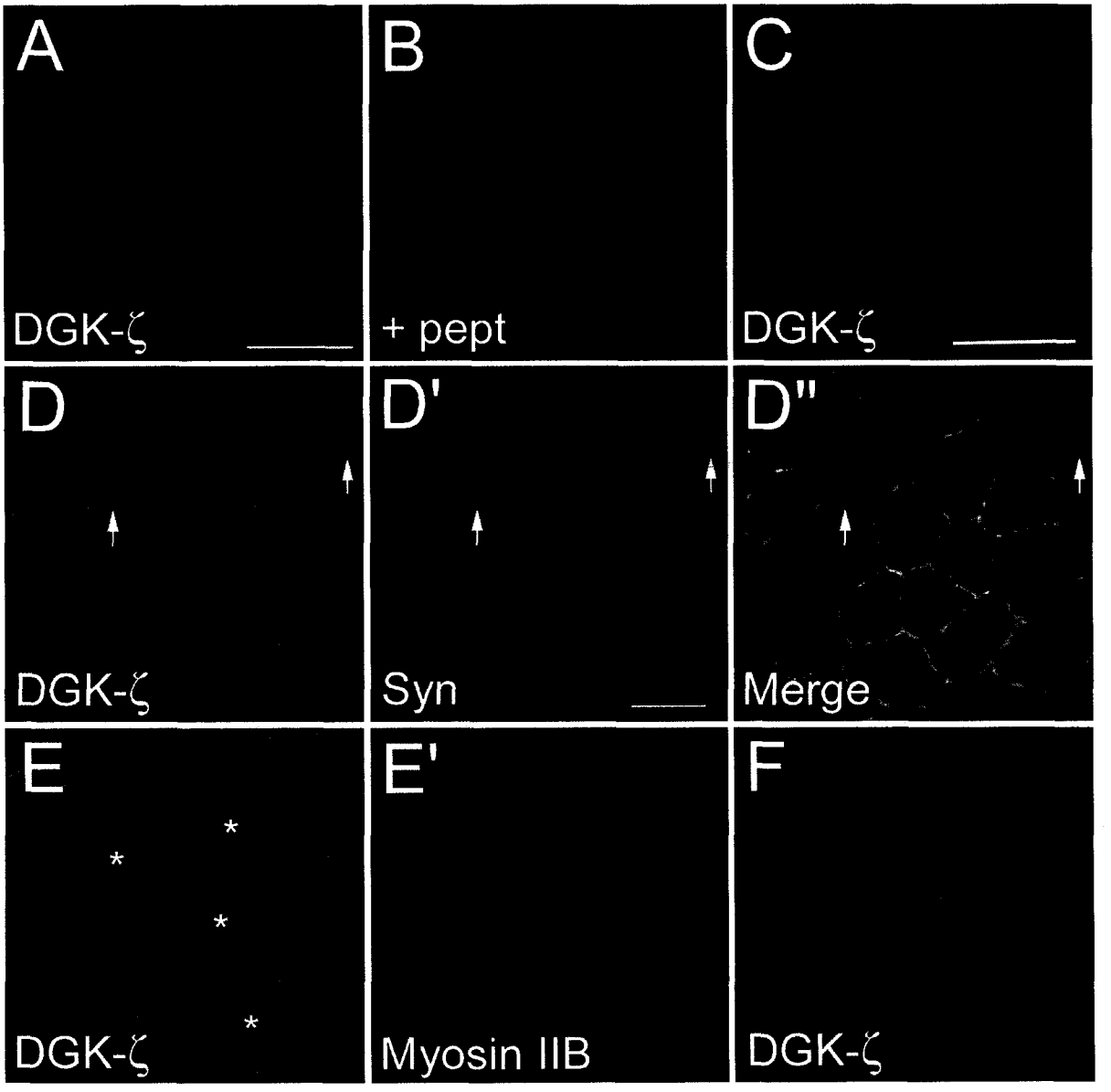

A
200
116
97
B
Input
GST
DGK-ζ
97
67
45
C
IP
Input
IgG
DGK-ζ
DGK-ζ
Syn

Figure 2.2. Immunofluorescence localization of DGK-ζ in skeletal muscle and colocalization with syntrophins. (A-C) Transverse sections of mouse tibialis anterior (TA) muscle were fixed and stained with affinity-purified antibodies to DGK-ζ and FITC-conjugated secondary antibodies. At low magnification (A) DGK-ζ immunoreactivity was observed in a subset of muscle fibers. (B) The specificity of the DGK-ζ immunolabeling was confirmed by preincubation of the antibody with its immunogenic peptide, which completely eliminated the signal. (C) Higher magnification revealed diffuse cytoplasmic and strong sarcolemmal labeling. (D-D") Mouse TA muscle was double labeled for DGK-ζ (D) and syntrophins (D'). Syntrophins were visualized with mAb 2101 and Texas-Red-conjugated secondary antibodies. (D") The merged image shows syntrophins and DGK-ζ colocalize at the sarcolemma. The *arrows* indicate regions where DGK-ζ staining is absent and a corresponding reduction in syntrophin staining. (E-E'). In mouse TA muscle, DGK-ζ is absent or greatly reduced in type IIB fibers. (F) DGK-ζ is expressed in all fibers of the soleus muscle (*lower left*), but not in the adjacent plantaris muscle (*upper right*). The *asterisks* indicate DGK-ζ-negative fibers stained for myosin IIB. Scale bars = 100 μm (A) and 25 μm (C-F).


A
DGK-ζ
B
+ pept
C
DGK-ζ
D
DGK-ζ
D'
Syn
D"
Merge
E
DGK-ζ
E'
Myosin IIB
F
DGK-ζ

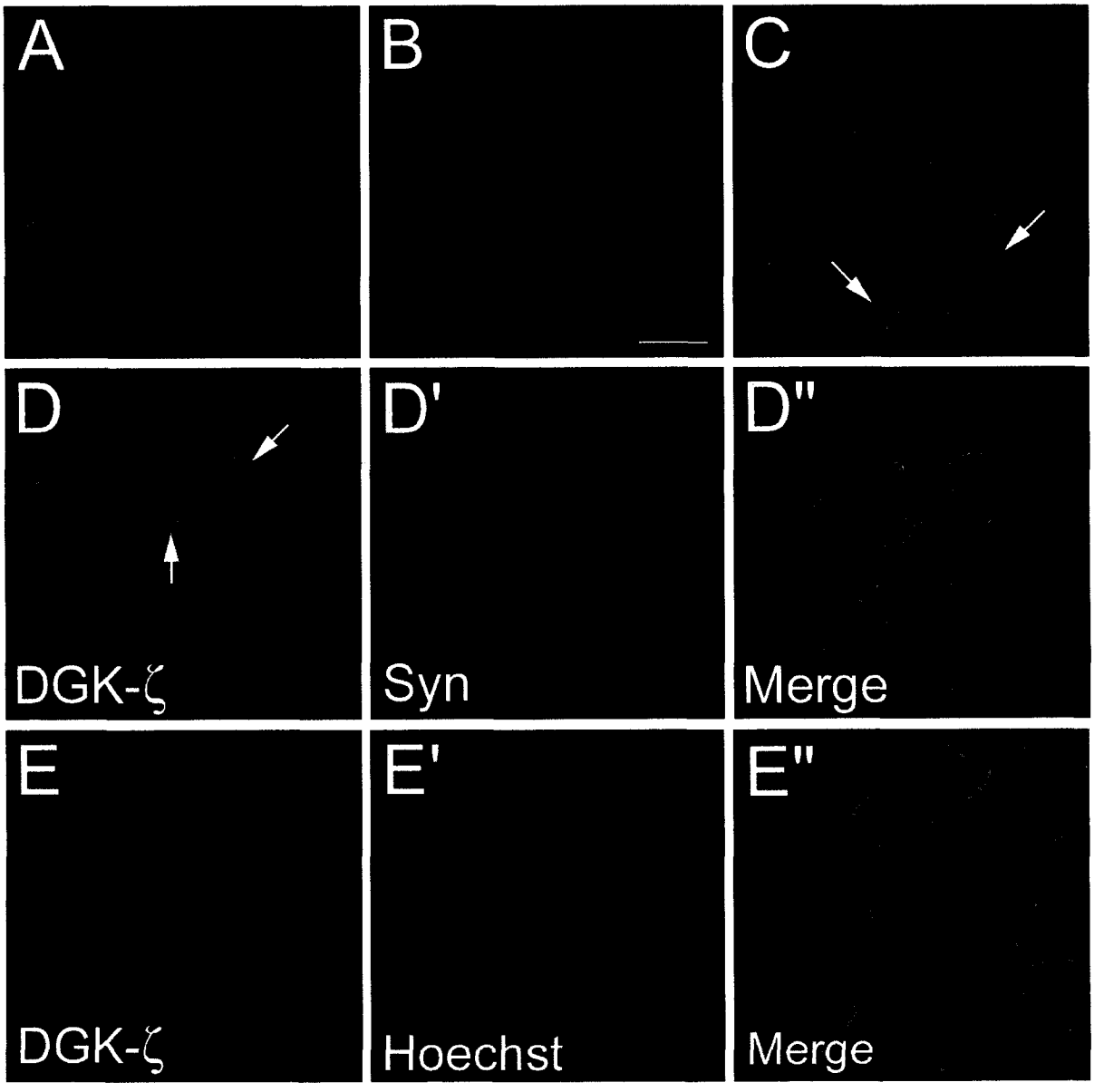
(Figure 2.2C). In double-labeled sections, DGK-ζ and syntrophins were colocalized at the sarcolemma (Figure 2.2D-D"). Combined with the biochemical data above, these results suggest DGK-ζ and syntrophins form a complex at the sarcolemma of skeletal muscle fibers.

The mouse TA muscle contains both oxidative (types IIA and IIX) and glycolytic (type IIB) fibers. In the latter, DGK-ζ staining was undetectable (Figure 2.2E and E'). In plantaris muscle, which also contains a mix of fiber types, a mosaic pattern of DGK-ζ labeling was observed (Figure 2.2F, *upper right*). However, all fibers were labeled in soleus muscle, which contains only type I and IIA fibers (Figure 2.2F, *lower left*). These results suggest DGK-ζ is found on the sarcolemma of all fibers types except IIB.

DGK-ζ is Mislocalized in *mdx* Muscle

In *mdx* mouse muscle, the absence of dystrophin results in a dramatic reduction of sarcolemmal syntrophins (Froehner *et al.*, 1987). Compared to control muscle fibers (Figure 2.3A), *mdx* fibers had substantially lower levels of DGK-ζ, although detectable sarcolemmal staining remained (Figure 2.3B). Interspersed among these were groups of small caliber regenerating fibers where DGK-ζ and syntrophin staining was noticeably increased (Figure 2.3C and D-D"). Central patches of DGK-ζ immunoreactivity were observed in *mdx* (Figure 2.3E), but not control fibers (Figure 2.3A). Hoechst staining revealed they correspond to central nuclei and the merged image shows that virtually every nucleus was associated with DGK-ζ immunoreactivity (Figure 2.3E-E''). In normal muscle, there was no obvious enrichment of DGK-ζ in large punctate structures at the sarcolemma, suggesting the enzyme is not normally associated with peripheral myofiber or satellite cell nuclei. Thus, these

Figure 2.3. DGK-ζ is reduced on the sarcolemma of *mdx* myofibers and is associated with central nuclei. Sections of TA muscle from normal (A) and *mdx* (B) mice were stained for DGK-ζ and photographed with identical exposure times. (C) In *mdx* muscle, occasionally groups of small caliber fibers (*arrows*) with increased DGK-ζ expression were observed. (D-D") The increased DGK-ζ immunoreactivity in small caliber fibers coincided with increased syntrophin expression. (E-E") In *mdx* TA muscle fibers, DGK-ζ was associated with central nuclei revealed by Hoechst stain (E'). Scale bar = 25 μm.


A
B
C
D
DGK-ζ
D'
Syn
D"
Merge
E
DGK-ζ
E'
Hoechst
E"
Merge

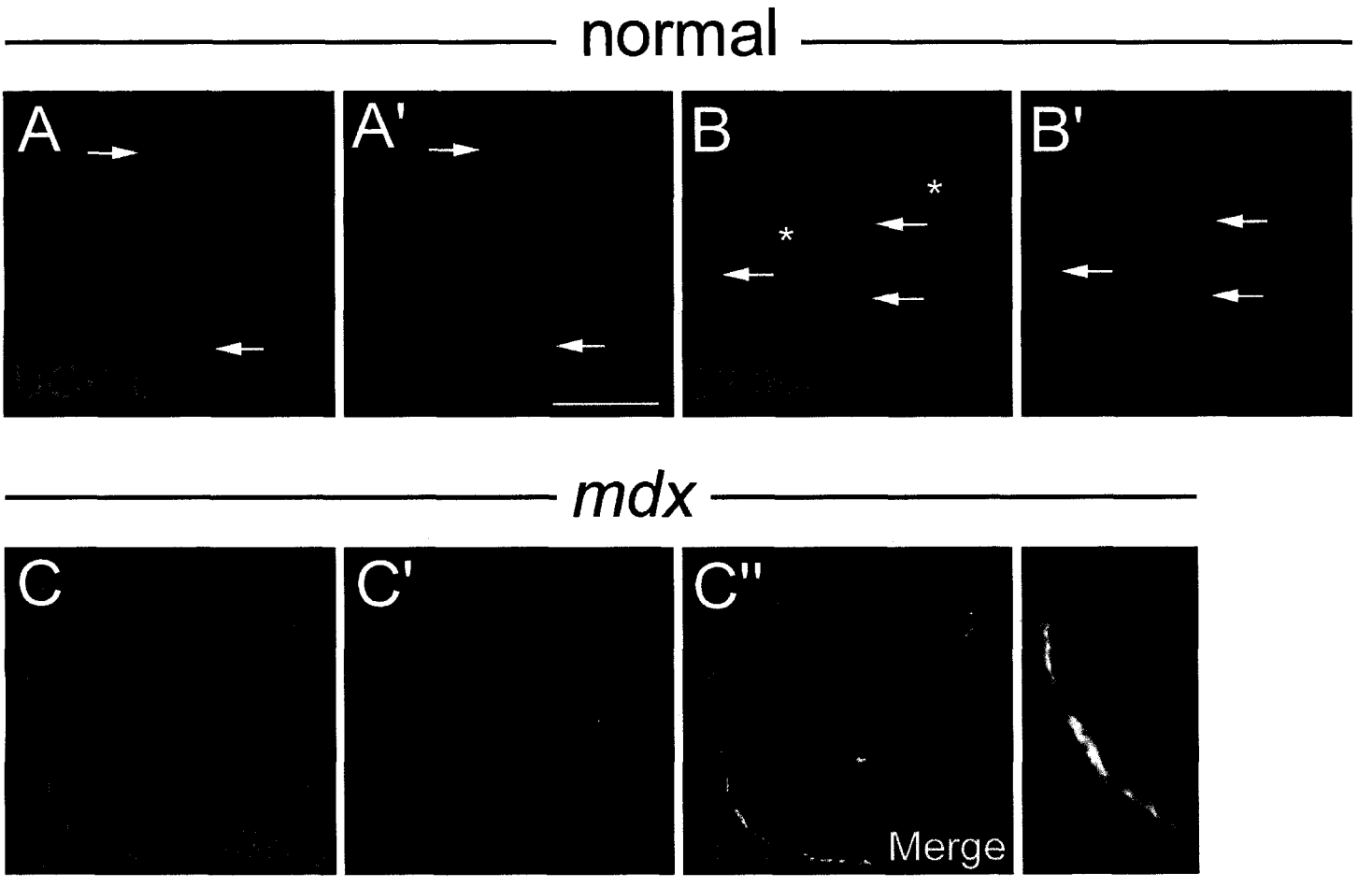
results suggest DGK-ζ accumulates in the nucleus of muscle fibers in the absence of sarcolemmal syntrophin and dystrophin.

DGK-ζ is Concentrated at NMJs

Three syntrophin isoforms are enriched at NMJs in normal muscle: β2-syn is restricted to the postsynaptic membrane, while α1- and β1-syn are additionally present on the extrajunctional sarcolemma (Peters *et al.*, 1997; Peters *et al.*, 1994). In fibers from control mice, sarcolemmal DGK-ζ labeling was noticeably increased at NMJs revealed by staining with α-BgTx, which specifically labels nicotinic acetylcholine receptors (AChRs) (Figure 2.4A and A', *arrows*). However, in type IIB fibers (indicated by *asterisks* in Figure 2.4B), DGKζ immunoreactivity was localized exclusively at NMJs (Figure 2.4B and B', *arrows*).

Although DGK-ζ staining was dramatically reduced on the extrajunctional sarcolemma of *mdx* fibers, it remained concentrated at synaptic sites (Figure 2.4C and C'), likely due to its association with syntrophins, which are specifically retained at NMJs (Peters *et al.*, 1997; Peters *et al.*, 1994; Yang *et al.*, 1995). Notably, DGK-ζ immunoreactivity extended beyond the AChR-rich regions and into the surrounding perisynaptic membrane (Figure 2.4C", *boxed region and panel at right*).

Figure 2.4. DGK-ζ is specifically targeted to NMJs in type IIB fibers and retained at NMJs in *mdx* muscle. TA muscles were labeled with an antibody to DGK-ζ and with α-BgTx, which labels AChRs. (A, A') In most fibers, DGK-ζ was present on the extrajunctional sarcolemma and was concentrated at NMJs (*arrows*). (B, B') In type IIB fibers (indicated by *asterisks*), DGK-ζ was restricted to junctional regions (*arrows*). (C-C") In *mdx* myofibers, DGK-ζ is absent from extrajunctional regions, but remains concentrated at synaptic sites. Higher magnification of the boxed region in C" (*bottom right*) shows DGK-ζ extends beyond the AChR-rich regions. Scale bar = 25 μm.


normal
A
A'
B
B'
*
*
mdx
C
C'
C''
Merge

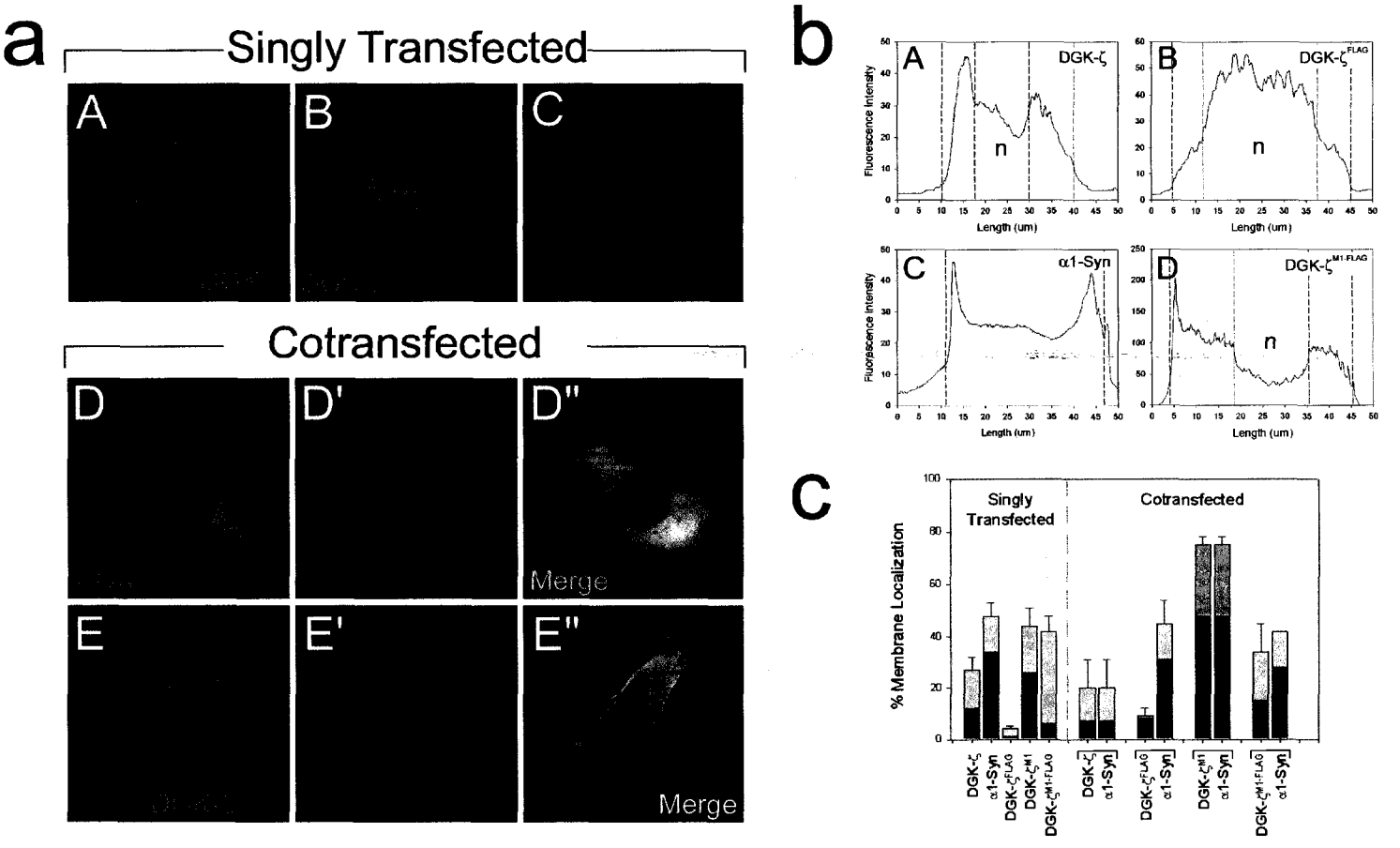
Reciprocal Interactions Regulate the Localization of DGK-ζ and Syntrophin in Muscle Cells

To gain insight into the molecular mechanisms regulating the membrane localization of DGK-ζ in muscle cells, we transiently transfected C2 myoblasts with a plasmid encoding wild type DGK-ζ with three N-terminal HA epitope tags. Wild type DGK-ζ was associated with the plasma membrane in 27 ± 5% of the cells examined, with only 12% demonstrating complete membrane localization (Figure 2.5c). In cells with a cytoplasmic distribution of DGK-ζ, the protein was diffusely distributed throughout and was largely excluded from the nucleus (Figure 2.5a, *panel* A).

We have previously shown that a C-terminal FLAG epitope tag blocks the binding of the DGK-ζ C-terminus to the PDZ domain of syntrophin (Hogan *et al.*, 2001). In C2 cells transfected with DGK-ζ^{FLAG}, only 4 ± 1% of the cells had any discernable membrane localization, and only 1% of the cells showed complete membrane localization (Figure 2.5c). DGK-ζ^{FLAG} also accumulated in the nucleus (Figure 2.5a, *panel* B), as previously observed in HeLa cells (Hogan *et al.*, 2001). Thus, in the absence of syntrophin interaction, DGK-ζ is decreased at the plasma membrane and accumulates in the nucleus.

PDZ-containing proteins act as scaffolds for the assembly of protein complexes at the plasma membrane (Sheng and Sala, 2001). Thus, we reasoned that α1-syntrophin, the predominant isoform in skeletal muscle (Adams *et al.*, 1993), might recruit DGK-ζ to the plasma membrane. By itself, myc-tagged α1-syntrophin (α1-Syn) was localized at the plasma membrane (Figure 2.5a, *panel C*) in 48 ± 5% (34% complete) of transfected C2 cells (Figure 2.5c). Surprisingly, DGK-ζ and α1-syn were colocalized in the

Figure 2.5. Reciprocal interactions regulate the localization of DGK-ζ and α1-syn in muscle cells. (a) C2 myoblasts were singly (A-C) or doubly transfected (D-E") with cDNAs encoding the indicated epitope-tagged proteins. The cells were fixed and labeled with antibodies specific for each epitope tag. (b) Representative profile plots of the fluorescence intensity of transfected C2 myoblasts labeled for the indicated constructs showing cytoplasmic (A), nuclear (B), complete membrane localization (C), and partial membrane localization (D). The profile plot in (D) is taken from the cell shown in Figure 2.6a, *panel B*. The vertical lines indicate transition points in the fluorescence intensity between cellular compartments. (c) The graph shows the percentage of cells with membrane localized protein in C2 myoblasts expressing the indicated constructs. Membrane localization was quantified as described in Materials and Methods. Grey bars indicate the percentage of cells with any membrane localization and black bars indicate complete membrane localization. The data are the average of three independent experiments. Error bars indicate S.E.M. n, nucleus.


a
Singly Transfected
A
B
C
Cotransfected
D
D'
D"
Merge
E
E'
E"
Merge
b
A
DGK-ζ
B
DGK-ζ FLAG
C
α1-Syn
D
DGK-ζ M1-FLAG
n
Fluorescence Intensity
Length (um)
c
Singly Transfected
Cotransfected
% Membrane Localization
DGK-ζ
α1-Syn
DGK-ζ FLAG
DGK-ζ M1
DGK-ζ M1-FLAG

cytoplasm in the majority of cotransfected cells (Figure 2.5a, *panels* D-D"). Only 20 ± 11% (7% complete) of the cells showed any membrane localization, which is comparable to cells expressing DGK-ζ alone and substantially lower than for α1-syn alone (Figure 2.5c).

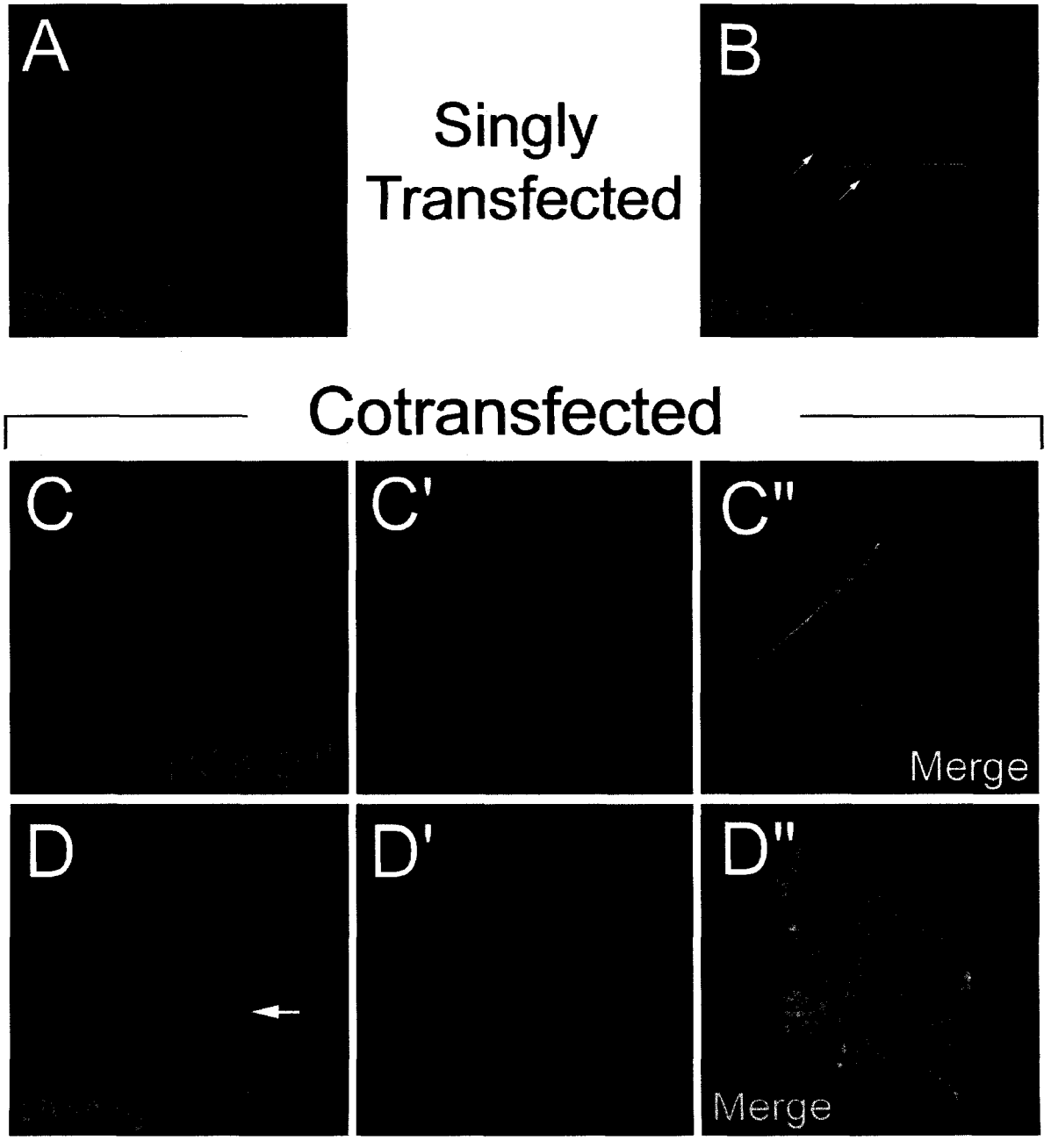
In contrast, DGK-ζ^{FLAG} failed to redistribute α1-Syn from the plasma membrane, even though it was expressed at similar levels to DGK-ζ (Figure 2.5a, *panels* E-E"). In 45 ± 9% (31% complete) of the doubly transfected cells, α1-syn was localized at the membrane, whereas in the same cells only 9 ± 3 % (8% complete) had observable DGK-ζ at the membrane (Figure 2.5c). Moreover, as noted earlier, DGK-ζ^{FLAG} amassed in the nucleus (Figure 2.5a, *panel* E). These results suggest that the redistribution of α1-Syn from the plasma membrane occurs by a specific interaction with the DGK-ζ C-terminus and argue against the idea that it is an artifact of overexpression.

Phosphorylation Targets DGK-ζ and Syntrophin to the Plasma Membrane

To determine if phosphorylation of the MARCKS domain regulates the association of DGK-ζ with the plasma membrane, we generated a mutant (DGK-ζ^{M1}) mimicking phosphorylation by substituting Asp for Ser. DGK-ζ^{M1} was membrane-associated (Figure 2.6A) in 44 ± 7% (26% complete) of the transfected cells (Figure 2.5c), approximately a 2-fold increase compared to cells expressing wild type DGK-ζ. These results suggest phosphorylation of the MARCKS domain targets DGK-ζ to the plasma membrane of muscle cells.

DGK-ζ^{M1} with a C-terminal FLAG tag (DGK-$\zeta^{M1\text{-}FLAG}$) was membrane-associated (Figure 2.6B) in 42 ± 6% of the cells, similar to DGK-ζ^{M1}, however, only 6% of the cells showed complete membrane localization (Figure 2.5c). A plot of the immunofluorescence

Figure 2.6. MARCKS domain-phosphorylation regulates DGK-ζ localization. (a) C2 myoblasts expressing the indicated constructs were fixed and visualized as described in Figure 2.5. The *arrow* in D indicates nuclear DGK-ζ staining.


A
Singly
Transfected
B
Cotransfected
C
C'
C''
Merge
D
D'
D''
Merge

intensity is shown in Figure 2.5b (*panel* D). These results suggest syntrophin is not absolutely required for the plasma membrane localization of DGK-ζ, but may stabilize (phosphorylated) DGK-ζ at the plasma membrane.

Syntrophins Increase the Membrane Localization of Phosphorylated DGK-ζ

To test this idea, we coexpressed DGK-ζ^{M1} with α1-syn in C2 cells. Both proteins were membrane associated in 75 ± 3% of the cotransfected cells, a substantial increase compared to cells expressing either protein alone (Figure 2.5c). In 48% of the cells, the two proteins were highly concentrated at the plasma membrane (complete membrane localization) (Figure 2.6C-C"). These results suggest that phosphorylation of the MARCKS domain signals the complex of DGK-ζ and syntrophin to move to the plasma membrane.

As a control, we coexpressed DGK-$\zeta^{M1\text{-}FLAG}$ with α1-syn. As expected, α1-syn did not increase the membrane localization of DGK-$\zeta^{M1\text{-}FLAG}$ and the percentage of cells with membrane localization was similar to DGK-$\zeta^{M1\text{-}FLAG}$ expressed alone (Figure 2.5c). Moreover, the two proteins were not exactly colocalized (Figure 2.6D-D"). Collectively, our results suggest syntrophins promote the stable association of phosphorylated DGK-ζ with the plasma membrane through PDZ interactions, which may be critical for the proper targeting of DGK-ζ to specialized membrane domains in muscle cells.

Endogenous DGK-ζ and Syntrophin are Concentrated in Regions of the Plasma Membrane Undergoing Actin Reorganization

Since overexpressed proteins may not reflect the location of their native counterparts, we determined the subcellular distribution of endogenous DGK-ζ and syntrophins in C2

myoblasts. Punctate DGK-ζ labeling was found throughout most of the cytoplasm and was largely excluded from the nucleus, similar to the overexpressed HA-tagged protein (Figure 2.7A). DGK-ζ was also concentrated in specific regions of the cell periphery including membrane ruffles and the leading edge of lamellipodia (Figure 2.7A-C, *small arrows*). At these sites, DGK-ζ immunoreactivity overlapped with F-actin (Figure 2.7A', *small arrows*), but it did not colocalize with stress fibers (*large arrows*) or with cortical F-actin (*arrowheads*). It also colocalized with syntrophins in these regions (Figure 2.7B-B", *small arrows*) and with Rac, a Rho-family GTPase that mediates actin reorganization underlying membrane ruffling and lamellipodia formation (Figure 2.7C-C", *arrows*) (Etienne-Manneville and Hall, 2002). Together, these results suggest DGK-ζ and syntrophins participate in the assembly or organization of the actin cytoskeleton.

Ras/ERK MAP kinase Phosphorylation Regulates DGK-ζ Association with the Cytoskeleton

Subcellular fractionation revealed that the two DGK-ζ species detected in immunoblots of muscle extracts have different localizations (Figure 2.8A). The upper band was present in the cytosolic fraction, while the lower band was recovered in the detergent-insoluble cytoskeleton fraction (Figure 2.8A). Neither band was detected in the membrane fraction. In contrast, syntrophins were detected in every fraction.

To investigate the basis for the difference in the size and subcellular localization of the two DGK-ζ species, we analyzed potential phosphorylation sites in a proline-rich sequence near the C-terminus (Figure 2.8B). Every Ser and Thr residue in the sequence was changed to Ala except for the one shown at the top of the corresponding lane of the immunoblot in

Figure 2.7. DGK-ζ colocalizes with actin, syntrophin and Rac1 in membrane ruffles and at the leading edge of lamellipodia. C2 myoblasts were stained with an antibody to DGK-ζ and with either phalloidin to detect F-actin (A-A"), or with antibodies to syntrophin (B-B") or Rac1 (C-C"). (A-A") *Small arrows* indicate membrane ruffles and lamellipodia leading edges, vertical *arrowheads* actin stress fibers and horizontal *arrowheads* cortical actin. Scale bar = 20 μm.

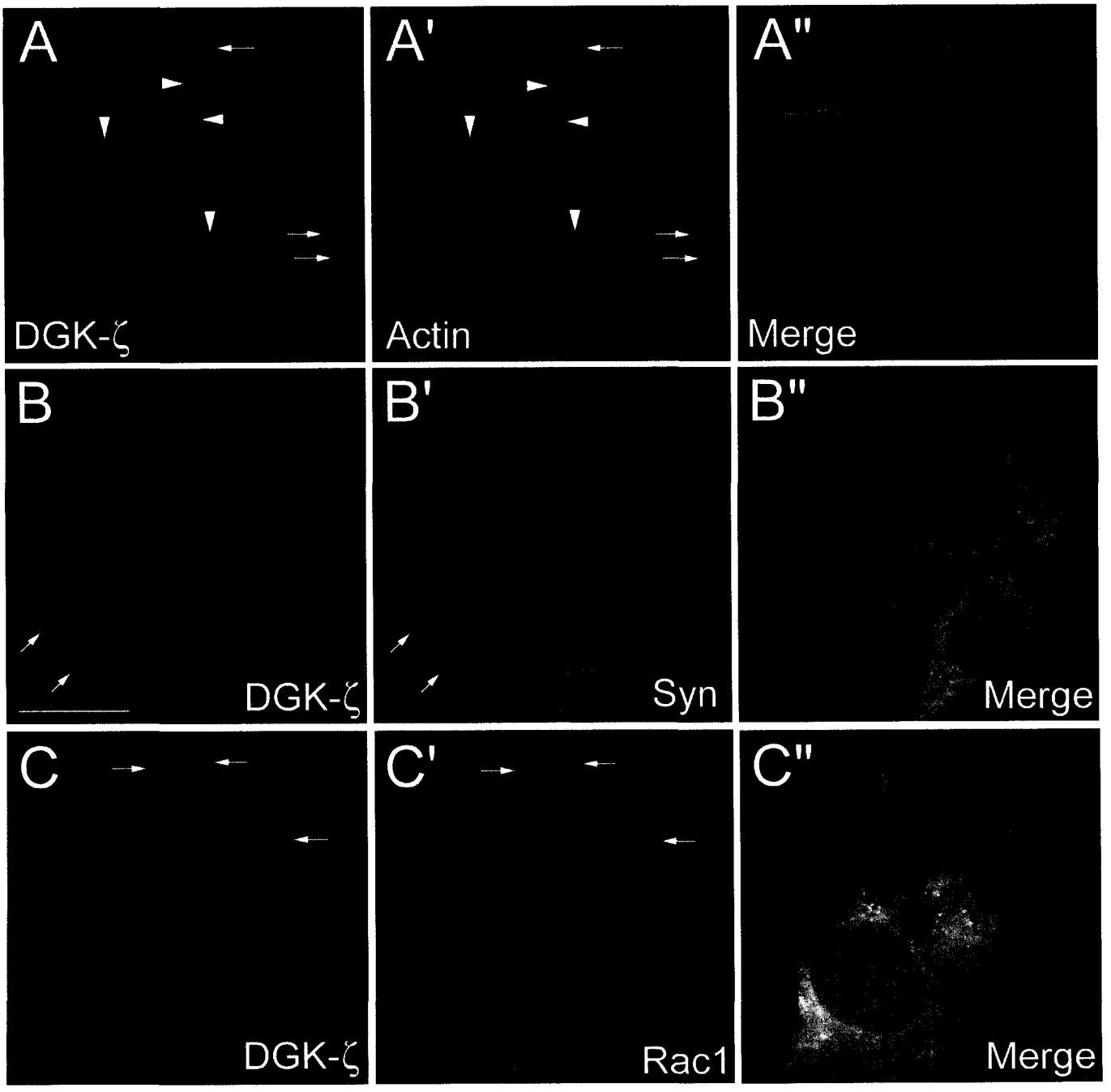

A
DGK-ζ
A'
Actin
A"
Merge
B
DGK-ζ
B'
Syn
B"
Merge
C
DGK-ζ
C'
Rac1
C"
Merge

Figure 2.8. ERK phosphorylation regulates the association of DGK-ζ with the cytoskeleton. (A) C2 myoblast lysates were fractionated as described in Materials and Methods and equal amounts of protein from each fraction were analyzed by SDS-PAGE and immunoblotting for DGK-ζ and syntrophin. (B) Schematic of DGK-ζ showing the domain organization, the sequence of the proline-rich region near the ankyrin repeats (*I-IV*) and the ERK docking-site consensus sequence. The N-terminal (*open box*), C1 (*ellipses*), MARCKS and catalytic domains are indicated. (C) COS-7 cells were transfected with DGK-ζ constructs in which all the Ser and Thr residues in the proline-rich sequence were mutated to Ala except the one shown above the corresponding lane of the immunoblot. The presence of a slightly higher shifted band in the lanes marked by an *asterisk* indicates that the residue is phosphorylated. (D) COS-7 cells were transfected with cDNAs encoding wild type DGK-ζ (*wt*), a mutant in which all the Ser/Thr residues shown in (B) were changed to alanines (*AAA*), or mutants in which the three Ser residues indicated by asterisks were changed to Asp (*DDD*) or Asn (*NNN*). The lysates were analyzed by SDS-PAGE and immunoblotting for DGK-ζ. (E) COS-7 cells were transfected with cDNAs encoding DGK-ζ and either Raf-ER or a control vector. 24 h later the cells were treated for 10 h with 1 μM estrogen or control vehicle. (F) COS-7 cells expressing DGK-ζ, Raf-ER and either the ERK phosphatase MKP3 or a control vector were treated with estrogen or vehicle as described above then analyzed for DGK-ζ.

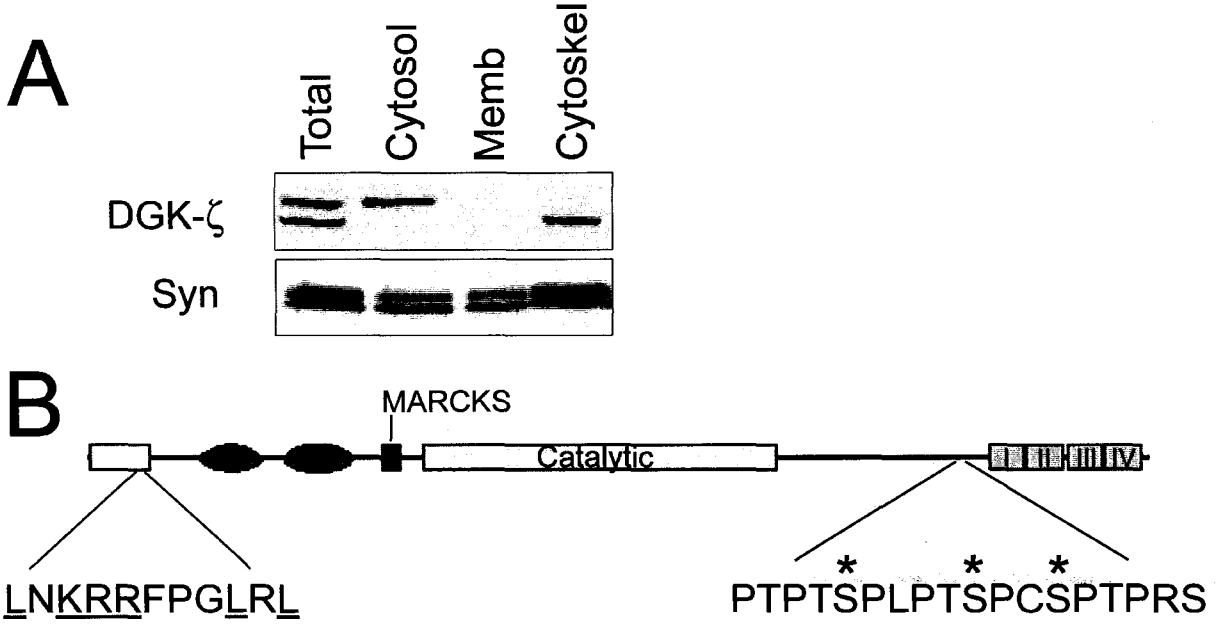

A
Total
Cytosol
Memb
Cytoskel
DGK-ζ
Syn
B
MARCKS
Catalytic
I II III IV
LNKRRFPGLRL
PTPTSPLPTSPCSPTPRS
C
wt T T S T S S T S
D
wt AAA DDD NNN
E
Raf-ER: + + -
Estrogen: - + +
F
Raf-ER: + + + +
MKP3: + + - -
Estrogen: - + - +

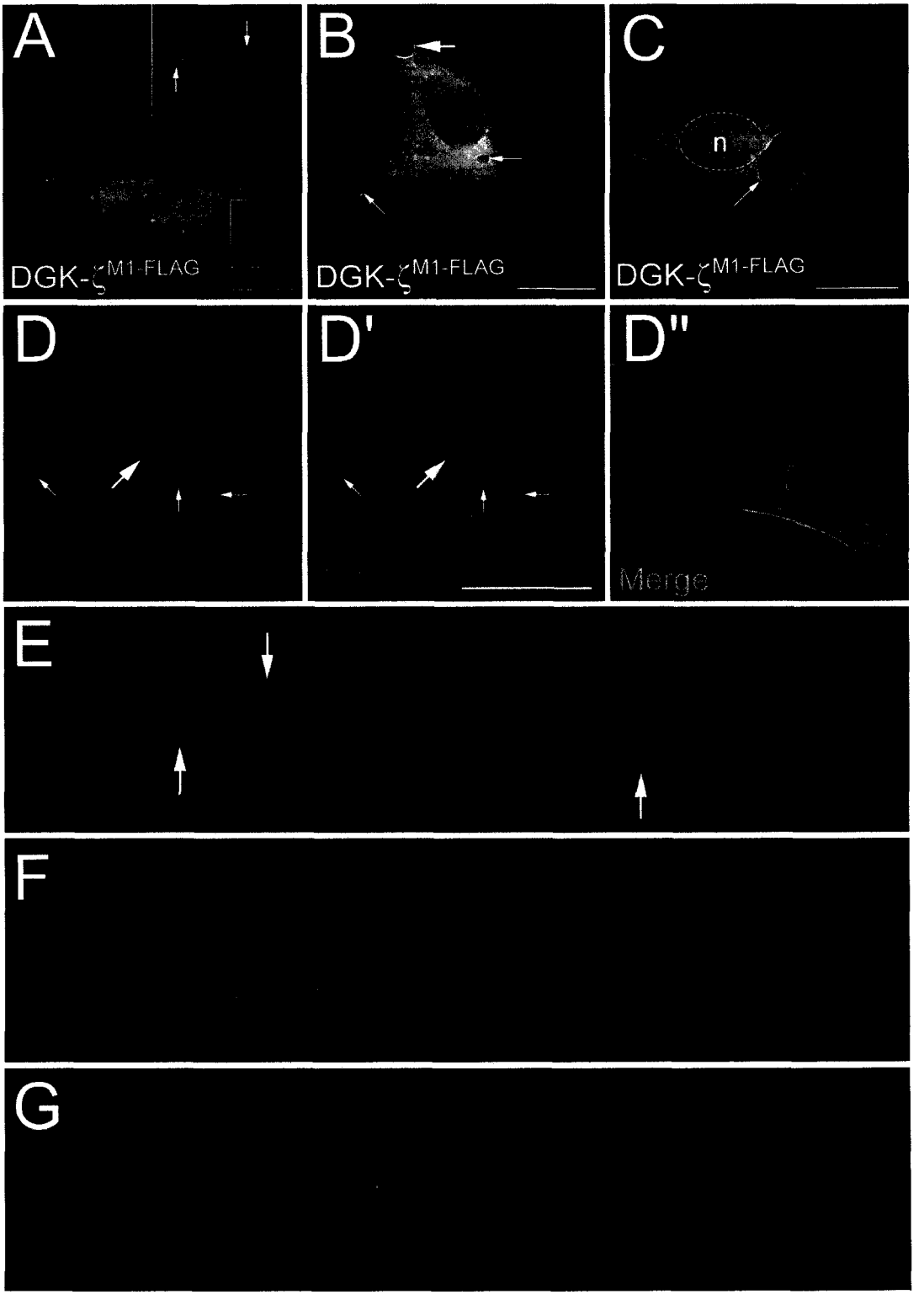
Figure 2.8C. Mutation of the three Ser residues indicated by asterisks gave rise to a second, more slowly migrating species, indicating that they can be phosphorylated. The three Ser residues were changed to Asp to mimic phosphorylation or to Asn, which is structurally similar but uncharged at physiological pH. Immunoblotting revealed that the Asp mutant (*DDD*) comigrates with the upper band, while the Asn mutant (*NNN*) comigrates with the lower band (Figure 2.8D). An additional mutant, in which all the Ser and Thr residues were changed to Ala (*AAA*) also comigrated with the lower band suggesting there are no additional phosphorylation sites within the proline-rich sequence.

The sequence identified above contains motifs conforming to the minimal MAP kinase phosphorylation consensus sequence (Ser/Thr-Pro). In addition, the N-terminus of DGKζ contains an ERK docking-site consensus sequence (Figure 2.8B) (Sharrocks *et al.*, 2000). To activate the Ras/ERK signaling pathway, we used a chimeric cDNA consisting of the hormone-binding domain of the estrogen receptor fused to an oncogenic form of Raf-1 (Samuels *et al.*, 1993; Topham and Prescott, 2001). Activation of the chimeric protein (Raf-ER) by estrogen in COS-7 cells induced phosphorylation of DGK-ζ, indicated by the accumulation of the slowly migrating band (Figure 2.8E). In contrast, coexpression of stress-activated protein kinase (SAPK) did not alter the migration of either DGK-ζ band (not shown). Co-expression of Raf-ER with MKP3, a dual Tyr-Ser/Thr phosphatase that inactivates ERK (Zhou *et al.*, 2002), completely attenuated the band shift even in the presence of estrogen, indicating that phosphorylation of DGK-ζ occurs downstream of ERK activation (Figure 2.8F). Another ERK phosphatase, CL100, had a similar effect (not shown). These results suggest ERK-dependent phosphorylation of DGK-ζ negatively regulates its association with the cytoskeleton.

DGK-$\zeta^{M1\text{-}FLAG}$ Induces Changes in the Actin Cytoskeleton

C2 myoblasts expressing DGK-$\zeta^{M1\text{-}FLAG}$ displayed prominent membrane ruffles enriched in both DGK-ζ (Figure 2.9A, *inset*, *arrows*) and F-actin (Figure 2.9D-D", *small arrows*). In addition, they often contained large vesicles and invaginations of the plasma membrane that were occasionally as large as the nucleus (Figure 2.9B-D). DGK-ζ labeling on the perimeter of these structures was colocalized with actin (Figure 2.9D-D", *arrows*). Large vesicles were also observed in C2 myotubes expressing DGK-$\zeta^{M1\text{-}FLAG}$ but not in myotubes expressing either wild type DGK-ζ (Figure 2.9G, *arrows*) or DGK-ζ^{M1} (not shown), suggesting the DGK-ζ/syntrophin interaction is important for proper control of actin cytoskeleton organization. Consistent with this interpretation, overexpression of α1-syntrophin lacking the PDZ domain (α1-Syn$^{\Delta PDZ}$) (Adams *et al.*, 2001) produced a similar phenotype (Figure 2.9F).

Figure 2.9. Expression of DGK-$\zeta^{M1\text{-}FLAG}$ in C2 cells induces F-actin-rich membrane ruffles and large vesicles. (A-C) Representative images of C2 myoblasts expressing DGK-$\zeta^{M1\text{-}FLAG}$. (A and inset) DGK-ζ was concentrated in membrane ruffles (*arrows*). (B) DGK-ζ was localized on the perimeter of large invaginations of the plasma membrane (*large arrow*) and vesicles ranging in size from 2 to 5 μm (*small arrows*). In some cells, vesicles as large as the nucleus were observed (C, *arrow*). (D-D") DGK-$\zeta^{M1\text{-}FLAG}$-induced membrane ruffles (*small arrows*) and invaginations (*large arrow*) were enriched in F-actin, which colocalized with DGKζ. (E-G) Representative images of C2 myotubes expressing the indicated constructs. DAPI stained nuclei are shown in blue. Vesicles were present in myotubes expressing DGK-$\zeta^{M1\text{-}FLAG}$ (E, *arrows*), α1-$Syn^{\Delta PDZ}$ (F), but not wild type DGK-ζ (G). n, nucleus. Scale bars = 20 μm.


A
DGK-ζM1-FLAG
B
DGK-ζM1-FLAG
C
n
DGK-ζM1-FLAG
D
D'
D''
Merge
E
F
G

Discussion

Recent studies indicate that the regulation of DGK activity depends on the correct subcellular localization of the enzyme (Topham *et al.*, 1998; Santos *et al.*, 2002; Imai *et al.*, 2002). Since DGK-ζ is found in the cytoplasm, at the plasma membrane and in the nucleus, the mechanisms regulating its localization are likely to be complex. Here, we investigated the role of syntrophins in the control of DGK-ζ localization in skeletal muscle. We demonstrated that DGK-ζ and syntrophins form a stable complex in muscle cells, in agreement with our previous studies showing the DGK-ζ C-terminus binds to the PDZ domains of syntrophin isoforms (α1, β1 and β2) expressed in skeletal muscle (Hogan *et al.*, 2001). In addition, we showed DGK-ζ and syntrophin reside in part on the sarcolemma of adult muscle fibers. Syntrophins appear to promote a more stable association of DGK-ζ with the plasma membrane, which in our studies, lead to an increase in cells with membrane-associated DGK-ζ. In agreement with this idea, there was a substantial loss of sarcolemmal DGK-ζ in *mdx* muscles, where the absence of dystrophin results in decreased syntrophin expression and mislocalization of the remaining membrane-associated protein to the cytosol (Froehner *et al.*, 1997). The latter finding suggests the membrane localization of DGK-ζ in skeletal muscle fibers depends on the formation of a complex containing both syntrophin and dystrophin.

Syntrophins Stabilize Active DGK-ζ at the Plasma Membrane and Anchor the Inactive Enzyme in the Cytoplasm

Recently, Santos et al. (2002) showed PKC-dependent phosphorylation of the MARCKS domain signals DGK-ζ translocation to the plasma membrane of T-cells. Our results confirm and extend their findings by showing that the localization of DGK-ζ and

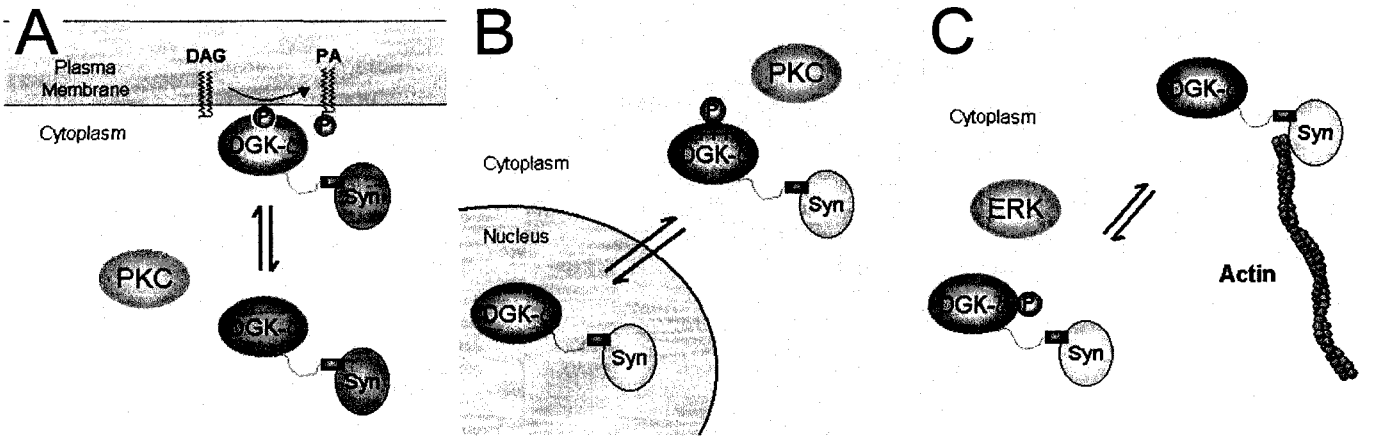
syntrophin is coordinately regulated by phosphorylation. Immunofluorescence data demonstrated that constitutive phosphorylation of the MARCKS domain increased the association of DGK-ζ with the plasma membrane relative to the wild-type protein. Moreover, coexpression of phosphorylated DGK-ζ with α1-syn leads to an increase in syntrophin localization at the plasma membrane. An interesting possibility is that phosphorylation of DGK-ζ causes a conformational change in syntrophin that increases its affinity for dystrophin. This could serve as a mechanism for targeting syntrophin/DGK-ζ complexes to the sarcolemma.

Syntrophins have been regarded as scaffold proteins that function exclusively at the plasma membrane (Froehner *et al.*, 1997), however substantial amounts are found in the cytosolic fraction of skeletal muscle cell and tissue lysates (Figure 2.8; S.G., unpublished observations), implying they have functional roles in the cytoplasm. Our data raise the possibility that cytoplasmic syntrophins sequester DGK-ζ in the cytoplasm of resting cells, away from sources of DAG. In this scheme, phosphorylation of the MARCKS domain by PKC would allow DGK-ζ and syntrophin to translocate to the plasma membrane, thereby regulating DGK-ζ activity (Figure 2.10A). These findings imply that syntrophin localization is dynamically regulated in muscle cells.

Regulation of Nuclear Localization

Consistent with our previous studies (Hogan *et al.*, 2001), we showed that DGK-ζ accumulates in the nucleus of C2 muscle cells in the absence of syntrophin interaction. We also observed DGK-ζ immunoreactivity associated with central nuclei of *mdx* muscles fibers, raising the possibility that the absence of dystrophin and the secondary loss of syntrophins

Figure 2.10. Model depicting the regulation of DGK-ζ localization in muscle cells. (A) PKC-dependent phosphorylation of the MARCKS domain regulates the association of DGK-ζ and syntrophin with the plasma membrane. (B) Syntrophin interaction and PKC-dependent phosphorylation regulate the nuclear-cytoplasmic shuttling of DGK-ζ. (C) ERK-dependent phosphorylation of DGK-ζ negatively regulates its association with the cytoskeleton.


A
Plasma Membrane
DAG
PA
Cytoplasm
DGK-ζ
Syn
PKC
B
PKC
Cytoplasm
Nucleus
C
Cytoplasm
ERK
Actin

cause the inappropriate accumulation of DGK-ζ in the nucleus. Since DGK-ζ regulates the levels of DAG in the nucleus (Topham *et al.*, 1998), which in turn regulates PKC-dependent changes in cell growth, inappropriate levels of DGK-ζ in nuclei of *mdx* muscles could affect the regulation of muscle cell growth. However, more detailed studies are required to verify that there are differences in nuclear DGK-ζ levels in normal versus *mdx* muscles. Nevertheless, these results, together with our previous studies, reinforce the idea that syntrophins are important for the stable retention of DGK-ζ in the cytoplasm and the regulation of nuclear DGK-ζ levels.

Modulation of the Actin Cytoskeleton

We found that DGK-ζ and syntrophins associate with actin in regions undergoing cytoskeletal rearrangements, suggesting they play a role in modulating actin dynamics. Consistent with this interpretation, DGK-ζ was colocalized with Rac, a member of the Rho family of small GTPases that control the assembly and organization of F-actin (Etienne-Manneville and Hall, 2002). Moreover, Tolias et al. (1998) demonstrated that Rac immunoprecipitates contain DGK activity with biochemical characteristics like those of DGK-ζ. Thus, DGK-ζ and syntrophins may function in a complex with Rac to control actin cytoskeleton remodeling, possibly by facilitating Rac activation and/or its localization to the cell surface. Activation of Rac produces lamellipodia and membrane ruffles, which serve as sites of actin polymerization, endocytosis, receptor tyrosine kinase signaling, and protease activity (Small *et al.*, 2002). Consistent with a role in regulating actin dynamics, overexpression of DGK-$\zeta^{M1\text{-}FLAG}$, which does not interact with syntrophins, produced dramatic changes in cell morphology and actin organization, including the formation of

membrane ruffles and large vesicles. This finding suggests that the control of DGK-ζ localization by syntrophins is important for regulating actin cytoskeleton rearrangements. For example, syntrophins might direct the assembly of Rac/DGK-ζ complexes to the leading edges of lamellipodia where other actin regulatory proteins are assembled.

This study demonstrates that ERK-dependent phosphorylation near the C-terminus of DGK-ζ negatively regulates its association with the cytoskeleton (Figure 2.10C). The mechanism by which this occurs is unclear, but DGK-ζ likely interacts with F-actin, since the two were colocalized in ruffles and at the leading edge of lamellipodia. The MARCKS domain might mediate this interaction directly, as demonstrated for the MARCKS protein (Hartwig *et al.*, 1992). Alternatively, syntrophins might mediate the interaction with the cytoskeleton via dystrophin family members, which bind actin at their N-terminus (Rybakova *et al.*, 1996; Galkin *et al.*, 2002). Moreover, at least one isoform, α1-syn, has been reported to bind directly to actin (Iwata *et al.*, 1998). Because the phosphorylated residues in DGK-ζ lie close to the C-terminus, ERK phosphorylation might affect the binding of DGK-ζ to syntrophin, thereby modulating its interaction with the cytoskeleton. Interestingly, ERK is concentrated at the leading edges of lamellipodia in migrating cells where it promotes the phosphorylation of myosin light chain kinase, leading to activation of the actin/myosin motor and cell migration (Howe *et al.*, 2002). Therefore, DGK-ζ may play a role in the cascade of signals that regulate actin reorganization at the leading edge of migrating muscle cells.

The Role of DGK-ζ at NMJs

DGK-ζ was concentrated at NMJs in normal myofibers and its localization at NMJs was unaffected in *mdx* muscle, suggesting it can associate with syntrophins bound to utrophin, a dystrophin homologue found primarily at synaptic sites. Additional observations suggest a role for utrophin in the localization of DGK-ζ. Utrophin is present at low levels on the extrasynaptic sarcolemma of slow twitch muscle fibers (Gramolini *et al.*, 2000), which may explain the residual sarcolemmal DGK-ζ observed in *mdx* muscles. In type IIB fibers, DGK-ζ was present exclusively at NMJs. In these fibers, sarcolemmal dystrophin levels are normal and utrophin is restricted to synaptic sites (Gramolini *et al.*, 2000), supporting the idea that DGK-ζ associates with utrophin at NMJs. Finally, utrophin is upregulated in regenerating fibers, accounting for the observed increase in DGK-ζ and syntrophin expression in a subset of *mdx* muscle fibers.

The function of DGK-ζ at adult NMJs is not known, however its close association with AChRs suggests it could play a role in the maintenance of postsynaptic AChR density through its effects on actin organization. At the developing NMJ, agrin secreted by motor neurons induces the clustering of AChRs and other proteins at synaptic sites (Sanes and Lichtman, 1999). This process involves extensive changes in the organization of the subsynaptic cytoskeleton, which anchors and stabilizes AChRs in the membrane. Studies using cultured myotubes have established that actin polymerization is necessary for the formation of AChR clusters (Dai *et al.*, 2000) and that agrin activates Rac, which is required for cluster formation (Weston *et al.*, 2000). Thus, DGK-ζ could have a role in the signaling pathways leading to the initial clustering of synaptic proteins at developing NMJs or in the maintenance of their density in the adult. In this regard, it is interesting to note that NMJs

from α1-syn null mice have reduced levels and an abnormal distribution of AChRs (Adams *et al.*, 2000).

Mislocalization of DGK-ζ in Dystrophic Muscle

Our results showing the mislocalization of DGK-ζ leads to alterations in the actin cytoskeleton suggest a basis for the widespread changes in the organization of the membrane-associated cytoskeleton observed in *mdx* myofibers (Williams and Bloch, 1999). These changes are thought to contribute to the fragility of the sarcolemma of dystrophic muscle and may ultimately contribute to the pathogenesis of the disease. Furthermore, DGK-ζ mislocalization could affect the regulation of DAG- or PA-activated proteins. In support of this idea, mutations in the *Drosophila* retinal degeneration A gene, which encodes a DGK isoform closely related to DGK-ζ, result in a severe retinal phenotype (Hardie and Raghu, 2001). The degeneration is caused by Ca^{2+} influx through constitutively active transient receptor potential channels (TRPC), which are regulated by DAG and its metabolites (Raghu *et al.*, 2000). Interestingly, dystrophic muscles have chronically elevated intracellular Ca^{2+} levels caused by a persistent influx through Ca^{2+}-permeable channels of the TRPC family (Franco, Jr. and Lansman, 1990; Vandebrouck *et al.*, 2002). This is thought to activate Ca^{2+}-dependent proteases, which contribute to muscle degeneration.

Conclusions

Recent studies suggest that the association of DGK-ζ and its target proteins into organized complexes is a mechanism to spatially regulate DAG signals (Topham and Prescott, 2001; Luo *et al.*, 2003a). To function optimally, these complexes must be targeted

to regions of the membrane where DAG is produced. Our findings suggest syntrophins participate in the assembly and organization of DGK-ζ signaling complexes at the plasma membrane of skeletal muscle fibers, which may be critical for the proper control of actin reorganization. In dystrophic muscle, mislocalized DGK-ζ may cause signaling and cytoskeletal changes that contribute to disease pathogenesis.

Chapter 3

Regulation of neurite outgrowth in N1E-115 cells through PDZ-mediated recruitment of diacylglycerol kinase ζ

Yury Yakubchyk[1,3¶], Hanan Abramovici[1,3], Jean-Christian Maillet[1,3], Elias Daher[1,3], Christopher Obagi[1,3], Robin Parks[2,3,4], Matthew K. Topham[5] and Stephen H. Gee*[1,3]

*Corresponding author

[1]Department of Cellular and Molecular Medicine
University of Ottawa
451 Smyth Rd
Ottawa, ON K1H 8M5
Canada

[2]Molecular Medicine Program, Ottawa Health Research Institute, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada

[3]Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON, Canada

[4]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada

[5]Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112 USA

Running Title: DGK-ζ promotes syntrophin-dependent neurite outgrowth

Characters: 60,040

Published in Molecular and Cellular Biology 25: 7289-7302.

Contribution from authors

Hanan Abramovici and Stephen Gee co-wrote the manuscript. Hanan Abramovici contributed significantly to the intellectual input and generated reagents required for experiments shown in Figure 3.7A and B, and Figure 3.8. Hanan Abramovici performed experiments presented in Figures 3.7A and B.

Acknowledgements

The authors would like thank John Collard for providing the GST-PAK1 PBD construct, Salvatore Carbonetto for purified laminin and Andrew Thorburn for Myc-tagged Rac1 constructs. Thanks also to Christopher Carpenter for sharing unpublished results, Pierre-Yves Gougeon and Andrew Ridsdale for help with confocal microscopy and deconvolution, and Nafiseh Sabri for critically evaluating the manuscript. The monoclonal antibody 3A10 developed by Thomas Jessell and Jane Dodd was obtained from the Development Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. This work was supported by the Neuromuscular Research Partnership Program, an alliance of the Amyotrophic Lateral Sclerosis Society of Canada, the Muscular Dystrophy Association of Canada and the Canadian Institutes of Health Research (CIHR). S.G. is supported by a CIHR New Investigator Award. H.A. is supported in part by a J. Armand Bombardier Foundation scholarship and an Ontario Graduate Scholarship.

Abstract

Syntrophins are scaffold proteins that regulate the subcellular localization of diacylglycerol kinase-ζ (DGK-ζ), an enzyme that phosphorylates the lipid second messenger diacylglycerol to yield phosphatidic acid. DGK-ζ and syntrophins are abundantly expressed in neurons of the developing and adult brain, but their function is unclear. Here, we show that they are present in cell bodies, neurites and growth cones of cultured cortical neurons and differentiated N1E-115 neuroblastoma cells. Overexpression of DGK-ζ in N1E-115 cells induced neurite formation in the presence of serum, which normally prevents neurite outgrowth. This effect was independent of DGK-ζ kinase activity but dependent on a functional C-terminal PDZ-binding motif, which specifically interacts with syntrophin PDZ domains. DGK-ζ mutants with a blocked C-terminus acted as dominant-negative inhibitors of outgrowth from serum-deprived N1E-115 cells and cortical neurons. Several lines of evidence suggest DGK-ζ promotes neurite outgrowth through association with the GTPase Rac1. DGK-ζ colocalized with Rac1 in neuronal processes and DGK-ζ-induced outgrowth was inhibited by dominant-negative Rac1. Moreover, DGK-ζ directly interacts with Rac1 through a binding site located within its C1 domains. Together with syntrophin, these proteins form a tertiary complex in N1E-115 cells. A DGK-ζ mutant that mimics phosphorylation of the MARCKS domain was unable to bind an activated Rac1 mutant ($Rac1^{V12}$) and PMA-induced PKC activation inhibited the interaction of DGK-ζ with $Rac1^{V12}$, suggesting PKC-mediated phosphorylation of the MARCKS domain negatively regulates DGK-ζ binding to active Rac1. Collectively, these findings suggest DGK-ζ, syntrophin and Rac1 form a regulated signaling complex that controls polarized outgrowth in neuronal cells.

Introduction

Nervous system function depends upon highly specific connections that form between neurons during development. Before these connections can be established however, neurons must first elaborate cellular processes called neurites that eventually become a single axon and multiple dendrites. Dynamic remodeling of the actin-based cytoskeleton is fundamental to neurite outgrowth (Luo, 2000; Luo, 2002).

The Rho GTPases are key regulators of actin cytoskeleton organization in neurons and have been shown to play important roles in many aspects of neuronal morphogenesis (Luo, 2000). The best studied members of this family; RhoA, Rac1, and Cdc42 regulate the formation of distinct actin structures. RhoA controls the formation of stress fibers, whereas Rac1 and Cdc42 control the formation of lamellipodia and filopodia, respectively (Etienne-Manneville and Hall, 2002). The latter structures play key roles in the elongation of neurites. Accordingly, Cdc42 and Rac1 are generally considered positive regulators of neurite outgrowth, whereas RhoA normally inhibits neurite extension (Etienne-Manneville and Hall, 2002).

Rho GTPases cycle between the inactive GDP-bound and active GTP-bound states (Etienne-Manneville and Hall, 2002). Studies in neuronal cell lines have shown that inactive mutants of Cdc42 and Rac1 act as dominant-negative inhibitors of neurite outgrowth (van Leeuwen *et al.*, 1997; Kozma *et al.*, 1997; Daniels *et al.*, 1998; Sarner *et al.*, 2000). Constitutively active mutants of Cdc42 and Rac1 induce the formation of filopodia and lamellipodia in developing growth cones (Kozma *et al.*, 1997), but activation of these GTPases is not sufficient to induce the outgrowth of neurites [(Daniels *et al.*, 1998; Sarner *et al.*, 2000); but see (van Leeuwen *et al.*, 1997)]. Thus, the activity of these GTPases appears

to be necessary but not sufficient for neurite outgrowth, prompting the question whether there are associated proteins that work in concert with these GTPases to regulate process outgrowth. Another unresolved question is how the activity of these GTPases is correctly localized to the tips of growing neurites (Jaffe and Hall, 2003). For example, in response to nerve growth factor, active Rac1 is rapidly recruited to actin-rich cellular protrusions that are destined to become neurites in PC12 cells (Yasui *et al.*, 2001), but the molecular mechanism underlying this translocation is unclear. Scaffold proteins are likely to play an important role in this process.

Scaffold proteins facilitate the proper intracellular localization of their partner proteins, including establishing interaction with the plasma membrane and targeting proteins to specific membrane domains (Burack and Shaw, 2000). Many signaling proteins contain C-terminal amino acid sequences that confer binding to PDZ domain-containing scaffold proteins (Sheng and Sala, 2001). The Rho GTPases however, do not appear to contain any obvious PDZ-interaction motifs. Thus, they are either localized by other means or they indirectly associate with scaffolding proteins.

In this regard, Rac1 has been reported to associate with a complex of proteins that includes a phosphatidylinositol-4-phosphate 5-kinase (PIP5K), a Rho guanine nucleotide dissociation inhibitor (RhoGDI) and a diacylglycerol kinase (DGK) (Tolias *et al.*, 1998). DGKs phosphorylate the lipid second messenger diacylglycerol (DAG), which transiently accumulates in cells following stimulation by growth factors and other agonists (Hodgkin *et al.*, 1998). The DGK associated with Rac1 had biochemical properties like those of the zeta (ζ) isoform, but was not identified conclusively (Hogan *et al.*, 2001). We have previously shown that the subcellular localization of DGK-ζ is regulated by the interaction of its C-

terminal PDZ-binding motif with the PDZ domain of syntrophins, scaffolding proteins that interact with the dystrophin family of cytoskeletal proteins (Hogan *et al.*, 2001; Abramovici *et al.*, 2003). Therefore, such interactions may provide a mechanism for localizing Rac1 in cells.

The syntrophin family consists of five structurally related isoforms (α1, β1, β2, γ1, and γ2) that are products of separate genes. Each isoform consists of two tandem pleckstrin homology (PH) domains, a single PDZ domain and a C-terminal region unique to syntrophins (Froehner *et al.*, 1997). The C-terminal half of syntrophin binds to members of the dystrophin family of cytoskeletal proteins, leaving the N-terminal half, including the PDZ domain, available to interact with signaling proteins (Albrecht and Froehner, 2002). Since certain dystrophin family members bind directly to actin (Keep, 2000), syntrophins provide a link between signaling proteins and the actin cytoskeleton. Consistent with a potential role in actin organization, we showed that DGK-ζ and syntrophins colocalize with Rac1 in membrane ruffles and at the leading edge of lamellipodia in muscle cells (Abramovici *et al.*, 2003).

Here, we sought to identify functional roles for DGK-ζ and syntrophins in actin cytoskeleton regulation in neuronal cells. We show that DGK-ζ expression induces neurite outgrowth in N1E-115 neuroblastoma cells. DGK-ζ-induced neurite outgrowth is largely independent of DGK-ζ catalytic activity and is inhibited by dominant-negative Rac1. DGK-ζ mutants that do not interact with syntrophins not only fail to promote neurite outgrowth, but also act as dominant-negative inhibitors of neurite outgrowth. We show that DGK-ζ interacts directly with Rac1 and provide evidence that the two proteins form a regulated signaling complex with syntrophin that is localized to growth cones and sites of early process

formation. Collectively, our results suggest syntrophins function as scaffolds to regulate the subcellular localization of a DGK-ζ/Rac1 complex that catalyzes actin cytoskeleton rearrangements associated with neurite outgrowth.

Materials and Methods

Reagents

Laminin purified from Engelbreth-Holm-Swarm tumor was a gift from Salvatore Carbonetto (McGill University, PQ). Polyethyleneimine was from Sigma-Aldrich (Saint-Louis, MO). Monoclonal antibody (mAb) 1351 was a gift from Stanley Froehner (U. Washington, Seattle). Affinity purified polyclonal antibodies to DGKζ were made as described previously (Abramovici *et al.*, 2003). Rac1 antibodies were purchased from BD Transduction Laboratories (Mississauga, ON) and Upstate Biotechnologies (Charlottesville, VA). Rabbit polyclonal anti-HA was purchased from Zymed Laboratories (San Francisco, CA). mAb 9E10 against c-myc was from Roche Diagnostics Corp. (Indianapolis, IN). Anti-FLAG M2 antibody and anti-neurofilament 200 (clone NE-14) were from Sigma-Aldrich. mAb 3A10 was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Protease inhibitors were from BioShop Canada (Burlington, ON). Glutathione Sepharose 4B beads were from Amersham Biosciences AB (Uppsala, Sweden). Fetal Bovine Serum (FBS) was purchased from Wisent Canadian Laboratories (St-Bruno, QC). Dulbecco's Modified Eagle Medium (DMEM), Neurobasal medium, minimal essential medium (MEM), L-glutamine, penicillin-streptomycin, N2 and B27 supplements were from GIBCO-Invitrogen (Carlsbad, CA). Plasmids encoding wild type DGK-ζ, DGK-ζ^{M1}, DGK-$\zeta^{\Delta ATP}$, DGK-ζ^{FLAG}, DGK-$\zeta^{M1\text{-}FLAG}$ have all been described previously (Topham *et al.*, 1998; Abramovici *et al.*, 2003). A plasmid encoding the p21-binding domain (PBD) domain of PAK1 fused to GST was a gift from Dr. John Collard (The Netherlands Cancer Institute, Amsterdam) (Sander *et al.*, 1998).

Recombinant Adenoviral Vectors and Infection

A mutant of human DGK-ζ with all four Ser residues in the MARCKS domain changed to aspartates (DGK-ζ^{M1}) has been described previously (Topham *et al.*, 1998; Abramovici *et al.*, 2003). An adenoviral construct encoding DGK-ζ^{M1} with three tandem N-terminal hemagglutinin (HA) epitope tags was generated by subcloning from the pCMV-HA vector. AdDGK-ζ^{M1} contains the DGK-ζ^{M1} cDNA under the regulation of the cytomegalovirus immediate-early promoter/enhancer (CMV) and bovine growth hormone polyadenylation sequence (BGHpA). In these viruses, the DGK-ζ^{M1} expression cassette replaced the E1-region and transcription is directed rightward, relative to the conventional human adenovirus serotype 5 map. Similar cloning strategies were used to make adenoviral constructs of wild type DGK-ζ (AdDGK-ζ) and DGK-ζ^{FLAG} (AdDGK-ζ^{FLAG}). The E1-deleted, first-generation Ad vectors used in these studies were constructed using a combination of conventional cloning techniques and RecA-mediated recombination (Chartier *et al.*, 1996; He *et al.*, 1998), and were grown and titered on HEK 293 cells, as previously described (Ng and Graham, 2002). HEK 293 cells were grown as a monolayer in MEM supplemented with 100 U/ml penicillin-streptomycin, 2.5 mg/ml Fungizone, and 10% fetal bovine serum (complete medium). The multiplicity of infection (MOI) indicates the number of plaque-forming units added per cell.

Primary Cultures of Cortical Neurons

The isolation of cortical neurons from mouse embryos was based on the method described by Ghosh et al. (1995) for rat cultures and modified according to Brewer et al. (Brewer *et al.*, 1993). Cortices were collected from gestational day 13-14 CD1 mouse embryos and triturated following a 30 min digestion with trypsin in Hanks balanced salt

solution. The cells were plated on 18 mm glass coverslips coated sequentially with poly-L-lysine and 20 μg/ml laminin at a density of 2 x 10^5 per coverslip. The cells were maintained in Neurobasal medium with 1% N2 and B27 supplements, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin.

N1E-115 Neuroblastoma Cell Cultures

N1E-115 mouse neuroblastoma cells (CRL-2263) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. The cells were transfected according to the method of Köhrmann et al. (Kohrmann *et al.*, 1999) or with FuGENE^{TM} 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. For adenoviral infection, N1E-115 cells were grown for 24 h then were infected at a MOI of 100 for 1 h in DMEM, after which the medium was replaced with DMEM + 10% FBS. For pull-down assays, N1E-115 cells expressing wild-type DGK-ζ or DGK-ζ^{M1} for 24 h were stimulated with 90 nM phorbol 12-myristate 13-acetate (PMA) or vehicle for 30 min. Where indicated, 500 nM Gö 6983 was added for 10 min before PMA stimulation.

Neurite-like Extension Scoring Assay

N1E-115 cells were grown on glass coverslips coated with polyethylenimine or laminin in 35 mm tissue culture dishes and transfected with the indicated expression vectors. 24-48 h after transfection, the cells were fixed, labeled and visualized by immunofluorescence microscopy. Transfected cells bearing a neurite-like structure with a

length of at least one cell body were counted and scored as the percentage of total transfected cells. Cells bearing a neurite-like structure with a length of at least one cell diameter were identified by immunofluorescence microscopy using epitope tag-specific antibodies, counted, then expressed as the percentage of total transfected cells (van Leeuwen *et al.*, 1997; Kranenburg *et al.*, 1999). At least 300 cells were counted for each condition and the experiments were repeated a minimum of three times. The images were taken with a charge-coupled device camera mounted on a Zeiss Axioskop 2 microscope equipped with a 63 X oil-immersion objective.

Confocal Microscopy and Deconvolution

All confocal images were acquired with a Bio-Rad MRC1024 confocal microscope equipped with a 60x, 1.4 numerical aperture oil-immersion objective. Z-series were obtained using a 0.3 μm step and image stacks were deconvolved with computational optical sectioning microscopy (COSM) software compiled for a PC, using an expectation maximization algorithm (Conchello *et al.*, 1994). The point spread function was calculated according to the method of Gibson and Lanni (1991).

Pull-down Assays

Glutathione S-transferase fusion proteins of $Rac1^{V12}$, $Rac1^{N17}$ and RhoA were produced as described previously (Gee *et al.*, 1998; Gee *et al.*, 2000; Hogan *et al.*, 2001) and purified according to method of Tolias et al. (1998). The fusion protein purity was determined by Coomassie blue staining of SDS-polyacrylamide gels and the protein concentration determined by the Bradford method (Bradford, 1976). Undifferentiated N1E-115 cells plated on laminin were infected with AdDGK-ζ and grown for 48 h then were

extracted with chilled lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1% NP40) including a protease inhibitor cocktail (antipain, leupeptin, pepstatin A, AEBSF HCl, and Benzamidine HCl). The lysates were centrifuged at 12,000 rpm for 5 min to remove cell debris and nuclei. An aliquot (5-10%) of the supernatant was set aside and constituted the "Input". Equivalent amounts of GST, GST-Rac1^{V12}, GST-Rac1^{N17} and GST-RhoA fusion proteins bound to glutathione-Sepharose 4B beads were incubated with 600-800 μg of supernatant for 2 h at 4°C on a rocker. The tubes were centrifuged at 2000 rpm for 2 min to collect the beads. The beads were then washed 3-5 times with 1 ml of lysis buffer, then resuspended in 1X reducing sample buffer and boiled for 5 min. The samples were analyzed by SDS-PAGE and immunoblotting.

Blot Overlay Assays

Rac1^{V12} and Rac^{N17} were subcloned into the *Bam HI* and *EcoR I* sites of the pET-32a expression vector (Novagen, Inc., Madison, WI), which contains sequences encoding thioredoxin, an N-terminal 15-amino acid S tag, and a hexahistidine nickel-binding motif. The expressed fusion proteins were recognized by the S-protein HRP conjugate (Novagen) and were purified from the soluble fraction on nickel-Sepharose columns according to the manufacturer's instructions. Blot overlay assays were carried out as described previously (Gee *et al.*, 2000). Rac1^{V12} and Rac^{N17} were used at a final concentration of 300 nM.

Results

Localization of DGK-ζ and syntrophins in distal neurites and growth cones

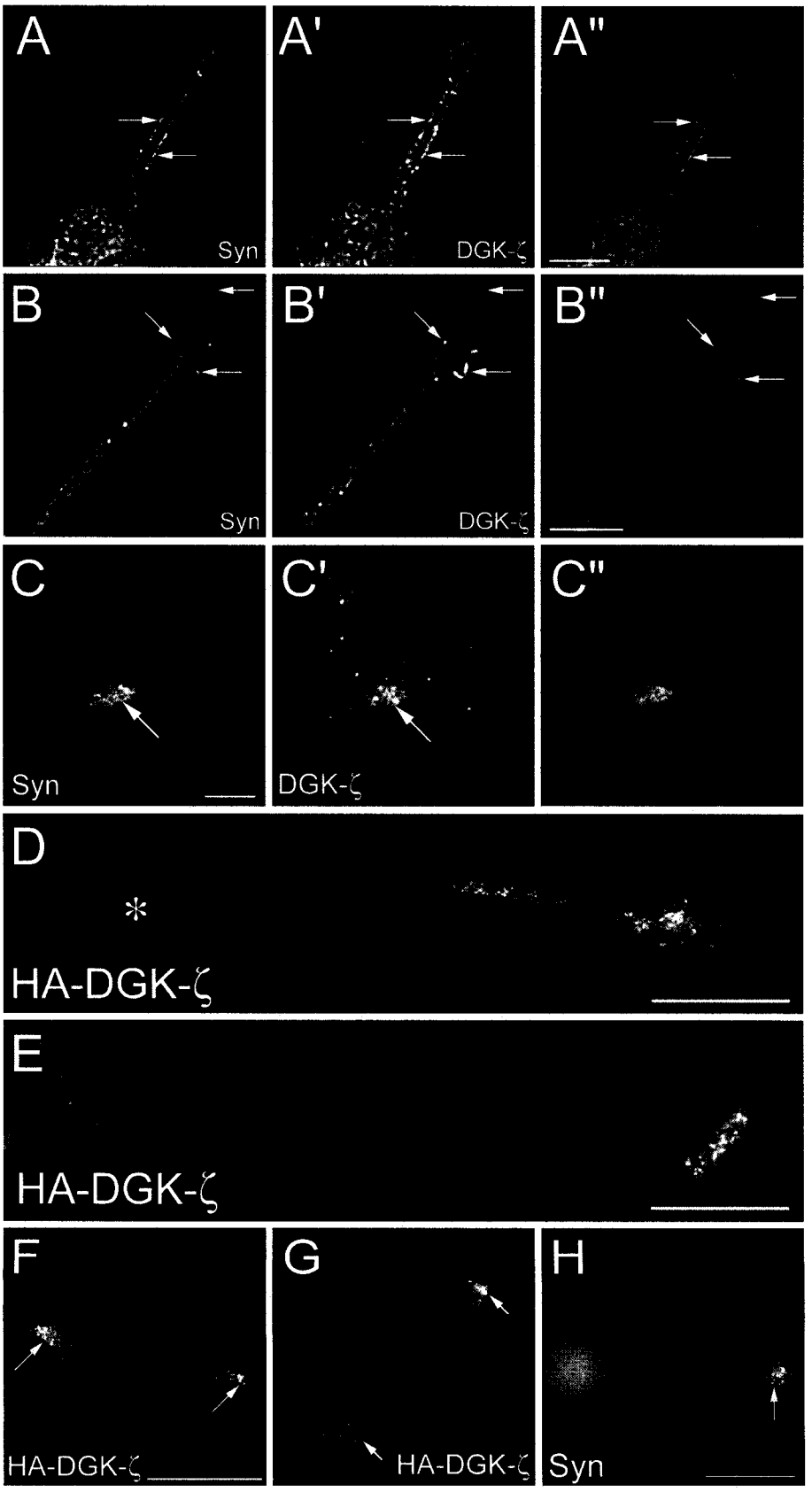
DGK-ζ is abundantly expressed in the central nervous system and is present in neurons of the olfactory bulb, hippocampus and cerebral and cerebellar cortices in the developing and adult brain, suggesting it may be involved in neuronal development and function (Goto and Kondo, 1996; Ding *et al.*, 1998; Hogan *et al.*, 2001; Hozumi *et al.*, 2003). Syntrophins are also abundantly expressed in the brain, with several isoforms being expressed in neurons in the same regions as DGK-ζ (Gorecki *et al.*, 1997; Piluso *et al.*, 2000; Hogan *et al.*, 2001; Brunig *et al.*, 2002).

To begin to investigate the function of DGK-ζ and syntrophins in neurons, we assessed their subcellular distribution in primary mouse cortical neurons double labeled with a pan-specific mAb (1351) to syntrophins and affinity purified anti-DGK-ζ antibodies, followed by AlexaFluor 488 (green)- and AlexaFluor 594 (red)-conjugated secondary antibodies. Confocal microscopy was used to obtain a Z-series of images, which were subsequently deconvolved as described in Materials and Methods.

Single optical sections show punctate immunoreactivity for DGK-ζ and syntrophins in cell bodies, along neurites and in growth cones (Figure 3.1A - B"). The *arrows* indicate regions of the neurite shaft (A – A") and of the growth cone (B – B") where punctate accumulations of both proteins overlap.

Additional localization studies were carried out in differentiated mouse N1E-115 neuroblastoma cells, which are commonly used to study aspects of neuronal differentiation *in vitro*. N1E-115 cells can be transfected with high efficiency (Kranenburg *et al.*, 1995) and

Figure 3.1. Colocalization of DGK-ζ and syntrophins in mouse cortical neurons and N1E-115 neuroblastoma cells. (A - B") Mouse cortical neurons cultured for 2-3 days *in vitro* were fixed and labeled with affinity purified anti-DGK-ζ antibodies and with the pan-specific mAb 1351 against syntrophins, followed by AlexaFluor 594 (red) and AlexaFluor 488 (green) secondary antibodies, respectively. A Z-series obtained by confocal immunofluorescence microscopy was deconvolved as described in "Materials and Methods". Single optical sections from the red and green channels are shown. Regions of the neurite where syntrophin and DGK-ζ immunoreactivity overlap are indicated by *arrows* and by the yellow color in the merged images. (C – C") N1E-115 cells differentiated in low serum conditions for 48 h were fixed and processed as above and visualized by epifluorescence microscopy. The *arrows* indicate a bright patch of syntrophin and DGK-ζ immunoreactivity at the leading edge of a neuronal process. (D - E) N1E-115 cells transfected with HA-tagged DGK-ζ were differentiated for 48 h in low serum conditions, then were fixed and stained with anti-HA antibodies. HA-DGK-ζ accumulated in the distal half of the neurite and at the neurite tip. The asterisk in (D) indicates the cell body. (F and G) Transfected N1E-115 cells differentiated for 24 h had high concentrations of HA-DGK-ζ within neurite buds (*arrows).* (H) Syntrophin immunoreactivity in an untransfected N1E-115 cell differentiated for 24 h. The *arrow* indicates strong syntrophin immunoreactivity at the tip of a small neuronal process. Scale bars, 20 μm, except for C – C", 5μm.

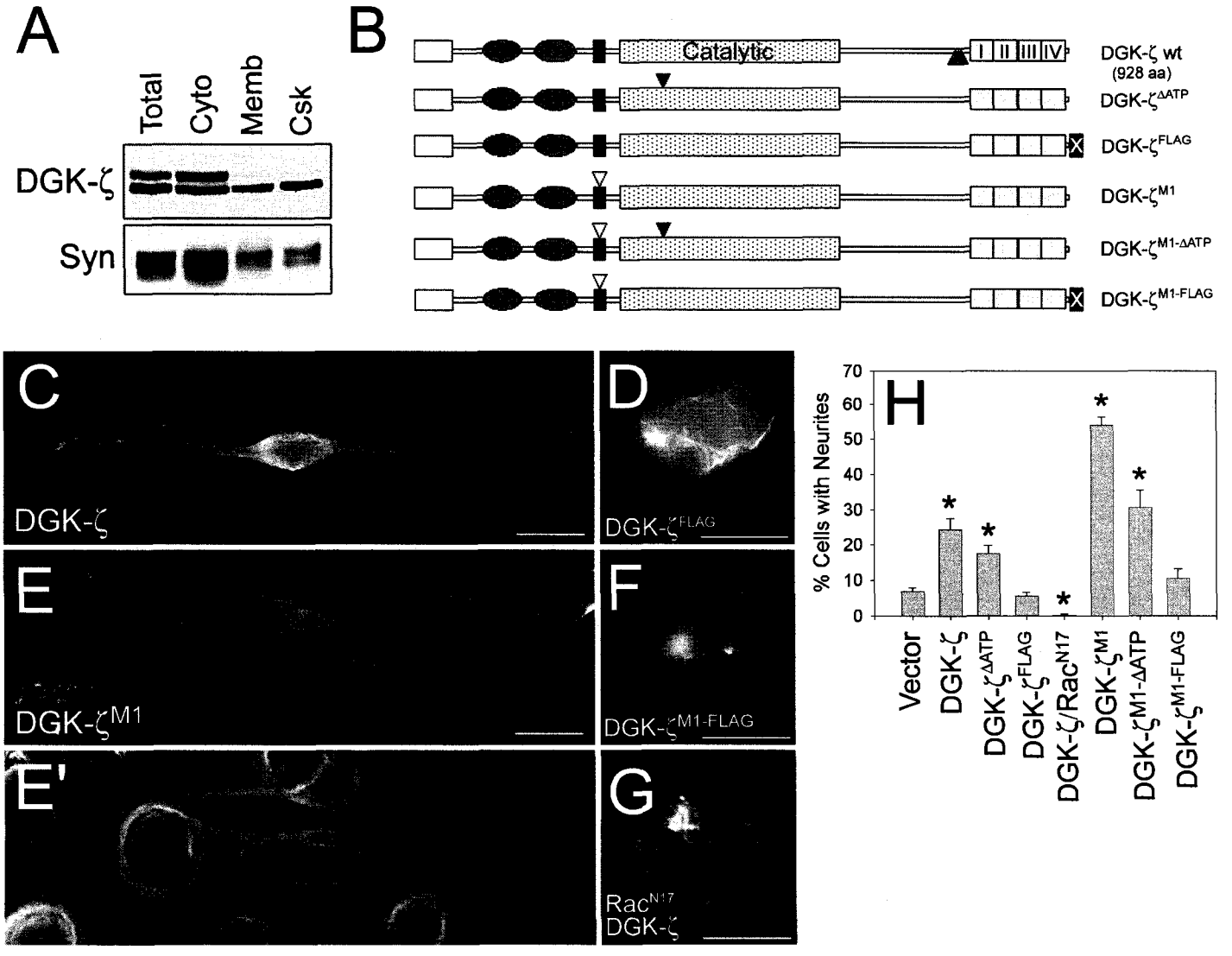

A
Syn
A'
DGK-ζ
A"
B
Syn
B'
DGK-ζ
B"
C
Syn
C'
DGK-ζ
C"
D
HA-DGK-ζ
E
HA-DGK-ζ
F
HA-DGK-ζ
G
HA-DGK-ζ
H
Syn

are rapidly induced to spread and extend neurites after serum withdrawal. Western blotting of subcellular fractions from undifferentiated N1E-115 cells confirmed that both DGK-ζ and syntrophins are expressed in these cells (Figure 3.2A). No difference in the level or relative distribution of either protein was detected upon differentiation (Y.Y and S.G, unpublished observations). Figure 3.1C – C" shows endogenous DGK-ζ and syntrophin concentrated in a patch at the leading edge of a growth cone in a differentiated N1E-115 cell, although elsewhere the signals do not colocalize exactly.

The lack of complete overlap of syntrophin and DGK-ζ immunoreactivity is not surprising because the pan-specific syntrophin antibody recognizes at least three different syntrophin isoforms. Moreover, mAb 1351 recognizes an epitope within the PDZ domain and competes with the binding of C-terminal peptide ligands (S.G., unpublished observations). Therefore, the antibody may only weakly recognize syntrophins with bound ligand. Nonetheless, these results suggest that some DGK-ζ and syntrophins are colocalized in neurites and growth cones.

To confirm and extend these findings, we examined DGK-ζ localization in transiently transfected N1E-115 cells induced to differentiate by serum deprivation. Shortly after serum withdrawal, neuroblastoma cells flatten and produce filopodia and lamellipodia around their circumference, which gradually polarize to one or two discreet regions called neurite buds, which then give rise to neurites (Sarner *et al.*, 2000). In N1E-115 cells differentiated for 48 h, recombinant HA-tagged DGKζ accumulated in distal neurite shafts and at neurite tips (Figure 3.1D and E). At earlier times after differentiation (≤ 24 h), HA-DGK-ζ was highly concentrated in neurite buds of polarized N1E-115 cells (Figure 3.1F and

Figure 3.2. DGK-ζ induces the formation of neurite-like extensions in N1E-115 cells. (A) N1E-115 cell lysates were fractionated as described in Materials and Methods and equal amounts of protein from each fraction were analyzed by SDS-PAGE and immunoblotting for DGK-ζ (*top panel*) and syntrophin (*bottom panel*). (B) Schematic showing the location of various protein domains within DGK-ζ and the organization of the various DNA constructs used. The N-terminal (*open box*), C1 (*ellipses*), MARCKS (*filled box*) and catalytic domains are indicated. Also shown are the four ankyrin repeats (I-IV) at the C-terminus and three MAP kinase phosphorylation sites (*grey triangles*). The filled inverted triangle indicates a Gly to Asp mutation in the catalytic domain, which completely eliminates DGKζ activity (DGK-$\zeta^{\Delta ATP}$). The open inverted triangle indicates four Ser to Asp mutations in the MARCKS domain, which mimic phosphorylation at these sites. The filled box containing an *X* represents a FLAG epitope tag, which blocks binding to syntrophins. (C-F) Representative images of N1E-115 cells grown on laminin in serum-containing medium and expressing the indicated DGK-ζ constructs with N-terminal HA (C, E, and G) or C-terminal FLAG (D and F) epitope tags. The cell shown in (G) was cotransfected with myc-tagged $Rac1^{N17}$. After 24 h, the cells were fixed and stained with epitope tag-specific antibodies. In (G), only the anti-myc labeling is shown. Comparison of the image in (E) with the corresponding phase contrast image (E') shows untransfected cells in the same field remained round. (H) The graph shows the percentage of cells bearing neurite-like processes, quantified as described in Materials and methods. Error bars represent the S.E.M. for at least three independent experiments in which at least 300 cells were counted per condition. Scale bars, 20 μm. An *asterisk* indicates a statistically significant difference from the vector control ($p<0.05$, two-tailed *t* test).


A
Total
Cyto
Memb
Csk
DGK-ζ
Syn
B
Catalytic
I II III IV
DGK-ζ wt (928 aa)
DGK-ζΔATP
DGK-ζFLAG
DGK-ζM1
DGK-ζM1-ΔATP
DGK-ζM1-FLAG
C
DGK-ζ
D
DGK-ζFLAG
E
DGK-ζM1
F
DGK-ζM1-FLAG
E'
G
RacN17
DGK-ζ
H
% Cells with Neurites
70
60
50
40
30
20
10
0
Vector
DGK-ζ
DGK-ζΔATP
DGK-ζFLAG
DGK-ζ/RacN17
DGK-ζM1
DGK-ζM1-ΔATP
DGK-ζM1-FLAG

G, *arrows*). Punctate (endogenous) syntrophin immunoreactivity was also concentrated in these early processes (Figure 3.1H, *arrow*). Collectively, these results show that syntrophins and DGK-ζ are present at the tips of neurites and neurite-like protrusions and raise the possibility that they play a role in process outgrowth.

DGK-ζ induces process outgrowth in N1E-115 cells

To explore potential roles of DGK-ζ in neurite outgrowth, we transiently transfected N1E-115 cells grown in the presence of serum with cDNAs encoding wild type or mutant versions of DGK-ζ. The constructs are shown schematically in Figure 3.2B. Expression of HA-tagged wild-type DGK-ζ in N1E-115 cells caused the cells to flatten and induced the formation of neurite-like extensions (Figure 3.2C and H). In contrast, N1E-115 cells transfected with vector alone appeared similar to untransfected cells; they remained round and only a small proportion had processes (Figure 3.2H). These findings were obtained with cells plated on laminin, but similar results were obtained for cells plated on other substrates such as polyethylenimine (not shown). Thus, DGK-ζ expression induces morphological changes in the presence of serum, conditions that normally prevent process outgrowth.

To examine whether the kinase activity of DGK-ζ is necessary for its ability to induce morphological changes, we used a mutant (DGK-$\zeta^{\Delta ATP}$) in which Asp was substituted for Gly 355, a critical residue in the ATP binding site. *In vitro* lipid kinase analysis of recombinant DGK-$\zeta^{\Delta ATP}$ has shown that it is inactive (Topham *et al.*, 1998). Similar to the wild type protein, DGK-$\zeta^{\Delta ATP}$ expression induced process outgrowth (Figure 3.2H), indicating that this effect is mostly independent of DGK-ζ kinase activity.

Recent studies have shown that protein kinase C (PKC)-dependent phosphorylation of the DGK-ζ MARCKS domain induces it to translocate to the plasma membrane (Santos *et al.*, 2002; Abramovici *et al.*, 2003). Since a mutant that mimics constitutive phosphorylation of the MARCKS domain (DGK-ζ^{M1}) shows increased association with the plasma membrane (Abramovici *et al.*, 2003), we hypothesized that it might be more biologically active in N1E-115 cells, even though it has only about half the catalytic activity of wild type DGK-ζ (Luo *et al.*, 2003b). Consistent with this idea, DGK-ζ^{M1} induced a ~2-fold increase in the number of cells with processes compared to wild type DGK-ζ (Figure 3.2E and H). A constitutively phosphorylated, but catalytically inactive mutant (DGK-$\zeta^{M1\text{-}\Delta ATP}$) was less effective than DGK-ζ^{M1}, but was more effective than wild type DGK-ζ, confirming that kinase activity is not required for the induction of process outgrowth (Figure 3.2H). In addition, these results suggest targeting DGK-ζ to the plasma membrane may contribute to the induction of outgrowth.

DGK-ζ-induced process outgrowth requires PDZ interactions

To assess the involvement of syntrophins in DGK-ζ-induced outgrowth, we used a wild type construct, but with a C-terminal FLAG epitope tag (DGK-ζ^{FLAG}), which blocks the interaction with the syntrophin PDZ domain (Hogan *et al.*, 2001; Abramovici *et al.*, 2003). We have previously shown that this DGK-ζ mutant causes aberrant changes in the actin cytoskeleton when expressed in muscle cells (Abramovici *et al.*, 2003). When expressed in N1E-115 cells, DGK-ζ^{FLAG} failed to induce neurite-like extensions (Figure 3.2D and H). Even the membrane-associated mutant DGK-ζ^{M1} did not induce process outgrowth when its C-terminal PDZ-binding motif was similarly blocked (DGK-$\zeta^{M1\text{-}FLAG}$; Figure 3.2F and H).

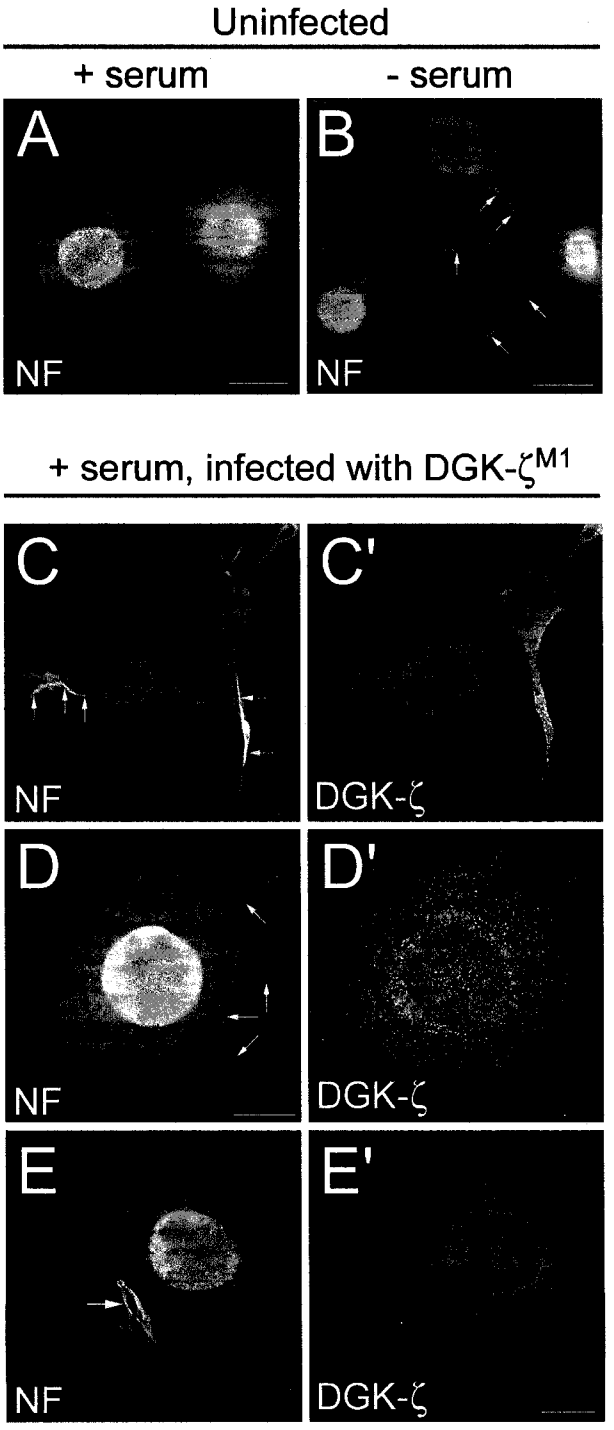
These results demonstrate that the C-terminal PDZ-binding motif is necessary for DGK-ζ-induced process outgrowth and suggest DGK-ζ triggers outgrowth by a mechanism that requires interaction with syntrophins.

DGK-ζ induces neurofilament expression

To determine if the cellular processes induced by DGK-ζ expression in N1E-115 cells have neurite-like properties, we used an antibody against the phosphorylated 200 kDa neurofilament subunit, which is specifically expressed in differentiated neuronal cells. We first tested the specificity of the antibody by staining undifferentiated N1E-115 cells grown in the presence of serum and N1E-115 cells induced to differentiate by serum deprivation. Undifferentiated cells showed background nuclear and cytoplasmic staining, but no filamentous staining (Figure 3.3A). In contrast, differentiated N1E-115 cells displayed prominent staining of neurofilaments in cellular processes (Figure 3.3B, *arrows*).

For these experiments, N1E-115 cells grown in the presence of serum were infected with an adenoviral vector encoding DGK-ζ^{M1} (AdDGK-ζ^{M1}; see Materials and Methods). Neurite-like extensions induced by expression of DGK-ζ^{M1} showed bright neurofilament staining (Figure 3.3C, *arrows* and C'). Even round, flattened cells with fibroblast-like morphologies had radially-oriented bundles of neurofilaments in multiple small cellular extensions (Figure 3.3D, *arrows* and D'). Interestingly, large flattened cells were occasionally observed with disordered bundles of neurofilaments in the cytoplasm (Figure 3.3E, *arrow*) suggesting DGK-ζ-induced expression of neurofilaments does not always result in productive process outgrowth. Nonetheless, these results suggest DGK-ζ induces the formation of cellular processes with neurite-like properties.

Figure 3.3. DGK-ζ-induced cellular processes contain neurofilaments. N1E-115 cells were grown in the presence of serum (A and C - E') or were differentiated for 48 h by serum deprivation (B). Uninfected cells (A and B), or cells infected with an adenoviral vector engineered to express HA-tagged DGK-ζ^{M1} (see Materials and Methods), were fixed and stained with anti-phospho-neurofilament (NF) and anti-HA antibodies (DGK-ζ). The arrows indicate bundles of neurofilaments. (E) Note the large neurofilament bundle in a cell with no neurites. Scale bars, 20 μm.


Uninfected
+ serum
- serum
A
NF
B
NF
+ serum, infected with DGK-ζ^{M1}
C
NF
C'
DGK-ζ
D
NF
D'
DGK-ζ
E
NF
E'
DGK-ζ

DGK-ζ mutants that do not interact with syntrophin inhibit outgrowth

Since C-terminally FLAG-tagged versions of DGK-ζ failed to stimulate outgrowth in serum-containing media, we tested their ability to inhibit neurite outgrowth induced by serum deprivation of N1E-115 cells. Expression of either DGK-ζ^{FLAG} or DGK-$\zeta^{M1\text{-}FLAG}$ dramatically inhibited neurite outgrowth (Figure 3.4A, E and F). Interestingly, these constructs occasionally induced the formation of large intracellular vesicles (Figure 3.4E and F, *arrows*). Dominant-negative Rac1 ($Rac1^{N17}$; see below) inhibited neurite outgrowth to a similar extent (Figure 3.4A), but vesicles were not observed (Figure 3.4G). Vector alone, wild type DGK-ζ or DGK-$\zeta^{\Delta ATP}$ did not inhibit serum deprivation-induced neurite outgrowth (Figure 3.4A-D). Collectively, these results suggest the DGK-ζ-syntrophin interaction is important for serum deprivation-induced neurite outgrowth in N1E-115 cells.

We next determined whether process outgrowth from cultured cortical neurons is affected by exogenous expression of DGK-ζ. Cortical neurons were infected 3 h after plating with adenoviral constructs encoding either wild type DGK-ζ or DGK-ζ^{FLAG}. After 24 h, the cells were fixed and double labeled with anti-HA antibodies and mAb 3A10 against neurofilament-associated proteins (Harada *et al.*, 1999). Cells infected with DGK-ζ showed robust neurite outgrowth, and often had several long, branched neurites (Figure 3.5A-A" and C). In contrast, cortical neurons infected with DGK-ζ^{FLAG} almost never extended neurites and were rounded, even though cells in the same field had neurites (Figure 3.5B-B" and D). In addition, cells infected with DGK-ζ^{FLAG} (D), but not wild type DGK-ζ (C), had globular accumulations of actin in their cell bodies, suggesting a possible defect in actin organization.

Figure 3.4. DGK-ζ mutants that do not interact with syntrophins inhibit serum starvation-induced neurite outgrowth. N1E-115 cells transfected with the indicated DGK-ζ constructs were induced to differentiate by serum withdrawal for 48 h. The cells were fixed, stained and assessed for neurite outgrowth as in Figure 3.2. (A) The graph shows the percentage of cells bearing neurites for each construct indicated. The error bars represent the S.E.M. for at least three independent experiments (n = 300 cells per condition). An *asterisk* indicates a statistically significant difference from the vector control ($p<0.05$, two-tailed *t* test). (B-G) Representative photomicrographs showing the morphology of N1E-115 cells expressing the indicated constructs with either N-terminal HA (C and D), C-terminal FLAG (E and F), or myc (G) epitope tags. The cell in (B) is expressing green fluorescent protein. The *arrows* in (E) and (F) indicate large cytoplasmic vesicles or vacuoles. Scale bars, 20 μm.

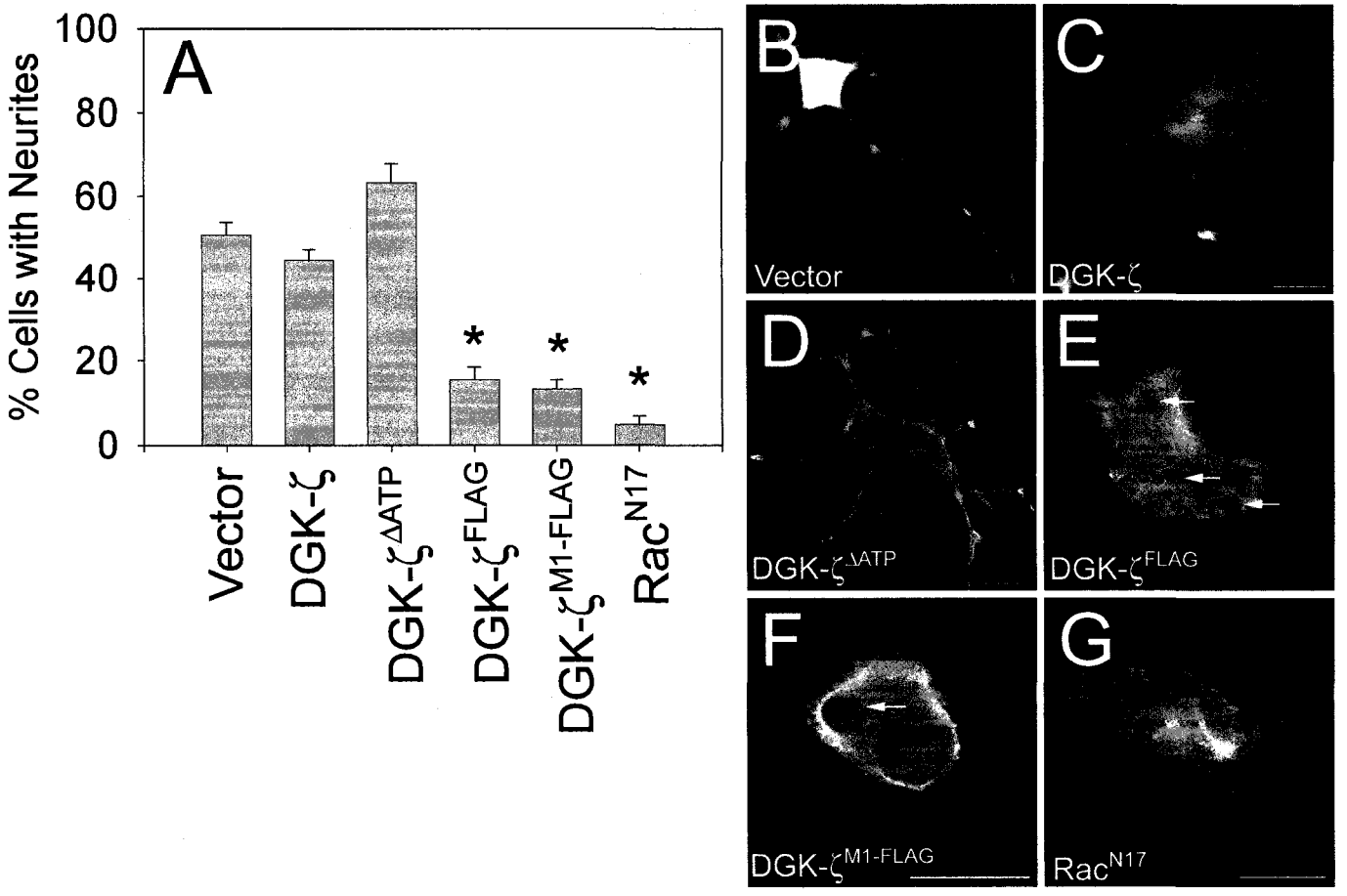
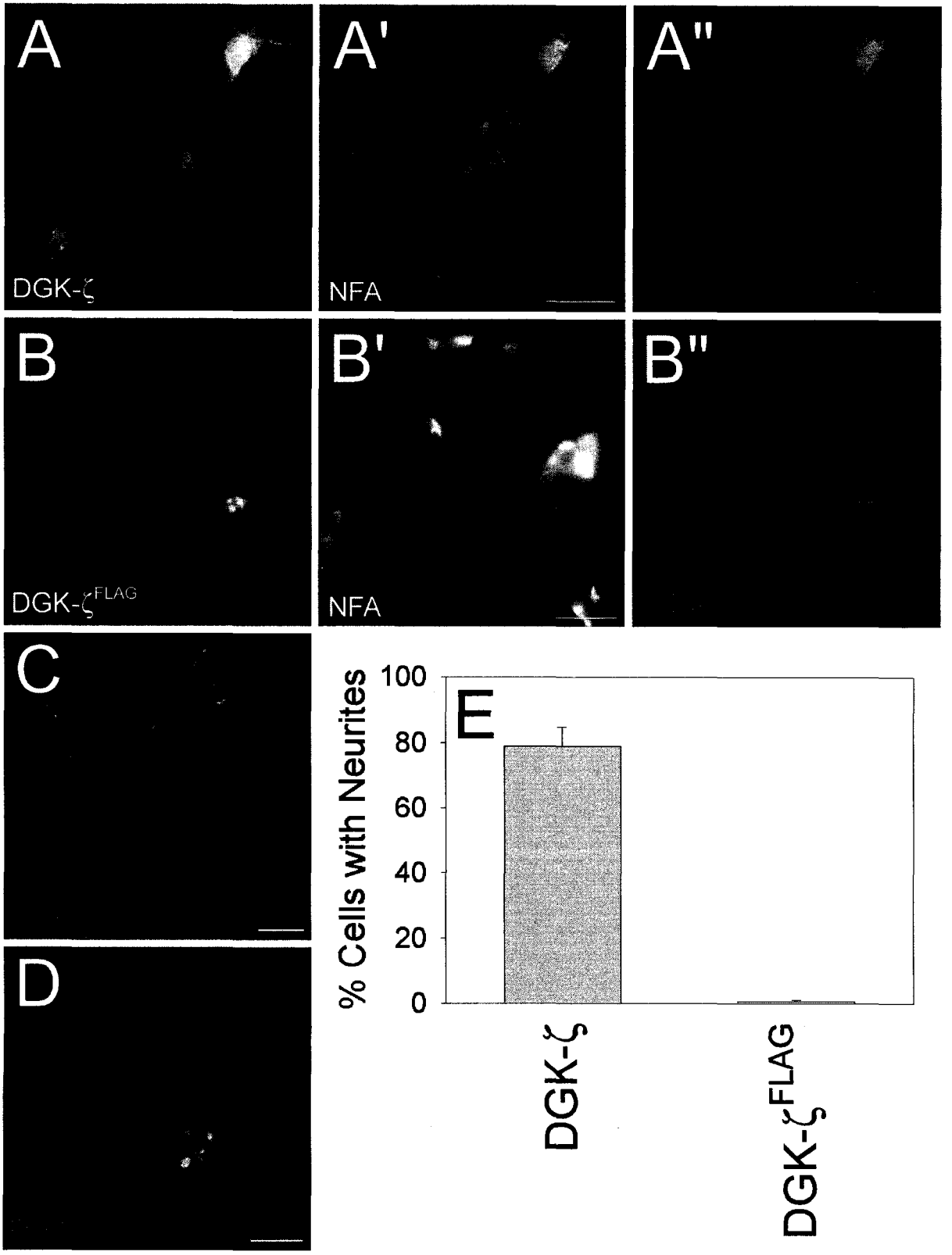

A
% Cells with Neurites
100
80
60
40
20
0
Vector
DGK-ζ
DGK-ζΔATP
DGK-ζFLAG
DGK-ζM1-FLAG
RacN17
*
*
*
B
Vector
C
DGK-ζ
D
DGK-ζΔATP
E
DGK-ζFLAG
F
DGK-ζM1-FLAG
G
RacN17

Figure 3.5. DGK-ζ^{FLAG} inhibits cortical neuron process outgrowth. Embryonic mouse cortical neurons were plated on laminin-coated coverslips, allowed to adhere for 3 h, then were infected with adenoviral constructs encoding either HA-tagged wild type DGK-ζ or DGK-ζ^{FLAG}. After 24 h, the cells were fixed and double labeled with epitope-tag specific antibodies and either mAb 3A10 against neurofilament-associated proteins (Harada *et al.*, 1999) (NFA; A – B") or AlexaFluor 594-conjugated phalloidin (C and D). (A-A") Representative images of cortical neurons infected with wild type DGK-ζ, which have several long, branched neurites. (B-B") Cells infected with DGK-ζ^{FLAG} were generally round and lacked neurites. However, note the neurites from other cells in the same field. Higher power confocal images show the morphology of neurons expressing DGK-ζ (C) and DGK-ζ^{FLAG} (D). Labeling for actin showed considerable overlap with wild type DGK-ζ in the soma and neurites and revealed globular accumulations in the cell expressing DGK-ζ^{FLAG}. Scale bars, 50 μm (A-A"), 25 μm (B-B") and 20 μm (C and D). (E) The graph shows the percentage of cells bearing neurites for each construct indicated. The error bars represent the S.E.M. for three independent experiments (n = 100 cells per condition).


A
A'
A"
DGK-ζ
NFA
B
B'
B"
DGK-ζFLAG
NFA
C
D
E
% Cells with Neurites
100
80
60
40
20
0
DGK-ζ
DGK-ζFLAG

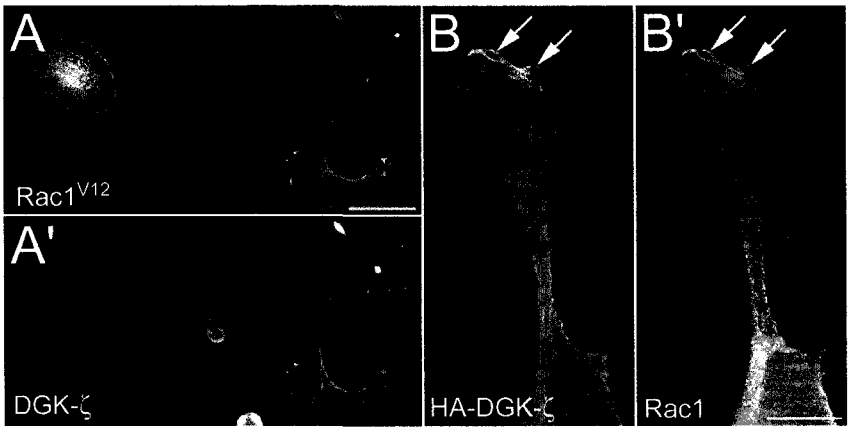
Thus, as in N1E-115 cells, DGK-ζ^{FLAG} prevents neurite extension in cortical neurons, supporting the idea that DGK-ζ-syntrophin interactions play an important role in neurite outgrowth.

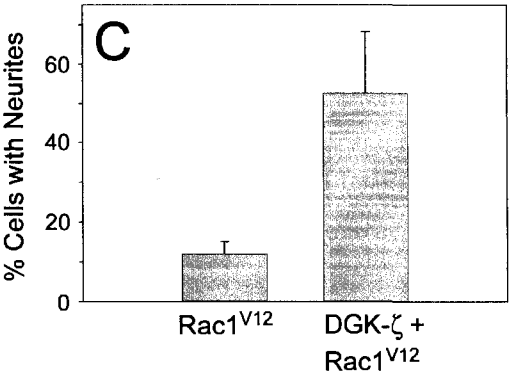
DGK-ζ-induced morphological changes are Rac1-dependent

Studies using a dominant-negative mutant ($Rac1^{N17}$) have established a key role for Rac1 in lamellipodia formation and neurite outgrowth (Kozma *et al.*, 1997). $Rac1^{N17}$ is mainly GDP bound, and blocks endogenous Rac1 function by sequestering regulatory molecules required to activate the wild-type protein. In N1E-115 cells, coexpression with $Rac1^{N17}$ completely blocked process outgrowth induced by either wild type DGK-ζ (Figure 3.2G and H) or DGK-ζ^{M1} (data not shown). These results suggest the induction of neurites by DGK-ζ is dependent on Rac1 and tentatively place DGK-ζ upstream of Rac1 activation.

To further investigate the relationship of DGK-ζ to Rac1 in neurite outgrowth, N1E-115 cells grown in the presence of serum were cotransfected with DGK-ζ and a constitutively-active Rac1 mutant ($Rac1^{V12}$). Previous studies have shown that $Rac1^{V12}$, which persists in a GTP-bound state and activates downstream Rac1 effectors, has dramatic effects on the morphology of neuronal cells, but does not induce neurite outgrowth. In agreement with these studies, we found that cells expressing $Rac1^{V12}$ had a characteristic round, flattened appearance, but did not form neurite-like extensions (Figure 3.6A, A'- *left*, and C). Thus, Rac1 activity alone appears to be insufficient for process outgrowth, at least in N1E-115 cells. In contrast, cells expressing both wild type DGK-ζ and $Rac1^{V12}$ extended long processes within 24 h and had large lamellipodia at their leading edges (Figure 3.6A, A' - *right*, and C). Furthermore, in cells expressing HA-tagged DGK-ζ alone, endogenous Rac1

Figure 3.6. Morphological characteristics of N1E-115 cells expressing DGK-ζ and constitutively active Rac1. (A-A') Cells grown in the presence of serum were cotransfected with myc-Rac1^{V12} and HA-DGK-ζ. After 24 h, the cells were fixed and double-labeled with anti-myc (A) and anti-HA (A') antibodies. Cells expressing Rac1^{V12} had a characteristic round, flattened appearance (A, *left*) while those expressing both Rac1^{V12} and DGK-ζ had neurite-like extensions and had prominent lamellipodia (A and A', *right*). (B and B') Cells expressing HA-DGK-ζ were double-labeled with anti-HA and anti-Rac1 antibodies. The arrows indicate close colocalization of endogenous Rac with DGK-ζ in membrane ruffles at the leading edge of a process. Scale bars = 50 μm (A and A'), 20 μm (B and B'). (C) The graph shows the percentage of cells bearing neurite-like extensions for each construct indicated. The error bars represent the S.E.M. for at least three independent experiments. An *asterisk* indicates a statistically significant difference from the vector control ($p<0.05$, two-tailed *t* test).


A
Rac1V12
A'
DGK-ζ
B
HA-DGK-ζ
B'
Rac1


C
% Cells with Neurites
0
20
40
60
Rac1V12
DGK-ζ +
Rac1V12

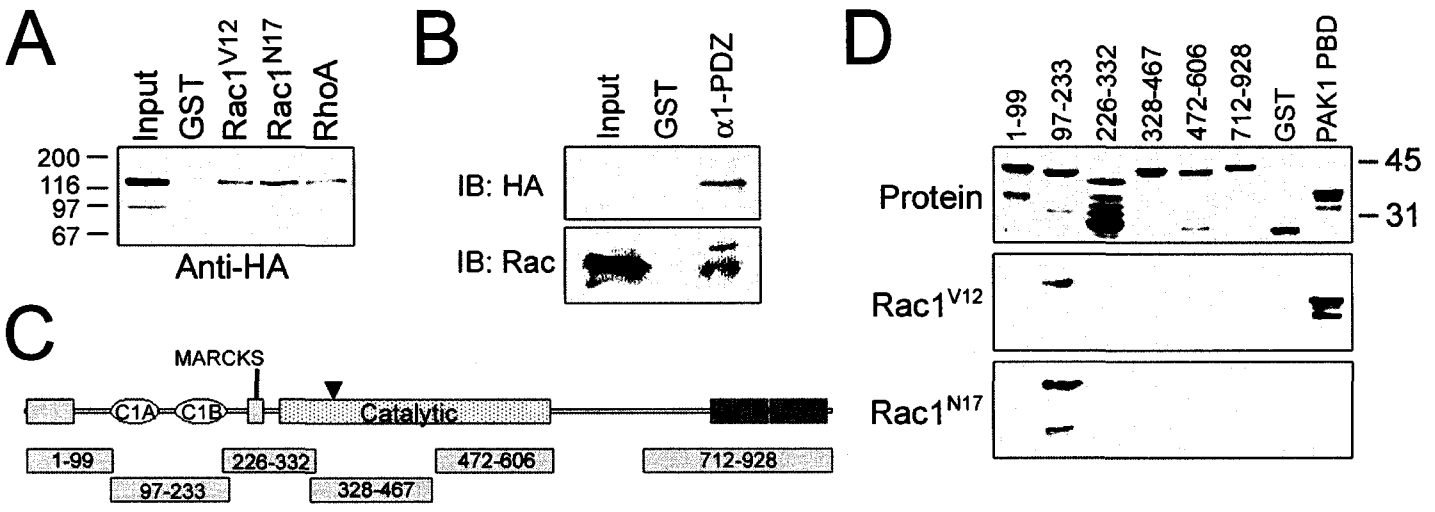
closely colocalized with DGK-ζ in membrane ruffles at the leading edge of cellular extensions (Figure 3.6B and B', *arrows*). Together, these data raise the possibility that DGK-ζ and Rac1 act in a coordinate manner to regulate process outgrowth.

DGK-ζ interacts with Rac1 and Rho

We next determined whether the observed effects of DGK-ζ on neurite outgrowth are the result of a cooperative interaction between DGK-ζ and Rac1. We first performed affinity precipitation experiments using N1E-115 cell lysates that had been infected with an adenoviral construct encoding wild type HA-tagged DGK-ζ. Beads charged with GST or GST fusion proteins of $Rac1^{V12}$ and $Rac1^{N17}$ were incubated with the lysates. Bound DGK-ζ was detected by immunoblotting with an anti-HA antibody. We included GST-RhoA as a control, expecting that DGK-ζ would not interact with it. Surprisingly, we found that $Rac1^{V12}$, $Rac1^{N17}$ and RhoA all captured DGK-ζ from the lysate, while none was captured by control GST beads (Figure 3.7A).

Since DGK-ζ was closely colocalized with Rac1 and not RhoA in cortical neurons (discussed below), we further characterized its interaction with Rac1. To determine if the DGK-ζ that is bound to Rac1 is also able to bind syntrophin, beads charged with a GST fusion protein of the α1-syntrophin PDZ domain (α1-PDZ) were used to capture HA-DGK-ζ from N1E-115 cell lysates (Figure 3.7B, *top panel*). Endogenous Rac1 was coprecipitated with DGK-ζ (Figure 3.7B, *bottom panel*). Since PDZ domains generally only bind a single ligand at a time, the most likely interpretation of these results is that the captured Rac1 was bound to DGK-ζ, which itself bound to α1-PDZ through its C-terminal PDZ-binding motif.

Figure 3.7. Rac1 binds directly to the C1 domains of DGK-ζ. (A) N1E-115 cells were infected with an adenoviral vector encoding HA-tagged DGK-ζ. After 48 h, the cells were lysed and the extracts were incubated with beads charged with GST, GST-Rac1^{V12}, GST-Rac1^{N17} or GST-RhoA. The bound proteins were analyzed by immunoblotting with anti-HA antibodies. *Input* represents 10% of the starting material. (B) Lysates of N1E-115 cells transfected with HA-DGK-ζ were incubated with GST or a GST fusion protein of the α1-syntrophin PDZ domain (α1-PDZ) and the bound proteins analyzed by immunoblotting with anti-HA (*top panel*) and anti-Rac1 (*bottom panel*) antibodies. (C) The indicated regions of DGK-ζ were fused to the C-terminus of GST. The schematic of DGK-ζ is as described in Figure 3.2. (D) The constructs shown in (C) were expressed in bacteria, purified, analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. GST, and a GST fusion protein of the p21-binding domain of PAK1 (PAK1 PBD) were included as negative and positive controls, respectively. The top panel (*protein*) is a Ponceau S-stained blot showing the positions and amounts of the various fusion proteins. The same blot was probed with purified recombinant Rac1^{V12} containing an S-Tag epitope, followed by S-Protein conjugated to HRP (*middle panel*). A duplicate blot was probed in the same manner with Rac1^{N17} (*bottom panel*).

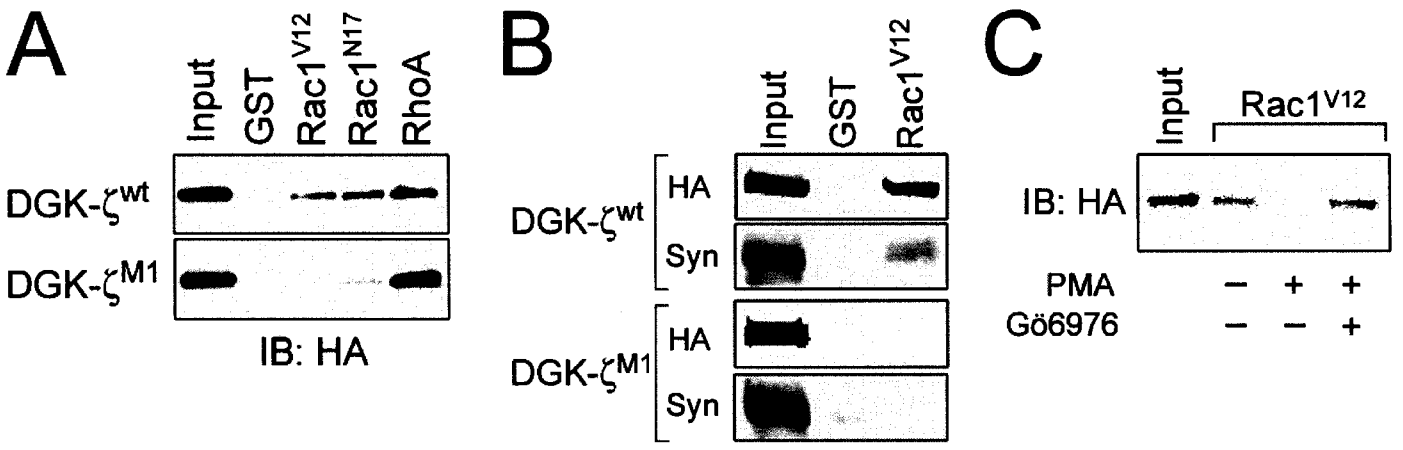

A
Input
GST
Rac1V12
Rac1N17
RhoA
200
116
97
67
Anti-HA
B
Input
GST
α1-PDZ
IB: HA
IB: Rac
C
MARCKS
C1A
C1B
Catalytic
1-99
97-233
226-332
328-467
472-606
712-928
D
1-99
97-233
226-332
328-467
472-606
712-928
GST
PAK1 PBD
Protein
Rac1V12
Rac1N17
45
31

To test whether DGK-ζ binds directly to Rac1, soluble His_6 fusion proteins of $Rac1^{V12}$ and $Rac1^{N17}$ (containing S-Tag epitopes) were used to overlay various GST fusion proteins of DGK-ζ, shown schematically in Figure 3.7C. As shown in Figure 3.7D, both $Rac1^{V12}$ and $Rac1^{N17}$ bound specifically to a DGK-ζ fusion protein containing amino acids 97-233, which includes two cysteine-rich regions homologous to the C1A and C1B motifs of PKCs (Hurley *et al.*, 1997). That both Rac1 mutants displayed comparable binding to DGK-ζ suggests the interaction is not significantly dependent on the activation state of the GTPase. As expected, only the $Rac1^{V12}$ mutant bound to a GST fusion protein corresponding to the p21-binding domain of PAK1 (PAK1 PBD), indicating that the two mutants retained their binding specificity for this particular Rac1 effector. Neither Rac1 mutant bound to GST or to other DGK-ζ domains, further demonstrating the specificity of the interaction.

Phosphorylation of the MARCKS domain causes DGK-ζ and Rac1 to dissociate

The increased activity of $DGK\text{-}\zeta^{M1}$ in neurite outgrowth assays prompted us to determine whether phosphorylation of the MARCKS domain affects the interaction with Rac1. To begin to address this possibility, we compared the ability of wild-type DGK-ζ and $DGK\text{-}\zeta^{M1}$ to bind to Rac1 in pull-down assays as described for Figure 3.7A. As before, $Rac1^{V12}$, $Rac1^{N17}$ and RhoA, but not GST efficiently bound wild-type DGK-ζ (Figure 3.8A, *top panel*). In contrast, $DGK\text{-}\zeta^{M1}$ did not bind to $Rac1^{V12}$ and weakly bound to $Rac1^{N17}$, whereas binding to RhoA was unaffected (Figure 3.8A, *bottom panel*).

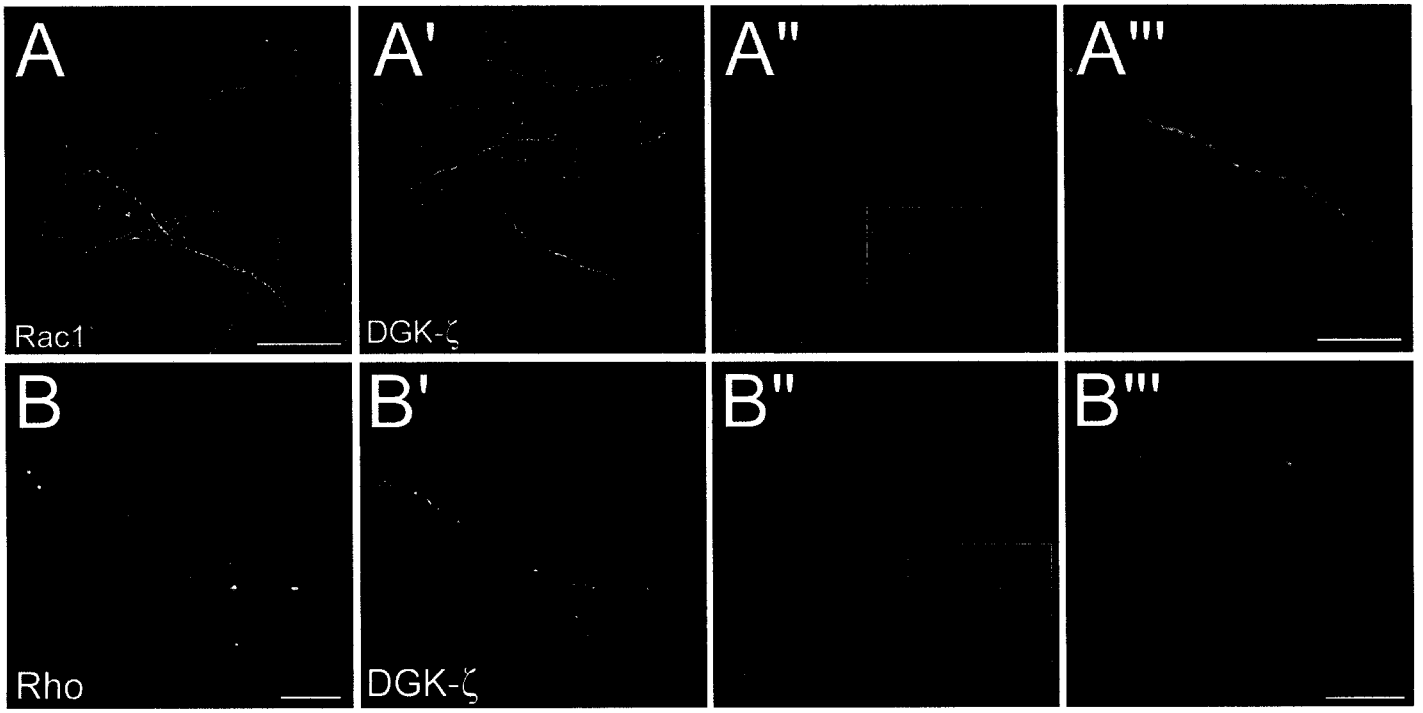
Figure 3.8. Phosphorylation of the MARCKS domain induces the dissociation of DGK-ζ and Rac1. (A) A pull-down assay as in Figure 3.7A comparing the ability of wild-type DGK-ζ (*top panel*) and DGK-ζ^{M1} (*bottom panel*) to bind to GST or GST fusion proteins of $Rac1^{V12}$, $Rac1^{N17}$ and RhoA. The bound proteins were analyzed by immunoblotting with anti-HA antibodies. *Input* represents 10% of the starting material. (B) Lysates of HeLa cells transiently transfected with HA-DGK-ζ or HA-DGK-ζ^{M1} were incubated with beads charged with GST or with GST-$Rac1^{V12}$. The bound proteins were analyzed by immunoblotting with anti-HA and anti-syntrophin antibodies. (C) N1E-115 cells infected with HA-DGK-ζ were stimulated with PMA or vehicle for 30 min. The cell extracts were incubated with GST-$Rac1^{V12}$ beads and the bound DGK-ζ was analyzed by blotting with anti-HA antibodies. To inhibit PKC activity, cells were treated with Gö6976 for 10 min before PMA stimulation.


A
Input
GST
Rac1V12
Rac1N17
RhoA
DGK-ζwt
DGK-ζM1
IB: HA
B
Input
GST
Rac1V12
DGK-ζwt
HA
Syn
DGK-ζM1
HA
Syn
C
Input
Rac1V12
IB: HA
PMA
Gö6976

We repeated this experiment using lysates of transiently transfected HeLa cells. In this cell type as well, GST- $Rac1^{V12}$ captured wild-type DGK-ζ but not DGK-$ζ^{M1}$ from the offered extracts (Figure 3.8B, *HA*). Moreover, endogenous syntrophins were captured in the presence of wild-type DGK-ζ but not in the presence of DGK-$ζ^{M1}$ (Figure 3.8B, *Syn*). These data suggest phosphorylation of the MARCKS domain attenuates the interaction of DGK-ζ and syntrophins with Rac1. To further test this possibility, we examined whether phorbol esters, potent activators of PKC, affected the interaction of DGK-ζ with Rac1. We monitored the binding of DGK-ζ to $Rac1^{V12}$ in lysates of N1E-115 cells infected with wild-type DGK-ζ and observed that treating the cells with PMA completely inhibited their interaction (Figure 3.8C). A PKC inhibitor abolished this inhibition, indicating that PKC activity was required to attenuate the binding. Collectively, these results suggest PKC-mediated phosphorylation of the MARCKS domain causes DGK-ζ and syntrophin to dissociate from active Rac1.

To provide additional evidence that DGK-ζ and Rac1 specifically associate in neurons, cultures of mouse cortical neurons double labeled for DGK-ζ and Rac1 were visualized by confocal microscopy. Single optical sections from a deconvolved Z-series of images showed that DGK-ζ and Rac1 were closely colocalized in neurites (Figure 3.9A - A"'). In contrast, DGK-ζ immunoreactivity did not overlap with labeling for RhoA. Moreover, punctate RhoA labeling was mainly present in areas depleted of DGK-ζ labeling (Figure 3.9B - B"'). Thus, these results support the idea that DGK-ζ and Rac1 physically interact in neurons.

Figure 3.9. Colocalization of DGK-ζ with Rac1, but not RhoA, in cultured cortical neurons. Mouse cortical neurons cultured for 3 days *in vitro* were fixed and double labeled for DGK-ζ and Rac1 (A-A''') or DGK-ζ and RhoA (B-B''') followed by AlexaFluor 488 (*green*) and AlexaFluor 594 (*red*) secondary antibodies, respectively. Z-series of the red and green channels were obtained by confocal microscopy. The image stacks were deconvolved as described in Materials and Methods. Shown are single 0.3 μm optical sections from each channel. The merged image shows extensive overlap of the DGK-ζ and Rac1 signals in neurites, as indicated by the yellow color. Note that not all neurites appear yellow due to differences in the intensity of the red and green labeling. (A''' and B''') Magnified images of the regions indicated by *boxes*. Scale bars, 20 μm (A and B), 5 μm (A''') and 10 μm (B''').


A
A'
A''
A'''
Rac1
DGK-ζ
B
B'
B''
B'''
Rho
DGK-ζ

Discussion

The results presented here establish a key role for DGK-ζ in the process of neurite outgrowth in N1E-115 cells. Expression of DGK-ζ induced outgrowth on laminin (and other substrates) in the presence of serum, which normally prevents neurite outgrowth. Surprisingly, the ability of DGK-ζ to stimulate neurite formation was largely independent of its lipid kinase activity, since a catalytically inactive DGK-ζ mutant (DGK-$\zeta^{\Delta ATP}$) (Topham *et al.*, 1998) induced outgrowth almost to the same extent as the wild type protein. This result, combined with the finding that DGK-$\zeta^{\Delta ATP}$ did not inhibit serum deprivation-induced neurite outgrowth, suggests DGK-ζ activity is not required for neurite formation. It is possible though, that DGK-ζ catalytic activity impinges on an aspect of neurite morphology, such as branching, that was not quantified in our studies.

In previous studies, we demonstrated that DGK-ζ mutants with a blocked C-terminus do not interact with syntrophins and are associated with the plasma membrane less often (Hogan *et al.*, 2001; Abramovici *et al.*, 2003). In the present study, these mutants (DGK-ζ^{FLAG} and DGK-$\zeta^{M1\text{-}FLAG}$) were unable to induce neurite outgrowth in N1E-115 cells, despite the fact that they were expressed at levels equivalent to the wild type construct (H.A. and S.G., unpublished observations). They also inhibited neurite outgrowth of cultured cortical neurons and of serum-deprived N1E-115 cells. These findings demonstrate that the C-terminal PDZ-binding motif of DGK-ζ is necessary for neurite outgrowth.

While we cannot exclude the possibility that the effect of these mutants is due in part to disruption of interactions with PDZ-containing proteins other than syntrophins, our previous studies showing that the C-terminus of DGK-ζ specifically binds to syntrophin PDZ domains and not to other closely related PDZ domains (Hogan *et al.*, 2001), strongly suggest

it is the interaction with syntrophins that is required for neurite outgrowth. Indeed, accumulating evidence suggests syntrophins are necessary for recruiting and organizing their protein partners into specialized complexes at the plasma membrane (Brenman *et al.*, 1996a; Kachinsky *et al.*, 1999; Kameya *et al.*, 1999; Adams *et al.*, 2000; Ort *et al.*, 2001).

How do DGK-ζ mutants that do not bind to syntrophins exert a dominant-negative effect on neurite outgrowth? In principle, they should not block the interaction of endogenous DGK-ζ with syntrophins because they do not bind them. The mutants might interfere with endogenous DGK-ζ function by sequestering effector proteins such as Rac1 (see below). If signaling complexes formed by DGK-ζ^{FLAG} are not directed to specialized domains of the plasma membrane by syntrophins, then they might be non-productive and, consequently, no morphological changes would ensue. Alternatively, indiscriminate targeting of DGK-ζ^{FLAG} complexes to the plasma membrane could result in spatially unrestricted activity, leading to gross, unpolarized changes in cell morphology.

At first glance, our finding that dominant-negative Rac1 completely blocked DGK-ζ-induced neurite outgrowth argues that DGK-ζ functions upstream of Rac1. However, this interpretation may be overly simplistic because constitutively active Rac1 was unable to induce neurite outgrowth by itself. Instead, expression of $Rac1^{V12}$ produced highly flattened and spread cells, often with edge ruffles, similar to its effects on other cell types. These findings agree with two previous reports which showed that Rac1 induces dramatic morphological changes, but does not result in polarized outgrowth of neuron-like cells (Daniels *et al.*, 1998; Sarner *et al.*, 2000).

We favor the idea that Rac1 functions cooperatively with DGK-ζ to induce neurite outgrowth. Several lines of evidence support this interpretation. First, DGK-ζ and Rac1

together induced ~5-fold more neurite-like processes in N1E-115 cells than Rac1 alone and both proteins were colocalized at the leading edge of cellular extensions in these cells. Second, biochemical experiments demonstrated that Rac1 directly interacts with DGK-ζ. Both the constitutively active and the dominant-negative mutants displayed comparable interaction with DGK-ζ, suggesting the interaction is not significantly dependent on the activation state of Rac1. Finally, DGK-ζ and Rac1 were closely colocalized in neurites of cultured cortical neurons. Evidence for the specificity of the interaction comes from our finding that a DGK-ζ mutant that mimics constitutive phosphorylation of the MARCKS domain (DGK-ζ^{M1}) did not bind to $Rac1^{V12}$ and that PMA-induced activation of PKC inhibited the interaction of wild-type DGK-ζ with $Rac1^{V12}$. Collectively, these results strongly indicate that DGK-ζ and Rac1 exist in a regulated signaling complex that controls neurite outgrowth.

Additionally, we provide evidence that DGK-ζ and syntrophin form a tertiary complex with Rac1 in N1E-115 cells. We favor the idea that DGK-ζ mediates the interaction between syntrophin and Rac1, because DGK-ζ binds directly to both proteins and because syntrophin was not captured by $Rac1^{V12}$ in the presence of DGK-ζ^{M1}, which itself does not interact with $Rac1^{V12}$. The association with DGK-ζ and syntrophins provides a potential mechanism for targeting Rac1 to specialized membrane domains within neurons.

Syntrophins bind directly to members of the dystrophin family of cytoskeletal proteins (Froehner *et al.*, 1997). Dystrophin, in turn, associates with a complex of intracellular, transmembrane and extracellular proteins collectively referred to as the dystrophin glycoprotein complex, which forms a transmembrane axis that tightly links the extracellular matrix to the actin cytoskeleton. Together, these interactions provide a

connection between DGK-ζ/Rac1 complexes, the actin cytoskeleton and the extracellular matrix. In this way, Rac1 activity can be spatially regulated and changes in actin organization mediated by Rac1 may be transmitted to the extracellular matrix to coordinately regulate intracellular and extracellular structural changes during neurite outgrowth.

Receptor-induced recruitment of effector proteins to the plasma membrane is a major theme in signaling (Hunter, 2000) and is particularly important for enzymes that act upon lipid second messengers, because it brings them into close proximity to their substrates. Membrane translocation appears to be a common paradigm for regulating DGK activity, but the molecular mechanisms underlying this process are not well understood (Sanjuan *et al.*, 2001; Imai *et al.*, 2002). Recently, Santos et al. (2002) showed DGK-ζ rapidly translocates to the plasma membrane of T-cells in response to activation of an exogenously expressed muscarinic receptor. They demonstrated that the kinase activity of DGK-ζ is not necessary for this translocation, but that PKC-driven phosphorylation of the MARCKS domain is required.

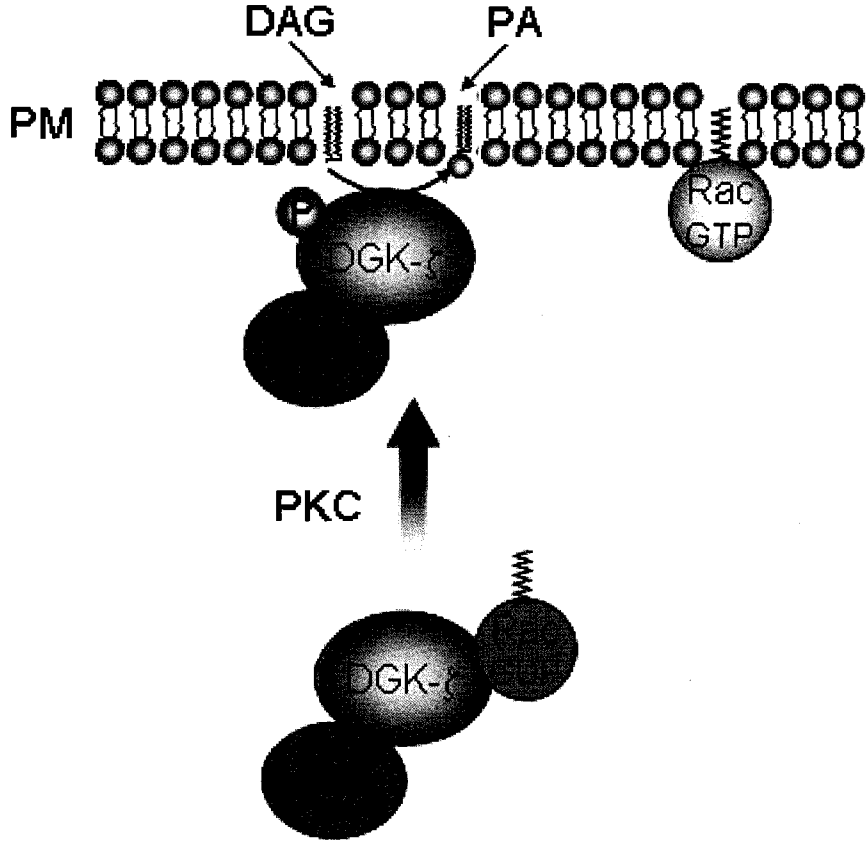
In agreement with their findings, we recently found that phosphorylation-mimicking DGK-ζ (DGK-ζ^{M1}) exhibits increased association with the plasma membrane of C2C12 muscle cells compared to the wild type protein (Abramovici *et al.*, 2003). The present study demonstrates that DGK-ζ^{M1} is ~2 fold more effective than the wild type protein at eliciting neurite outgrowth from N1E-115 cells. This effect is not due to enhanced kinase activity because DGK-ζ^{M1} is ~50% less active than the wild type enzyme (Luo *et al.*, 2003b). In addition, catalytically inactive DGK-ζ^{M1} (DGK-$\zeta^{M1\text{-}\Delta ATP}$) was as effective as the wild type protein at inducing neurite outgrowth, confirming that kinase activity is not essential for this

process. Taken together, these findings suggest membrane localization is an important aspect of DGK-ζ's ability to induce neurite outgrowth.

How might translocation of DGK-ζ from the cytosol to the plasma membrane drive neurite outgrowth? Our finding that phosphorylation of the MARCKS domain dissociates DGK-ζ from Rac1 (especially $Rac1^{V12}$) offers a possible mechanistic basis for this effect. We propose a model where, in the basal state, DGK-ζ and syntrophin associate with Rac1-GDP in the cytoplasm (Figure 3.10). Signals that stimulate neurite outgrowth cause PKC-mediated phosphorylation of the MARCKS domain, which induces the complex to translocate to the plasma membrane. This allows for nucleotide exchange on Rac1 (GTP for GDP) and its subsequent dissociation from DGK-ζ and syntrophin. In this scenario, Rac1 is delivered to the plasma membrane, activated and then released, which may account for the lack of complete overlap between DGK-ζ and Rac1 in cortical neurons.

This model implies that the MARCKS domain of DGK-ζ is a physiologic target of PKC. Indeed, it is the predominant nuclear localization signal of DGK-ζ and its phosphorylation by PKC reduces nuclear accumulation of DGK-ζ, which alters nuclear DAG levels (Topham *et al.*, 1998). In addition, a recent study showed that DGK-ζ directly associates with PKCα and negatively regulates its protein kinase activity (Luo *et al.*, 2003a). This association was abolished when the MARCKS domain of DGK-ζ was phosphorylated by PKCα, relieving the inhibition of PKCα activity. Thus, it appears that PKCα-mediated phosphorylation of the MARCKS domain causes DGK-ζ to dissociate from both PKCα and Rac1. Presumably, phosphorylation of the MARCKS domain causes a conformational change in DGK-ζ that alters its binding to different effector proteins. In support of this idea

Figure 3.10. Model for the regulation of the interaction between DGK-ζ and Rac1. In the basal condition, DGK-ζ and GDP-bound Rac1 form a complex with syntrophin in the cytoplasm. Upon stimulation, PKC-mediated phosphorylation of the MARCKS domain induces the complex to translocate to the plasma membrane where Rac1 is activated by exchange of GTP for GDP. This causes activated Rac1 to dissociate from DGK-ζ and syntrophin, which may allow it to bind to effector proteins that promote neurite outgrowth.


DAG
PA
PM
P
Rac
GTP
PKC

we found that MARCKS domain-phosphorylation increases the binding of DGK-ζ to syntrophin PDZ domains (E.D. and S.G., unpublished observations).

We have yet to find evidence for DGK-ζ-induced activation of Rac1, as measured by the binding of GTP-bound Rac1 to the PBD of PAK1 (H.A. and S.G, unpublished observations). How can this result be reconciled with our finding that dominant-negative Rac1 inhibits DGK-ζ-induced neurite outgrowth? One possibility is that DGK-ζ activates Rac1, but the GTP-bound Rac1 remains bound to the cytoskeleton and is "overlooked" by the PAK1 PBD assay, which only measures soluble GTP-bound Rac1 (Wojciak-Stothard and Ridley, 2003). Consistent with this possibility, a significant fraction of both DGK-ζ and syntrophins is associated with the insoluble cytoskeleton (Figure 3.2A). Alternatively, DGK-ζ might not directly activate Rac1, but a basal level of Rac1 activity may be necessary to induce the actin cytoskeletal changes that underlie DGK-ζ-induced neurite outgrowth.

Several studies have shown that inhibition or sequestration of Rho induces neurite outgrowth (Govek *et al.*, 2005). Interestingly, Houssa et al. (1999) showed that activated RhoA binds to DGK-θ in N1E-115 cells and inhibits its catalytic activity, although it was not determined what effect this inhibition has on neurite outgrowth. Because we found that DGK-ζ bound to RhoA in pull-down assays, it is possible that it induces neurite outgrowth by inhibiting RhoA. However, this does not seem to be a likely mechanism because it does not easily account for the results obtained with the different DGK-ζ constructs (Figure 3.2). In addition, DGK-ζ and Rho immunoreactivity did not overlap in cortical neurons (Figure 3.8B-B"'). Thus, further studies are needed to more thoroughly characterize the association of DGK-ζ with RhoA and to determine whether this interaction has any functional consequences.

The region of DGK-ζ that binds to Rac1 (amino acids 97-233) comprises two tandem C1 domains, C1A and C1B. C1 domains were initially identified as the targets of diacylglycerol and phorbol ester binding in conventional PKC isozymes. Indeed, many C1 domain-containing proteins bind phorbol esters, but intriguingly a large number do not (Hurley *et al.*, 1997). The C1 domains in DGK-ζ differ substantially in sequence from "typical" C1 domains and do not fit the profile for phorbol ester binding. Interestingly, among the C1 domains that do not bind phorbol esters, several have been shown to bind to small G-proteins (Hurley *et al.*, 1997). Moreover, there are several reports describing interactions of DGKs with Rho GTPases (Tolias *et al.*, 1998; Houssa *et al.*, 1999; Tsushima *et al.*, 2004), thus it is interesting to speculate that "atypical" C1 domains in DGKs may represent a common motif for interaction with this subfamily of small G-proteins.

A recent study by Tsushima et al. (2004) showed that DGK-γ functions as an upstream suppressor of Rac1 in NIH 3T3 cells. In contrast to DGK-ζ, DGK-γ kinase activity was required to inhibit Rac1 activation. Expression of a kinase dead DGK-γ acted as a dominant-negative mutant, inhibiting endogenous DGK-γ activity and inducing lamellipodia and membrane ruffling in the absence of growth factor stimulation. Although it was not determined whether DGK-γ binds directly to Rac1, it is interesting to speculate that the C1 domains in this isoform function in an analogous manner to those in DGK-ζ. Since DGK-γ is abundantly expressed in neurons (Kai *et al.*, 1994; Goto *et al.*, 1994), an interesting possibility is that the two DGK isoforms compete for binding to Rac1 and provide fine tuning of growth and retraction signals that regulate process formation.

In conclusion, our results suggest syntrophins provide localization cues that direct DGK-ζ-containing complexes to specialized plasma membrane domains and that this activity

is required for neurite formation. We propose that DGK-ζ is an integral part of signal transduction pathways in neurons that translate receptor activation by extracellular ligands into cytoskeletal rearrangements and local growth. In the absence of localization cues, DGK-ζ may fail to properly regulate actin cytoskeleton rearrangements that are required for neurite formation.

Chapter 4

Morphological changes and spatial regulation of diacylglycerol kinase-ζ, syntrophins and Rac1 during myoblast fusion

Hanan Abramovici[1] and Stephen H. Gee*[1]

*Corresponding author

[1]Department of Cellular and Molecular Medicine
Center for Neuromuscular Disease
University of Ottawa
451 Smyth Rd
Ottawa, ON K1H 8M5
Canada

Running Title: Regulation of DGK-ζ and syntrophins during myoblast fusion

Keywords: Syntrophin, diacylglycerol kinase, myoblast fusion, Rho GTPase, localization, adhesion zipper

Words: 8,754

Published in Cell Motility and the Cytoskeleton 64: 549-567.

Contribution from authors

Hanan Abramovici and Stephen Gee co-wrote the manuscript. Hanan Abramovici was responsible for the majority of the intellectual input and performed all the experiments.

Acknowledgements

This work was funded by the Muscular Dystrophy Association USA. S.G. is supported by a CIHR New Investigator Award. H.A. is supported by an Ontario Graduate Scholarship. Thanks to Matthew Topham and Stanley Froehner for providing antibodies and to Yury Yakubchyk for technical assistance. We are grateful to Nafiseh Sabri for critically reviewing the manuscript.

Abstract

The fusion of mononuclear myoblasts into multinucleated myofibers is essential for the formation and growth of skeletal muscle. Myoblast fusion follows a well-defined sequence of cellular events, from initial recognition and adhesion, to alignment, and finally plasma membrane fusion. These processes depend upon coordinated remodeling of the actin cytoskeleton. Our recent studies suggest diacylglycerol kinase-zeta (DGK-ζ), an enzyme that metabolizes diacylglycerol to yield phosphatidic acid, plays an important role in the actin reorganization. Here, we investigated whether DGK-ζ has a role in the fusion of cultured C2C12 myoblasts. We show that DGK-ζ and syntrophins, scaffold proteins of the dystrophin glycoprotein complex that bind directly to DGK-ζ, are spatially regulated during fusion. Both proteins accumulated with the GTPase Rac1 at sites where fine filopodia mediate the initial contact between myoblasts. In addition, DGK-ζ codistributed with the Ca^{2+}-dependent cell adhesion molecule N-cadherin at nascent, but not previously established cell contacts. We provide evidence that C2 cells are pulled together at cell-cell junctions by N-cadherin-containing filopodia reminiscent of epithelial adhesion zippers, which guide the advance of lamellipodia from apposing cells. At later times, vesicles with properties of macropinosomes formed close to cell-cell junctions. Reconstruction of confocal optical sections showed these form dome-like protrusions from the dorsal surface of contacting cells. Collectively, these results suggest DGK-ζ and syntrophins play a role at multiple stages of the fusion process. Moreover, our findings provide a potential link between changes in the lipid content of the membrane bilayer and reorganization of the actin cytoskeleton during myoblast fusion.

Introduction

The formation of multinucleated muscle fibers occurs during embryogenesis through the fusion of multiple immature muscle cells called myoblasts. The fusion of myoblasts with mature muscle fibers continues throughout life in response to the needs that arrive through growth, exercise and traumatic injury. Consequently, the precise regulation of myoblast fusion is essential to produce muscles with the correct shape, orientation and myotube (muscle fiber) number, and later in life for maintaining desired muscle mass. Light and electron microscopic observations of cultured vertebrate muscle cells have provided much insight into the morphological changes that occur throughout the fusion process (Knudsen, 1992). More recent genetic studies in *Drosophila* have provided additional details of the fusion process and have begun to characterize the molecular events that underlie the various steps, outlined below (reviewed in (Dworak and Sink, 2002; Abmayr *et al.*, 2003; Chen and Olson, 2004).

Myoblast fusion can be thought of as an orderly sequence of events that begins with differentiation, and is followed by cell-cell recognition, adhesion, alignment and finally fusion of the membrane bilayers (Knudsen and Horwitz, 1978; Wakelam, 1985). In the differentiation step, myoblasts begin to express proteins that make the cells competent to fuse. Myoblasts then locate and recognize an appropriate target for fusion, which can be another myoblast or an existing myotube. Then they develop a tear-shaped morphology with a single long extension or pseudopod. This asymmetrical shape is characteristic of migrating cells and may be a response to signals from the target cell that encourage the fusion competent myoblast to move towards it (Wakelam, 1985; Dworak and Sink, 2002). Myoblasts then adhere to each other through specific Ca^{2+}-dependent and Ca^{2+}-independent

processes (Knudsen and Horwitz, 1977). There is considerable evidence implicating members of the cadherin family of homophilic cell-cell adhesion molecules in the Ca^{2+}-dependent adhesion of myoblasts (Krauss *et al.*, 2005). After adhesion, myoblasts assume a bipolar morphology and align along their long axes. The aligned plasma membranes come in close apposition and local membrane fusion events form small areas of cytoplasmic continuity between the cells (Kalderon and Gilula, 1979).

The union of myoblast membranes involves specific changes in the lipid content of the bilayers during the fusion process. Upon the initiation of fusion, there is a rapid breakdown of phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] and a concomitant increase in the lipid second messengers diacylglycerol (DAG) and phosphatidic acid (PA) (Wakelam and Pette, 1982; Wakelam, 1983). DAG is known to activate several signaling proteins including classical and novel protein kinase C (PKC) isoforms (Ron and Kazanietz, 1999; Kazanietz, 2000); Ras guanyl nucleotide-releasing proteins (Ras-GRPs) (Lorenzo *et al.*, 2000); and chimaerins (i.e. Rac GTPase activating proteins) (Ahmed *et al.*, 1993), while PA modulates the activity of enzymes like phosphatidylinositol-4-phosphate 5-kinase, Raf-1 kinase, atypical PKCs and others (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000). These specific changes in lipid content parallel an increased fluidity of the plasma membrane that precedes fusion (Prives and Shinitzky, 1977; Wakelam, 1983), thus it is likely that they activate intracellular signal transduction pathways that directly stimulate membrane fusion events.

The transient increase in both DAG and PA at the onset of membrane union raises the possibility that diacylglycerol kinases (DGKs), enzymes that phosphorylate DAG to yield PA, have a role in regulating their levels. By metabolizing DAG, DGKs effectively terminate DAG-mediated signaling; however, they also yield a product, PA with its own signaling

functions. Thus, DGKs might be responsible for attenuating and/or initiating specific lipid signaling pathways that regulate fusion. There are nine different mammalian DGK isozymes that differ in their structure, patterns of tissue expression, and enzymatic properties (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000). Of these, DGK-ζ is highly expressed in the somites of mouse embryos (Ding *et al.*, 1998), in developing skeletal muscle (Bunting *et al.*, 1996; Ding *et al.*, 1997; Abramovici *et al.*, 2003) and the C2C12 skeletal muscle cell line (Abramovici *et al.*, 2003). In skeletal muscle and other tissues, DGK-ζ interacts with syntrophins, PDZ domain-containing scaffold proteins that link signaling proteins to the dystrophin family of cytoskeletal proteins (Hogan *et al.*, 2001; Abramovici *et al.*, 2003). Our previous studies suggest syntrophins and DGK-ζ form a regulated signaling complex that is localized at specialized membrane domains in muscle cells.

In this report, we used immunofluorescence microscopy of cultured C2 myoblasts, visualized at various times after serum withdrawal, to follow the spatial distribution of DGK-ζ, syntrophins and Rac1 during myoblast fusion. We found that these proteins were concentrated in or around structures formed at the border of contacting myoblasts during different stages of the fusion process. We also compared the localization of DGK-ζ to that of N-cadherin, a cell-cell adhesion molecule shown previously to have a role in fusion. DGK-ζ codistributed with N-cadherin at forming and newly formed contacts but not at more established contacts, where DGK-ζ was absent and N-cadherin remained. In the course of these experiments, we documented several previously unrecognized features of fusing cells, including fine actin-based cellular extensions; structures analogous to adhesion zippers of epithelial cells and small and large sized macropinosome-like vesicles at the border of contacting myoblasts. Collectively, our findings suggest DGK-ζ and syntrophins participate

in several steps of the fusion process and provide additional insight into the mechanism of fusion.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium was purchased from GIBCO-Invitrogen (Carlsbad, CA). Fetal bovine serum and penicillin-streptomycin-glutamine were from SIGMA (St. Louis, MO). Matrigel was from BD Transduction Laboratories (Mississauga, Ontario). 24-well plates were from Sarstedt (Montreal, Quebec). Paraformaldehyde and Triton X-100 were from BioShop (Burlington, Ontario). Bovine serum albumin (BSA) fraction V was from Roche (Indianapolis, IN). Anti-HA rabbit polyclonal antibody was obtained from Zymed (San Francisco, CA). Monoclonal antibody 9E10 against C-myc was from Roche (Indianapolis, IN). Monoclonal antibody 1351 was a gift from Stanley Froehner (University of Washington, Seattle). AlexaFluor-conjugated secondary antibodies and phalloidin were obtained from Molecular Probes-Invitrogen (Carlsbad, CA). Monoclonal antibodies to N-cadherin and Rac1 were purchased from BD Transduction Laboratories. Affinity-purified polyclonal antibodies to DGK-ζ polyclonal antibody were made as described previously (Abramovici *et al.*, 2003). Fluoromount G was from Electron Microscopy Services-Southern Biotech (Birmingham, AL).

Immunocytochemistry

C2C12 myoblasts were plated on Matrigel-coated glass coverslips placed in 24-well plates, and grown overnight in a humidified incubator at 37°C with 5% CO_2. The next day, cells were serum starved for either 4-6 h or 24 h in DMEM at 37°C as indicated, and then fixed in either 4% or 0.5% paraformaldehyde (for immunolabeling of syntrophins) for 30 min-1 h at 37°C. All subsequent steps were carried out at room temperature and all washes,

blocking, and antibody dilutions were carried out in blocking/washing solution (a filter sterilized solution of 1% BSA in phosphate buffered saline (PBS), pH 7.2) unless indicated otherwise. Diluted antibodies were prepared by mixing the antibodies with blocking/washing solution followed by centrifugation at 10,000 rpm for 3 minutes to precipitate protein aggregates. Coverslips were washed twice with PBS then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Cells were then washed 3 more times, and blocked for 1 hour with blocking/washing solution. Cells were incubated for 1 hour with the appropriate primary antibodies, washed 3 times, stained with secondary antibodies for 1 hour and washed three more times. Coverslips were then mounted on glass slides with Fluoromount G and sealed with nail polish. All primary antibodies were used at a dilution of 1:100, secondary antibodies at 1:300, and phalloidin at 1:1000. Images were taken with a Zeiss AxioCam charge-coupled device camera mounted on a Zeiss Axioskop 2 microscope equipped with a 63X oil-immersion objective.

Dextran Uptake Experiments

C2C12 myoblasts were grown on glass coverslips coated with Matrigel placed in 24 well dishes. Cells were seeded at a density of 30,000 cells / well. The next day, myoblast growth media was replaced with differentiation media (2% fetal calf serum in DMEM) and Texas-Red dextran (70 kDa, lysine fixable) was added to each well at a final concentration of 0.5 mg/ml. At the indicated time points, the dextran-containing differentiation media was removed, cells were washed with 1 X PBS twice and 4% paraformaldehyde was added to the cells for 30 minutes. Coverslips were then stained with appropriate antibodies.

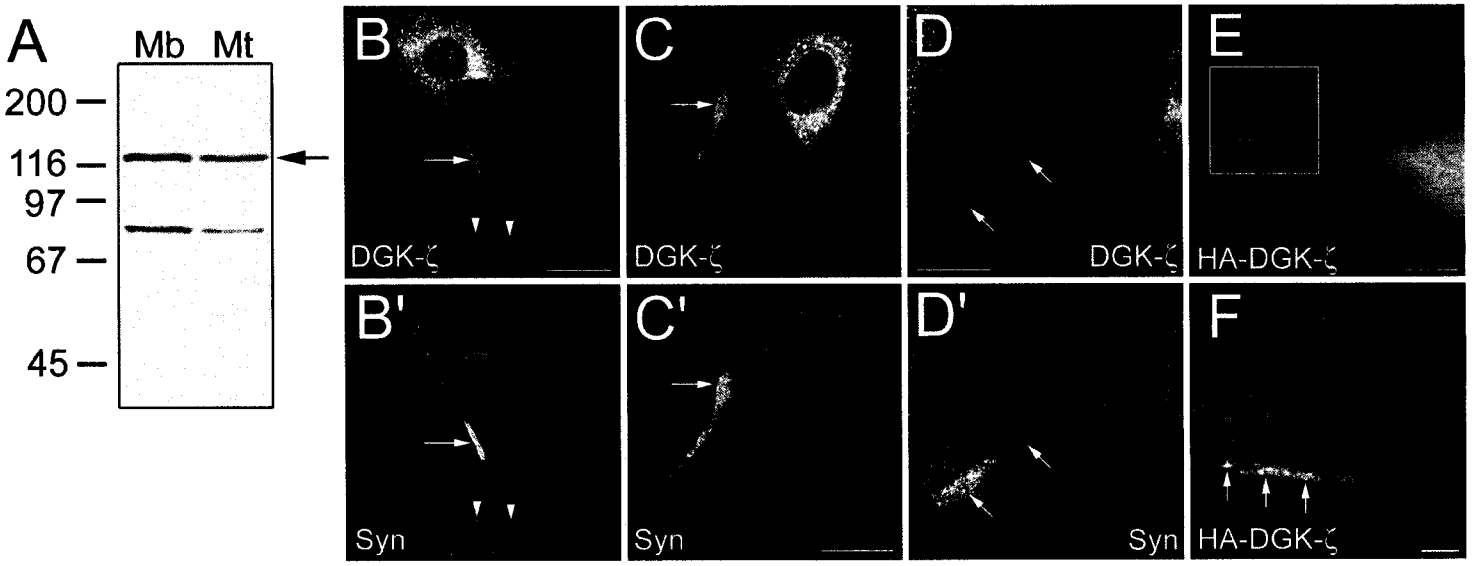
Results

DGK-ζ, syntrophins and Rac1 accumulate at intercellular contacts mediated by filopodia

To study the role of DGK-ζ and syntrophins in myoblast fusion we used C2C12 muscle cells, which proliferate in serum-containing media and, upon serum withdrawal, differentiate and fuse into multinucleated myotubes. Soon after switching to low-serum conditions, myoblasts generally extend a single, prominent pseudopodium that establishes contact with neighboring myoblasts to initiate fusion (Dworak and Sink, 2002). In the following experiments, C2 myoblasts were plated at relatively low densities to facilitate the visualization of DGK-ζ immunoreactivity at individual cell-to-cell contacts. We first confirmed by immunoblotting that DGK-ζ is endogenously expressed in C2 myoblasts (Figure 4.1A). An antibody to the N-terminus of DGK-ζ recognized a single prominent band at ~120 kDa in myoblast and myotube extracts, consistent with previous reports (Bunting *et al.*, 1996; Ding *et al.*, 1998; Abramovici *et al.*, 2003). Syntrophins are also abundantly expressed in C2 myoblasts (Abramovici *et al.*, 2003).

In cells serum-starved for 4 h, DGK-ζ and syntrophins were often concentrated at the distal tips of pseudopodia close to neighboring myoblasts (Figure 4.1B - C', *arrows*). In some cases, though, DGK-ζ and syntrophin accumulated along an extended area of a cell close to a pseudopodium from a neighboring cell, which itself had no obvious accumulation of either protein (Figure 4.1D and D', *arrows*). Hereafter; we refer to the cell extending the pseudopodium as the "sender" and the one being contacted by the pseudopodium as the "recipient". We found no instances in which both the sender and the recipient contained high levels of DGK-ζ and syntrophins. In other words, at one intercellular junction, either the

Figure 4.1. DGK-ζ and syntrophins accumulate at the distal tips of pseudopodia in serum-starved myoblasts. (A) A western blot of C2 myoblast (Mb) and myotube (Mt) lysates with the anti-DGK-ζ antibody reveals a single prominent band at ~120 kDa (*arrow*) and another at ~80 kDa. The latter is likely a proteolytic fragment or a non-specific band since its intensity varied in different experiments. (B-D') C2C12 myoblasts were serum-starved for 4 h then were fixed and double-labeled with affinity purified antibodies to DGK-ζ and with the pan-specific mAb 1351 against syntrophins, followed by AlexaFluor 594 and AlexaFluor 488 secondary antibodies, respectively. Under these conditions, the myoblasts usually extended a single, prominent pseudopodium. Both DGK-ζ and syntrophins were highly concentrated at the distal tips of pseudopodia that were in close proximity to other myoblasts (B - C', *arrows*). In general, there was no corresponding accumulation of protein in the apposing area of the "receiving" myoblast (C and C'), although some labeling is visible along the leading edge of the cell in B and B' (*arrowheads*). (D and D') DGK-ζ and syntrophins are concentrated in the cytoplasm (*arrows*) along the edge of a cell "receiving" a pseudopod from a neighboring cell. Note that in this case there is no accumulation of label in the distal pseudopodium. Bars, 20 μm. (E) C2 myoblasts were transfected with HA-tagged DGK-ζ, serum-starved for 4 h, then fixed and stained with an anti-HA antibody. The image shows a transfected cell (*right*) with a long pseudopodium that makes contact with another myoblast (*boxed region*). (F) A close-up view of the boxed region in (E) shows an accumulation of DGK-ζ in the pseudopodium where it contacts the neighboring cell (*arrowheads*).

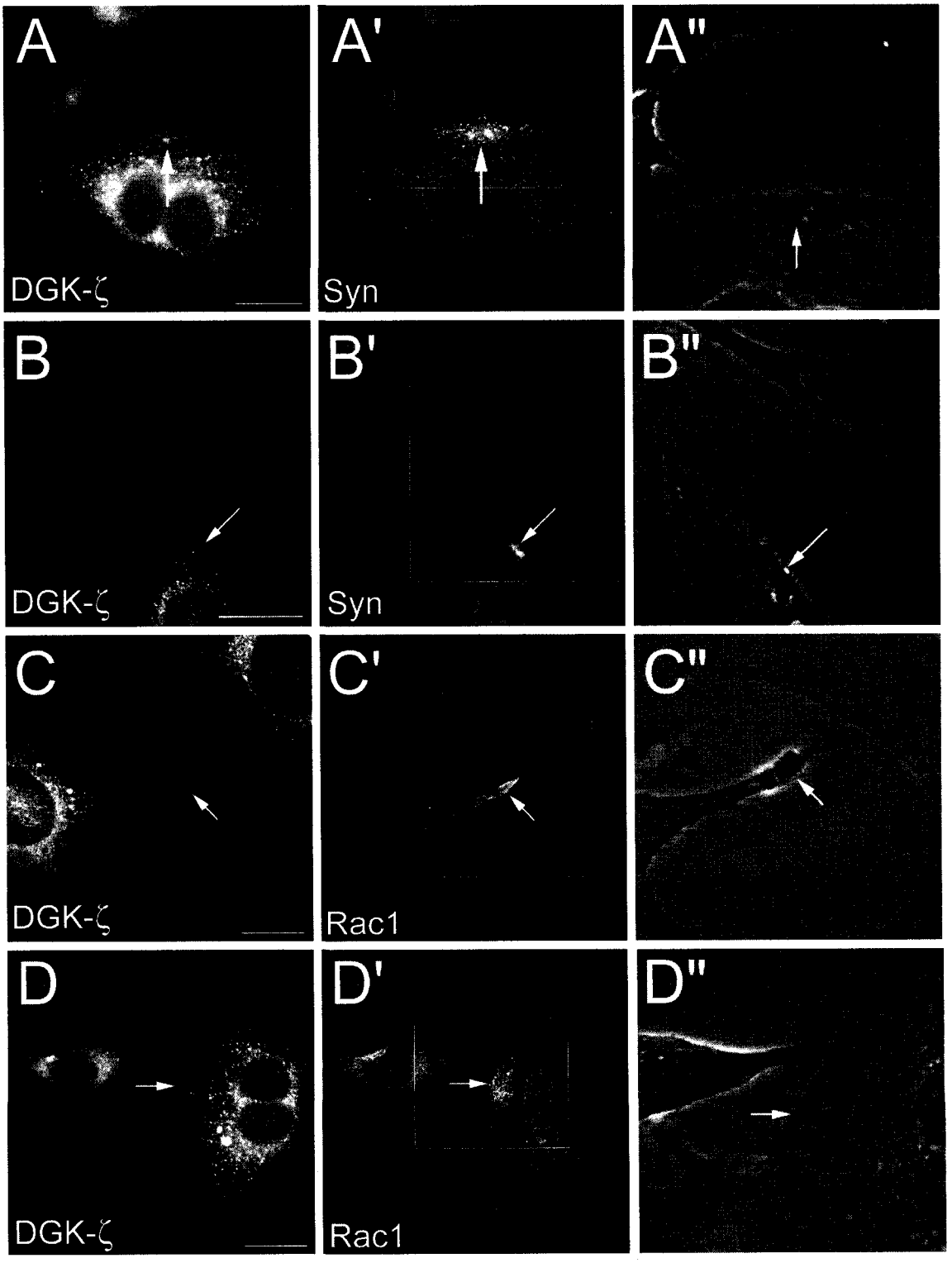

A
Mb
Mt
200
116
97
67
45
B
DGK-ζ
C
DGK-ζ
D
DGK-ζ
E
HA-DGK-ζ
B'
Syn
C'
Syn
D'
Syn
F
HA-DGK-ζ

sender or recipient, but not both, had locally elevated levels of these proteins. Since the intensity of the DGK-ζ labeling was somewhat weaker than that of syntrophin, we compared endogenous DGK-ζ localization to HA-tagged DGKζ introduced by transient transfection. We found that exogenous DGK was similarly localized at the tips of pseudopodia (Figure 4.1E and F).

Occasionally, we observed local accumulations of DGK-ζ and syntrophins along the edge of cells that were not in close proximity to a pseudopodium (Figure 4.2A) or where the pseudopodium was oriented away from the cell (Figure 4.2B). Closer inspection of the corresponding phase contrast images in these cases revealed ultrafine filopodial processes connecting the neighboring myoblasts (Figure 4.2A" and B", *arrowheads*). The points at which these processes intersected with the cell body coincided with local accumulations of DGK-ζ and syntrophins (Figure 4.2A – B", *arrows*). Here again, only one of the two cells accumulated these proteins at contact sites.

In an earlier study, we showed that Rac1 colocalized with DGK-ζ at membrane ruffles in C2 myoblasts (Abramovici *et al.*, 2003). More recently, we demonstrated that Rac1 directly interacts with DGK-ζ (Yakubchyk *et al.*, 2005). Here, we found that Rac1 was concentrated along with DGK-ζ at the distal tips of pseudopodia that made contact with neighboring cells via fine filopodia (Figure 4.2C and C'). Rac1 was also colocalized with DGK-ζ in accumulations along the border of recipient cells (Figure 4.2D and D'). In addition, phase-contrast images revealed that the areas in contact with filopodial processes were often darker that the surrounding regions of the cell, suggesting intercellular contacts are associated with specialized membrane domains (Figure 4.2A" and B"). Collectively, these results suggest myoblasts extend long, thin filopodia that connect to neighboring cells.

Figure 4.2. Polarized accumulation of DGK-ζ, syntrophins and Rac1 at sites of contact between neighboring myoblasts mediated by fine filopodia. (A – D') Immunofluorescence microscopy images of serum-starved C2 myoblasts that were prepared as described in the legend to Figure 4.1. Cells were stained for DGK-ζ and syntrophins (A – B') or DGK-ζ and Rac1 (C – D'). The *white arrows* indicate accumulations of the indicated proteins. (A" – D") Phase contrast images of the regions bordered by the boxes in A'-D' reveal fine filopodia connecting the neighboring cells (*dark arrowheads*) as well as thicker processes (*dark arrows*). The phase-dark regions correspond to regions where the proteins have accumulated (white arrows in A"-D"). Bars, 20 μm (A – D and A' – D'); 10 μm (A", C" and D"); 5 μm (B").

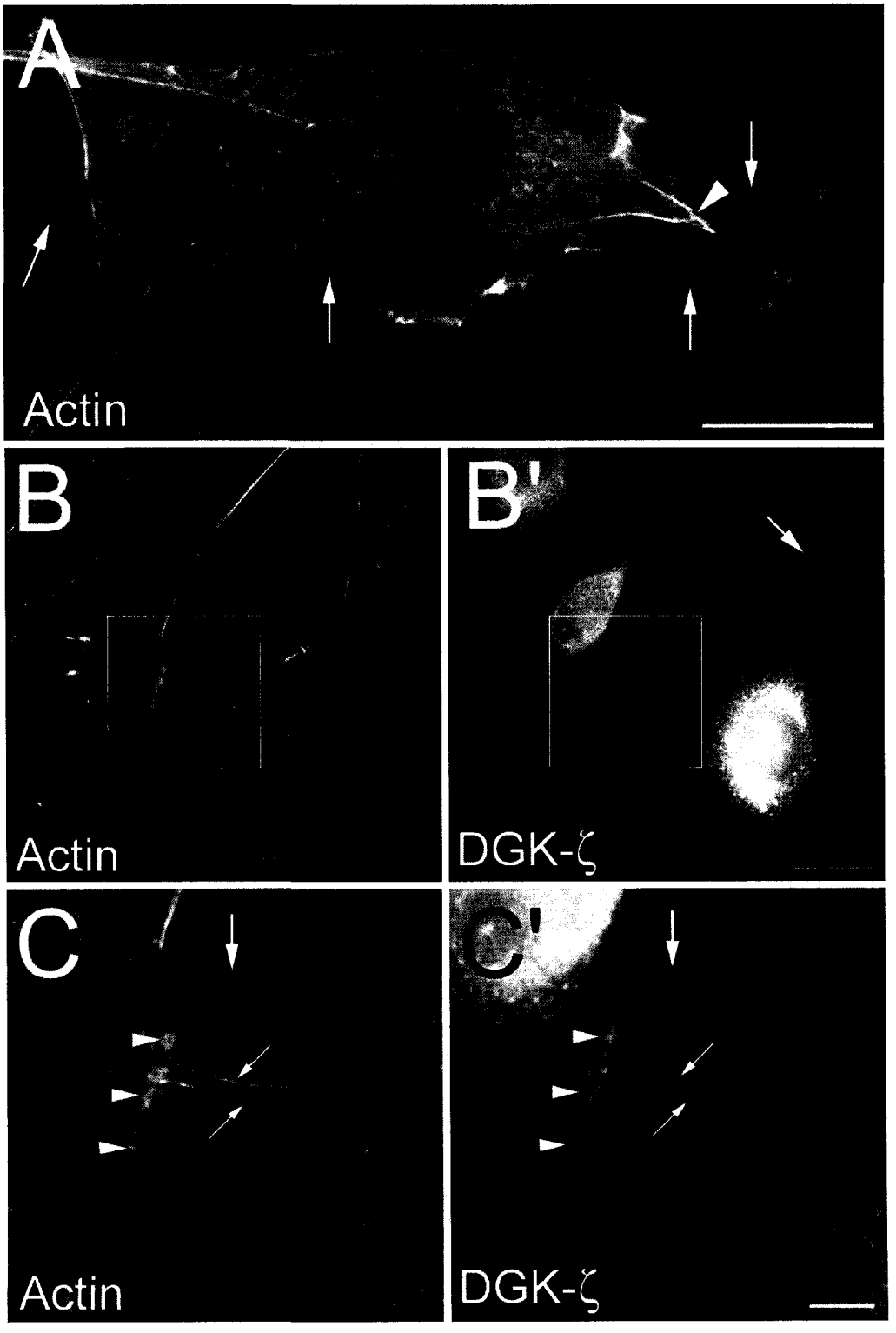

A
A'
A"
DGK-ζ
Syn
B
B'
B"
DGK-ζ
Syn
C
C'
C"
DGK-ζ
Rac1
D
D'
D"
DGK-ζ
Rac1

These intercellular contact sites coincide with the local accumulations of DGK-ζ, syntrophins and Rac1 in potentially distinct membrane domains.

Filopodia between contacting myoblasts contain actin and DGK-ζ

Next, we examined the distribution of F-actin at intercellular contacts by staining serum-starved C2 myoblasts with AlexaFluor 488-conjugated phalloidin. As shown in Figure 4.3A (*arrows*), faint strands of actin fluorescence bridged neighboring myoblasts. In addition, there was increased F-actin staining along the edges of the cells at contact sites (Figure 4.3A, *arrowhead*), which coincided with a local increase in DGK-ζ immunoreactivity (Figure 4.3B and B' boxed region and 3C and C', *arrowheads*). Again, this local accumulation of actin and DGK-ζ was generally only present in one of a pair of contacting cells. In general, larger diameter cellular extensions also contained microtubules (S.G. and H.A., unpublished observations). Close-up views of the contacting filopodia revealed DGK-ζ puncta along the length of bridging actin filaments and at the tips of non-connecting filopodia (Figure 4.3C and C', *arrows*). These results suggest DGK-ζ codistributes with local accumulations of F-actin at contact sites and that intercellular filopodial bridges contain both F-actin and DGK-ζ. Moreover, these results are consistent with previous studies showing that fine filopodia between contacting epithelial cells are composed exclusively of actin filaments (Yonemura *et al.*, 1995; Vasioukhin *et al.*, 2000).

Figure 4.3. Filopodia between contacting myoblasts contain actin and DGK-ζ. C2 myoblasts prepared as described in the legend to Figure 4.1 were fixed and labeled with AlexaFluor 488-conjugated phalloidin to reveal F-actin (A, B, and C) and with anti-DGK-ζ (B' and C'). (A) Fine filopodial processes connect adjacent myoblasts. (B and B') F-actin and DGK-ζ are concentrated along the border of a cell where actin-rich filopodia form a bridge with an adjacent myoblast (*boxed regions*). The *arrow* at the top right of the image in B' indicates DGK-ζ staining in the distal part of a pseudopodium that makes contact with the cell on the lower right. (C, C') Close-up views of the boxed regions in B and B', respectively, show an accumulation of F-actin and DGK-ζ at the border of the cell on the left (*arrowheads*) and the lack of corresponding accumulations in the cell on the right. Two thicker processes (*small arrows*) bridge the two cells but several other smaller processes emanate from either cell (e.g., *large arrow*), some of which appear to have been broken. Bars, 20 μm (A, B and B'); 5 μm (C and C').

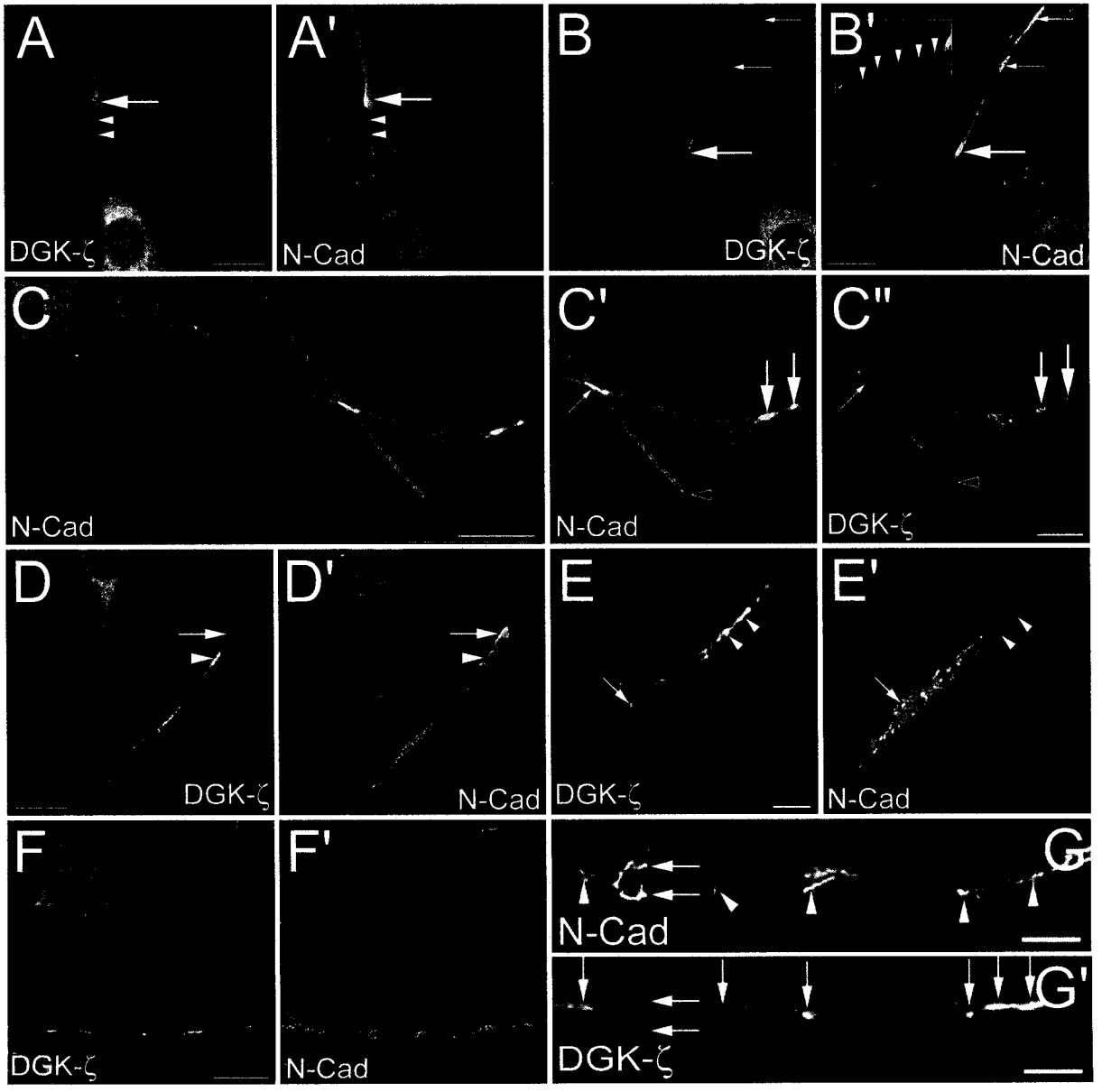

A
Actin
B
Actin
B'
DGK-ζ
C
Actin
C'
DGK-ζ

DGK-ζ codistributes with N-cadherin at nascent, but not established, intercellular contacts

The recognition and contact stage of myoblast fusion depends on cell-cell interactions. Cadherins are transmembrane cell adhesion molecules that interact in a Ca^{2+}-dependent manner with similar cadherins on neighboring cells via their ectodomains and with the actin-based cytoskeleton via their cytoplasmic domains. Several lines of evidence suggest cadherin-mediated interactions are involved in the regulation of myogenesis (Charrasse *et al.*, 2003; Krauss *et al.*, 2005). N-cadherin is a major isoform in muscle cells and accumulates at sites of contact between fusing myoblasts (Mege *et al.*, 1992). Furthermore, clustering or immobilization of N-cadherin on the surface of myoblasts directly triggers signaling events that promote the activation of a myogenic differentiation program (Goichberg and Geiger, 1998). These findings suggested to us that N-cadherin signaling might recruit DGK-ζ to contact sites between neighboring C2 myoblasts at early times after serum-withdrawal. In agreement with this idea, we found that N-cadherin staining was concentrated at the distal tips of pseudopodia with DGK-ζ (Figure 4.4A – C", *large arrows*), although not all pseudopodia accumulated DGK-ζ and N-cadherin at their tips (Figure 4.4C' and C", *open arrowhead*). In those with bright staining, there was a clear spatial correspondence between local high concentrations of DGK-ζ and N-cadherin (Figure 4.4C' and C", *large arrows*). Close-up views of merged images showed the two signals did not precisely overlap (not shown), however punctate DGK-ζ immunoreactivity generally appeared directly adjacent to regions with membrane-associated N-cadherin staining suggesting DGK-ζ has a role in delivering N-cadherin to the plasma membrane or functions downstream of N-cadherin signaling. In many instances, pseudopodia with high levels of

Figure 4.4. DGK-ζ transiently accumulates with N-cadherin at nascent sites of intercellular contact. C2 myoblasts serum-starved for 4 h were fixed and double labeled with antibodies to DGK-ζ and N-cadherin. (A and A') Representative micrographs showing strong immunoreactivity for DGK-ζ and N-cadherin at the distal tip of a pseudopodium (*arrow*). The *arrowheads* indicate a small filopodial process connecting the two cells. (B and B') DGK-ζ is not concentrated at established cell-cell contacts. A myoblast process indicated by the *arrows* originated from a cell at the top right of the panel. There is strong N-cadherin immunoreactivity but no significant DGK-ζ labeling along the process where it makes contact with another underlying myoblast (*small arrows*). However, both DGK-ζ and N-cadherin are concentrated at the tip of the process (*large arrow*). The inset in B' shows several fine processes originating from the cell at the *left* of the panel, at least one of which forms a link with the tip of the pseudopodium (*arrowheads*). (C-C") A second example showing DGK-ζ is absent from an established contact site. C' and C" are close-up views of the boxed region in C. Strong N-cadherin staining remains (C", *small arrow*) where two pseudopodia have crossed paths, but there is no corresponding DGK-ζ staining (C', *small arrow*). The *large arrows* indicate areas with strong DGK-ζ and N-cadherin immunoreactivity at the tip of one pseudopodium. Note the lack of accumulated DGK-ζ and N-cadherin at the tip of the other pseudopodium (*open arrowhead*). (D, D') In this example, a bright patch of DGK-ζ immunoreactivity (*arrowhead*) lies close to, but does not overlap with intense N-cadherin staining (*arrow*) at the tip of a pseudopodium in contact with a neighboring cell. Additional bright immunoreactivity is associated with cell contacts from a different neighboring cell (*boxed regions*). (E – E') Close-up views of the boxed regions in D and D', respectively. Here again, bright DGK-ζ patches generally lie close to, but do not

overlap with, N-cadherin (*arrowheads*). Some patches did overlap, however (*arrows*). (F and F') Multiple N-cadherin-positive contacts form along the length of two adjacent cellular processes (boxed areas). A pseudopod from the cell at the top of the image makes contact with them at one point. (G and G') Close-up views of the boxed regions in F and F', respectively. The *vertical arrows* indicate bright patches of DGK-ζ immunoreactivity opposite accumulations of N-cadherin (*arrowheads*) at contact sites. Note that the DGK-ζ patches are confined to the upper cellular process. The *horizontal arrows* indicate bright N-cadherin patches but no corresponding DGK-ζ staining at a point where a pseudopod makes contact with the two parallel processes. Bars, 20 μm (A, B, C, D and F); 10 μm (C'); 5 μm (E and G).


A
DGK-ζ
A'
N-Cad
B
DGK-ζ
B'
N-Cad
C
N-Cad
C'
N-Cad
C''
DGK-ζ
D
DGK-ζ
D'
N-Cad
E
DGK-ζ
E'
N-Cad
F
DGK-ζ
F'
N-Cad
G
N-Cad
G'
DGK-ζ

DGK-ζ and N-cadherin at their tips made contact with neighboring cells via fine filopodia (*arrowheads* in Figure 4.4A and A' and Figure 4.4B', *inset*).

As shown in Figure 4.4B' and C" (*small arrows*), bright N-cadherin staining remained at points where pseudopodia had previously come into contact with neighboring cells. Interestingly, there is no corresponding accumulation of DGK-ζ at these sites (Figure 4.4B and C', *small arrows*), even though the same pseudopodia have noticeable accumulations of DGK-ζ at their tips that colocalize with N-cadherin (Figure 4.4C' and C", *large arrows*). Some images revealed intense DGK-ζ immunoreactivity close to, but not overlapping with N-cadherin staining at the tips of pseudopodia fully contacting neighboring cells (Figure 4.4D and D', E and E'). In one case, where two pseudopodia formed multiple N-cadherin-positive contacts along their lengths, each contact was associated with a bright patch of DGK-ζ staining (Figure 4.4F and F', *boxed regions*). Close-up views of these regions revealed that the DGK-ζ patches were only present in one pseudopodium (Figure 4.4G', *vertical arrows*) and were in register with accumulations of N-cadherin in the other (Figure 4.4G, *arrowheads*). Interestingly, DGK-ζ was not concentrated at N-cadherin-positive contacts with a pseudopodium emanating from a second neighboring cell (Figure 4.4F and F', *top*; Figure 4.4G and G', *horizontal arrows*). Collectively, these results suggest DGK-ζ codistributes with N-cadherin at forming and newly formed intercellular contacts, but not at more established contacts. This raises the possibility that DGK-ζ plays a transient role in N-cadherin-mediated recognition and adhesion.

The adhesion zipper as an intermediate step in myoblast fusion

Following initial recognition and adhesion, myoblast plasma membranes become closely apposed; a process that requires tight adhesion between neighboring cells (Knudsen, 1992). Studies in cultured epithelial cells have defined a dynamic mechanism for intercellular adhesion, whereby E-cadherin complexes cluster at filopodia tips on closely apposed cells, generating a two-rowed "adhesion zipper" that is hypothesized to pull the membranes of the cells together (Yonemura *et al.*, 1995; Vasioukhin *et al.*, 2000). To our knowledge, no such mechanism has been described for the adhesion of myoblasts. We found similar N-cadherin-positive structures in serum-starved C2 cells. As shown in Figure 4.5A (*boxes* and *arrow*), anti-N-cadherin labeled a series of fine, regularly spaced filaments at sites of intercellular contact. These filaments appeared continuous, and extended 2-3 μm past the boundary of either cell, suggesting they are anchored at a point behind the cell's leading edge (Figure 4.5B). In addition, bright N-cadherin puncta were present at the ends of the filaments (Figure 4.5B, *arrowheads*). In contrast, no significant accumulations of DGK-ζ were observed either along the filaments or at their base (Figure 4.5A' and B'). A few filaments were visible in phase contrast images of the same regions (Figure 4.5C, *arrowheads*). We were fortunate to capture some intercellular contacts at the mid-zippering point. In Figure 4.5D, an adhesion zipper (*arrowheads*) narrows then merges to form a continuous seam of N-cadherin staining (*arrow*) along the border between the two cells. This linear sequence likely recapitulates what normally occurs as a temporal sequence in living cells. In this case, the cells appear to have been fixed at a point in time when they were zippering from right to left. Collectively, these data suggest adhesion zippers are an

Figure 4.5. N-Cadherin-positive filaments zip together adjacent myoblasts.

C2 myoblasts serum-starved for 4 h were fixed and double-labeled for N-cadherin and DGK-ζ. (A and A') Filaments connecting adjacent cells in a group of closely-spaced myoblasts were strongly labeled for N-cadherin (*boxes* and *arrow*). Punctate DGK-ζ immunoreactivity was present throughout the cells, but was not concentrated in the filaments. (B and B') Close-up views of the regions enclosed by the *upper boxes* in A and A', respectively. Several regularly spaced filaments labeled bridge the gap between the two cells in (B). Note the focal accumulation of N-cadherin at either end of the filaments (*arrowheads*), which appear anchored several μm from the edge of the gap. Faint wisps of filamentous DGK-ζ labeling are apparent against the dark background of the gap in (B'). (C) At least two filaments (*arrowheads*) are clearly observed in a phase contrast micrograph of the same region. (D) Close-up view of the region enclosed by the *lower* box in (A) shows a seam of strong N-cadherin immunoreactivity along the border of two myoblasts (*arrow*). To the left, N-cadherin-positive filaments (*arrowheads*) bridge a gap that narrows as it approaches the seam, similar to a zipper. (E - E") The cell on the upper right has extended several filopodia toward the cell on the lower left, which has a number of lamellipodia in the opposite direction. (F - F") Close-up views of the boxed regions in E - E", respectively, show that the lamellipodia from the one cell lie along a track formed by filopodia (F- F", *arrows*) from the apposing cell and come to a point where they meet the filopodia (F' and F", arrowheads). Punctate N-cadherin labeling is found along the length of the filopodia (F, *arrowheads*). (G-G") Regularly spaced filopodia originating from the cell on the left make contact with the cell on the right. The dashed lines in G" indicate areas of overlap between the two cells. (H-

H") Close-up views of the boxed regions in G-G", respectively, show N-cadherin puncta aligned along the length of filopodia. The arrows in H" indicate the base of the filopodia. Bars, 20 μm (A and A'); 5 μm (B - H").

A
N-Cad
A'
DGK-ζ
B
N-Cad
B'
PGK-ζ
C
D
N-Cad
E
N-Cad
E'
Actin
E"
F
N-Cad
F'
Actin
F"
G
N-Cad
G'
Actin
G"
H
N-Cad
H'
Actin
H"

intermediate in the process of myoblast fusion that precedes the formation of a linear stripe of N-cadherin between contacting cells.

During dorsal closure in the developing *Drosophila* embryo, epithelial sheets sweep towards each other and zip together to seal the epidermis along the midline (Jacinto and Wolpert, 2001). Several studies indicate that actin-based structures play a critical role during this process (Young *et al.*, 1993; Edwards *et al.*, 1997; Jacinto *et al.*, 2000; Jacinto *et al.*, 2002). One study showed that front-row cells on opposing epithelial faces extend dynamic filopodia and lamellipodia that contact, engage and pull together the two epithelial edges (Jacinto *et al.*, 2000). This process is required to establish the correct alignment of cells at the midline. Given the parallels between epithelial and myoblast adhesion we have uncovered so far, we explored whether filopodia and lamellipodia play similar dynamic roles during myoblast recognition and adhesion. Careful analysis of high magnification phase-contrast and phalloidin-stained images of adhesion zippers made it possible in some cases to distinguish cells extending the filopodial processes (senders) from the recipients. Filopodia were usually wider at the base of sender cells (Figure 4.5F – F" and H - H", *arrows*) and sometimes extended over the surface of recipient cells (Figure 4.5E – H). Interestingly, recipient cells extended lamellipodia that closely followed the long axis of the filopodia, suggesting they use filopodia from the senders to guide their advance (Figure 4.5E – F"). This idea is reinforced by the observation that the lamellipodia come to a point along the axis of the filopodia and further suggests they are tethered and pulled along by the filaments (Figure 4.5F' and F", *arrowheads*). Together, these data suggest myoblasts elaborate filopodia at intercellular contacts, which function as guidewires to direct the advance of lamellipodia from neighboring cells.

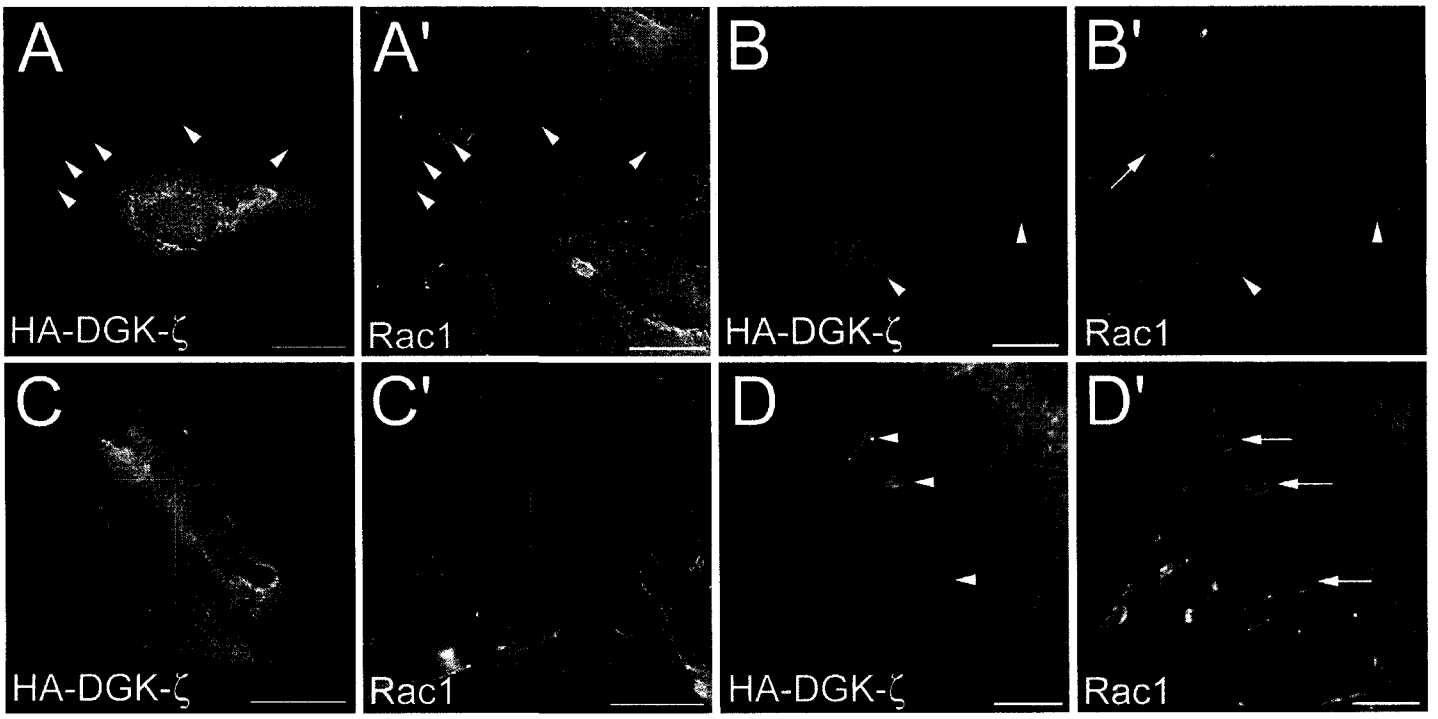
We more closely examined the localization of N-cadherin at these intercellular contact sites. We found N-cadherin was distributed in distinct, bright puncta along the length of filopodial processes between contacting cells (Figure 4.5F and H, *arrowheads*). This pattern of labeling raises the possibility that N-cadherin is vesicular and is transported along actin filaments to or from forming contacts. Alternatively, the labeling could be accounted for by the local accumulation of non-vesicular N-cadherin at sites of contact between overlapping filopodia. Further studies will be required to differentiate between these and other possibilities. Nevertheless, these results suggest actin filaments form adhesion zippers between fusing myoblasts and may additionally facilitate the assembly of N-cadherin complexes at adhesion sites.

DGK-ζ and Rac1 are concentrated in discrete microdomains at the base of intercellular filopodial contacts

When we examined the localization of HA-tagged DGK-ζ in transiently transfected C2 cells, we noticed it was concentrated in patches at the base of filopodia making intercellular contacts (Figure 4.6A – D). The boundary of these patches was often sharply demarcated from the surrounding cytoplasm, suggesting DGK-ζ partitions into discrete microdomains (Figure 4.6B and D). We were not able distinguish similar zones at the base of filopodia using an antibody to detect endogenous DGK-ζ, perhaps because of the comparatively weak signal or because the epitope recognized by the antibody is masked by an associated protein. By contrast, endogenous Rac1 was visibly concentrated in microdomains and colocalized with HA-DGK-ζ in transfected cells (Figure 4.6B' and D', *arrowheads*). Rac1 was present in similar microdomains in untransfected cells

Figure 4.6. Protein microdomains are present at the base of filopodia at intercellular contacts.

C2 myoblasts transfected with HA-tagged wild type DGK-ζ were serum starved for 4 h then were fixed and labeled with anti-HA (A – D) and anti-Rac1 (A'-D') antibodies. The panels at the bottom are close-up views of the boxed regions in the panels at the top, respectively. Arrowheads indicate overlapping patches of HA and Rac1 immunoreactivity at the base of filopodia at intercellular contacts. The arrow in (B') indicates a Rac1 microdomain in an untransfected cell. Bars, 20 μm (A and A'); 5 μm (B and B').


A
A'
B
B'
HA-DGK-ζ
Rac1
HA-DGK-ζ
Rac1
C
C'
D
D'
HA-DGK-ζ
Rac1
HA-DGK-ζ
Rac1

(Figure 4.6B', *arrow*) suggesting they are not an artifact of DGK-ζ overexpression. These results raise the possibility that there is a specialized, local pool of DGK-ζ and Rac1 associated with the base of filopodia at intercellular contacts.

DGK-ζ, syntrophins and Rac1 define a macropinocytic compartment at the junction of fusing myoblasts

We next examined the localization of DGK-ζ, syntrophins and Rac1 by immunofluorescence microscopy in C2 cells that had been serum-starved for 24 h. We found large areas virtually devoid of DGK-ζ immunoreactivity at the interface of closely apposed and/or fusing myoblasts (Figure 4.7A-H). Bright DGK-ζ puncta were clustered on the perimeter of these zones, forming an annular pattern (Figure 4.7B, D, F, and H, *arrows*). Intense syntrophin staining was also observed along the border of these zones (Figure 4.7A' – D', *boxes* and *arrows*). Surprisingly, Rac1 immunoreactivity was sometimes concentrated within, as well as surrounding the areas of depleted DGK-ζ staining (Figure 4.7E' - H', *boxes* and *arrows*).

To study this compartment in more detail, series of optical sections were taken through the Z-axis of myoblasts stained for both DGK-ζ and Rac1. Three-dimensional image reconstruction revealed that certain DGK-ζ-depleted, Rac1-enriched zones formed dome-like structures that conspicuously protrude from the dorsal surface of the cells (Figure 4.8A, *arrows*). 2-D confocal images and plane projections clearly show that DGK-ζ is excluded from these zones (Figure 4.8B). More rarely, we observed areas protruding from the dorsal membrane that were stained exclusively for DGK-ζ (Figure 4.8A, *arrowhead* and 8B, *asterisk*). These areas were not readily apparent in 2-D images, thus their significance

Figure 4.7. Syntrophins and DGK-ζ delineate a vesicular compartment between contacting myoblasts.

C2 myoblasts were serum-starved for 24 h then were fixed and double-labeled for DGK-ζ (A – H) and either syntrophin (A' – D') or Rac1 (E' – H'). The left two columns are representative images of contacting myoblasts showing large, roughly circular areas devoid of DGK-ζ staining (*boxes*). These were generally located at cell-cell junctions or in perinuclear regions facing cellular junctions. The *arrow* in (A') indicates a pseudopodium with bright syntrophin staining. Close-up views of the boxed regions are shown in the two columns at the right. Intense syntrophin staining (B' and D', *arrows*) and somewhat less intense DGK-ζ staining (B, D, F and H, *arrows*) is evident along the perimeter of DGK-ζ-depleted areas. Rac1 is sometimes concentrated on the perimeter of these zones, but more often it is concentrated within them (F' and H', *arrows*). Bars, 20 μm (A, C, E, and G); 5 μm (B, D, F and H).

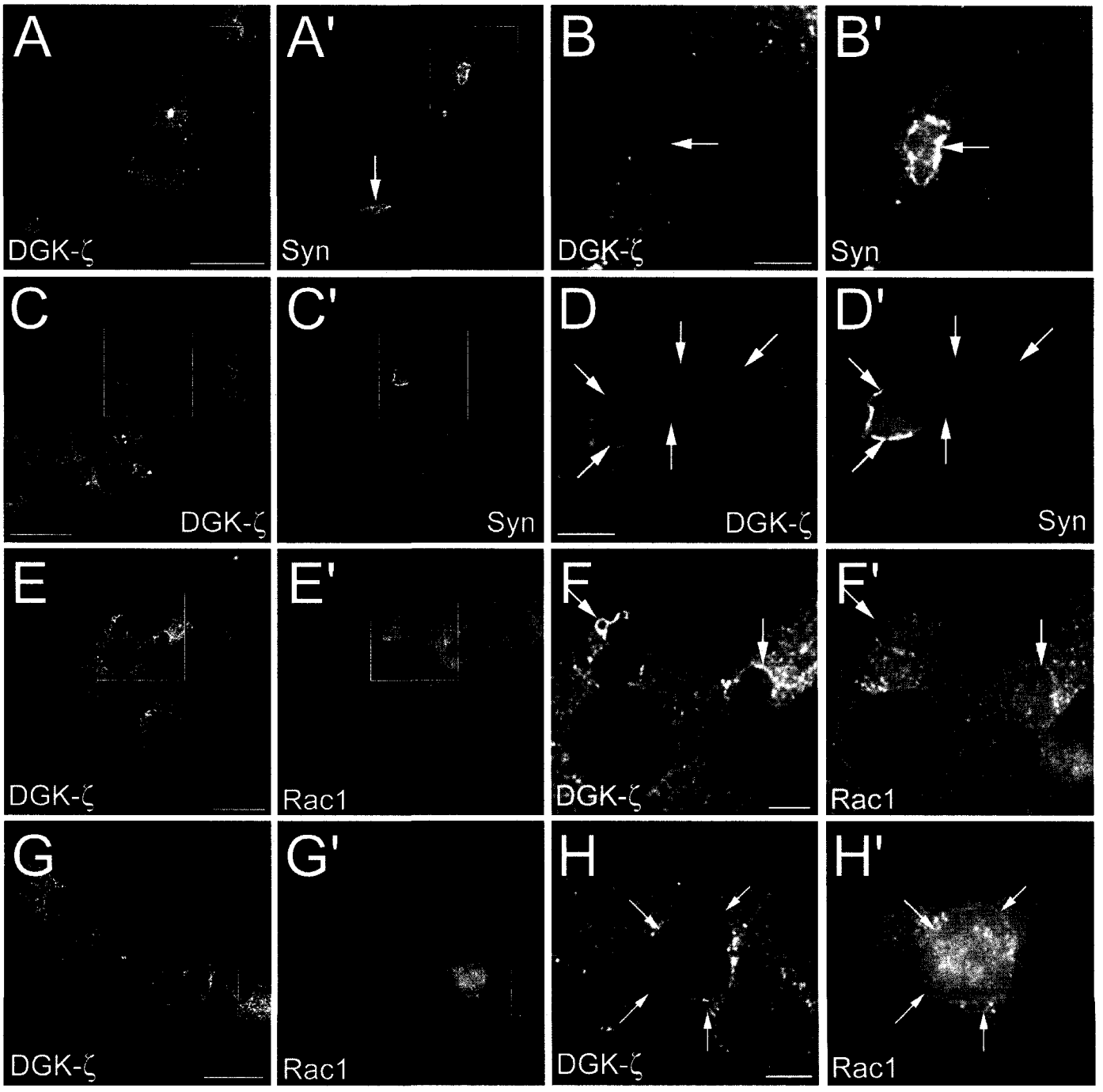
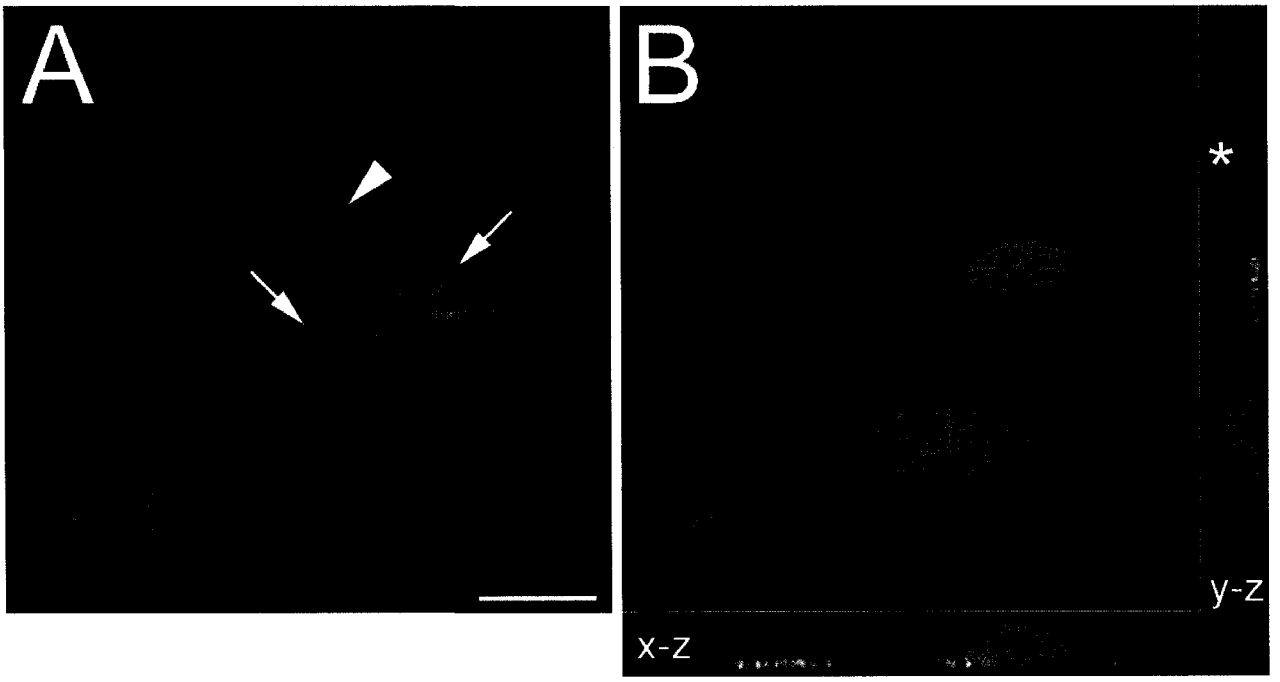

A
DGK-ζ
A'
Syn
B
DGK-ζ
B'
Syn
C
DGK-ζ
C'
Syn
D
DGK-ζ
D'
Syn
E
DGK-ζ
E'
Rac1
F
DGK-ζ
F'
Rac1
G
DGK-ζ
G'
Rac1
H
DGK-ζ
H'
Rac1

Figure 4.8. DGK-ζ-depleted, Rac1-enriched zones form dome-like protrusions from the dorsal surface of contacting myoblasts.

C2 myoblasts were serum-starved for 24 h then were fixed and double-labeled for DGK-ζ and Rac1. Confocal laser-scanning microscopy was used to acquire z-series of optical sections, which were used to reconstruct 3-dimensional images. (A) A representative 3-D image showing sharply defined areas where Rac1 staining protrudes from the dorsal surface of two closely contacting myoblasts, forming dome-like structures (*arrows*). The *arrowhead* indicates a raised area that is uniquely stained for DGK-ζ; these were only rarely observed. (B) The corresponding *x*-z and *y*-*z* projections show that DGK-ζ staining is excluded from the protruding areas of Rac1 staining. Note that the signal intensity was the lowest at the cell edge, making it appear that the cells are separated by several μm. Bar, 10 μm.


A
B
*
y-z
x-z

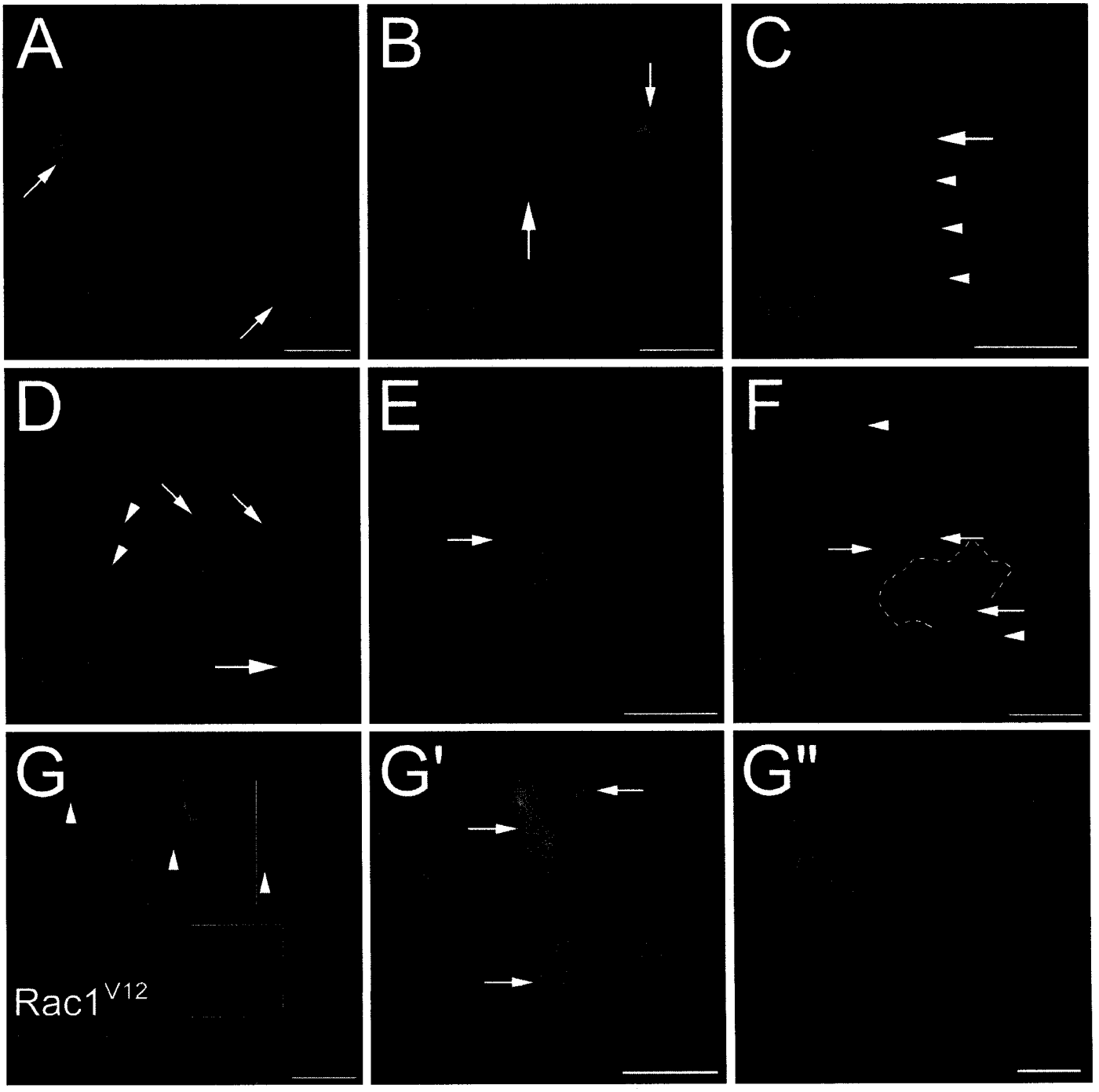
is not yet clear. Nevertheless, these data suggest fusing myoblasts create specialized domains depleted of DGK-ζ, but enriched in Rac1, which protrude from the dorsal plasma membrane.

The large size of the compartments bounded by DGK-ζ, syntrophins and Rac1 suggested they might be macropinosomes, relatively large vesicles that provide an efficient route for the non-selective endocytosis of solute macromolecules (Swanson and Watts, 1995). To explore this possibility, serum-starved myoblasts were incubated for 3 h with Texas Red-conjugated 70,000 M.W. dextran, then were fixed and stained for DGK-ζ, syntrophins or Rac1. We found that closely contacting myoblasts contained large vesicles filled with dextran (Figure 4.9A-G). Dextran-filled vesicles were often located at the junction of two cells (Figure 4.9B, F and G', *arrows*) or at the ends of narrow cellular processes connecting the cells (Figure 4.9A, C and D, *arrows*). We quantified the occurrence of such dextran-filled structures in serum-starved myoblasts to determine if they are indeed associated with contact sites. When viewed using phase-contrast optics, we found that the majority (75 ± 2 %) of cells with at least one large dextran-containing macropinosome contacted a neighboring myoblast. Only 25 ± 2 % of the cells with macropinosomes did not make obvious contact with another myoblast. Collectively, these data indicate that cell-to-cell contact induces macropinocytosis in myoblasts at sites where they interact.

Rac1, syntrophins and DGK-ζ were concentrated around the perimeter of most dextran-filled vesicles (*arrows* in Figure 4.9D, E and F, respectively); however, some appeared to have no significant accumulations of DGK-ζ (Figure 4.9A, *arrows*). This is most clearly illustrated in Figure 4.9B, where there is intense DGK-ζ labeling surrounding a large membrane invagination at the junction of two closely apposed cells that is not filled with dextran (*large arrow*), but significantly less DGK-ζ around a similarly sized, dextran-filled

Figure 4.9. Dextran uptake at contact sites between serum-starved myoblasts.

Untransfected C2 myoblasts (A – E) or C2 myoblasts transfected with HA-tagged DGK-ζ alone (F) or with myc-Rac1^{V12} (G-G") were serum starved for 24 h and then incubated with 0.5 mg/ml lysine-fixable, Texas Red-conjugated 70 kDa dextran for 3 h, followed by several washes in PBS. After fixation, the cells were stained with antibodies to DGK-ζ (A and B), Rac1 (C and D), syntrophin (E) or HA (F – G"). *Small arrows* indicate substantial accumulations of Texas Red dextran (A – E) in large vesicles. The *large arrow* in (B) indicates a large invagination at the junction of two cells with bright DGK-ζ staining, but no significant dextran uptake. (G') and (G") are magnified images of the upper and lower boxes in (G), respectively. The *arrowheads* in (C) and (D) indicate processes bridging adjacent myoblasts and in (G), extensive membrane ruffling along the border of two closely contacting cells. The dashed line in (F) traces the outline of the cell at the bottom of the image, which lies underneath the cell at the top. Bars, 20 μm (A – G); 10 μm (G'); 5 μm (G").

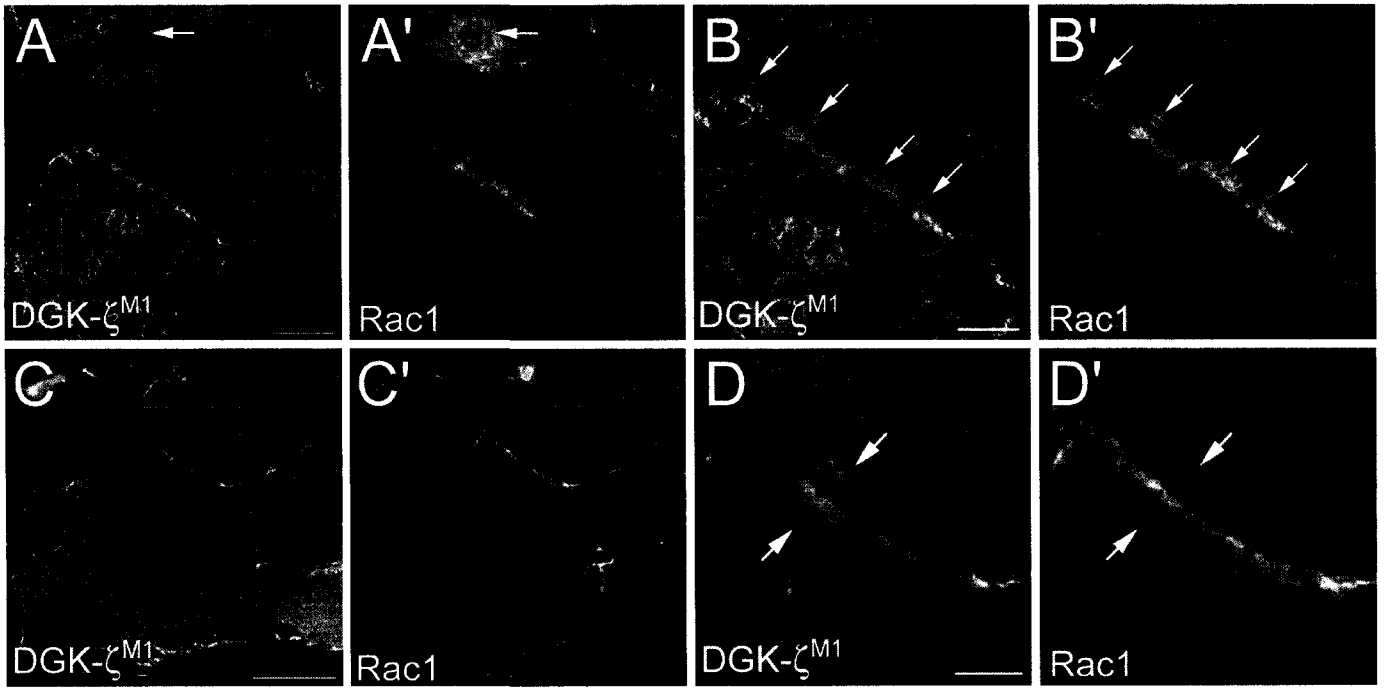

A
B
C
D
E
F
G
G'
G''
Rac1V12

vesicle in the same cell (*small arrow*). Based on many such observations, DGK-ζ appears to be a prominent component of forming or newly formed dextran-containing vesicles at the periphery, but is less abundant on more centrally located vesicles. It should be noted, however, that some DGK-ζ-positive vesicles took up no observable dextran (Figure 4.9F, *arrowheads*). Nevertheless, these results reveal that the DGK-ζ-depleted, Rac1-enriched compartments take up dextran and thus, in this respect, they are similar to macropinosomes.

To further explore the relationship of DGK-ζ to macropinocytosis, we analyzed dextran uptake by fluorescence microscopy of C2 cells cotransfected with HA-tagged DGK-ζ and myc-tagged $Rac1^{V12}$, a constitutively active Rac1 mutant that has been shown to induce macropinocytosis when overexpressed in cultured cells (Ridley *et al.*, 1992). In cultures double-labeled for HA-DGK-ζ and rhodamine-dextran, cells transfected with $Rac1^{V12}$ were identified on the basis of morphology; those expressing high levels were flatter and more spread than cells expressing DGK-ζ alone and exhibited extensive membrane ruffling (H.A. and S.G., unpublished observations). In the two closely apposed cells shown in Figure 4.9G, bright labeling for DGK-ζ was apparent in membrane ruffles at sites of cell-cell contact (*arrowheads*) and surrounding large dextran-filled vesicles located directly opposite one another on either side of the border (*upper box* and Figure 4.9G', *arrows*). DGK-ζ was also present on several large, unfilled vesicles in the central region of the cell (Figure 4.9G, *lower box* and G"), which may have formed before the start of dextran uptake.

In C2 cultures cotransfected with $Rac1^{V12}$ and DGK-ζ^{M1}, a DGK-ζ mutant that mimics constitutive phosphorylation of the MARCKS domain (Abramovici *et al.*, 2003), we again observed DGK-ζ-depleted, Rac1-enriched zones at intercellular junctions (Figure 4.10A and A', *arrows*). In addition, we observed clusters of vesicles at cell-cell junctions

Figure 4.10. DGK-ζ and Rac1 are present in clusters of vesicles at the border of contacting myoblasts. C2 myoblasts cotransfected with HA-tagged DGK-ζ^{M1} and myc-tagged $Rac1^{V12}$ were serum-starved for 48 h then were fixed and stained with epitope-tag specific antibodies. Both proteins were concentrated in ruffling membranes at sites of cell-cell contact (A and A'; C and C', *boxed regions).* Higher magnification images reveal groups of small (1-2 μm) vesicles along the border of the contacting cells (B and B', D and D', *arrows*). In (D), DGK-ζ^{M1} is clearly visible on the perimeter of vesicles that appear to be aligned on opposite sides of the cell junction. Also note the large DGK-ζ-depleted region in (A, *arrow*) that is intensely stained for Rac1 (A', *arrow*). Bars, 20 μm (A and C); 5 μm (B and D).


A
A'
B
B'
DGK-ζM1
Rac1
DGK-ζM1
Rac1
C
C'
D
D'
DGK-ζM1
Rac1
DGK-ζM1
Rac1

whose size (1-2 μm) and association with membrane ruffles are consistent with macropinosomes (Figure 4.10B and B'; D and D', *arrows*). Some of these vesicles contained both Rac1 and DGK-ζ (Figure 4.10B and B', arrows), while others appeared to be labeled only for DGK-ζ (Figure 4.10D and D', *arrows*). In one case, the vesicles appeared to be aligned opposite one another on either side of the border of two closely apposed cells (Figure 4.10D, *arrows*). Taken together, these results suggest DGK-ζ and Rac1 have a role in the formation of macropinosome-like vesicles at sites of cell-cell contact between closely apposed myoblasts.

Discussion

The molecular and cellular events that occur during vertebrate myoblast fusion, although long studied, are still unclear, in part because fusion is such a protracted process in mammals. The shorter time frame for myoblast fusion, the much simpler architecture of the somatic musculature, and its ease of genetic manipulation have made the fruit fly *Drosophila melanogaster* a powerful model system for the study of fusion (Dworak and Sink, 2002). Similar morphological changes take place in vertebrate and *Drosophila* muscle cells during fusion and many of the genes essential for fusion in *Drosophila* have mammalian counterparts, suggesting the molecular and cellular events of myoblast fusion are evolutionarily conserved (Knudsen, 1992; Doberstein *et al.*, 1997). In *Drosophila*, mutants of these genes arrest at distinct stages of the fusion process, allowing functional roles to be assigned to specific gene products (Chen and Olson, 2004).

The fact that many of the genes essential for fusion encode proteins that impinge on actin reorganization suggest this is a central event during myoblast fusion. This is consistent with earlier cell culture studies showing that the cytoskeleton is extensively reorganized in fusing myoblasts (Fulton *et al.*, 1981) and that interfering with the formation of actin filaments inhibits fusion (Sanger *et al.*, 1971; Sanger and Holtzer, 1972; Constantin *et al.*, 1995). Indeed, cytoskeletal rearrangement is a prerequisite for the formation of pseudopodia, the migration of myoblasts towards their fusion partners, and for the alignment of plasma membranes in juxtaposed cells prior to membrane merger.

There is considerable evidence linking phospholipid signaling pathways to actin cytoskeleton reorganization. Phosphoinositides and their lipid second messengers bring about changes in actin organization by directly activating actin-binding proteins and by

modulating signaling pathways regulated by GTPases (Janmey and Lindberg, 2004). In cultured myoblasts, there is a rapid breakdown of $PI(4,5)P_2$ and a concomitant increase in DAG and PA upon the initiation of membrane fusion (Wakelam and Pette, 1982; Wakelam, 1983). Proteins that transduce these specific changes in membrane lipid composition into actin cytoskeleton rearrangements are likely to have an important role in the fusion process. DGK-ζ, which metabolizes DAG to PA, is a strong candidate to mediate such changes, provided it is appropriately localized within fusing myoblasts. Its interaction with syntrophins may be particularly important for targeting DGK-ζ to the proper subcellular domains. Below, we discuss our findings on DGK-ζ and syntrophin localization in fusing myoblasts with respect to the different steps of fusion.

Recognition and adhesion

In *Drosophila*, myoblast fusion involves the initial recognition and adhesion of a few founder myoblasts, which establish muscle identity and position, and many fusion competent myoblasts (Dworak and Sink, 2002). Founder cells are thought to initiate fusion through the expression of two related transmembrane immunoglobulin (Ig) domain-containing receptors (Duf/Kirre and Roughest) that function as chemoattractants for fusion competent myoblasts (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001). Fusion competent myoblasts, which migrate and extend pseudopodia towards founder cells, express a different Ig superfamily member called Sticks and Stones (SNS) (Bour *et al.*, 2000). It is thought that these recognition molecules mediate the initial attraction between the two cell types. Consistent with this interpretation, fusion competent myoblasts in both *duf* and *sns* mutants extend pseudopodia without any preferred orientation and fail to migrate to, and aggregate with,

founder cells (Ruiz-Gomez *et al.*, 2000; Bour *et al.*, 2000). Because both Duf and SNS are transmembrane receptors, it is not clear how they mediate the long-range attraction of fusion competent myoblasts to founder cells, which may be several cell diameters apart. It has been suggested that they communicate via long, cytoplasmic extensions (Ruiz-Gomez *et al.*, 2000).

Our experiments using cultures of serum starved C2 cells showed that DGK-ζ, syntrophins and Rac1 were concentrated at sites where fine, filopodia-like extensions bridged neighboring cells. These structures were observed as early as 4 h after serum withdrawal, raising the possibility that they mediate the initial attraction and recognition of myoblasts during fusion. Filopodia were often seen at the distal tips of comparatively larger pseudopodia where DGK-ζ, syntrophins and Rac1 accumulated, but were also present elsewhere on the cell body. Frequently, cells were connected by multiple filopodia, but only some coincided with the local accumulations of DGK-ζ, syntrophin and Rac1 (Figure 4.2). This might reflect transient increases of these proteins at intercellular contacts, which were captured at specific time points. Therefore, we suspect that all filopodia were at some point associated with accumulations of DGK-ζ, syntrophins and Rac1. However, additional studies using living cells will be needed to confirm this hypothesis.

Similar cellular extensions have been observed in a variety of cellular contexts and have been variously referred to as pseudopodia, filopodia, cytonemes or tunneling nanotubules (Bryant, 1999; Ramirez-Weber and Kornberg, 2000; Rorth, 2003; Rustom *et al.*, 2004). Moreover, two recent studies suggest muscle cells develop filopodia-like extensions called 'myopodia' that cluster at sites of motor neuron innervation (Ritzenthaler *et al.*, 2000; Uhm *et al.*, 2001). While many types of cells send out such extensions, little is known about

how they form or what their function is (Rorth, 2003). Recent evidence suggests they mediate the mutual exchange of signals over long distances (Ramirez-Weber and Kornberg, 1999). However, determining the direction of signal propagation and designating the sending and receiving cells is tricky without a complete picture of the participating molecules (Rorth, 2003). Indeed, in our studies the static images of fixed cells that were obtained made it difficult to differentiate sending cells from recipients. Moreover, it is not clear whether protein accumulation occurred before a filopodial connection was established or whether it was induced by contact-mediated signaling events. Because of these limitations, there are several possible interpretations for the finding that DGK-ζ, syntrophin and Rac1 were invariably concentrated at the junction of only one of a pair of interacting cells (i.e. at one end of a filopodium). One possibility is that the proteins are involved in the local reorganization of the actin cytoskeleton required for filopodia extension from the sending cell. In support of this idea, we recently demonstrated that DGK-ζ and syntrophins form a complex with Rac1 that functions in neurite outgrowth from cultured neurons (Yakubchyk *et al.*, 2005). Since neurites can be considered a prototype cellular extension, it is conceivable that a similar Rac1-dependent mechanism of actin reorganization controls the outgrowth of cellular extensions in muscle and other cell types.

Alternatively, DGK-ζ, syntrophin and Rac1 might be recruited to contact sites in response to signals generated in either the sending or recipient cell. Consistent with this possibility, genetic studies in *Drosophila* have identified genes essential for fusion whose protein products belong to a possible signaling cascade downstream of cell recognition by Duf/SNS (Chen and Olson, 2004). These proteins mechanistically converge on the activation of Rac, which is itself required for fusion (Luo *et al.*, 1994; Hakeda-Suzuki *et al.*, 2002). For

example, *myoblast city* encodes a protein with homology to human DOCK180 (Rushton *et al.*, 1995; Erickson *et al.*, 1997), which together with a second protein called ELMO, forms a complex that acts as an unconventional guanine nucleotide exchange factor (GEF) for Rac (Brugnera *et al.*, 2002; Lu *et al.*, 2004). Another gene essential for fusion, *loner*, encodes a putative GEF for the *Drosophila* homologue of ARF6, a small GTPase that regulates cytoskeletal rearrangements by controlling the subcellular localization of Rac1 (Chen *et al.*, 2003). Thus, Rac appears to play a pivotal role in myoblast fusion. The present results, together with our previous finding that DGK-ζ and syntrophin form a complex with Rac1 (Yakubchyk *et al.*, 2005), suggest these proteins function together to regulate local cytoskeletal changes induced by contact-mediated signals.

Adhesion zippers

Our results suggest myoblasts are pulled together and establish close contact by a mechanism that is intrinsically similar to that described for epithelial cells (Jacinto and Wolpert, 2001). In keratinocyte cultures, interdigitating actin-based filopodia embed into neighboring cells forming adhesion zippers that pull the opposing membranes together (Vasioukhin *et al.*, 2000). These structures are characterized by two parallel rows of E-cadherin puncta at filopodia tips, which together with other adherens junction proteins, serve as membrane attachment sites to the cortical actin cytoskeleton. In myoblasts, the comparable distribution of N-cadherin suggests it performs a similar function, although more definitive proof will come with demonstration that other adherens junction proteins are also present at filopodia tips. Although DGK-ζ and Rac1 were not concentrated in adhesion zippers *per se*, both were found in a well-delineated zone at the base of the filopodia.

Interestingly, studies suggest cadherin-catenin complexes are rapidly recruited from cytoplasmic pools to these initial filopodial contacts, which subsequently evolve into mature adherens junctions (Costa *et al.*, 1998; Raich *et al.*, 1999). Thus, the pools of DGK-ζ and Rac1 could function as a reserve of signaling molecules when rapid reorganization of the actin cytoskeleton is required. Consistent with this idea, Rac1 plays a prominent role in dorsal closure of the *Drosophila* embryo (Harden *et al.*, 1995; Harden *et al.*, 1999). Our studies further suggest that advancing lamellipodia from one myoblast are guided by filopodia from the apposing cell, lending support to the idea that filopodia and lamellipodia have dynamic roles at these intercellular junctions (Jacinto *et al.*, 2000). Interestingly, we also found multiple N-cadherin puncta along the length of filopodia, raising the possibility that it is transported along actin filaments to filopodia tips.

Membrane union and the formation of cytoplasmic continuity

Perhaps the least understood process in myoblast fusion is the merger of the membrane bilayers to establish cytoplasmic continuity. Early studies showed that the fusion of cultured vertebrate muscle cells takes place within phospholipid-enriched, protein-depleted domains of the plasma membrane (Kalderon and Gilula, 1979; Fulton *et al.*, 1981). These membrane domains were closely associated with cytoplasmic, lipid-enriched vesicles that were shown to be essential components of the fusion process (Lipton and Konigsberg, 1972; Kalderon and Gilula, 1979; Engel and David, 1985). An apparently similar situation arises following the adhesion of fusion competent myoblasts with founders during *Drosophila* embryogenesis, when electron dense vesicles collect in the cytoplasm adjacent to sites of cell-cell contact (Doberstein *et al.*, 1997). In these "pre-fusion complexes", pairs of

vesicles align with each other across the apposing plasma membranes. In both systems, electron-dense plaques form in areas where membrane breakdown ultimately occurs, presumably through the fusion of the vesicles with the plasma membrane (Engel and David, 1985; Doberstein *et al.*, 1997). This step is followed by elongation and alignment of the myoblasts along their long axes, then by membrane breakdown and pore formation, which establishes cytoplasmic continuity between the fusing cells. The excess plasma membrane in the fusion area vesiculates and is eliminated, while the plasma membrane outside of the fusion area remains intact, resulting in the formation of a single multinucleated myofiber (Rash and Fambrough, 1973; Kalderon and Gilula, 1979; Doberstein *et al.*, 1997).

Several of our results bear on these fusion-related processes. In C2 myoblasts coexpressing DGK-ζ^{M1} and $Rac1^{V12}$, groups of vesicles were found along the interface of juxtaposed cells (Figure 4.9G-G" and Figure 4.10). In some cases, these vesicles appeared to be aligned on opposite sides of the membrane interface, raising the possibility that they are related to *Drosophila* pre-fusion complexes (Doberstein *et al.*, 1997). However, one obvious difference is the much larger size (1-2 μm) of the vesicles observed in our studies, which is typical of macropinosomes. Two additional criteria support the identification of these vesicles as macropinosomes: their ability to take up fluorescent dextran from the extracellular milieu and their association with membrane ruffles. The exact role of macropinosomes at these intercellular junctions is unclear. However, in addition to providing a route for the non-selective endocytosis of solute macromolecules, macropinosomes also modulate the expression of cell surface molecules (Swanson and Watts, 1995), so it is possible that they regulate the cell surface expression of proteins required for fusion. A

possible candidate is N-cadherin, which we found was closely associated with DGK-ζ at earlier stages of the fusion process.

The exact nature and function of the larger domains that were devoid of DGK-ζ and syntrophins but enriched for Rac1 (see Figure 4.7), is less obvious. Some of these domains formed dome-like structures that conspicuously protruded from the dorsal surface of the cells (Figure 4.8). An interesting possibility is that these domains are, in fact, precursors to the large dextran-containing compartments found at cell contacts. Rac might cause the membrane to protrude at these sites, which subsequently folds back in on itself, and in the process, takes up dextran from the extracellular milieu. Once formed, Rac would no longer be needed; hence the lack of Rac in dextran-filled vesicles (Figure 4.9C and D).

Dextran-containing compartments were often found at either end of long, narrow cellular extensions that appeared to be continuous with both contacting cells (Figure 4.9A). An intriguing idea is that these cellular extensions mediate the initial fusion of myoblasts. Consistent with this possibility, electron microscopic studies showed that the earliest fusion site in quail myoblasts is a small cytoplasmic bridge, approximately 120 nm in diameter (Lipton and Konigsberg, 1972). The authors noted then that only a single point of cytoplasmic continuity is found between fusing cells, suggesting that fusion is initiated at a focal site that subsequently enlarges to provide cytoplasmic confluence. However, since the formation of cytoplasmic continuity cannot be established with certainty in this case, we cannot be sure whether the myoblasts had indeed undergone fusion. Additional experiments are necessary to examine this compartment in more detail.

Chapter 5

Diacylglycerol kinase ζ regulates actin cytoskeleton reorganization through dissociation of Rac1 from RhoGDI

Hanan Abramovici[1,3], Parmiss Mojtabaie[1,3], Robin J. Parks[2,3,4], Xiao-Ping Zhong[6], Gary A. Koretzky[7], Matthew K. Topham[5] and Stephen H. Gee[1,3]*

*Corresponding author

[1]Department of Cellular and Molecular Medicine
University of Ottawa
451 Smyth Rd
Ottawa, ON K1H 8M5
Canada

[2]Molecular Medicine Program, Ottawa Health Research Institute, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada

[3]Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON, Canada

[4]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada

[5]Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112 USA

[6]Departments of Pediatrics and Immunology, Duke University Medical Center, Durham, NC 27710

[7]The Signal Transduction Program, the Abramson Family Cancer Research Institute, Philadelphia, Pennsylvania, 19104 USA

Characters: 39,540

Running Title: DGKζ regulates Rac1-RhoGDI dissociation

Submitted to: Molecular Biology of the Cell
Status: Accepted with minor revisions

Contribution from authors

Hanan Abramovici and Stephen Gee co-wrote the manuscript. Hanan Abramovici contributed the majority of the intellectual input and performed all the experiments with the exception of those shown in Figure 5.3 panels B, C and D which were performed by Parmiss Mojtabaie. Matthew Topham generated the immortalized mouse embryonic fibroblasts. Xiao-Ping Zhong and Gary Koretzky generated the DGKζ knockout mice. Robin Parks produced the DGKζ and GFP-control adenoviruses.

Acknowledgements

We thank Drs. Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA) and Kaoru Goto (Yamagata University School of Medicine, Yamagata, Japan) for providing affinity-purified antibodies to PAK1 and DGKι, respectively. The paxillin antibody was a gift from Dr. John Copeland (University of Ottawa, ON). The Rhotekin-RBD construct was a gift from Dr. Martin Alexander Schwartz (University of Virginia, Charlottesville, VA). The PAK1 GST-PBD construct was a gift from Dr. John Collard (The Netherlands Cancer Institute, Amsterdam). This work was supported by the Muscular Dystrophy Association USA and by the Cancer Research Society. We are grateful to Radoslav Zinoviev and James Cheng for technical assistance.

Abstract

Activation of Rac1 GTPase signaling is stimulated by phosphorylation and release of RhoGDI by the effector PAK1, but it is unclear what initiates this potential feed-forward mechanism for regulation of Rac activity. Phosphatidic acid (PA) activates PAK1. Here, we investigated whether PA produced by DGKζ initiates RhoGDI release and Rac1 activation. In DGKζ-deficient fibroblasts PAK1 phosphorylation and Rac1-RhoGDI dissociation were attenuated, leading to reduced Rac1 activation following PDGF stimulation. The cells showed defective Rac1-regulated behaviors including lamellipodia formation, membrane ruffling, migration, and spreading. Wild-type DGKζ, but not a kinase-dead mutant, or exogenous PA, rescued Rac activation. DGKζ stably associated with PAK1 and RhoGDI, suggesting these proteins form a complex that functions as a Rac1-selective RhoGDI dissociation factor. These results define a pathway that links diacylglycerol, DGKζ and PA to the activation of Rac1: The PA generated by DGKζ activates PAK1, which dissociates RhoGDI from Rac1 leading to changes in actin dynamics that facilitate the changes necessary for cell motility.

Introduction

Rho GTPases regulate gene transcription, cell-cycle progression, vesicular traffic, and cell polarity (Jaffe and Hall, 2005; Ridley, 2006) but are best known for their ability to coordinate alterations in cellular actin networks that regulate cell morphology. Such changes are necessary for directed cell migration during embryogenesis, inflammation, wound healing and tumor metastasis (Burridge and Wennerberg, 2004). In mammalian cells, **Rac1 promotes** actin polymerization **and focal complex assembly** leading to lamellipodia protrusion and membrane ruffle formation, Cdc42 regulates filopodial extension, and Rho promotes the assembly of actin stress fibers and focal adhesions (Ridley *et al.*, 1992; Ridley and Hall, 1992; Nobes and Hall, 1995).

All Rho GTPases cycle between inactive GDP-bound and active GTP-bound conformations; the active versions interact with specific downstream effectors to elicit distinct biological responses. This cycle is tightly regulated by guanine nucleotide exchange factors (GEFs), which activate 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. A third class of proteins, guanine nucleotide dissociation inhibitors (GDIs), regulates Rho GTPase function in as many as three distinct ways. RhoGDI, the best characterized member, (1) prevents GDP dissociation, (2) inhibits intrinsic or GAP-stimulated GTP hydrolysis (Chuang *et al.*, 1993b), and (3) regulates GTPase partitioning between the cytosol and plasma membrane (Olofsson, 1999). The latter task is accomplished by sequestering the GTPases as soluble cytosolic complexes in which the C-terminal membrane-targeting lipid moiety is prevented from interacting with membranes (Dermardirossian and Bokoch, 2005; Dovas and Couchman, 2005). Dissociation of GTPases

from RhoGDI is a prerequisite for membrane association and activation by GEFs. Because the vast majority of Rho GTPase protein exists in a biologically inactive cytosolic complex with RhoGDI in resting cells, this a major point of regulation of Rho GTPase activity and function (Chuang *et al.*, 1993a; Olofsson, 1999).

The selective activation of individual Rho GTPases by growth and/or adhesive factors implies that separate mechanisms must exist to dissociate them from cytosolic RhoGDI complexes and target them to the appropriate membrane domain. The means by which Rho family proteins are released from RhoGDI is poorly understood and, so far, no common mechanistic theme has emerged. Dissociation seems to use several different mechanisms including direct phosphorylation by protein kinases (Bourmeyster *et al.*, 1992; Mehta *et al.*, 2001; Dermardirossian *et al.*, 2004) and protein displacement factors such as integrins, ERM proteins and the p75 NGF receptor (Takahashi *et al.*, 1997; del Pozo *et al.*, 2002; Yamashita and Tohyama, 2003).

Accumulating evidence suggests negatively charged phospholipids play an important role in RhoGDI release (Bourmeyster *et al.*, 1992; Chuang *et al.*, 1993a; Faure *et al.*, 1999; Dovas and Couchman, 2005; Ugolev *et al.*, 2006). Among those shown to dissociate RhoGDI, phosphatidic acid (PA) is particularly interesting because it also activates p21-activated kinase 1 (PAK1) (Bokoch *et al.*, 1998), a downstream effector of activated Rac and Cdc42 implicated in the regulation of multiple cellular activities including cytoskeletal dynamics (Bokoch, 2003). Recently, PAK1 was shown to bind to and phosphorylate RhoGDI, resulting in the selective release of Rac1 (Dermardirossian *et al.*, 2004). Thus, PA-mediated activation of PAK1 might initiate Rac signaling but precisely how this occurs and the source of PA are unclear.

Diacylglycerol kinases (DGKs) phosphorylate the lipid second messenger diacylglycerol (DAG) to yield PA and are thus a potential source for PA-mediated Rac activation. Supporting this possibility, Tolias et al.(1998) found that Rac/RhoGDI exists in a complex with a DGK and we recently demonstrated that the ubiquitously expressed ζ isoform binds directly to Rac1 (Yakubchyk *et al.*, 2005). Here, we investigated the possibility that DGKζ might be part of a Rac/PAK1 complex and a source of PA for PAK1 activation by analyzing the phenotype and biochemical properties of embryonic fibroblasts from DGKζ-null mice. Our findings demonstrate that DGKζ is part of a signaling complex that functions as a Rac1-selective RhoGDI dissociation factor and define a direct link between lipid second messengers and regulation of the actin cytoskeleton.

Materials and Methods

Antibodies

Monoclonal HA, actin, tubulin, and alpha-actinin antibodies were from Sigma (St. Louis, MO). Monoclonal Rac1, BD Biosciences (San Jose, CA). Monoclonal RhoA, polyclonal RhoGDI, polyclonal PAK1, Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal PAK1, Cell Signaling Technologies (Danvers, MA). Polyclonal phospho-Akt Ser473 and Akt, Cell Signaling Technologies. Monoclonal phospho-tyrosine (clone 4G10), Upstate Biotech-Millipore (Temecula, CA). A polyclonal antibody raised against the N-terminus of DGKζ is as described (Topham *et al.*, 1998). mAb 1351 was a gift from Dr. Stanley Froehner (University of Washington, Seattle, WA). AlexaFluor 488 and 594-conjugated secondary antibodies and phalloidin, Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondaries, Jackson Immunoresearch (West Grove, PA).

Reagents

Matrigel was from BD Biosciences. FuGENE 6 transfection reagent, Roche (Indianapolis, IN). Dulbecco's modified Eagle's medium (DMEM), Invitrogen. Protein A agarose, Upstate Biotech-Millipore. 1,2-Dioctanoyl-*sn*-Glycero-3-Phosphate (08:0 PA), 1,2-Dioctanoyl-*sn*-glycerol (08:0 DG), and 1-Hexanoyl-2-Hydroxy-*sn*-Glycero-3-Phosphate (06:0 LPA), Avanti Polar Lipids (Alabaster, AL). All other reagents were from Sigma.

Plasmids and constructs

The PAK1-PBD and Rhotekin-RBD constructs were as described (Sander *et al.*, 1998) and (Ren *et al.*, 1999). N-terminal myc-tagged $Rac1^{V12}$ in pEFmPLINK was a gift from Dr. Andrew Thorburn (University of Colorado, CO). N-terminal YFP-tagged $Rac1^{V12}$ was generated by subcloning $Rac1^{V12}$ from pEFmPLINK into the Kpn1 and Apa1 sites of pEYFP-C1 (Clontech, Mountain View, CA). The GST-RhoGDI construct was generated by subcloning mouse RhoGDI from pcDNA 3.1 (UMR cDNA resource center; www.cdna.org) into the EcoR I and Xho I cloning sites of pGEX-4T-1.

Cell Culture and transfection /infection

Primary mouse embryonic fibroblasts (MEFs) were isolated from wild type (+/+) and DGKζ-deficient (-/-) embryos (13.5 days post coitus) as described (Robertson, 1987). MEFs were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, and grown at 37 °C, 5% CO_2. Immortalized cell lines were generated by transfecting MEFs with pcDNA3.1 harboring an SV40 large T antigen expression cassette and the *Sh ble* antibiotic resistance gene. Stable clones were selected in media containing 200 μg ml^{-1} Zeocin.

For $Rac1^{V12}$ experiments, cells were transfected with either myc- or YFP-tagged $Rac1^{V12}$ constructs for 24 h. Cells were fixed in 4% paraformaldehyde (PFA), then processed for immunocytochemistry (for myc-$Rac1^{V12}$) or live microscopy (for YFP-$Rac1^{V12}$).

Cloning and production of adenoviral constructs encoding GFP, wt DGKζ, $DGK\zeta^{FLAG}$, or the kinase-dead mutant have been described (Yakubchyk *et al.*, 2005). For rescue experiments, fibroblasts were infected with adenoviruses at a multiplicity of infection

(MOI) of 100-250 (for ruffling rescue and Rac1 activity assays) or 1000 (for wounding assays) for 1 h at 37°C. Cells were further incubated for 24-36 h under standard growth conditions.

Immunofluorescence microscopy and quantification of actin-based structures

Fibroblasts were seeded onto Matrigel-coated coverslips, serum-starved overnight then stimulated with either PDGF-BB (Sigma) or vehicle (0.14M HCl, 0.1% BSA). Cells were fixed and processed as described (Abramovici and Gee, 2007). Images were obtained using a charge-coupled device (CCD) camera on a Zeiss Axioskop 2 microscope. To quantify actin based structures, fixed cells were labeled with AlexaFluor (488 or 594)-conjugated phalloidin. A lamellipodium was an area of the cell with thick peripheral F-actin within a broad, sheet-like region; a peripheral ruffle was a curvilinear accumulation of F-actin at the cell edge; circular ruffles were circular accumulations of F-actin on the dorsal cell surface, and filopodia finger-like, actin-rich protrusions. For rescue experiments, all cells were assumed to have been infected. Cell and ruffle surface areas were measured using the "outline" tool function in Zeiss AxioVision 4.5.

Adhesion assays

Adhesion assays were performed as described (Wagner *et al.*, 2002). Cells were plated at a density of 2 x 10^4/coverslip for the indicated times and processed for immunofluorescence microscopy as above.

Wounding assays

Cell monolayers on 12 mm coverslips were scraped with a 20 gauge needle, generating wounds approximately 0.2 x 12 mm. Three wounds were made on each coverslip.

Cells were fixed with 0.5% PFA immediately (0 h) or following 2 h incubation at 37 °C. Cells were stained with AlexaFluor-conjugated phalloidin (or indicated antibodies) and prepared for immunofluorescence microscopy. Photomicrographs were taken at 500 μm intervals along the length of the wound, images exported into Zeiss AxioVision 4.5 software, and the extent of cell migration quantified by two independent methods, both yielding similar results. The wound width was determined by lines drawn perpendicular to the wound edge at 50 μm intervals. The percentage wound closure was calculated by subtracting wound width at 2 h from 0 h and dividing the resulting number by wound width at 0 h. In the second method, the area devoid of cells was manually traced with the "outline" tool function and the wound area automatically calculated by the software. Measurements at 2 h were then subtracted from the wound area at 0 h and divided by wound area at 0 h. Data was exported into Microsoft Excel, then graphed using SigmaPlot software (SPSS, Chicago, IL).

Transwell migration assays

Modified Boyden chamber assays were performed as described (Sander *et al.*, 1998). Fibronectin coated filters were placed into the lower chamber containing either 0.1% BSA or 50 ng ml^{-1} PDGF-BB. Wild type or DGKζ-null MEFs (1×10^5) resuspended in 0.1% BSA in DMEM were added to the upper chamber and allowed to migrate for up to 12 h. Cells were fixed with 4% PFA for 30 min and stained with DAPI. 10 randomly selected fields were photographed and counted for each experiment.

Immunoprecipitation and GST pull-down experiments

Immunoprecipitations were carried out essentially as described (Hogan *et al.*, 2001). To measure Rac1 –RhoGDI dissociation, wt and DGKζ-null MEFs were serum-starved

overnight, then stimulated with 50 ng ml^{-1} PDGF for 5 min. Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 5% glycerol, 1 mM DTT, and protease inhibitors and centrifuged at 18,000 x g for 10 min at 4 °C. Equivalent amounts of protein (~1 mg) were incubated with 5 μg RhoGDI antibody or control rabbit IgG for 4 h at 4 °C. 40 μl of 50% Protein A agarose slurry was added for 1.5 h. The beads were washed with lysis buffer, resuspended in 1X reducing sample buffer, and analyzed for bound RhoGDI or Rac1 by immunoblotting. GST pull-down experiments were carried out as described (Yakubchyk *et al.*, 2005) using lysis buffer without $MgCl_2$. For some experiments, wt fibroblasts were infected with HA-DGKζ and the cells harvested 24 h later.

Rho GTPase activity assays

Levels of active Rac1 and Cdc42 were measured using a GST-PAK1 PBD pull-down assay (Sander *et al.*, 1998). Cells were serum-starved overnight, then stimulated with 50 ng ml^{-1} PDGF for 3 min for Rac1 assays, or 100 ng ml^{-1} bradykinin for 4 min for Cdc42 assays. For lipid assays, serum-starved cells were treated with 100 nM PA, DAG, LPA, or vehicle for 25 min at 37°C. After stimulation, media was removed and cells immediately harvested in chilled lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% TX-100, 20 mM $MgCl_2$, and protease inhibitors). Lysates were incubated for 10 min at 4°C, then centrifuged at 18,000 x g for 10 min. Equivalent amounts of protein were incubated with GST-PAK1 PBD beads for 30 min at 4 °C. The beads were washed with lysis buffer, boiled in reducing sample buffer, and eluted proteins assayed for bound Rac1 or Cdc42 by immunoblotting.

Quantification of phosphorylated PAK1

Fibroblasts were seeded onto 100 mm dishes and grown until confluent. Cells were treated as indicated, then lysed directly in reducing sample buffer. Lysates were boiled, sonicated, and analyzed by immunoblotting using an affinity-purified phospho-specific PAK1 antibody(Sells *et al.*, 2000), or a polyclonal anti-PAK1 antibody. Digital images were captured using a Kodak Image Station 440 CF (Rochester, NY) and band intensity measured by densitometric analysis. To calculate relative amounts of pPAK1, the intensity of the upper band of the pPAK doublet was normalized to total PAK and expressed as a percentage of the unstimulated, wt condition.

Live-cell imaging

Imaging was carried out using a Zeiss Axiovert 200 M microscope with a stage-fixed cell chamber at 37 °C, 5% CO_2. Equal numbers of wild-type or DGKζ-null fibroblasts were cultured in 35 mm dishes (MatTek, Ashland, MA) and were either serum-starved overnight, then stimulated with 50 ng ml^{-1} PDGF for 15 min, or transfected with YFP-$Rac1^{V12}$ for 24 h. For ruffling experiments, cells were imaged at a 100 X magnification using a DIC III filter, and filmed at 10 sec intervals for 15 min using an Axiocam HRm CCD camera. For YFP-$Rac1^{V12}$ experiments, cells were imaged at a 400 X magnification and filmed for 10 min at 10 second intervals using a Zeiss LSM5 scanning module with an argon laser at 488 nm.

Results

Generation of DGKζ-deficient mouse embryonic fibroblasts

To investigate potential roles for DGKζ in Rac1-regulated events, we established immortalized fibroblast cell lines derived from wild-type mice or mice in which the DGKζ gene was disrupted by homologous recombination (Zhong *et al.*, 2003). DGKζ-deficient mouse embryonic fibroblasts (MEFs) did not express detectable levels of the DGKζ protein (Figure S1A). To determine if the loss of DGKζ leads to compensatory up-regulation of other DGKζ family members, extracts of wt and null MEFs were immunoblotted with an antibody to DGKι, the most closely related isoform. No differences in total DGKι levels were observed (Figure S1B), suggesting fibroblasts do not compensate for the lack of DGKζ by modulating the expression of DGKι. The levels of several other relevant proteins remained unchanged in the DGKζ-null cells (Figure S1C).

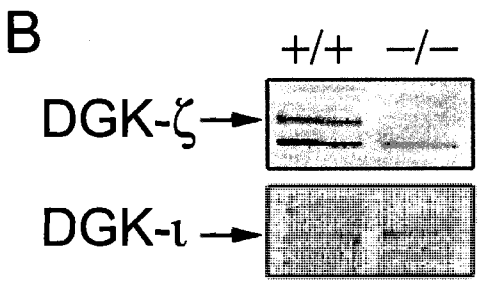
Decreased Rac1 activation in the absence of DGKζ

To determine if absence of DGKζ affects Rac1 activation, the level of GTP-bound (active) Rac1 was assayed in lysates of wt and null cells using an effector pull-down assay (Sander *et al.*, 1998). There was no significant difference in resting Rac1-GTP levels between serum-starved wt and null cell lysates (Figure 5.1A and data not shown). There was a substantial increase in Rac1-GTP levels in wt cell lysates following PDGF stimulation as expected, but only a small increase in null cell lysates (Figure 5.1A and B). Thus, these data reveal a dramatic decrease in PDGF-dependent Rac1 activation in the absence of DGKζ. There was no appreciable difference in Cdc42 activity between the wt and null lysates, either with or without bradykinin stimulation (Figure 5.1C).

Figure S1. Characterization of SV40-immortalized wild type and DGKζ-null fibroblasts.

(A) Schematic showing the location of various protein domains within DGKζ. The N-terminal (*open box*), C1 domains, MARCKS (*filled box*) and catalytic domains are indicated. Also shown are the four ankyrin repeats (I-IV) at the C-terminus. The extreme C-terminus contains a class I PDZ-binding motif. (B) Immunoblot analysis of wt and DGKζ-null fibroblasts. Equivalent total protein from cell lysates of wild-type (+/+) and DGKζ-null fibroblasts were separated by SDS-PAGE, transferred to a nitrocellulose membrane and confirmed by Ponceau S staining. The blot was probed with an antibody to the N-terminus of DGKζ (*top panel*) or to the closely related isoform DGKι (*bottom panel*). (C) Replicate blots were probed with antibodies to syntrophin (Syn), Rac1, and several focal adhesion proteins as indicated. Pax (paxillin).


A
MARCKS
PDZ binding
C1A
C1B
Catalytic
I
II
III
IV


B
+/+
–/–
DGK-ζ
DGK-ι

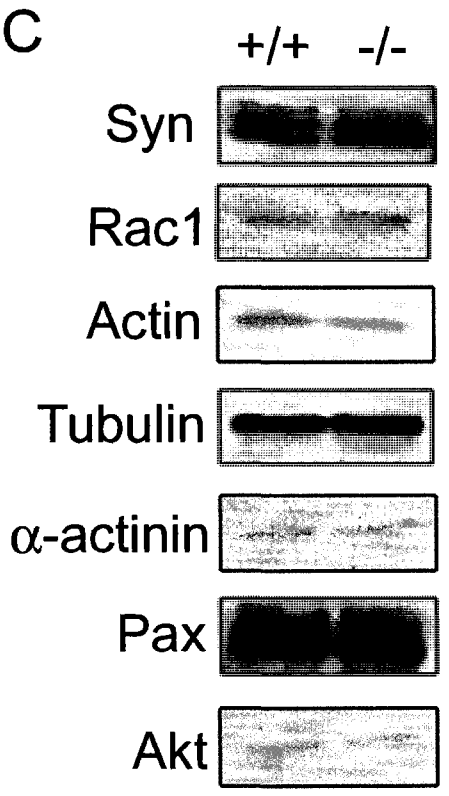
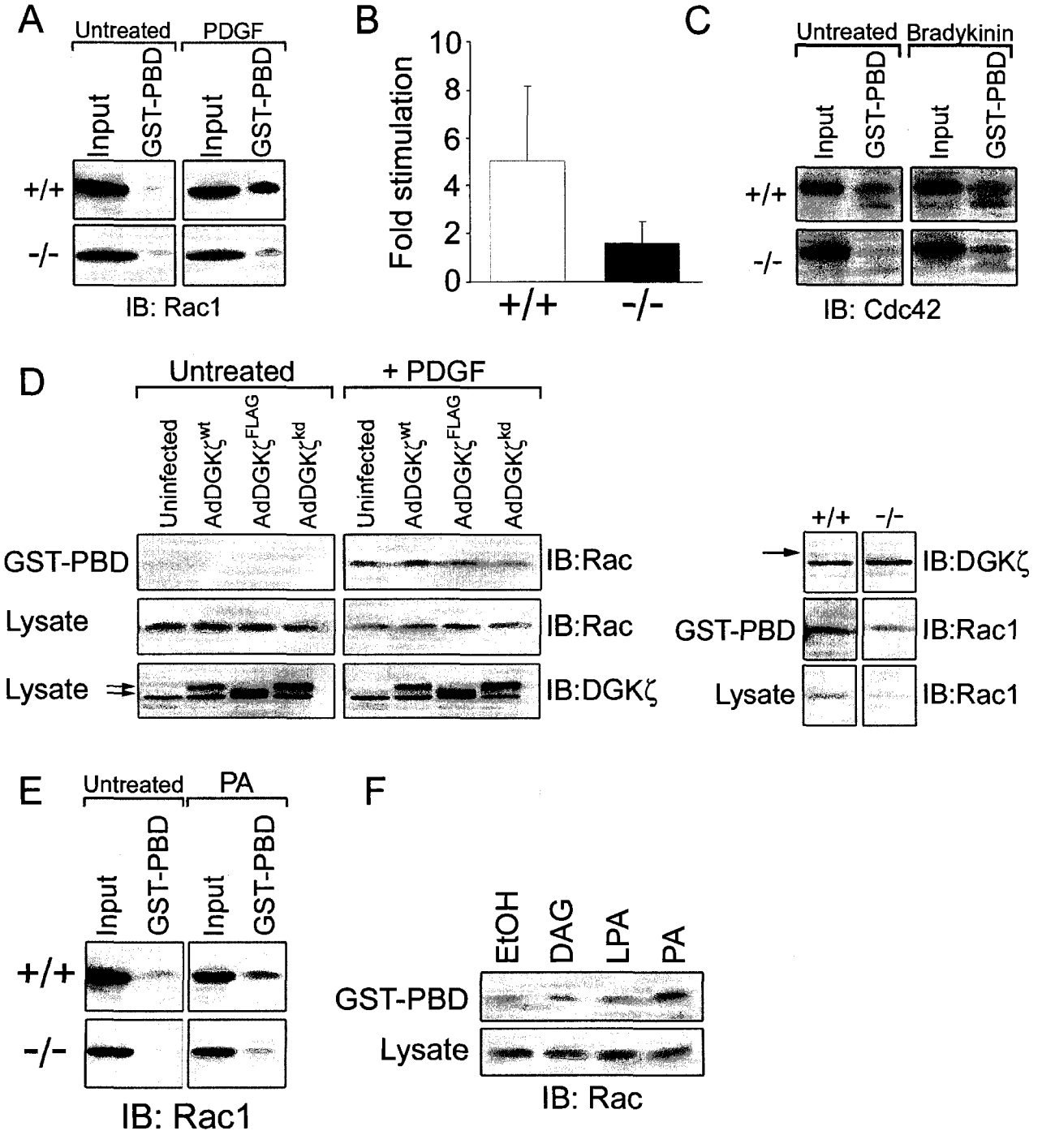

C
+/+
-/-
Syn
Rac1
Actin
Tubulin
α-actinin
Pax
Akt

Figure 5.1. Decreased Rac1 activation in DGKζ-null fibroblasts following PDGF stimulation.

(A) Assay of global Rac1 GTPase activity. Serum-starved wt (+/+) and DGKζ-null (-/-) fibroblasts were treated 5 min with PDGF or vehicle alone (Untreated). Cell lysates were incubated with immobilized GST or GST-PBD and the bound proteins were analyzed by immunoblotting (IB) for Rac1. Input represents ~5% of the starting material. (B) Quantification of active Rac levels by densitometric analysis of immunoblots. (C) Cdc42 activity was assayed as in (A) using fibroblasts treated 5 min with bradykinin or vehicle. (D) Rescue of Rac activation requires DGKζ catalytic activity. (*left*) DGKζ-null cells were infected with adenovirus bearing HA-tagged wt or kinase-dead (kd) DGKζ, or with DGKζ with a C-terminal FLAG tag (indicated by arrows, respectively). Rac activity and total Rac content in cell lysates were assayed as above. The level of endogenous DGKζ or infected DGKζ was assayed with an anti-DGKζ antibody. The lower MW species is a non-specific band migrating slightly faster than $DGK\zeta^{FLAG}$ and present at equivalent levels in all four samples. (*Right*) Rac activity, total Rac content and the level of endogenous DGKζ in the lysates of uninfected, PDGF-stimulated wt (+/+) and DGKζ-null (-/-) fibroblasts are shown for comparison. The *arrow* indicates endogenous DGKζ. (E) Serum-starved wt and DGKζ-null cells were treated for 25 min with 100 μM PA then Rac activity determined as above. (F) Serum-starved DGKζ-null cells were treated for 25 min with vehicle (EtOH) or 100 μM DAG, lysophosphatidic acid (LPA), or PA then Rac activity assayed as above.


A
Untreated
PDGF
Input
GST-PBD
Input
GST-PBD
+/+
-/-
IB: Rac1
B
Fold stimulation
10
8
6
4
2
0
+/+
-/-
C
Untreated
Bradykinin
Input
GST-PBD
Input
GST-PBD
+/+
-/-
IB: Cdc42
D
Untreated
+ PDGF
Uninfected
AdDGKζwt
AdDGKζFLAG
AdDGKζkd
Uninfected
AdDGKζwt
AdDGKζFLAG
AdDGKζkd
GST-PBD
IB:Rac
Lysate
IB:Rac
Lysate
IB:DGKζ
+/+
-/-
IB:DGKζ
GST-PBD
IB:Rac1
Lysate
IB:Rac1
E
Untreated
PA
Input
GST-PBD
Input
GST-PBD
+/+
-/-
IB: Rac1
F
EtOH
DAG
LPA
PA
GST-PBD
Lysate
IB: Rac

To demonstrate that the change in Rac activity is due directly to the loss of DGKζ, the wt protein was reintroduced into null cells by adenoviral infection (Figure 5.1D). Protein expression was verified by immunoblotting cell lysates with an anti-DGKζ antibody. PDGF-stimulated Rac1 activity in lysates of cells expressing wt DGKζ (AdDGKζ^{wt}) was noticeably increased compared to uninfected control cell lysates. Similar results were obtained with a DGKζ mutant (AdDGKζ^{FLAG}) that does not interact with syntrophins, but which has normal catalytic activity (Hogan *et al.*, 2001). In contrast, a kinase-dead mutant (AdDGKζ^{kd}) failed to increase active Rac levels despite being expressed at a 2-fold higher level than DGKζ^{wt}. These results suggest the Rac activation defect in null cells is due to loss of DGKζ activity.

To determine if the defect is due to decreased PA production by DGKζ and hence could be rescued by exogenous PA, wt and null cells were treated with a membrane permeable form of PA and the lysates subsequently analyzed for Rac activity (Figure 5.1E). Exogenous PA increased Rac activity in both wt and null cell lysates to a similar extent (~2.5 fold). In control experiments, neither DAG nor lysophosphatidic acid (LPA) caused a detectable increase in Rac activity in DGKζ-null cells (Figure 5.1F). Collectively, these results suggest DGKζ-derived PA contributes to the regulation of Rac activity following PDGF stimulation.

Decreased PAK1 phosphorylation in DGKζ-null cells

The p21-activated kinases (PAKs) are Ser/Thr protein kinases whose activity is regulated by binding of active Rac or Cdc42. Since Rac1 activity was decreased in DGKζ-null cells, we investigated whether there was a corresponding decrease in active PAK1.

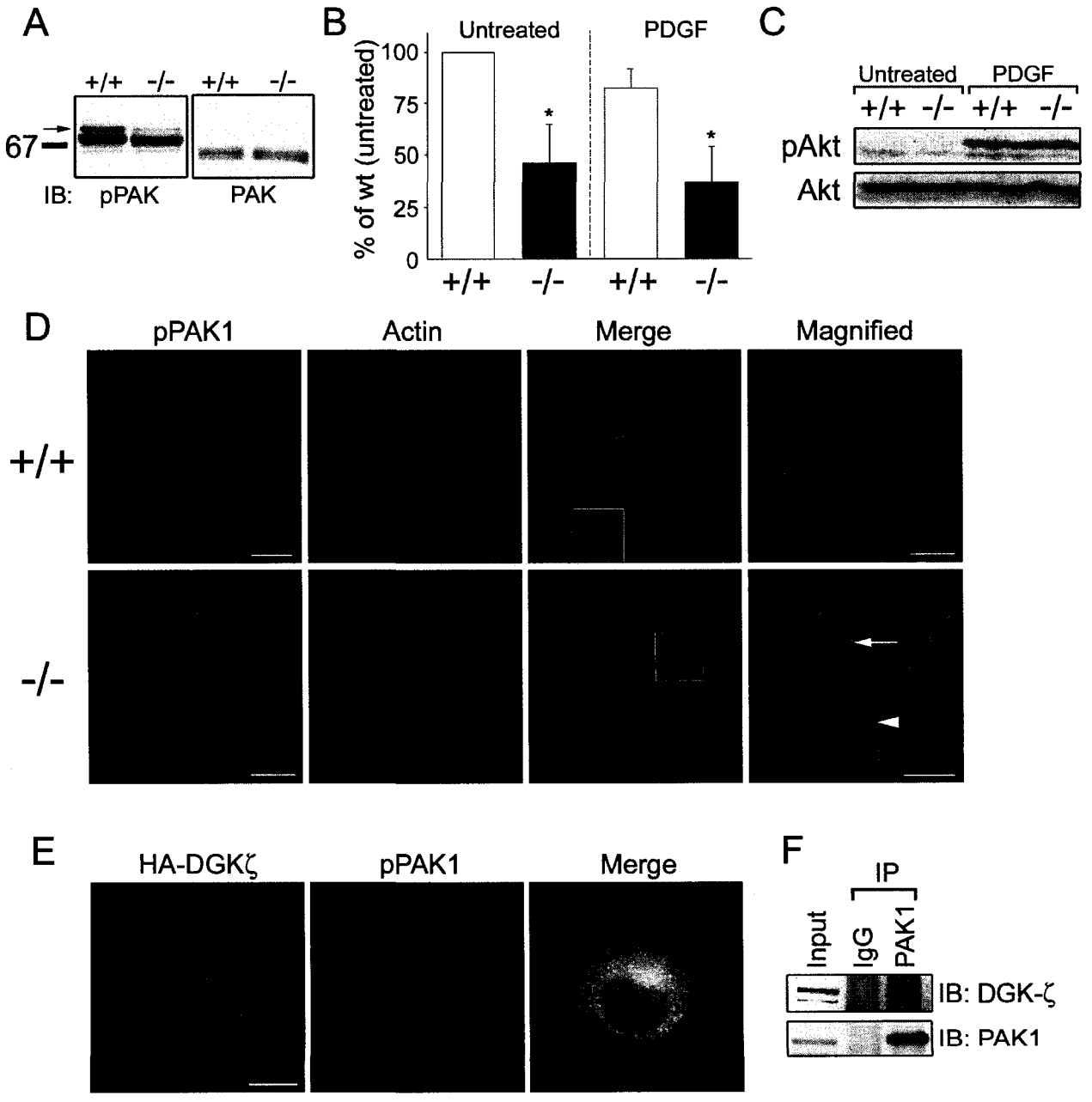
Binding of active Rac or Cdc42 to PAK1 relieves autoinhibition and stimulates autophosphorylation, leading to increased kinase activity (Bokoch, 2003). Thus, the level of pPAK, as determined by immunoblotting with a phospho-specific antibody, was used to gauge PAK activity. In unstimulated wt cells, two pPAK1 bands were evident (Figure 5.2A); the upper band most likely represents hyper-phosphorylated PAK1 and the lower band, a less phosphorylated version (Sells *et al.*, 2000). There was a substantial decrease in the levels of both bands in DGKζ-null cells. When normalized to total PAK1 levels, the intensity of the upper pPAK1 band was only about half that seen in wt cells (Figure 5.2B).

We next assayed pPAK levels in cells stimulated by PDGF, which is known to activate PAK1 (Dharmawardhane *et al.*, 1997). A previous report noted only a slight increase (28%) in total pPAK1 levels upon PDGF stimulation by measuring the fluorescence intensity of individual, immunolabeled fibroblasts (Sells *et al.*, 2000). Consistent with this, we found global pPAK levels in PDGF-treated wt cells were not significantly different from unstimulated cells (Figure 5.2B), despite the same concentration of PDGF being able to induce substantial membrane ruffling (not shown). It is likely that small differences in individual cells do not translate into a biochemically detectable difference among a large population. Nevertheless, as for unstimulated cells, PDGF-stimulated null cells had only half as much pPAK1 as wt cells (Figure 5.2B).

Extracellular stimuli can activate PAK1 via multiple independent pathways (Bokoch, 2003; Menard and Mattingly, 2003). Akt, a serine/threonine kinase which is rapidly phosphorylated following growth factor stimulation, phosphorylates PAK1 via a GTPase-independent mechanism (Welch *et al.*, 1998; Tang *et al.*, 2000). Therefore, we determined whether the Akt pathway is altered in DGKζ-null cells by assessing the activation status of

Figure 5.2. Decreased PAK1 phosphorylation in DGKζ-null fibroblasts.

(A) Detergent extracts prepared from wt and DGKζ-null cells grown in serum-containing medium were analyzed by immunoblotting for phosphorylated (p)PAK1 and total PAK1. (B) Quantification of pPAK1 levels by densitometric analysis of immunoblots. The upper pPAK1 band (arrow in a) was measured and normalized to total PAK levels. Values are means ± s.d. from six independent experiments. Asterisks denote a significant difference from untreated wt cells ($P<0.05$) by Student's *t*-test. (C) PDGF-induced Akt phosphorylation is unaffected by loss of DGKζ as assessed by immunoblotting wt and null cell extracts with a pAkt antibody. (D) Mislocalization of pPAK1 in DGKζ-null fibroblasts. Cells seeded onto fibronectin-coated coverslips were fixed 45 min after plating then double labeled with an anti-pPAK1 antibody (*green*) followed by AlexaFluor 488-conjugated secondary antibody and with AlexaFluor 594-conjugated phalloidin (*red*). Scale bar: 10 μm. (E) Colocalization of DGKζ and pPAK1 at lamellipodia edges. Fibroblasts transfected with HA-tagged DGKζ were fixed 30 min after replating then stained with anti-HA and anti-pPAK antibodies. Scale bar: 10 μm. (F) Interaction of endogenous DGKζ and PAK1. Detergent extracts of wt fibroblasts were incubated with normal rabbit IgG or an antibody to PAK1. Immune complexes were precipitated with protein G agarose then analyzed by immunoblotting with anti-PAK1 and anti-DGKζ antibodies. Input represents 3 % of starting material.


A
+/+ -/- +/+ -/-
67
IB: pPAK PAK
B
Untreated
PDGF
% of wt (untreated)
100
75
50
25
0
+/+ -/- +/+ -/-
C
Untreated PDGF
+/+ -/- +/+ -/-
pAkt
Akt
D
pPAK1
Actin
Merge
Magnified
+/+
-/-
E
HA-DGKζ
pPAK1
Merge
F
IP
Input
IgG
PAK1
IB: DGK-ζ
IB: PAK1

Akt by immunoblotting using an anti-pAkt antibody. The loss of DGKζ had no detectable effect on pAkt levels following PDGF stimulation (Figure 5.2C) suggesting signaling upstream of Akt is not impaired in DGKζ-deficient fibroblasts.

PAK1 localization

In resting cells, PAK1 is localized to membranous structures within the cytoplasm (Dharmawardhane *et al.*, 1997). However, active PAK1 preferentially accumulates in dynamic actin-based structures such as focal adhesions and membrane ruffles (Dharmawardhane *et al.*, 1997; Sells *et al.*, 1997; Sells *et al.*, 2000), most likely via its association with one or more binding partners (Bokoch *et al.*, 1996; Manser *et al.*, 1998; Bagrodia *et al.*, 1999; Manabe *et al.*, 2002). To determine if DGKζ influences the targeting of active PAK1 to adhesive sites, wt and DGKζ-null cells were stained for pPAK1 after replating cells for 45 min on fibronectin. As expected, pPAK1 labeling in wt cells was concentrated in focal adhesions (identified by their typical radiating actin bundles) on the cell's circumference (Figure 5.2D). In contrast, pPAK1 was confined to a perinuclear, cytoplasmic compartment in DGKζ-null cells. At higher magnification, pPAK1 foci in the null cells appear to be organized into circular profiles, suggesting they are associated with cytoplasmic vesicles. The localization of total PAK1 was mainly cytoplasmic and not obviously different in wt and null cells (Figure S2A). We verified that focal adhesions are formed normally in DGKζ-null cells using phosphotyrosine immunoreactivity as an indicator of focal adhesion formation (Figure S3A). In wt cells, DGKζ was not concentrated in the vast majority of focal adhesions, revealed by staining for phosphorylated FAK (FAK-pY861;

Figure S2. PAK localization in WT and DGKζ-null cells.

Indirect immunofluorescence localization of PAK in wild-type and DGKζ-null fibroblasts. Wild-type fibroblasts were fixed and double stained with an antibody to PAK1 followed by AlexaFluor 594-conjugated secondary antibody (*red*) and with AlexaFluor 488-conjugated phalloidin (*green*) to visualize actin. The pattern of PAK immunoreactivity was indistinguishable between wt and null cells. Note the numerous filopodia present in the DGKζ-null cells. Scale bars = 10 μm.

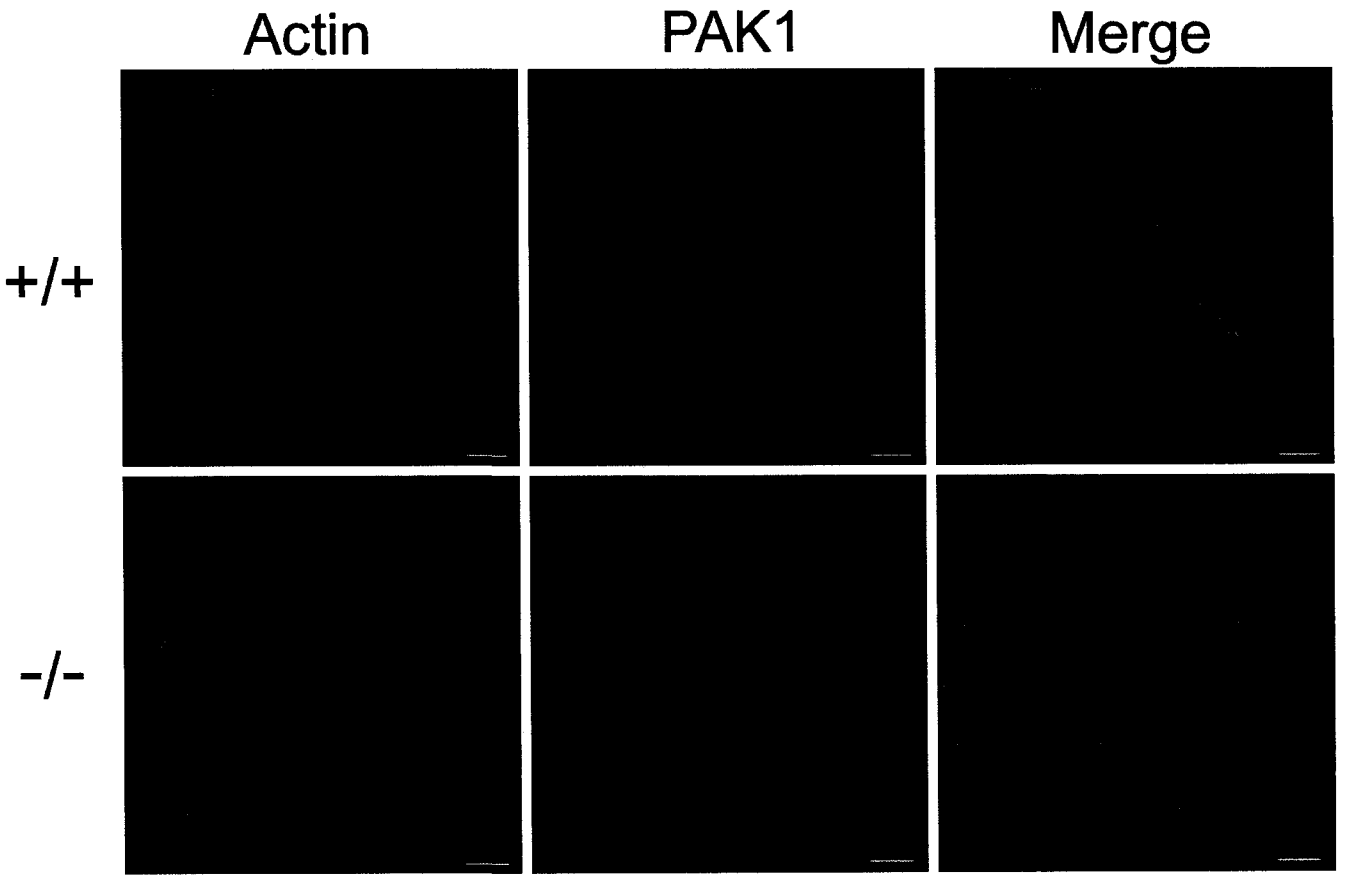

Actin
PAK1
Merge
+/+
-/-

Figure S3. Focal adhesion formation in WT and DGKζ-null cells.

(A) Immunofluorescence localization of actin (*green*) and phosphotyrosine (*pY, red*) after replating DGKζ-null cells on FN for 60 min. Arrowheads indicate focal adhesions. Scale bar: 20 μm. (B) Wt MEFs expressing HA-DGKζ were double stained with antibodies to HA and to tyrosine-phosphorylated FAK (FAK-pY861). The lower panels are magnified images of the area boxed area in the merged image. The *arrow* indicates a region of signal overlap. Scale bars = 10 μm. (C) A cell stained as in (B) showing an area of intense HA-DGKζ immunoreactivity that overlaps with diffuse FAK-pY861 staining. The lower panels are magnified images of the area boxed area in the merged image. The *arrowheads* delineate the area of intense staining. Scale bars = 10 μm.

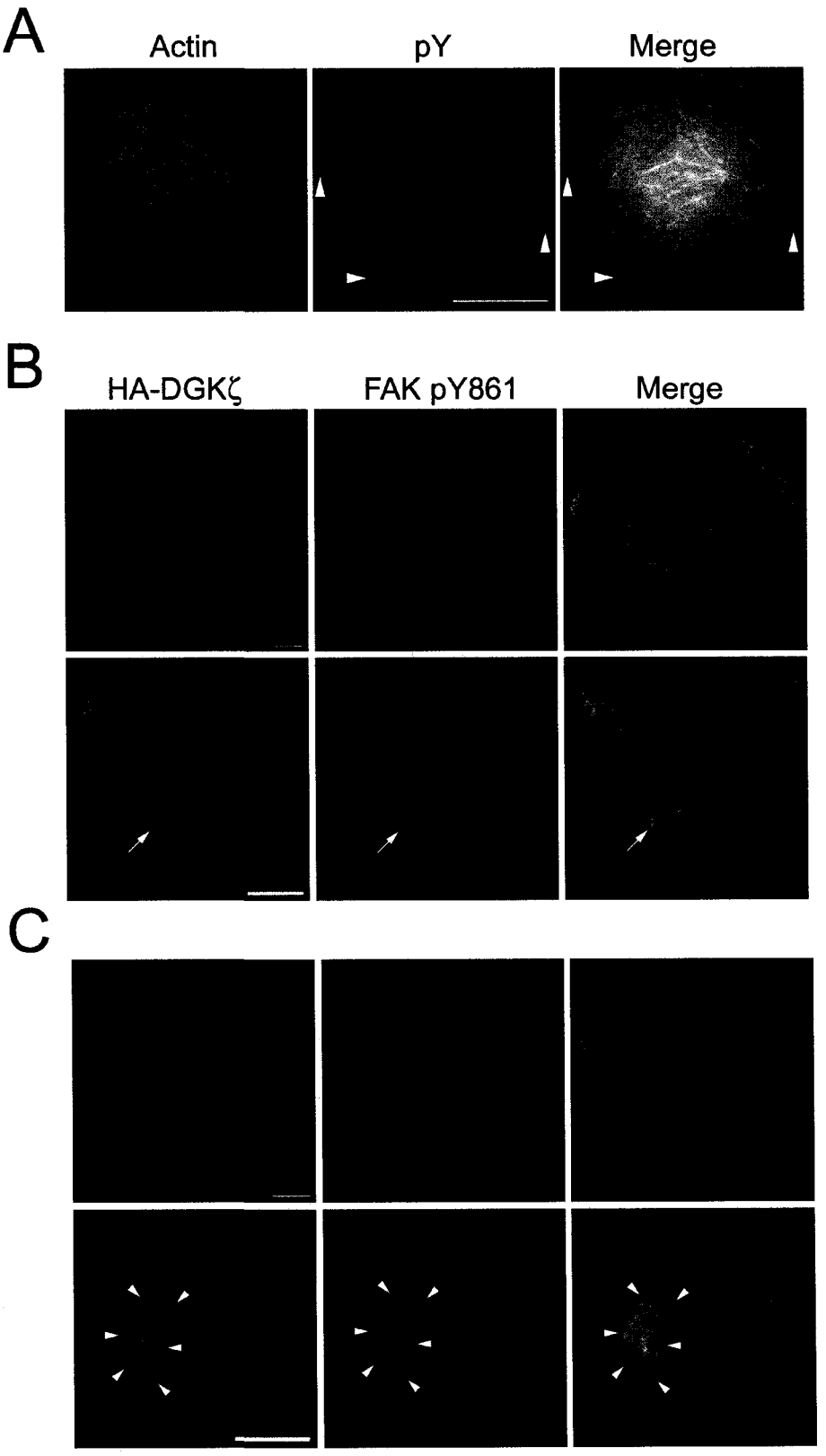

A
Actin
pY
Merge
B
HA-DGKζ
FAK pY861
Merge
C

Figure S3B). It was, however, codistributed with diffuse FAK-pY861 in areas rich in focal adhesions (Figure S3C). Presumably, these are areas in which focal adhesions are in dynamic flux. Collectively these data suggest DGKζ is required for the correct targeting of pPAK1 to focal adhesions but that it does not recruit pPAK to these sites.

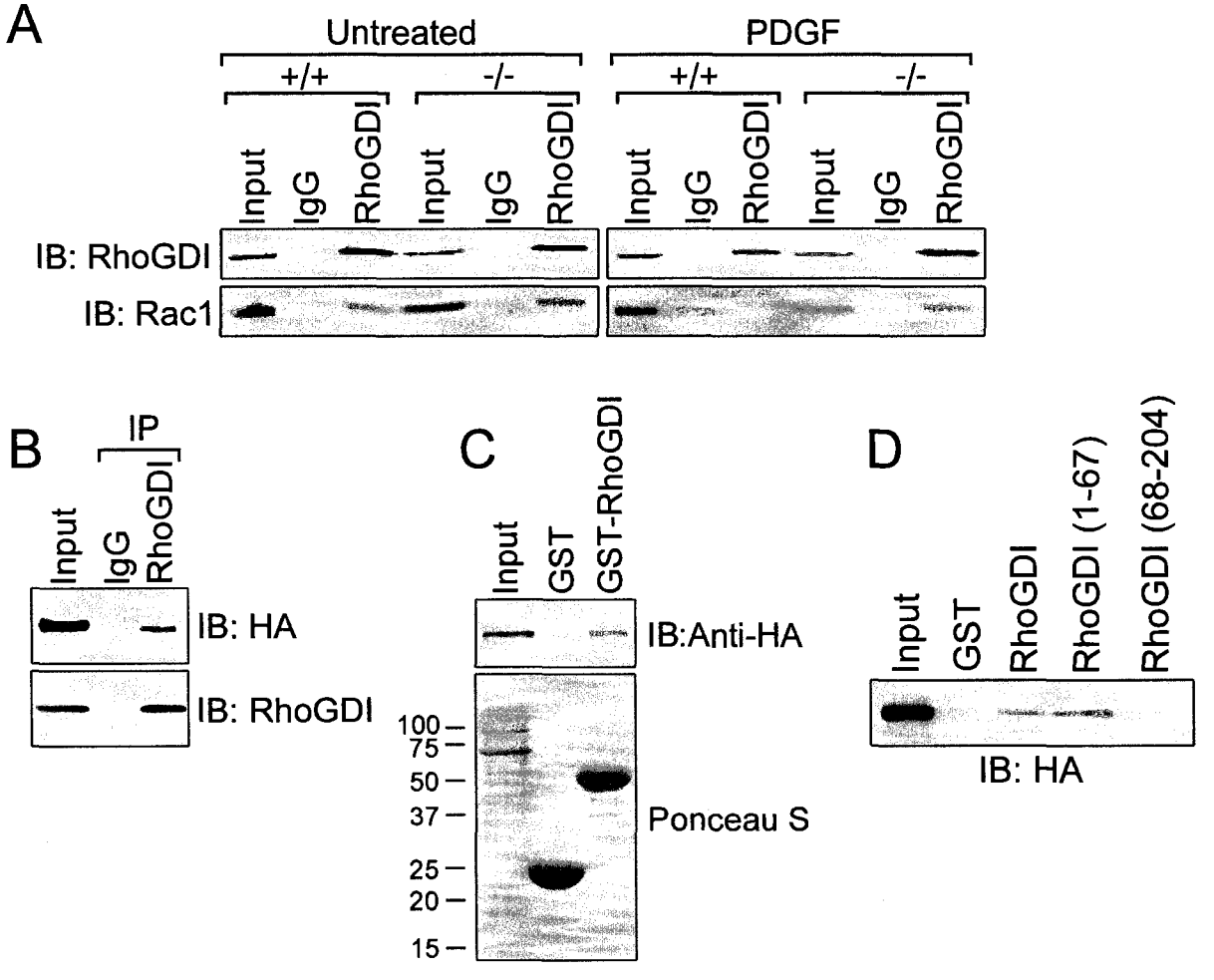
In support of an interaction between DGKζ and pPAK, we found that HA-DGKζ colocalized with pPAK at the edge of lamellipodia in fibroblasts that were transfected with HA-DGKζ for 24 h then replated for 30 min on FN (Figure 5.2E). DGKζ also coimmunoprecipitated with PAK1 from extracts of wt fibroblasts (Figure 5.2F), suggesting the two proteins form a stable complex in cells.

Impaired Rac1 release from RhoGDI in DGKζ-null cells

A recent study showed that following PDGF stimulation, PAK1 binds to and phosphorylates RhoGDI, which selectively dissociates from Rac1(Dermardirossian *et al.*, 2004). Since active PAK1 is decreased in DGKζ-null cells, we hypothesized that Rac1 release from RhoGDI would be attenuated following PDGF stimulation. To investigate this possibility, the amount of Rac1 bound to RhoGDI was assayed by coimmunoprecipitation from lysates of unstimulated and PDGF-stimulated wt and DGKζ-null cells (Figure 5.3A). Rac1 coprecipitated with RhoGDI from lysates of unstimulated but not stimulated, wt cells suggesting PDGF induces the dissociation of Rac from RhoGDI under these conditions. The amount of Rac1 that coimmunoprecipitated with RhoGDI from unstimulated null cell lysates was comparable to wt, however PDGF stimulation did not decrease the amount of Rac1 that coprecipitated with RhoGDI, suggesting a defect in the release of Rac from RhoGDI in the absence of DGKζ.

Figure 5.3. DGKζ is required for PDGF-induced dissociation of Rac1 from RhoGDI.

(A) Serum-starved wt (+/+) and DGKζ-null (-/-) cells treated 5 min with PDGF or vehicle were harvested and RhoGDI immunoprecipitated from the detergent-solubilized particulate fraction. Immune complexes were analyzed by immunoblotting for RhoGDI and Rac1. Results are representative of three independent experiments. (B) Detergent extracts of wt cells infected with adenovirus bearing HA-tagged, wt DGKζ were incubated with control rabbit IgG or RhoGDI antibody. Immune complexes were analyzed by immunoblotting with anti-HA and anti-RhoGDI antibodies. (C) Lysates prepared as in (B) were incubated with immobilized GST or GST-RhoGDI. The amount of each fusion protein is shown in the Ponceau S stained blot. Bound proteins were analyzed by immunoblotting with an anti-HA antibody. (D) GST fusion proteins corresponding to the N-terminal regulatory domain (a.a. 1-67) and the C-terminal immunoglobulin-like domain (a.a. 68-204) of RhoGDI were compared to full-length GST-RhoGDI for the ability to capture HA-DGKζ from extracts prepared as in (B). In each case, input represents ~5% of the extract.


A
Untreated
PDGF
+/+
-/-
Input
IgG
RhoGDI
IB: RhoGDI
IB: Rac1
B
IP
IB: HA
IB: RhoGDI
C
GST
GST-RhoGDI
IB:Anti-HA
100
75
50
37
25
20
15
Ponceau S
D
RhoGDI (1-67)
RhoGDI (68-204)
IB: HA

We next assessed potential interactions between DGKζ and RhoGDI. RhoGDI was immunoprecipitated from lysates of wt fibroblasts expressing HA-tagged DGKζ and the bound proteins were detected by immunoblotting. HA-DGKζ was coprecipitated by anti-RhoGDI but not by control IgG (Figure 5.3B). In addition, a GST-RhoGDI fusion protein, but not GST alone, captured HA-DGKζ from fibroblast extracts (Figure 5.3C). Amino acids 1-67 of RhoGDI, which correspond to the N-terminal regulatory arm of the protein (Dovas and Couchman, 2005), were sufficient to bind DGKζ (Figure 5.3D). Taken together, these results suggest DGKζ and RhoGDI form a stable complex in cells.

DGKζ-null fibroblasts have fewer lamellipodia and membrane ruffles

Rac1 regulates the formation of lamellipodia and membrane ruffles, structures that are driven by dynamic and spatially regulated changes in the actin cytoskeleton at the leading edge of migrating cells (Ridley *et al.*, 1992). Two types of ruffles can generally be distinguished in cells stimulated by growth factors: peripheral ruffles, which are associated with active lamellipodia and dorsal, circular ruffles (Abercrombie *et al.*, 1970). DGKζ is concentrated in peripheral ruffles, at the leading edge of lamellipodia (Topham and Prescott, 2001; Abramovici *et al.*, 2003; Luo *et al.*, 2004a) and in circular ruffles, along with syntrophin and Rac1 (Figure S4). To determine if the defect in Rac activation affects lamellipodia and/or membrane ruffle production, these structures were quantified in fixed wt and null cells. Lamellipodia were observed in 67 ± 7% of wt fibroblasts grown in serum-containing media, but only in 24 ± 2% of null cells grown under identical conditions (Figure 5.4A).

Figure S4. DGKζ and syntrophins are present in circular ruffles.

Wild-type fibroblasts were fixed and stained for the indicated endogenous proteins then visualized by indirect immunofluorescence. Arrows indicate circular ruffles. Scalebars: 20 μm.

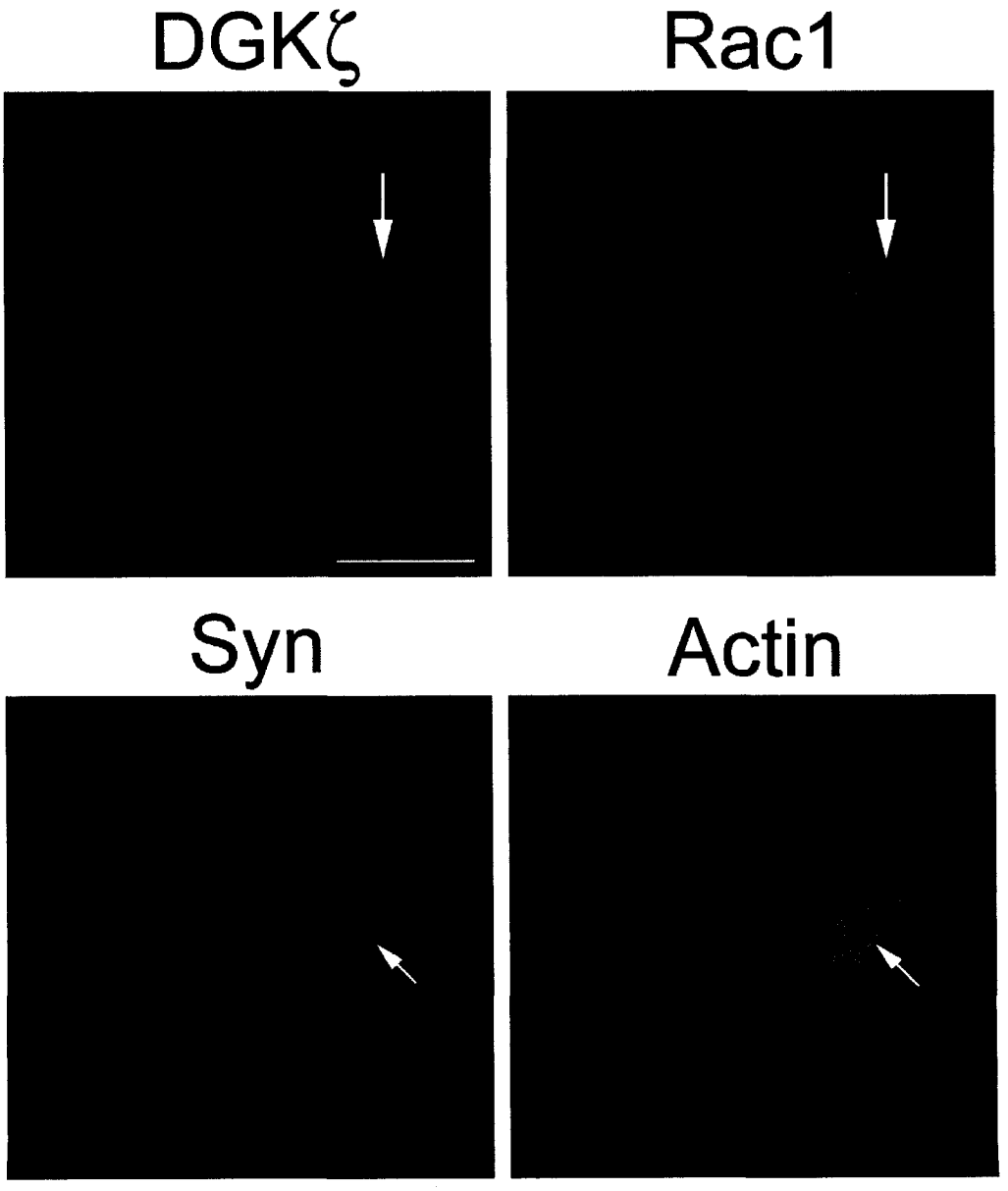
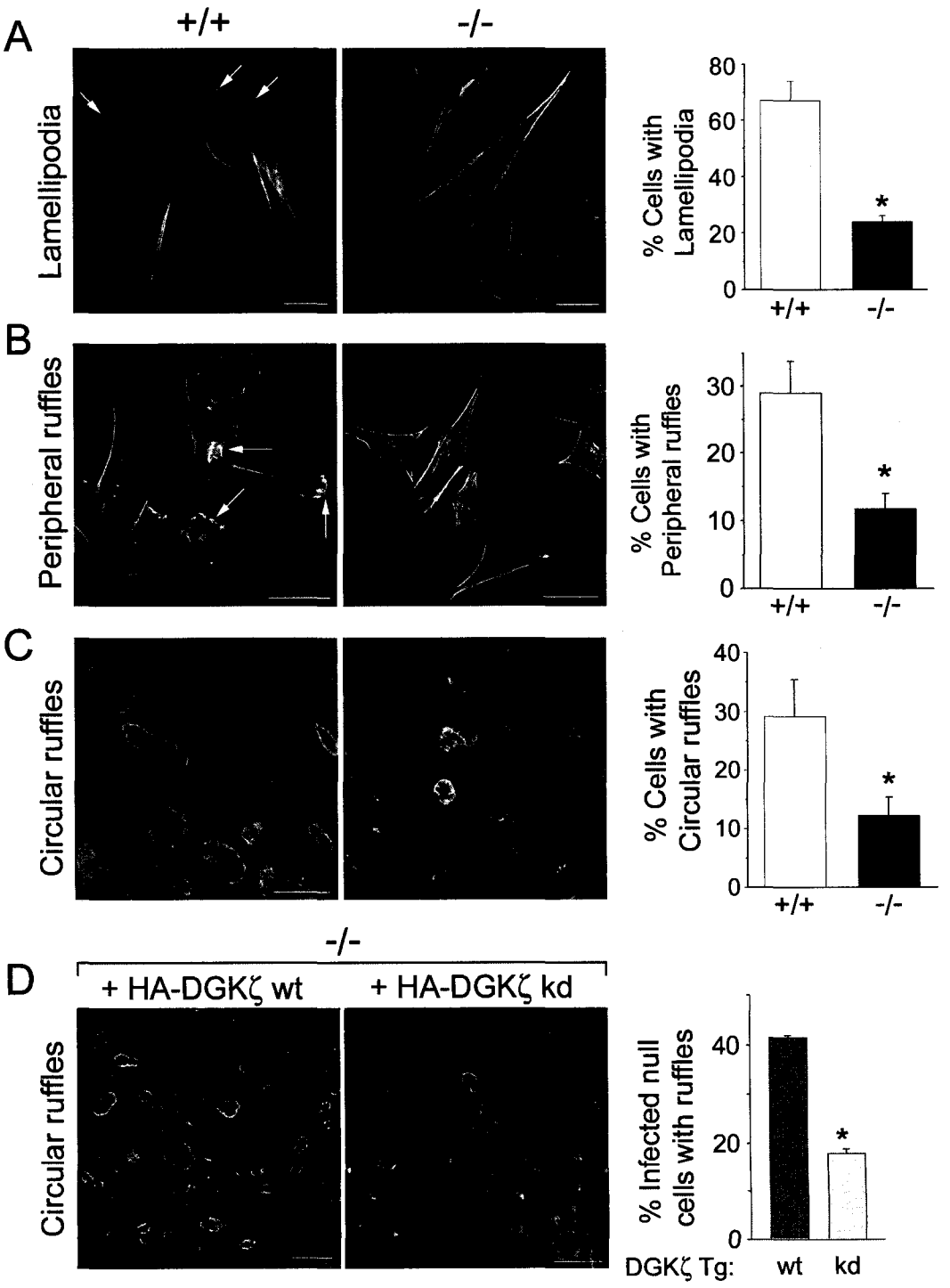

DGKζ
Rac1
Syn
Actin

Figure 5.4. Impaired ruffling and lamellipodia formation in DGKζ-null fibroblasts.

Representative images of wt (+/+) and DGKζ-null (-/-) fibroblasts stained with AlexaFluor 488-conjugated phalloidin showing (A) lamellipodia (*arrows*), (B) peripheral membrane ruffles (*arrows*) and (C) circular ruffles. The cells shown in (A) were grown in media containing 10% serum, while those in (B) and (C) were serum-starved overnight and then stimulated with 10 ng/ml PDGF for 5 min. Scale bars: 20 μm. The graph to the right of each pair of images shows the quantification of each structure in wt and null cells. In each case, values are the mean ± s.e.m. from at least three independent experiments. The asterisks denote a significant difference between wt and null cells ($P < 0.05$) by Student's *t*-test. (D) Reintroduction of wt DGKζ rescues circular ruffling. Representative images of DGKζ-null cells infected with adenoviruses bearing HA-tagged versions of wt DGKζ (HA-DGKζ wt) or a kinase-dead mutant (HA-DGKζ kd). Scale bars: 50 μm. The graph shows the quantification of circular ruffling in infected null cells fixed and stained to reveal actin. Values are the mean ± s.e.m. from two independent experiments. The asterisk denotes a significant difference ($P < 0.005$) by Student's *t*-test.


A
+/+
-/-
Lamellipodia
% Cells with Lamellipodia
80
60
40
20
0
*
+/+
-/-
B
Peripheral ruffles
% Cells with Peripheral ruffles
30
20
10
0
*
+/+
-/-
C
Circular ruffles
% Cells with Circular ruffles
40
30
20
10
0
*
+/+
-/-
D
-/-
+ HA-DGKζ wt
+ HA-DGKζ kd
Circular ruffles
% Infected null cells with ruffles
40
20
0
*
DGKζ Tg:
wt
kd

A similar deficiency in lamellipodia formation was noted following PDGF stimulation of serum-starved cells (data not shown). Similarly, PDGF-induced peripheral ruffling was significantly reduced in DGKζ-null cells (Figure 5.4B). Finally, there was a ~3–fold decrease in the frequency of PDGF-induced circular ruffles in null cells (Figure 5.4C). Circular ruffling in DGKζ-null cells was rescued by HA-tagged wt DGKζ but not by a kinase-dead mutant (Figure 5.4D), demonstrating that enzymatic activity is required. Taken together, these data indicate the formation of lamellipodia and ruffles is impaired by the loss of DGKζ.

To examine the possibility that ruffle formation is delayed rather than deficient, we compared living wt and null cells using time-lapse digital microscopy. We limited our study to circular ruffles since they are readily imaged using differential interference microscopy and follow a stereotypical time course (Buccione *et al.*, 2004). After ~2 min PDGF stimulation, virtually every wt cell initiated a transient wave of circular ruffling that began at the cell periphery and progressed inwards (Figure 5.5). The vast majority of wt cells had completed ruffling 10 min after the onset of stimulation. In contrast, circular ruffling was severely impaired in DGKζ-null cells (Figure 5.6). Only a few null cells formed ruffles and in those that did, the ruffles were delayed by 1-2 min and were usually smaller and much less conspicuous. No additional circular ruffling was initiated for the duration of the recording (15 min). Thus, video imaging reveals a profound defect in circular ruffling in DGKζ-null cells.

Figure 5.5. Time-lapse observation of wild type MEFs immediately following stimulation with 50 ng/ml PDGF. The cells were viewed using differential interference microscopy. Circular ruffles appear as dark rings that begin at the cell edge and progress inwards (*arrows*). Scale bar: 50 μm

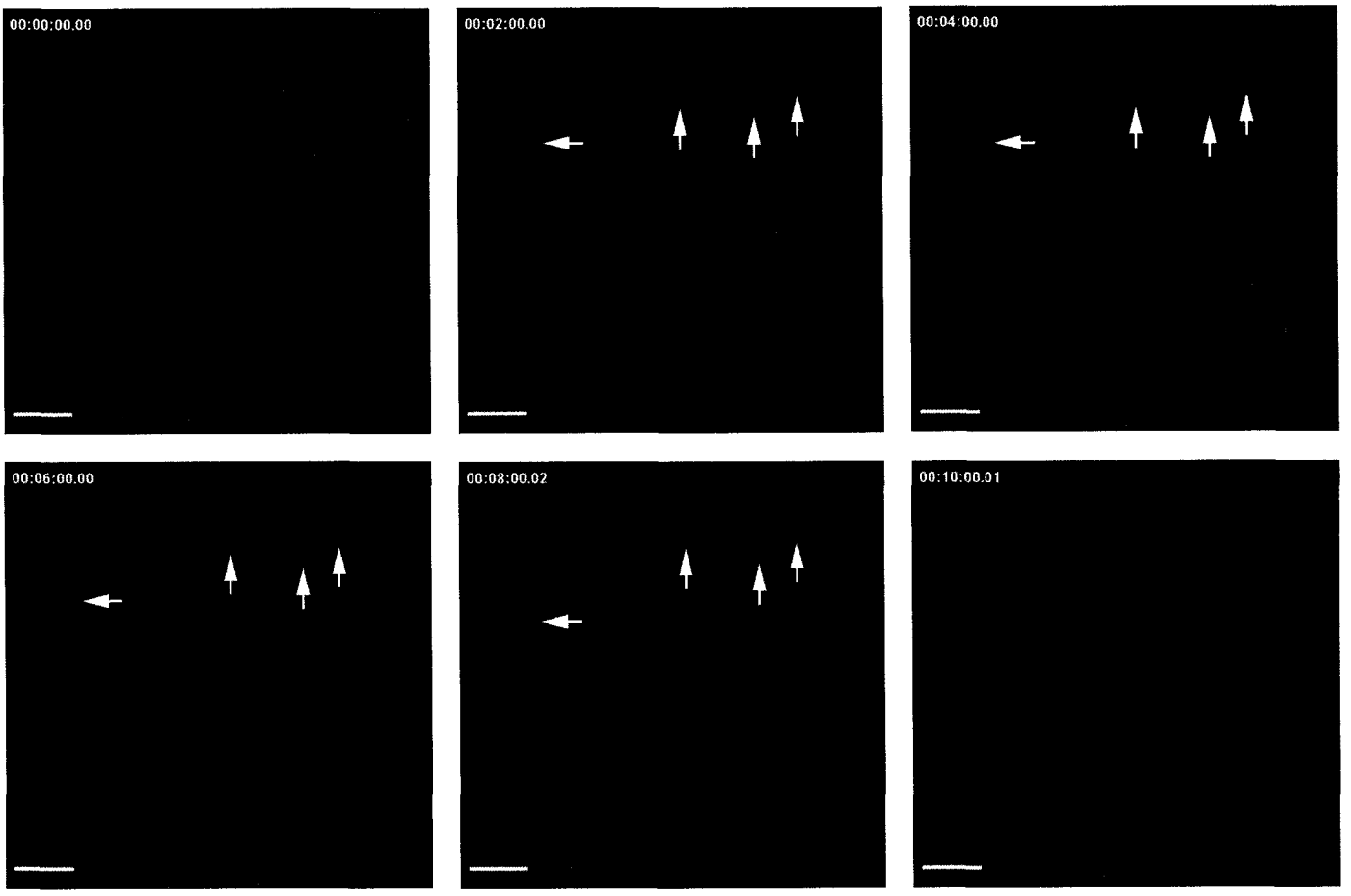
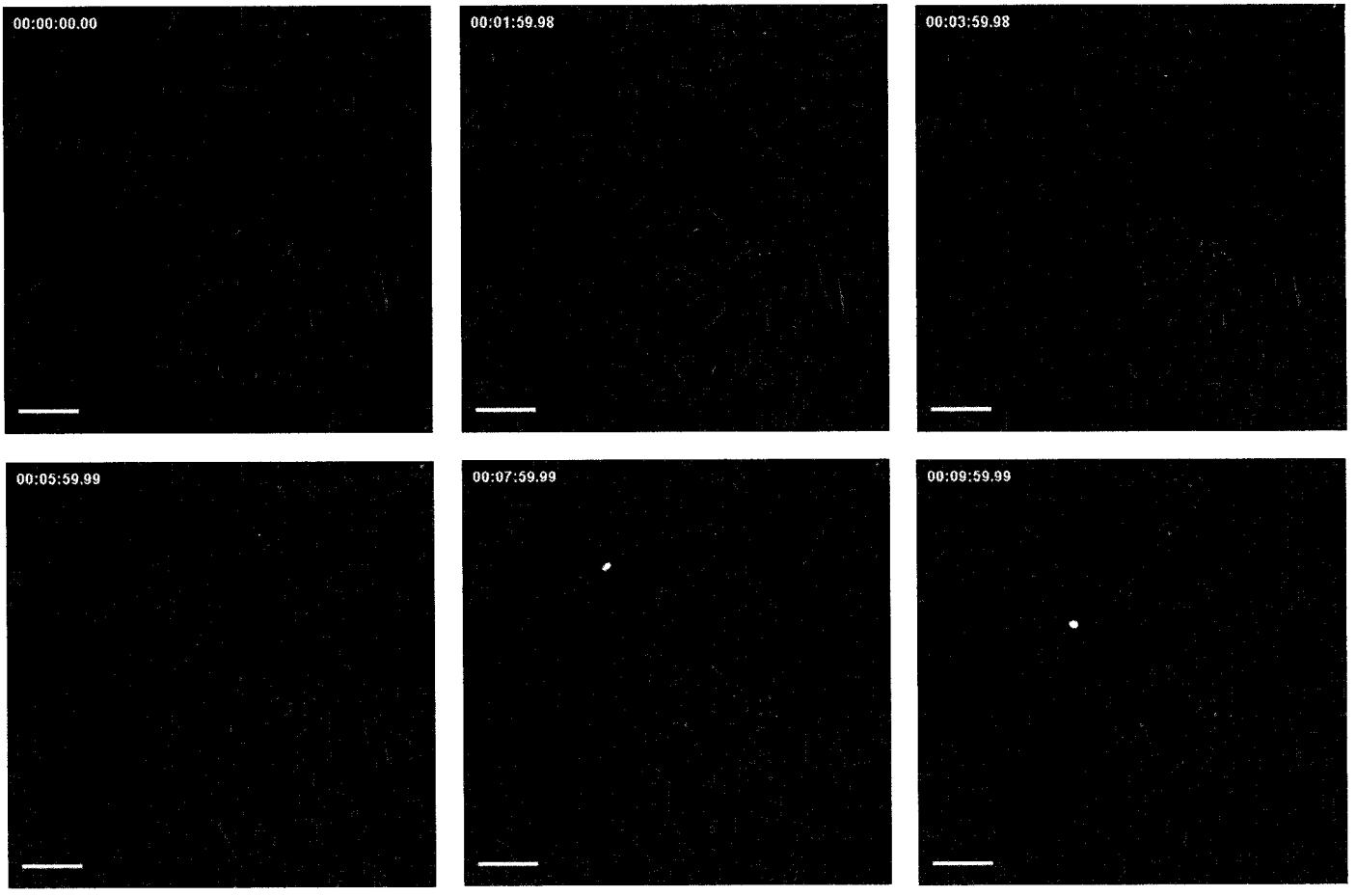

00:00:00.00
00:02:00.00
00:04:00.00
00:06:00.00
00:08:00.02
00:10:00.01

Figure 5.6. Time-lapse observation of DGKζ-null MEFs following stimulation with 50 ng/ml PDGF. The cells were viewed using differential interference microscopy. Scale bar: 50 μm.


00:00:00.00
00:01:59.98
00:03:59.98
00:05:59.99
00:07:59.99
00:09:59.99

Impaired lamellipodia formation in DGKζ-null cells

Given the failure of DGKζ-null cells to assemble PAK1 at focal adhesions, we evaluated the extent of integrin-mediated cell spreading and the morphology of the spreading cells by immunofluorescence microscopy at various times after replating on fibronectin.Wild-type cells stained for actin were generally cuboid and had numerous membrane ruffles (Figure 5.7A). After 60 min they had fewer ruffles and were generally well spread. In contrast, null cells were stellate, had numerous large filopodia and substantially fewer membrane ruffles. However, the extent of spreading at 60 min was not significantly different. Quantitative analysis revealed a substantial reduction in membrane ruffling in the null cells 15 and 30 min after replating and a proportional increase in filopodia. Thus, DGKζ-null cells seem to have a defect in lamellipodial formation but normal filopodial extension.

Migration is impaired in DGKζ-deficient fibroblasts

Wounding of a confluent cell monolayer induces lamellipodia formation and membrane ruffling at the leading edge; this requires Rac1 and Cdc42 activity(Nobes and Hall, 1999). We therefore assessed the ability of wt and DGKζ-null MEFs to undergo coordinated cell migration in a wound closure assay (Nobes and Hall, 1999; Fenteany *et al.*, 2000). DGKζ-null cells exhibited a marked reduction in wound closure compared with wt cells when assayed 2 h following wounding (Figure 5.8A and B). By 4 h post-wounding however, both wt and null MEFs had closed the wound, indicating that migration is not completely blocked in the null cells (not shown). These data suggest DGKζ-null MEFs migrate less than their wt counterparts.

Figure 5.7. The absence of DGKζ affects cell spreading on fibronectin.

Wt and DGKζ-null cells were plated on fibronectin-coated coverslips, incubated at 37°C for the indicated times, then fixed and stained with AlexaFluor 594-conjugated phalloidin. (A) Typical morphology of wt (+/+) and null (-/-) cells after replating. *Arrows* indicate membrane ruffles and *arrowheads* filopodia. Scale bars: 20 μm. (B) Random fields (n=10) of wt and null cells were scored for the presence of ruffles and filopodia. Data are displayed as the percentage (mean ± s.e.m.) of total cells per field. In each case, wt is significantly different from null ($P < 0.05$) by Student's *t*-test.

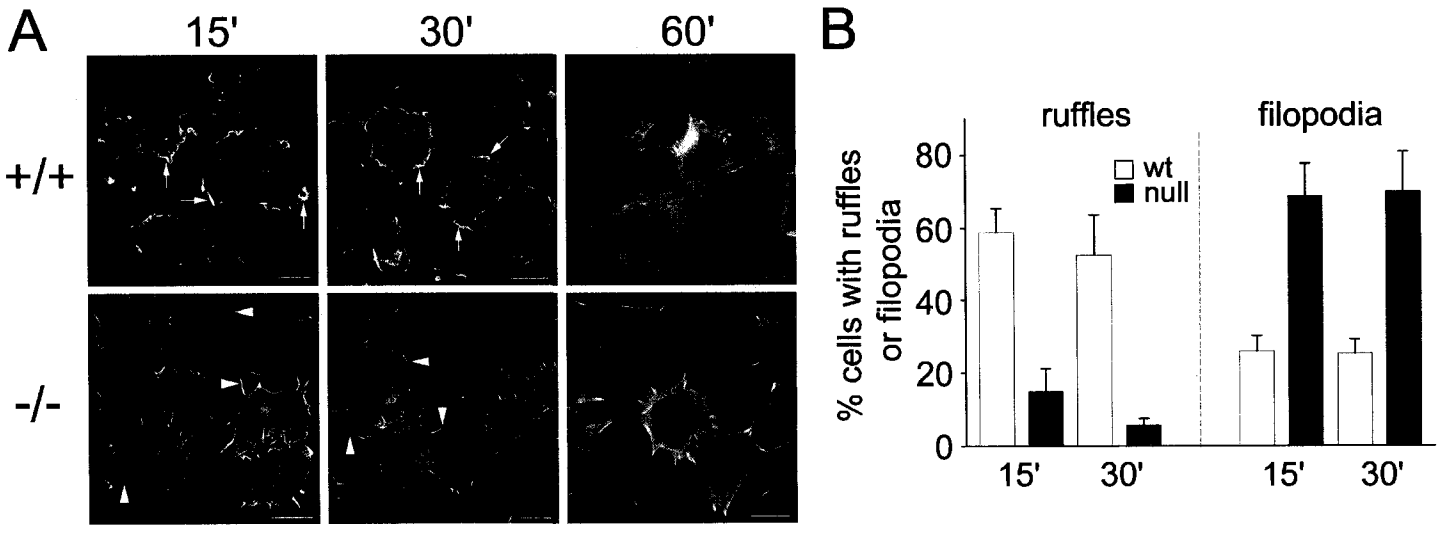
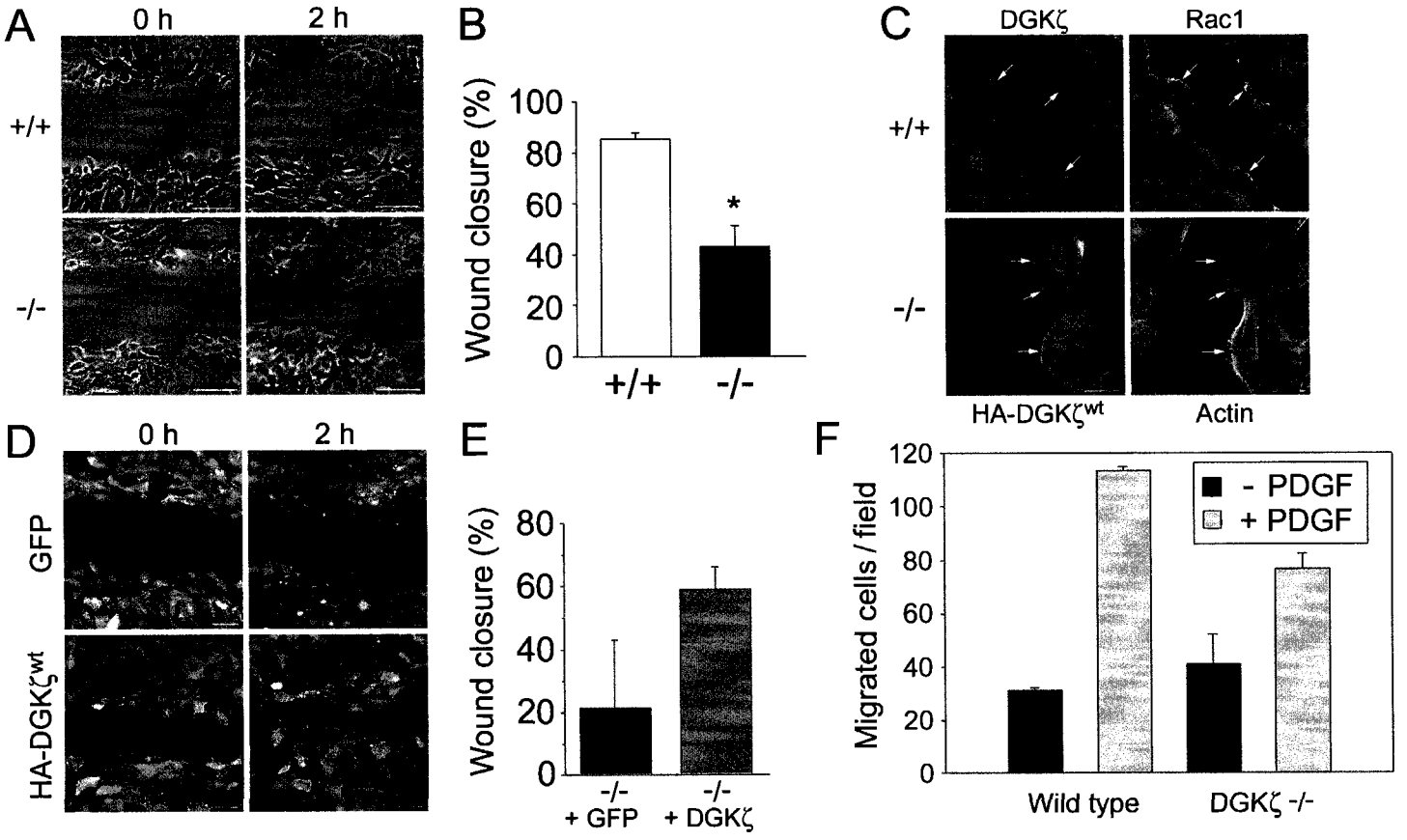

A
15'
30'
60'
+/+
-/-
B
ruffles
filopodia
wt
null
% cells with ruffles or filopodia
80
60
40
20
0
15'
30'
15'
30'

Figure 5.8. Reduced migration of DGKζ-null fibroblasts.

(A) Phase-contrast images of wounded wt (+/+) and DGKζ-null (-/-) fibroblast monolayers taken immediately after wounding (0 h) or 2 h later. Scale bars: 50 μm. (B) Quantification of the wounding results in (A). The graph shows the percent wound closure 2 h post-wounding. Values are the mean ± s.e.m. of four independent experiments. The asterisk denotes a significant difference from wt cells ($P < 0.005$) by Student's *t*-test. (C) DGKζ is concentrated at the border of cells facing the wound. WT fibroblasts were fixed and immunolabeled for endogenous DGKζ and Rac1 2 h post-wounding. Null fibroblasts infected with adenovirus bearing HA-tagged DGKζ were fixed 2 h after wounding and stained with an anti-HA antibody and AlexaFluor 488-conjugated phalloidin to visualize actin. *Arrows* indicate protein concentrated at the leading edge of cells migrating into the wound. Scale bars: 20 μm. (D) Rescue of the migration defect by DGKζ overexpression. DGKζ-null cells were infected with adenovirus bearing either GFP or HA-tagged DGKζ and fixed at the indicated times after wounding. Cells were fixed and stained with anti-GFP (*top*) or anti-HA (*bottom*). Scale bar: 50 μm. (E) Quantification of percent wound closure at 2 h. Values are the mean ± s.d. of three independent experiments. (F) Reduced three-dimensional migration of null fibroblasts. Wt and null fibroblasts were placed in the upper chamber of a Transwell migration plate and allowed to migrate across a porous membrane for 4 h in the absence (*black bars*) or presence (*grey bars*) of 10 ng/ml PDGF. The graph shows the number of migrated cells per field. The data are the mean ± s.e.m. of two independent experiments with 5-10 microscope fields scored per condition.


A
0 h
2 h
+/+
-/-
B
Wound closure (%)
100
80
60
40
20
0
*
+/+
-/-
C
DGKζ
Rac1
+/+
-/-
HA-DGKζwt
Actin
D
0 h
2 h
GFP
HA-DGKζwt
E
Wound closure (%)
80
60
40
20
0
-/-
+ GFP
-/-
+ DGKζ
F
Migrated cells / field
120
100
80
60
40
20
0
- PDGF
+ PDGF
Wild type
DGKζ -/-

To determine if the loss of DGKζ was the primary cause of the reduced migration, the wt protein was reintroduced into null MEFs. We verified that exogenously expressed DGKζ was properly localized in the null fibroblasts. In wt fibroblasts, endogenous DGKζ colocalized with Rac1 in lamellipodia along the edge of cells facing the wound (Figure 5.8C), consistent with previous reports (Abramovici *et al.*, 2003; Luo *et al.*, 2004a). Similarly, HA-DGKζ was concentrated at the leading edge of null MEFs migrating into the wound, where it colocalized with actin (Figure 5.8C). These data suggest exogenous DGKζ is correctly targeted to the front of migrating cells. Consistent with this finding, HA-DGKζ restored the migration of null MEFs to near wt levels, whereas expression of GFP in the null MEFs did not significantly increase wound closure (Figure 5.8D and E). Collectively, these data suggest the migration defect of the null MEFs is directly attributable to the loss of DGKζ and that proteins required for normal DGKζ localization are largely unaltered.

Three-dimensional migration was assessed using modified Boyden chamber assays. The number of DGKζ-null MEFs that randomly migrated through a porous membrane (haptotaxis) was not significantly different from wt MEFs after 4 h (Figure 5.8F). However, in response to a PDGF gradient, ~1.5-fold fewer null cells migrated across the membrane (chemotaxis), suggesting directional migration is impaired.

Discussion

The interaction of Rho family proteins with RhoGDI is a major point of regulation of GTPase activity. Because they share a common inhibitor, different mechanisms should exist to specifically dissociate Rho GTPases from RhoGDI. However, the signaling mechanisms responsible are poorly understood (Dermardirossian *et al.*, 2004; Dovas and Couchman, 2005). Our results suggest DGKζ is part of a signaling complex that functions as a Rac1-selective RhoGDI dissociation factor.

The finding that the basal Rac1-GTP level was unaffected in DGKζ-null cells, but that the PDGF-stimulated level was substantially decreased compared to wt cells, indicates a defect in Rac1 activation. Consistent with such a defect, the phenotype of the cells is remarkably similar to that of Rac1-null MEFs including; altered morphology, defects in lamellipodia formation and membrane ruffling, and a marked reduction in PAK phosphorylation (Nobes and Hall, 1999; Guo *et al.*, 2006; Vidali *et al.*, 2006). In contrast, Cdc42 activity did not appear to be impaired in the absence of DGKζ. Supporting this conclusion, DGKζ-null cells replated on FN contained numerous filopodia, which are regulated by integrin-induced activation of Cdc42 (Price *et al.*, 1998).

The finding that Rac and RhoGDI associate with enzymes regulating phosphoinositide metabolism, including DGK and PI5K, lead to the suggestion of a lipid kinase signaling complex that regulates Rac activation (Tolias *et al.*, 1998; Tolias and Carpenter, 2000). Our data showing DGKζ binds directly to Rac1 (Yakubchyk *et al.*, 2005) and associates in a complex with RhoGDI and PAK1 supports the idea that Rac1 resides in a regulated signaling complex whose function is to control the selective activation of Rac1 and the regulation of actin cytoskeletal dynamics. Moreover, because DGKζ also associates with

PLCγ1 (Luo *et al.*, 2004b), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to yield DAG and inositol triphosphate, it appears to occupy a central position in an inositol lipid signaling pathway leading to Rac activation.

Phosphatidic acid and Rac activity

The role phosphoinositides play in Rac activity is complex; they act both upstream and downstream of Rac to drive actin reorganization in response to extracellular signals (Burridge and Wennerberg, 2004). The available evidence suggests PA and other anionic phospholipids lie upstream of Rac activation. PA dissociates Rac1-RhoGDI complexes (Bourmeyster *et al.*, 1992; Chuang *et al.*, 1993a; Tolias *et al.*, 1998; Mesmin *et al.*, 2004; Ugolev *et al.*, 2006) and activates PAK1 (Bokoch *et al.*, 1998), which has been shown to phosphorylate RhoGDI and initiate Rac release (Dermardirossian *et al.*, 2004). Our results provide evidence that DGKζ-derived PA activates PAK1, initiating the release of Rac1 from RhoGDI. This conclusion is supported by the finding that in DGKζ-null cells both active Rac1 and pPAK1 were substantially reduced and Rac release from RhoGDI was impaired. That Rac activation in the null cells was rescued by wt DGKζ, but not by a kinase inactive mutant, suggests enzymatic conversion of DAG to PA is required. Finally, addition of exogenous PA, but not DAG or LPA, increased Rac1 activity in the DGKζ-null MEFs.

PA may play multiple roles in Rac activation. In addition to stimulating PAK activity, negatively charged PA may function as a lipid anchor by binding positively charged residues at the C-terminus of Rac1. Indeed, the polybasic region is required in addition to the C-terminal isoprenyl group of Rac1 for membrane targeting (Joseph *et al.*, 1994; Kreck *et al.*, 1996; Ueyama *et al.*, 2005). The interaction of the polybasic region with DGKζ-derived PA

could facilitate shuttling of the C-terminal isoprenyl group from the hydrophobic pocket in RhoGDI to the inner leaflet of the plasma membrane.

Regulation of PAK activity and localization

The absence of DGKζ affected both the level and targeting of pPAK, without affecting total PAK1. However, pPAK levels were not reduced to zero, suggesting other phosphorylation mechanisms remain active in DGKζ-null cells. Both PDK1 and Akt phosphorylate PAK1 in response to PDGF stimulation (Franke *et al.*, 1995; King *et al.*, 2000; Tang *et al.*, 2000) and Cdc42 can activate PAK upon adhesion to FN (Price *et al.*, 1998). Since Akt and Cdc42 activities were largely unaffected in the DGKζ-null MEFs, they could account for the residual PAK phosphorylation.

In addition to phosphorylation, subcellular localization likely plays a key role in the regulation of PAK1 activity. In resting cells PAK1 localizes to cytosolic vesicles, but in stimulated cells, the active form accumulates in membrane ruffles and focal adhesions (Dharmawardhane *et al.*, 1997; Manser *et al.*, 1997; Sells *et al.*, 2000). Targeting of active PAK to these structures influences cytoskeletal dynamics through phosphorylation of a variety of downstream effectors (Edwards *et al.*, 1999; Sanders *et al.*, 1999; Slack-Davis *et al.*, 2003). The spatial regulation of PAK1 involves a GIT1-PIX-paxillin complex that promotes autophosphorylation and recruits the kinase from the cytosol to focal adhesions and the leading edge (Zhao *et al.*, 2000; Manabe *et al.*, 2002; Brown *et al.*, 2002; Loo *et al.*, 2004). Our data suggest DGKζ is also necessary for the translocation of pPAK1 from the cytosol to focal adhesions. In contrast, targeting of pPAK1 to membrane ruffles at the

leading edge of the null cells was seemingly unaffected, so DGKζ does not appear to be required for this translocation event.

Integrin-mediated cell spreading

Integrins transduce signals from the extracellular matrix (ECM) to the actin cytoskeleton through pathways regulated by Rho GTPases (Clark *et al.*, 1998). Focal adhesions serve as convergence points for rapid integration of growth factor- and integrin-mediated signaling responses (Plopper *et al.*, 1995). Consistent with this idea, integrin engagement stimulates rapid assembly of structural and signaling components into focal adhesions, including both synthetic and degradative enzymes of the inositol lipid signaling cascade. Moreover, adhesion of fibroblasts to the ECM is required for PDGF-dependent hydrolysis of $PI(4,5)P_2$ into DAG and IP_3 (McNamee *et al.*, 1993). Consistent with a defect in integrin-mediated signaling events, DGKζ-null MEFs exhibited reduced spreading after plating on FN. However, after longer periods, the surface area of DGKζ-null cells was not significantly different from wt cells. Since both lamellipodia and membrane ruffling were substantially decreased in the null cells, the residual spreading may be mediated by blunt filopodia and/or pseudopodia, which have been suggested to mediate spreading of Rac1-null cells (Vidali *et al.*, 2006).

Integrins contribute to spatial regulation of Rac function by dissociating it from RhoGDI and enhancing its interaction with effectors at cell edges (del Pozo *et al.*, 2002). They also stimulate Rac membrane translocation by modulating the local lipid environment and preventing internalization of cholesterol-rich membrane domains (del Pozo *et al.*, 2004). Integrin activation stimulates $PI(4,5)P_2$ hydrolysis to DAG by activating PLCγ (Auer and

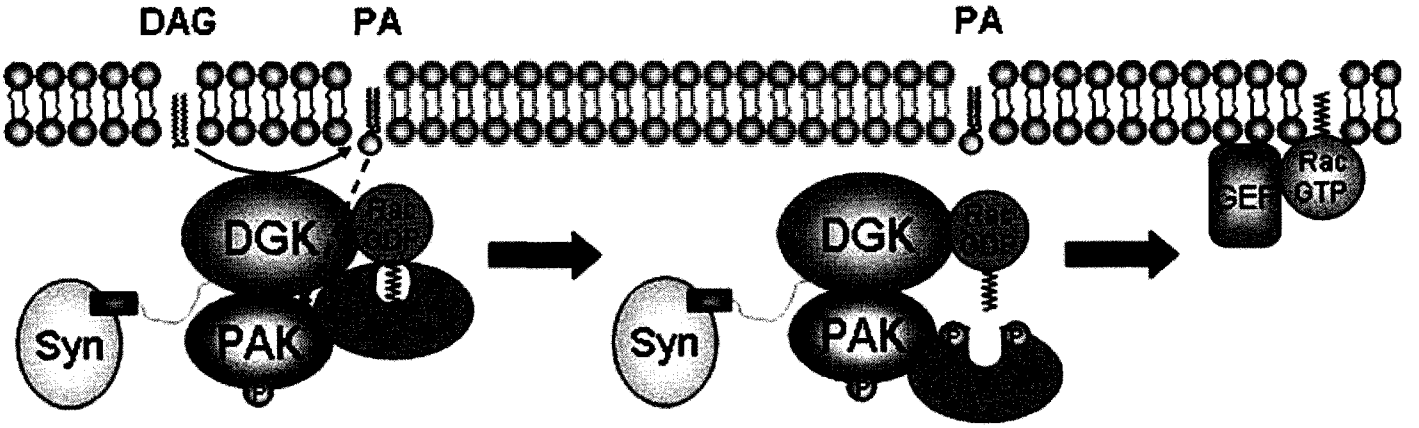
Jacobson, 1995); the latter reaction is enhanced by PDGF stimulation(McNamee *et al.*, 1993). In this regard, DGKζ is positioned to integrate signals from the two inputs and to generate an output (PA) that activates PAK1 and dissociates Rac1 from RhoGDI.

Proposed mechanism of Rac release from RhoGDI

On the basis of these and previous findings (Tolias *et al.*, 1998; Dermardirossian *et al.*, 2004) we propose DGKζ exists in a multi-protein signaling complex with syntrophin, Rac1, PAK1 and RhoGDI and that DGKζ-derived PA activates PAK1, which subsequently phosphorylates RhoGDI to trigger Rac1 release (Figure 5.9). Thus, DGKζ occupies a key position in the signaling pathway from $PI(4,5)P_2$ breakdown to Rac activation. Together, these results establish a mechanism whereby changes in lipid second messengers modulate the amount of active Rac and thus contribute to the regulation of the actin cytoskeleton.

Figure 5.9. Model for the release of Rac1 from RhoGDI.

(*Left*) DGKζ-catalyzed conversion of DAG to PA (*curved arrow*) activates PAK1 (*dashed arrow*) which undergoes autophosphorylation (P). The C-terminal lipid moiety of Rac1-GDP is sequestered in a hydrophobic pocket of RhoGDI. (*Center*) PAK1-mediated phosphorylation of RhoGDI at two sites releases the lipid group. DGKζ binding to Rac1 may facilitate insertion of the lipid group into the plasma membrane. (*Right*) Membrane translocation allows Rac to interact with a GEF and to become activated.


DAG
PA
PA
DGK
PAK
Syn
DGK
PAK
Syn
Rac
GTP

Chapter 6

General Discussion

Eukaryotic cells possess a remarkable ability to alter their shape in response to different extracellular stimuli. It is now well established that these stimuli promote the breakdown and synthesis of lipids in the plasma membrane as well as the recruitment and activation of actin-associated proteins, which together drive the remodeling of the actin cytoskeleton (Janmey, 1995). It is also becoming clear that restriction of these signaling processes to small subregions of the cell is necessary to effect localized changes in cell morphology required during events such as myoblast fusion, neurite outgrowth and cell migration (Teruel and Meyer, 2000; Massarwa *et al.*, 2007; Kim *et al.*, 2007).

$PI(4,5)P_2$ is a major lipid regulator of actin remodeling, but its breakdown product DAG is also important because it binds and activates PKCs, kinases with important roles in actin dynamics. Furthermore, PA, the product of DAG phosphorylation positively regulates other actin restructuring proteins such as PIP5K and PAK as well as promoting the dissociation of Rac1 from its inhibitor RhoGDI thereby contributing to Rac1 activation (Chuang *et al.*, 1993a). Thus, cells must be able to regulate the accumulation of these lipids to ensure that actin is reorganized appropriately in response to extracellular stimuli.

By phosphorylating DAG to yield PA, DGKs attenuate DAG-activated signaling and stimulate PA-mediated signaling. Studies by our collaborators showed that DGKζ forms biochemical complexes with PKCα and PIP5KIα (Luo *et al.*, 2003a; Luo *et al.*, 2004a). Another study detected DGK and PIP5K activity in Rac1 immunoprecipitates (Tolias *et al.*, 1998). Together, these findings raised the possibility that DGKζ plays a role in actin reorganization by controlling the activity of the DAG and PA-responsive proteins mentioned above.

Membrane Localization of DGKζ

Clustering of lipids and proteins into specific signaling complexes at the membrane in response to receptor activation is thought to be an important signal transduction mechanism for regulating actin remodeling and changes in cell shape (Janmey, 1995). However, many proteins reside in the cytoplasm or other compartments and must translocate to the membrane where they can bind to their regulators or effectors. DGKs are a good example because both the substrate and the product of the DGK reaction reside in the membrane, while the enzyme itself lies in the cytoplasm. Therefore to carry out their function, DGKs must move from the cytoplasm to the membrane and specific mechanisms must exist to regulate this movement.

DGKζ has a motif similar to the basic effector domain (BED) in the MARCKS protein, a major PKC target (Topham *et al.*, 1998). PKC-mediated phosphorylation of the MARCKS BED regulates the membrane localization of the MARCKS protein (McLaughlin and Aderem, 1995; Allen and Aderem, 1995; Seykora *et al.*, 1996). My studies show that a DGKζ mutant ($DGK\zeta^{M1}$) that mimics PKC-mediated phosphorylation of the DGKζ MARCKS domain was localized more often at the membrane in muscle cells (Chapter 2). Furthermore, this mutant greatly enhanced neurite outgrowth (Chapter 3), a process that requires the recruitment of proteins to the membrane of extending neurites (Futerman and Banker, 1996; Luo, 2002). Together, these findings suggest PKC-dependent phosphorylation of the DGKζ MARCKS motif drives the movement of DGKζ to the membrane.

Several possibilities exist to explain how phosphorylation of the DGKζ MARCKS motif promotes its membrane localization. It is known for example that PKC-mediated phosphorylation of the MARCKS BED in the MARCKS protein induces a conformational

change that blocks one of two actin-binding sites and diminishes the binding of MARCKS to actin (Hartwig *et al.*, 1992; Bubb *et al.*, 1999). Similarly, phosphorylation of the DGKζ MARCKS motif might reduce DGKζ binding to a cytoplasmic or cytoskeletal anchor (such as actin), thereby driving its movement to the membrane. It is also possible that phosphorylation of the DGKζ MARCKS domain increases the affinity of the enzyme for membrane-associated proteins, thereby promoting its localization at the membrane.

Yet another possibility involves the binding of DGKζ to syntrophins, membrane-associated PDZ scaffolding proteins. In Chapter 2, I showed that co-expression of DGKζ^{M1} and α-syntrophin increased the membrane localization of both proteins and our laboratory has preliminary unpublished evidence suggesting phosphorylation of the DGKζ MARCKS motif increases the binding of DGKζ to syntrophins. Phosphorylation of DGKζ may also induce a conformational change in syntrophin structure resulting in an increased affinity of syntrophin for dystrophin, other membrane-associated proteins, or even sub-membranous F-actin (Iwata *et al.*, 2004).

Regardless of the precise mechanism, my studies suggest PKC-mediated phosphorylation of the MARCKS domain of DGKζ, as well as binding of DGKζ to syntrophins, promote the translocation of DGKζ from the cytoplasm to the membrane. Many extracellular stimuli trigger the accumulation of DAG at the membrane thereby activating PKC. The phosphorylation of DGKζ by activated PKC could help explain how DAG recruits DGKζ to the membrane. In addition, the binding of DGKζ to syntrophins could anchor DGKζ at the membrane or restrict its localization at the membrane to areas where the DAPC is localized.

Subcellular fractionation experiments presented in Chapter 2 suggested the presence of DGKζ in the cytosolic as well as in the detergent-insoluble cytoskeletal fraction. Additional experiments showed that a proline-rich sequence near the C-terminus of DGKζ could be phosphorylated by ERK and raised the possibility that ERK-mediated phosphorylation of this region negatively regulated the association of DGKζ with the cytoskeleton. These conclusions were based in part on data obtained from immunoblots probed with an anti-DGKζ antibody which revealed the presence of an "upper" immunoreactive band in the cytosolic fraction and a "lower" immunoreactive band in the cytoskeletal fraction. Immunoblots of lysates from DGKζ knockout fibroblasts probed with this same antibody lacked the "upper" band but still showed a "lower" band (Chapter 5) suggesting that this faster migrating species was not likely DGKζ but rather a non-specific immunoreactive band. While these results do not directly challenge the notion that DGKζ can be phosphorylated by ERK, they do suggest a reevaluation of the idea that ERK-mediated phosphorylation of DGKζ regulates the association of the enzyme with the cytoskeleton.

Spatial Control of Lipid Signaling

Lipid-mediated signaling necessitates spatial compartmentalization of signaling assemblies because many lipids, their modifying enzymes, and downstream targets are shared by multiple lipid signaling pathways (Wymann and Schneiter, 2008). It is well established that PDZ proteins, such as syntrophins, direct the assembly of specific protein signaling complexes and regulate their spatial distribution, thereby playing an essential role in the spatial control of cell signaling (Zimmermann, 2006). Emerging evidence also

suggests PDZ proteins are involved in lipid signaling because PDZ domains bind phosphoinositides (Yan *et al.*, 2005; Zimmermann, 2006). Stimulation of cell surface receptors triggers the local synthesis and breakdown of phospholipids, which recruit and activate actin remodeling proteins. Therefore, PDZ proteins may also have a hand in regulating lipid signaling and actin dynamics by targeting actin remodeling proteins to membrane domains enriched in certain types of phospholipids.

DGK activity is topologically restricted and tightly associated with receptor stimulation, suggesting that DAG and PA signaling are spatially regulated (van der Bend *et al.*, 1994; Topham and Prescott, 1999; Topham and Prescott, 2001; Luo *et al.*, 2003a; Luo *et al.*, 2004a; Luo *et al.*, 2004b). Rather than entering a non-specific pool, DAG and its metabolite PA act locally to regulate target proteins complexed with DGKζ. This allows the precise regulation of intended DAG or PA targets without simultaneous activation of unintended targets, an important consideration in biological processes requiring spatially restricted remodeling of the actin cytoskeleton.

In fusing myoblasts, DGKζ and syntrophins accumulate at sites of cell-cell contact and colocalize with F-actin (Chapter 4). Preventing the binding of DGKζ to syntrophins decreases the amount of DGKζ at the membrane and results in dramatic changes in muscle cell shape (Chapter 2). In neuronal cells, these two proteins normally accumulate in growth cones and blocking DGKζ-syntrophin association prevents neurite outgrowth (Chapter 3). Blocking DGKζ-syntrophin binding also promotes the shuttling of DGKζ to the nucleus (Chapters 2 and 3). Therefore, my studies strongly suggest syntrophins are required for the proper spatial localization of DGKζ and that binding of syntrophins to DGKζ plays an important role in regulating cell shape and remodeling of the actin cytoskeleton.

DGKζ Regulates Actin Reorganization

A number of studies suggest DGKs contribute to the regulation of actin dynamics by controlling the activity of Rho GTPases (Tolias *et al.*, 1998; Houssa *et al.*, 1999; Tsushima *et al.*, 2004; Yasuda *et al.*, 2007). My studies are the first to show that DGKζ positively regulates the activity of the GTPase Rac1. The following points support this conclusion: First, DGKζ colocalizes with Rac1 in structures undergoing actin reorganization such as the distal tips of myoblast pseudopodia and sites of myoblast-myoblast contact, as well as the growth cones of neurites. Second, DGKζ associates directly with Rac1 and forms a biochemically stable complex. Third, DGKζ knockout fibroblasts have decreased Rac1 activity and correspondingly fewer Rac1-regulated cell specializations such as lamellipodia and membrane ruffles. They also display greatly decreased spreading and migration, cell behaviors controlled to a large extent by Rac1.

In Chapter 5, I explored the mechanism by which DGKζ regulates Rac1 activity. I showed that the decreased Rac1 activity observed in DGKζ knockout fibroblasts results from a defect in the dissociation of Rac1 from its inhibitor RhoGDI. This dissociation depended on the ability of DGKζ to produce PA, since a catalytically inactive DGKζ mutant did not rescue Rac1 activity while exogenous addition of PA increased it. These findings are consistent with studies showing that PA promotes the dissociation of Rac1 from RhoGDI (Chuang *et al.*, 1993a).

I also demonstrated that DGKζ forms an immunoprecipitable complex with PAK1, a PA-activated serine/threonine kinase that phosphorylates RhoGDI and drives its dissociation from Rac1 (Bokoch *et al.*, 1998; Dermardirossian *et al.*, 2004). In DGKζ knockout

fibroblasts, the localization of PAK1 was altered and its phosphorylation, and hence its activity, was also decreased. These findings raise the possibility that DGKζ regulates the dissociation of Rac1 from RhoGDI by affecting the localization and activity of PAK1.

A large body of evidence suggests a role for phosphoinositides, DAG and PA in the reorganization of the actin filament network, but concrete mechanisms to explain how these lipids do so remain relatively obscure. My studies have filled this gap in knowledge by providing strong evidence that suggests DGKζ functions as a RhoGDI displacement factor for Rac1. Moreover, the idea that DGKζ regulates PAK1 activity and localization provides an elegant and simple way to explain how PA, which activates PAK and is generated in response to growth factor stimulation, promotes the dissociation of Rac1 from RhoGDI and triggers Rac1-mediated remodeling of the actin cytoskeleton.

DGKζ and Other PDZ Proteins

My findings, along with those of Hogan et al. (2001) strongly suggest syntrophins, and not other closely related PDZ proteins, associate with and regulate the intracellular localization of DGKζ. Nevertheless, because of the sometimes promiscuous nature of PDZ-binding interactions (Walma *et al.*, 2004; Cui *et al.*, 2007), it is difficult to completely rule out a similar role for other PDZ proteins. For example, Fabre et al. (2000) detected an interaction between DGKζ and the PDZ protein TIP-15 in a yeast-two hybrid screen, although to date no physiological role has been ascribed to this association. It has however been suggested that the TIP-1 isoform acts as a dominant negative by competing with other PDZ proteins for ligand binding (Alewine *et al.*, 2006). TIP-15 may function in an analogous

manner by competing with syntrophins for binding to DGKζ, thereby modulating DGKζ localization and activity.

DGKζ also interacts with sorting nexin 27 (SNX27), a PDZ protein involved in membrane traffic and protein sorting (Rincon *et al.*, 2007). SNX27 may target DGKζ to endocytic recycling compartments (Rincon *et al.*, 2007) where DGKζ could regulate the levels of DAG and PA. These lipids recruit proteins to endosomes, affect membrane curvature and promote membrane fusion or fission (Burger, 2000; Andresen *et al.*, 2002; Kooijman *et al.*, 2003; Kooijman *et al.*, 2005; Barona *et al.*, 2005; Rincon *et al.*, 2007; Churchward *et al.*, 2008).

These findings do not diminish the importance of syntrophins as DGKζ binding partners. On the contrary, they suggest DGKζ may be recruited into a number of signaling pathways and play different roles by associating with more than one PDZ protein.

DGKζ in the Nucleus

Diacylglycerol regulates cell growth and differentiation and its transient accumulation in the nucleus may be particularly important in this context (Topham *et al.*, 1998). Many groups have reported the presence of DGKs in the nucleus (Goto and Kondo, 1996; Topham *et al.*, 1998; Bregoli *et al.*, 2001; Bregoli *et al.*, 2002; Hozumi *et al.*, 2003; Tabellini *et al.*, 2003; Matsubara *et al.*, 2006; Raben and Tu-Sekine, 2008). My studies and those of Hogan et al. (2001) show that blocking the association of DGKζ with syntrophins results in the accumulation of the protein in the nucleus. On the other hand, PKC-mediated phosphorylation of the DGKζ MARCKS domain, which contains a nuclear localization signal, keeps DGKζ out of the nucleus (Topham *et al.*, 1998). These findings suggest

phosphorylation of the DGKζ MARCKS domain and binding of DGKζ to syntrophins play opposing roles in regulating DGKζ localization.

In myoblasts, DGKζ has been proposed to play a role in cell cycle progression and differentiation required for myogenesis. The levels of nuclear DGKζ increase during myogenic differentiation in C2C12 myoblasts and induce the required withdrawal from the cell-cycle, whereas knocking-down DGKζ expression markedly impairs differentiation (Evangelisti *et al.*, 2006; Evangelisti *et al.*, 2007). Furthermore, DGKζ colocalizes with nuclear speckles, structures typically associated with gene transcription. DGKζ also associates with and is positively regulated by the retinoblastoma protein (pRB) (Los *et al.*, 2006). pRB negatively regulates cell cycle progression by inhibiting E2F-dependent gene transcription, and is best known as a tumor suppressor, although it also plays important roles in promoting myogenic differentiation and inhibiting apoptosis during myogenesis (Wang *et al.*, 1997; De Falco *et al.*, 2006; Du and Pogoriler, 2006). Collectively, these findings strongly suggest a role for nuclear DGKζ in myogenesis. Since both syntrophins and PKC-mediated phosphorylation influence the nuclear localization of DGKζ, they are also likely to regulate nuclear DGKζ activity and therefore to affect myogenesis. The striking accumulation of DGKζ in the nuclei of regenerating muscle fibers of dystrophic mice (Chapter 2) further supports the notion that DGKζ is involved in myogenesis and raises the possibility that its aberrant nuclear localization plays a role in the pathogenesis of muscular dystrophy.

Future Directions

The large number of DGKs and their ubiquitous tissue expression suggests important physiological roles for these proteins. Indeed, DGKs are involved in the proper functioning of the immune, nervous, and cardiovascular systems and may also play a role in cell growth and malignancy (Zhong *et al.*, 2003; Takahashi *et al.*, 2005; Lukiw *et al.*, 2005; McMullan *et al.*, 2006; Olenchock *et al.*, 2006; Musto and Bazan, 2006; Harada *et al.*, 2007; Niizeki *et al.*, 2007; Takeishi *et al.*, 2007; Baum, 2007). Dysregulation of DGK function could therefore contribute to certain pathological states (Merida *et al.*, 2008). In fact, recent discoveries suggest a role for DGKδ in diabetes (Chibalin *et al.*, 2008) and DGKζ in heart disease (Takeda *et al.*, 2001; Takahashi *et al.*, 2005; Arimoto *et al.*, 2006; Harada *et al.*, 2007; Niizeki *et al.*, 2007; Takeishi *et al.*, 2007) thus implicating these DGK isoforms in diseases that have a significant impact in Western populations. Given the emerging importance of DGKs in these disorders further studies of DGK function are indeed warranted.

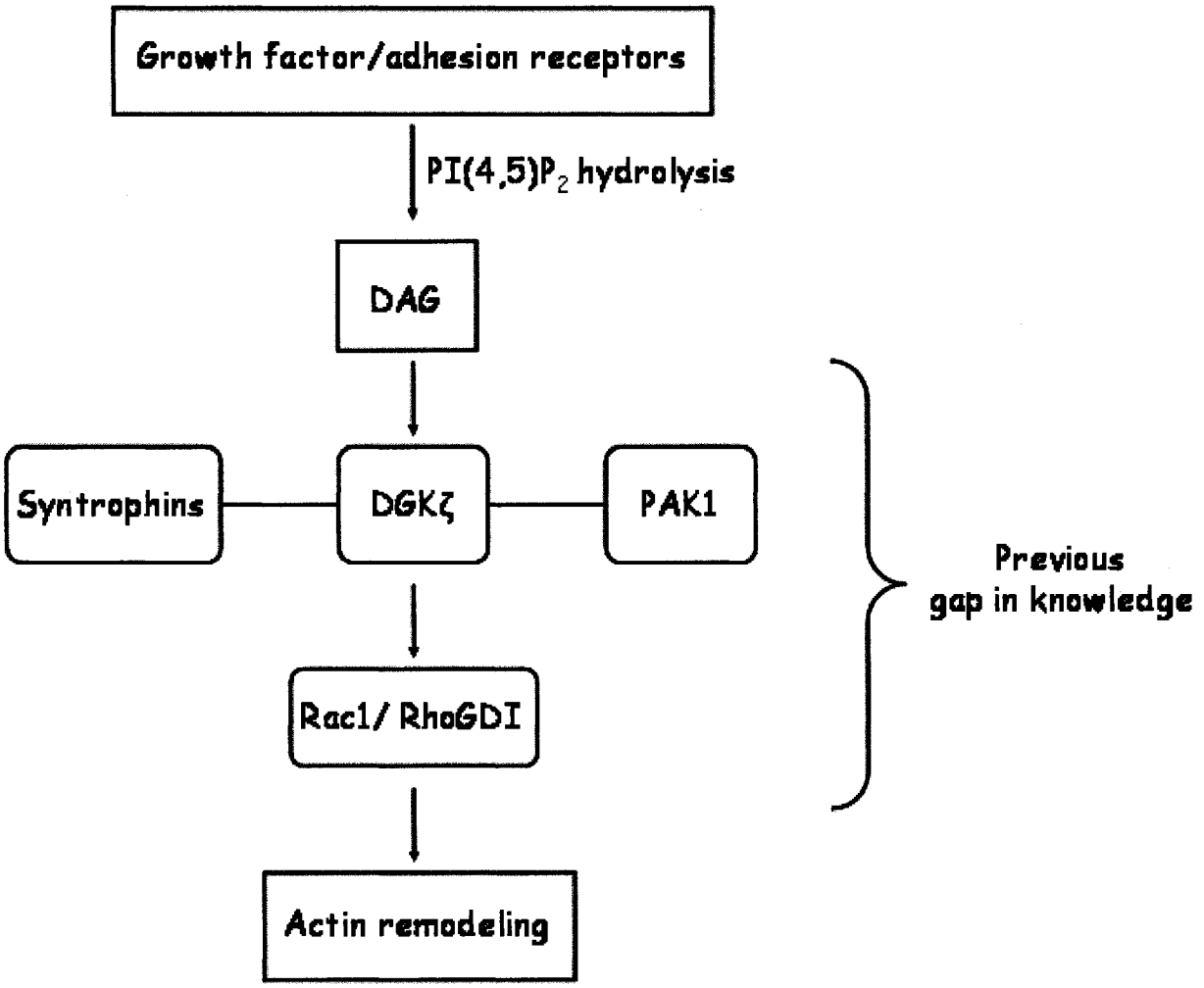
The isolation, purification and enrichment of primary myoblasts from the muscles of DGKζ knockout mice, or from mice expressing different DGKζ mutants, followed by differentiation of the cells *in vitro* could further clarify the role of DGKζ in myogenesis and myoblast fusion. In addition, the use of such mice in models of experimentally-induced muscle regeneration may also provide further information regarding the role of DGKζ in muscle development. The generation of DGKζ/dystrophin double knockout mice or DGKζ "knock-in"-dystrophin knockout mice may help elucidate the role of DGKζ in the pathogenesis of muscular dystrophy.

Similarly, the function of DGKζ in the nervous system, particularly in neurite outgrowth, may be further explored in murine models of nerve injury using DGKζ knockout or "knock-in" mice or in *in vitro* models of neurite outgrowth using primary neurons cultured from such mice.

The role of DGKζ in regulating cell migration raises the possibility that it may be involved in wound healing or even in tumor invasion and metastasis. Therefore, future studies could also examine the role of DGKζ in these processes *in vivo* by using the murine models described above.

In conclusion, my results strongly suggest that the lipid kinase DGKζ exists in a signaling complex with PAK1, Rac1 and RhoGDI, proteins with well known roles in actin cytoskeleton remodeling. This molecular arrangement enables changes in DAG and PA levels to be rapidly and efficiently translated into changes in Rac1 activity and restructuring of the actin filament network. Syntrophins, membrane-associated scaffolding proteins that also bind phospholipids, could regulate the spatial localization of DGKζ and associated proteins by targeting the signaling complex to sites of receptor activation and lipid generation. These novel and exciting findings provide a mechanism to explain how changes in the lipid composition of the plasma membrane are connected to the reorganization of the actin cytoskeleton and place DGKζ in a key position in the signaling pathway from $PI(4,5)P_2$ breakdown to Rac activation (Figure 6.1).

Figure 6.1. Flowchart summarizing the main findings and highlighting the gap in knowledge that existed at the start of my studies.


Growth factor/adhesion receptors
PI(4,5)P2 hydrolysis
DAG
Syntrophins
DGKζ
PAK1
Rac1/ RhoGDI
Actin remodeling
Previous
gap in knowledge

Chapter 7

Reference List

Abercrombie,M., Heaysman,J.E., Pegrum,S.M. (1970). The locomotion of fibroblasts in culture. I. Movements of the leading edge. Exp.Cell Res. *59*, 393-398.

Abmayr,S.M., Balagopalan,L., Galletta,B.J., Hong,S.J. (2003). Cell and molecular biology of myoblast fusion. Int.Rev Cytol. *225*, 33-89.

Abramovici,H., Gee,S.H. (2007). Morphological changes and spatial regulation of diacylglycerol kinase-zeta, syntrophins, and Rac1 during myoblast fusion. Cell Motility and the Cytoskeleton *64*, 549-567.

Abramovici,H., Hogan,A.B., Obagi,C., Topham,M.K., Gee,S.H. (2003). Diacylglycerol kinase-ζ localization in skeletal muscle is regulated by phosphorylation and interaction with syntrophins. Molecular Biology of the Cell *14*, 4499-4511.

Adams,M.E., Butler,M.H., Dwyer,T.M., Peters,M.F., Murnane,A.A., Froehner,S.C. (1993). Two forms of mouse syntrophin, a 58 kd dystrophin-associated protein, differ in primary structure and tissue distribution. Neuron. *11*, 531-540.

Adams,M.E., Dwyer,T.M., Dowler,L.L., White,R.A., Froehner,S.C. (1995). Mouse alpha 1- and beta 2-syntrophin gene structure, chromosome localization, and homology with a discs large domain. The Journal of Biological Chemistry *270*, 25859-25865.

Adams,M.E., Kramarcy,N., Krall,S.P., Rossi,S.G., Rotundo,R.L., Sealock,R., Froehner,S.C. (2000). Absence of alpha-syntrophin leads to structurally aberrant neuromuscular synapses deficient in utrophin. Journal of Cell Biology *150*, 1385-1398.

Adams,M.E., Mueller,H.A., Froehner,S.C. (2001). In vivo requirement of the alpha-syntrophin PDZ domain for the sarcolemmal localization of nNOS and aquaporin-4. Journal of Cell Biology *155*, 113-122.

Ahmed,S., Kozma,R., Monfries,C., Hall,C., Lim,H.H., Smith,P., Lim,L. (1990). Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. Biochem.J. *272*, 767-773.

Ahmed,S., Lee,J., Kozma,R., Best,A., Monfries,C., Lim,L. (1993). A novel functional target for tumor-promoting phorbol esters and lysophosphatidic acid. The p21rac-GTPase activating protein n-chimaerin. J Biol Chem. *268*, 10709-10712.

Ahn,A.H., Freener,C.A., Gussoni,E., Yoshida,M., Ozawa,E., Kunkel,L.M. (1996). The three human syntrophin genes are expressed in diverse tissues, have distinct chromosomal locations, and each bind to dystrophin and its relatives. The Journal of Biological Chemistry *271*, 2724-2730.

Ahn,A.H., Yoshida,M., Anderson,M.S., Feener,C.A., Selig,S., Hagiwara,Y., Ozawa,E., Kunkel,L.M. (1994). Cloning of human basic A1, a distinct 59-kDa dystrophin- associated protein encoded on chromosome 8q23-24. Proc.Natl.Acad.Sci.USA *91*, 4446-4450.

Aktories,K. (1994). Clostridial ADP-ribosylating toxins: effects on ATP and GTP-binding proteins. Mol.Cell Biochem. *138*, 167-176.

Aktories,K., Hall,A. (1989). Botulinum ADP-ribosyltransferase C3: a new tool to study low molecular weight GTP-binding proteins. Trends Pharmacol.Sci. *10*, 415-418.

Albrecht,D.E., Froehner,S.C. (2002). Syntrophins and dystrobrevins: defining the dystrophin scaffold at synapses. Neurosignals. *11*, 123-129.

Alessi,A., Bragg,A.D., Percival,J.M., Yoo,J., Albrecht,D.E., Froehner,S.C., Adams,M.E. (2006). gamma-Syntrophin scaffolding is spatially and functionally distinct from that of the alpha/beta syntrophins. Exp.Cell Res. *312*, 3084-3095.

Alewine,C., Olsen,O., Wade,J.B., Welling,P.A. (2006). TIP-1 has PDZ scaffold antagonist activity. Molecular Biology of the Cell *17*, 4200-4211.

Allen,L.A., Aderem,A. (1995). Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts. The EMBO Journal *14*, 1109-1121.

Anderson,J.L., Head,S.I., Rae,C., Morley,J.W. (2002). Brain function in Duchenne muscular dystrophy. Brain *125*, 4-13.

Andresen,B.T., Rizzo,M.A., Shome,K., Romero,G. (2002). The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. FEBS Lett. *531*, 65-68.

Araki,N., Egami,Y., Watanabe,Y., Hatae,T. (2007). Phosphoinositide metabolism during membrane ruffling and macropinosome formation in EGF-stimulated A431 cells. Exp.Cell Res. *313*, 1496-1507.

Arimoto,T., Takeishi,Y., Takahashi,H., Shishido,T., Niizeki,T., Koyama,Y., Shiga,R., Nozaki,N., Nakajima,O., Nishimaru,K., Abe,J., Endoh,M., Walsh,R.A., Goto,K., Kubota,I. (2006). Cardiac-specific overexpression of diacylglycerol kinase zeta prevents Gq protein-coupled receptor agonist-induced cardiac hypertrophy in transgenic mice. Circulation *113*, 60-66.

Auer,K.L., Jacobson,B.S. (1995). Beta 1 integrins signal lipid second messengers required during cell adhesion. Molecular Biology of the Cell *6*, 1305-1313.

Bagrodia,S., Bailey,D., Lenard,Z., Hart,M., Guan,J.L., Premont,R.T., Taylor,S.J., Cerione,R.A. (1999). A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. The Journal of Biological Chemistry *274*, 22393-22400.

Banks,G.B., Choy,P.T., Lavidis,N.A., Noakes,P.G. (2003). Neuromuscular synapses mediate motor axon branching and motoneuron survival during the embryonic period of programmed cell death. Dev.Biol. *257*, 71-84.

Bar-Sagi,D., Feramisco,J.R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science *233*, 1061-1068.

Barona,T., Byrne,R.D., Pettitt,T.R., Wakelam,M.J., Larijani,B., Poccia,D.L. (2005). Diacylglycerol induces fusion of nuclear envelope membrane precursor vesicles. The Journal of Biological Chemistry *280*, 41171-41177.

Bate,M. (1990). The embryonic development of larval muscles in Drosophila. Development *110*, 791-804.

Baum,C. (2007). I could die for you: new prospects for suicide in gene therapy. Mol.Ther. *15*, 848-849.

Beg,A.A., Sommer,J.E., Martin,J.H., Scheiffele,P. (2007). alpha2-Chimaerin is an essential EphA4 effector in the assembly of neuronal locomotor circuits. Neuron *55*, 768-778.

Bellon,A., Ortiz-Lopez,L., Ramirez-Rodriguez,G., Anton-Tay,F., Benitez-King,G. (2007). Melatonin induces neuritogenesis at early stages in N1E-115 cells through actin

rearrangements via activation of protein kinase C and Rho-associated kinase. J.Pineal Res. *42*, 214-221.

Benard,V., Bohl,B.P., Bokoch,G.M. (1999). Characterization of rac and cdc42 activation in chemoattractant- stimulated human neutrophils using a novel assay for active GTPases. The Journal of Biological Chemistry *274*, 13198-13204.

Benitez-King,G., Hernandez,M.E., Tovar,R., Ramirez,G. (2001). Melatonin activates PKC-alpha but not PKC-epsilon in N1E-115 cells. Neurochem.Int. *39*, 95-102.

Bhattacharya,B., Ciardiello,F., Salomon,D.S., Cooper,H.L. (1988). Disordered metabolism of microfilament proteins, tropomyosin and actin, in mouse mammary epithelial cells expressing the Ha-ras oncogene. Oncogene Res. *3*, 51-65.

Black,J.D. (2000). Protein kinase C-mediated regulation of the cell cycle. Front Biosci. *5*, D406-D423.

Blake,D.J., Hawkes,R., Benson,M.A., Beesley,P.W. (1999). Different dystrophin-like complexes are expressed in neurons and glia. Journal of Cell Biology *147*, 645-658.

Blake,D.J., Weir,A., Newey,S.E., Davies,K.E. (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev. *82*, 291-329.

Blau,H.M. (1993). Muscular dystrophy. Muscling in on gene therapy. Nature *364*, 673-675.

Blau,H.M., Epstein,C.J. (1979). Manipulation of myogenesis in vitro: reversible inhibition by DMSO. Cell *17*, 95-108.

Bloch,R.J., Gonzalez-Serratos,H. (2003). Lateral force transmission across costameres in skeletal muscle. Exerc.Sport Sci.Rev. *31*, 73-78.

Bokoch,G.M. (2003). Biology of the p21-activated kinases. Annu.Rev.Biochem. *72*, 743-781.

Bokoch,G.M., Bohl,B.P., Chuang,T.H. (1994). Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. Journal of Biological Chemistry *269*, 31674-31679.

Bokoch,G.M., Reilly,A.M., Daniels,R.H., King,C.C., Olivera,A., Spiegel,S., Knaus,U.G. (1998). A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. The Journal of Biological Chemistry *273*, 8137-8144.

Bokoch,G.M., Wang,Y., Bohl,B.P., Sells,M.A., Quilliam,L.A., Knaus,U.G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). The Journal of Biological Chemistry *271*, 25746-25749.

Bonsall,J., Rehder,V. (1999). Regulation of chick dorsal root ganglion growth cone filopodia by protein kinase C. Brain Res. *839*, 120-132.

Boonen,G.J., de Koster,B.M., Elferink,J.G. (1993). Activation of neutrophil migration by dioctanoyl-sn-glycerol and fMet-Leu-Phe is controlled by different pathways. Agents Actions *38 Spec No*, C130-C132.

Borm,B., Requardt,R.P., Herzog,V., Kirfel,G. (2005). Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. Exp.Cell Res. *302*, 83-95.

Bour,B.A., Chakravarti,M., West,J.M., Abmayr,S.M. (2000). Drosophila SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. Genes and Development *14*, 1498-1511.

Bourmeyster,N., Stasia,M.J., Garin,J., Gagnon,J., Boquet,P., Vignais,P.V. (1992). Copurification of rho protein and the rho-GDP dissociation inhibitor from bovine neutrophil cytosol. Effect of phosphoinositides on rho ADP-ribosylation by the C3 exoenzyme of Clostridium botulinum. Biochemistry *31*, 12863-12869.

Bradford,M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. *72*, 248-254.

Brandt,D., Gimona,M., Hillmann,M., Haller,H., Mischak,H. (2002). Protein kinase C induces actin reorganization via a Src- and Rho-dependent pathway. The Journal of Biological Chemistry *277*, 20903-20910.

Bray,D., Levin,M.D., Morton-Firth,C.J. (1998). Receptor clustering as a cellular mechanism to control sensitivity. Nature *393*, 85-88.

Bregoli,L., Baldassare,J.J., Raben,D.M. (2001). Nuclear diacylglycerol kinase-theta is activated in response to alpha-thrombin. The Journal of Biological Chemistry *276*, 23288-23295.

Bregoli,L., Tu-Sekine,B., Raben,D.M. (2002). DGK and nuclear signaling nuclear diacylglycerol kinases in IIC9 cells. Adv.Enzyme Regul. *42*, 213-226.

Brenman,J.E., Chao,D.S., Gee,S.H., McGee,A.W., Craven,S.E., Santillano,D.R., Wu,Z., Huang,F., Xia,H., Peters,M.F., Froehner,S.C., Bredt,D.S. (1996a). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell *84*, 757-767.

Brenman,J.E., Christopherson,K.S., Craven,S.E., McGee,A.W., Bredt,D.S. (1996b). Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. Journal of Neuroscience *16*, 7407-7415.

Brewer,G.J., Torricelli,J.R., Evege,E.K., Price,P.J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. *35*, 567-576.

Brown,M.C., West,K.A., Turner,C.E. (2002). Paxillin-dependent paxillin kinase linker and p21-activated kinase localization to focal adhesions involves a multistep activation pathway. Molecular Biology of the Cell *13*, 1550-1565.

Brugnera,E., Haney,L., Grimsley,C., Lu,M., Walk,S.F., Tosello-Trampont,A.C., Macara,I.G., Madhani,H., Fink,G.R., Ravichandran,K.S. (2002). Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. Nat.Cell Biol. *4*, 574-582.

Brunig,I., Suter,A., Knuesel,I., Luscher,B., Fritschy,J.M. (2002). GABAergic Terminals Are Required for Postsynaptic Clustering of Dystrophin But Not of GABAA Receptors and Gephyrin. Journal of Neuroscience *22*, 4805-4813.

Bryant,D.M., Kerr,M.C., Hammond,L.A., Joseph,S.R., Mostov,K.E., Teasdale,R.D., Stow,J.L. (2007). EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. J.Cell Sci. *120*, 1818-1828.

Bryant,P.J. (1999). Filopodia: fickle fingers of cell fate? Curr.Biol *9*, R655-R657.

Bubb,M.R., Lenox,R.H., Edison,A.S. (1999). Phosphorylation-dependent conformational changes induce a switch in the actin-binding function of MARCKS. The Journal of Biological Chemistry *274*, 36472-36478.

Buccione,R., Orth,J.D., McNiven,M.A. (2004). FOOT AND MOUTH: PODOSOMES, INVADOPODIA AND CIRCULAR DORSAL RUFFLES. Nature Reviews Molecular Cell Biology *5*, 647-657.

Buechler,C., Boettcher,A., Bared,S.M., Probst,M.C., Schmitz,G. (2002). The carboxyterminus of the ATP-binding cassette transporter A1 interacts with a beta2-syntrophin/utrophin complex. Biochemical and Biophysical Research Communications *293*, 759-765.

Bunting,M., Tang,W., Zimmerman,G.A., McIntyre,T.M., Prescott,S.M. (1996). Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. The Journal of Biological Chemistry *271*, 10230-10236.

Burack,W.R., Shaw,A.S. (2000). Signal transduction: hanging on a scaffold. Curr.Opin.Cell Biol. *12*, 211-216.

Burger,K.N. (2000). Greasing membrane fusion and fission machineries. Traffic. *1*, 605-613.

Burridge,K., Wennerberg,K. (2004). Rho and Rac take center stage. Cell *116*, 167-179.

Burry,R.W. (1998). PKC activators (phorbol ester or bryostatin) stimulate outgrowth of NGF-dependent neurites in a subline of PC12 cells. J.Neurosci.Res. *53*, 214-222.

Caloca,M.J., Fernandez,N., Lewin,N.E., Ching,D., Modali,R., Blumberg,P.M., Kazanietz,M.G. (1997). Beta2-chimaerin is a high affinity receptor for the phorbol ester tumor promoters. The Journal of Biological Chemistry *272*, 26488-26496.

Caloca,M.J., Garcia-Bermejo,M.L., Blumberg,P.M., Lewin,N.E., Kremmer,E., Mischak,H., Wang,S., Nacro,K., Bienfait,B., Marquez,V.E., Kazanietz,M.G. (1999). beta2-chimaerin is a novel target for diacylglycerol: binding properties and changes in subcellular localization mediated by ligand binding to its C1 domain. Proc.Natl.Acad.Sci.U.S.A *96*, 11854-11859.

Campanelli,J.T., Roberds,S.L., Campbell,K.P., Scheller,R.H. (1994). A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. Cell. *77*, 663-674.

Chardin,P., Boquet,P., Madaule,P., Popoff,M.R., Rubin,E.J., Gill,D.M. (1989). The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. The EMBO Journal *8*, 1087-1092.

Charrasse,S., Causeret,M., Comunale,F., Bonet-Kerrache,A., Gauthier-Rouviere,C. (2003). Rho GTPases and cadherin-based cell adhesion in skeletal muscle development. J Muscle Res.Cell Motil. *24*, 309-313.

Chartier,C., Degryse,E., Gantzer,M., Dieterle,A., Pavirani,A., Mehtali,M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J Virol. *70*, 4805-4810.

Chen,E.H., Olson,E.N. (2004). Towards a molecular pathway for myoblast fusion in Drosophila. Trends Cell Biol *14*, 452-460.

Chen,E.H., Olson,E.N. (2005). Unveiling the mechanisms of cell-cell fusion. Science *308*, 369-373.

Chen,E.H., Pryce,B.A., Tzeng,J.A., Gonzalez,G.A., Olson,E.N. (2003). Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. Cell *114*, 751-762.

Chen,Z., Hague,C., Hall,R.A., Minneman,K.P. (2006). Syntrophins regulate alpha1D-adrenergic receptors through a PDZ domain-mediated interaction. The Journal of Biological Chemistry *281*, 12414-12420.

Chibalin,A.V., Leng,Y., Vieira,E., Krook,A., Bjornholm,M., Long,Y.C., Kotova,O., Zhong,Z., Sakane,F., Steiler,T., Nylen,C., Wang,J., Laakso,M., Topham,M.K., Gilbert,M., Wallberg-Henriksson,H., Zierath,J.R. (2008). Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell *132*, 375-386.

Chockalingam,P.S., Gee,S.H., Jarrett,H.W. (1999). Pleckstrin homology domain 1 of mouse alpha 1-syntrophin binds phosphatidylinositol 4,5-bisphosphate. Biochemistry *38*, 5596-5602.

Choe,Y., Lee,B.J., Kim,K. (2002). Participation of protein kinase C alpha isoform and extracellular signal-regulated kinase in neurite outgrowth of GT1 hypothalamic neurons. Journal of Neurochemistry *83*, 1412-1422.

Choi,J.H., Yang,Y.R., Lee,S.K., Kim,I.S., Ha,S.H., Kim,E.K., Bae,Y.S., Ryu,S.H., Suh,P.G. (2007). Phospholipase C-gamma1 potentiates integrin-dependent cell spreading and migration through Pyk2/paxillin activation. Cell Signal. *19*, 1784-1796.

Chou,Y.H., Flitney,F.W., Chang,L., Mendez,M., Grin,B., Goldman,R.D. (2007). The motility and dynamic properties of intermediate filaments and their constituent proteins. Exp.Cell Res. *313*, 2236-2243.

Chuang,T.H., Bohl,B.P., Bokoch,G.M. (1993a). Biologically active lipids are regulators of Rac.GDI complexation. The Journal of Biological Chemistry *268*, 26206-26211.

Chuang,T.H., Xu,X., Knaus,U.G., Hart,M.J., Bokoch,G.M. (1993b). GDP dissociation inhibitor prevents intrinsic and GTPase activating protein-stimulated GTP hydrolysis by the Rac GTP-binding protein. The Journal of Biological Chemistry *268*, 775-778.

Churchward,M.A., Rogasevskaia,T., Brandman,D.M., Khosravani,H., Nava,P., Atkinson,J.K., Coorssen,J. (2008). Specific lipids supply critical intrinsic negative curvature - an essential component of native Ca2+-triggered membrane fusion. Biophys.J.

Clark,E.A., King,W.G., Brugge,J.S., Symons,M., Hynes,R.O. (1998). Integrin-mediated signals regulated by members of the rho family of GTPases. Journal of Cell Biology *142*, 573-586.

Clejan,S., Dotson,R.S., Wolf,E.W., Corb,M.P., Ide,C.F. (1996). Morphological differentiation of N1E-115 neuroblastoma cells by dimethyl sulfoxide activation of lipid second messengers. Exp.Cell Res. *224*, 16-27.

Cohen,R., Pacifici,M., Rubinstein,N., Biehl,J., Holtzer,H. (1977). Effect of a tumour promoter on myogenesis. Nature *266*, 538-540.

Conchello,J.-A., Kim,J.J., Hansen,E.W. (1994). Enhanced three-dimensional reconstruction from confocal scanning microscope images. II. Depth discrimination versus signal-to-noise ratio in partically confocal images. Appl.Opt. *33*, 3740-3750.

Connors,N.C., Adams,M.E., Froehner,S.C., Kofuji,P. (2004). The potassium channel Kir4.1 associates with the dystrophin-glycoprotein complex via alpha-syntrophin in glia. The Journal of Biological Chemistry *279*, 28387-28392.

Constantin,B., Cognard,C., Raymond,G. (1995). Myoblast fusion is not a prerequisite for the appearance of calcium current, calcium release, and contraction in rat skeletal muscle cells developing in culture. Exp.Cell Res. *217*, 497-505.

Costa,M., Raich,W., Agbunag,C., Leung,B., Hardin,J., Priess,J.R. (1998). A putative catenin-cadherin system mediates morphogenesis of the Caenorhabditis elegans embryo. J Cell Biol *141*, 297-308.

Cross,M.J., Roberts,S., Ridley,A.J., Hodgkin,M.N., Stewart,A., Claesson-Welsh,L., Wakelam,M.J. (1996). Stimulation of actin stress fibre formation mediated by activation of phospholipase D. Curr.Biol. *6*, 588-597.

Cui,H., Hayashi,A., Sun,H.S., Belmares,M.P., Cobey,C., Phan,T., Schweizer,J., Salter,M.W., Wang,Y.T., Tasker,R.A., Garman,D., Rabinowitz,J., Lu,P.S., Tymianski,M. (2007). PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. Journal of Neuroscience *27*, 9901-9915.

Cybulsky,A.V., Carbonetto,S., Cyr,M.D., McTavish,A.J., Huang,Q. (1993). Extracellular matrix-stimulated phospholipase activation is mediated by beta 1-integrin. Am.J.Physiol *264*, C323-C332.

da Silva,J.S., Dotti,C.G. (2002). Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. Nat.Rev.Neurosci. *3*, 694-704.

Dai,Z., Luo,X., Xie,H., Peng,H.B. (2000). The Actin-Driven Movement and Formation of Acetylcholine Receptor Clusters. The Journal of Cell Biology *150*, 1321-1334.

Dalkilic,I., Kunkel,L.M. (2003). Muscular dystrophies: genes to pathogenesis. Curr.Opin.Genet.Dev. *13*, 231-238.

Dan,C., Nath,N., Liberto,M., Minden,A. (2002). PAK5, a new brain-specific kinase, promotes neurite outgrowth in N1E- 115 cells. Mol.Cell Biol. *22*, 567-577.

Daniels,R.H., Hall,P.S., Bokoch,G.M. (1998). Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. The EMBO Journal *17*, 754-764.

Dartsch,P.C., Ritter,M., Haussinger,D., Lang,F. (1994). Cytoskeletal reorganization in NIH 3T3 fibroblasts expressing the ras oncogene. Eur.J.Cell Biol. *63*, 316-325.

David,S., Bouchard,C., Tsatas,O., Giftochristos,N. (1990). Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. Neuron. *5*, 463-469.

Davies,K.E., Nowak,K.J. (2006). Molecular mechanisms of muscular dystrophies: old and new players. Nat.Rev.Mol.Cell Biol. *7*, 762-773.

De Falco,G., Comes,F., Simone,C. (2006). pRb: master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription. Oncogene *25*, 5244-5249.

del Pozo,M.A., Kiosses,W.B., Alderson,N.B., Meller,N., Hahn,K.M., Schwartz,M.A. (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. Nat.Cell Biol. *4*, 232-239.

del Pozo,M.A., Alderson,N.B., Kiosses,W.B., Chiang,H.H., Anderson,R.G.W., Schwartz,M.A. (2004). Integrins Regulate Rac Targeting by Internalization of Membrane Domains. Science *303*, 839-842.

Dempsey,E.C., Newton,A.C., Mochly-Rosen,D., Fields,A.P., Reyland,M.E., Insel,P.A., Messing,R.O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. Am.J.Physiol Lung Cell Mol.Physiol *279*, L429-L438.

Dermardirossian,C., Bokoch,G.M. (2005). GDIs: central regulatory molecules in Rho GTPase activation. Trends Cell Biol. *15*, 356-363.

Dermardirossian,C., Rocklin,G., Seo,J.Y., Bokoch,G.M. (2006). Phosphorylation of RhoGDI by Src regulates Rho GTPase binding and cytosol-membrane cycling. Molecular Biology of the Cell *17*, 4760-4768.

Dermardirossian,C., Schnelzer,A., Bokoch,G.M. (2004). Phosphorylation of RhoGDI by Pak1 mediates dissociation of Rac GTPase. Mol.Cell *15*, 117-127.

Dharmawardhane,S., Sanders,L.C., Martin,S.S., Daniels,R.H., Bokoch,G.M. (1997). Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. Journal of Cell Biology *138*, 1265-1278.

Dharmawardhane,S., Schurmann,A., Sells,M.A., Chernoff,J., Schmid,S.L., Bokoch,G.M. (2000). Regulation of macropinocytosis by p21-activated kinase-1. Molecular Biology of the Cell *11*, 3341-3352.

Ding,L., Bunting,M., Topham,M.K., McIntyre,T.M., Zimmerman,G.A., Prescott,S.M. (1997). Alternative splicing of the human diacylglycerol kinase zeta gene in muscle. Proc.Natl.Acad.Sci.U.S.A *94*, 5519-5524.

Ding,L., McIntyre,T.M., Zimmerman,G.A., Prescott,S.M. (1998). The cloning and developmental regulation of murine diacylglycerol kinase zeta. FEBS Lett. *429*, 109-114.

Doberstein,S.K., Fetter,R.D., Mehta,A.Y., Goodman,C.S. (1997). Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. J Cell Biol. *136*, 1249-1261.

Dovas,A., Couchman,J.R. (2005). RhoGDI: multiple functions in the regulation of Rho family GTPase activities. Biochem.J. *390*, 1-9.

Dowrick,P., Kenworthy,P., McCann,B., Warn,R. (1993). Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. Eur.J.Cell Biol. *61*, 44-53.

Dransart,E., Olofsson,B., Cherfils,J. (2005). RhoGDIs revisited: novel roles in Rho regulation. Traffic. *6*, 957-966.

Du,W., Pogoriler,J. (2006). Retinoblastoma family genes. Oncogene *25*, 5190-5200.

Dulong,S., Goudenege,S., Vuillier-Devillers,K., Manenti,S., Poussard,S., Cottin,P. (2004). Myristoylated alanine-rich C kinase substrate (MARCKS) is involved in myoblast fusion through its regulation by protein kinase Calpha and calpain proteolytic cleavage. Biochem.J. *382*, 1015-1023.

Durbeej,M., Campbell,K.P. (2002). Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. Curr.Opin.Genet.Dev. *12*, 349-361.

Dworak,H.A., Sink,H. (2002). Myoblast fusion in Drosophila. Bioessays *24*, 591-601.

Ebinu,J.O., Bottorff,D.A., Chan,E.Y., Stang,S.L., Dunn,R.J., Stone,J.C. (1998). RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science *280*, 1082-1086.

Edwards,D.C., Sanders,L.C., Bokoch,G.M., Gill,G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat.Cell Biol. *1*, 253-259.

Edwards,K.A., Demsky,M., Montague,R.A., Weymouth,N., Kiehart,D.P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. Dev.Biol *191*, 103-117.

El Sayegh,T.Y., Arora,P.D., Ling,K., Laschinger,C., Janmey,P.A., Anderson,R.A., McCulloch,C.A. (2007). Phosphatidylinositol-4,5 bisphosphate produced by PIP5KIgamma

regulates gelsolin, actin assembly, and adhesion strength of N-cadherin junctions. Molecular Biology of the Cell *18*, 3026-3038.

Engel,L.C., David,J.D. (1985). Temperature-sensitive non-fusing myoblast variant and spontaneous revertant: isolation and characterization. Somat.Cell Mol.Genet *11*, 325-338.

Erickson,M.R., Galletta,B.J., Abmayr,S.M. (1997). Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. J Cell Biol. *138*, 589-603.

Eriksson,A., Siegbahn,A., Westermark,B., Heldin,C.H., Claesson-Welsh,L. (1992). PDGF alpha- and beta-receptors activate unique and common signal transduction pathways. The EMBO Journal *11*, 543-550.

Ervasti,J.M. (2003). Costameres: the Achilles' heel of Herculean muscle. The Journal of Biological Chemistry *278*, 13591-13594.

Etienne-Manneville,S., Hall,A. (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

Evangelisti,C., Riccio,M., Faenza,I., Zini,N., Hozumi,Y., Goto,K., Cocco,L., Martelli,A.M. (2006). Subnuclear localization and differentiation-dependent increased expression of DGK-zeta in C2C12 mouse myoblasts. J.Cell Physiol *209*, 370-378.

Evangelisti,C., Tazzari,P.L., Riccio,M., Fiume,R., Hozumi,Y., Fala,F., Goto,K., Manzoli,L., Cocco,L., Martelli,A.M. (2007). Nuclear diacylglycerol kinase-zeta is a negative regulator of cell cycle progression in C2C12 mouse myoblasts. FASEB J. *21*, 3297-3307.

Evers,E.E., Zondag,G.C., Malliri,A., Price,L.S., ten Klooster,J.P., van der Kammen,R.A., Collard,J.G. (2000). Rho family proteins in cell adhesion and cell migration. Eur.J.Cancer *36*, 1269-1274.

Fabre,S., Reynaud,C., Jalinot,P. (2000). Identification of functional PDZ domain binding sites in several human proteins. Mol.Biol.Rep. *27*, 217-224.

Faix,J., Rottner,K. (2006). The making of filopodia. Curr.Opin.Cell Biol. *18*, 18-25.

Falcone,S., Cocucci,E., Podini,P., Kirchhausen,T., Clementi,E., Meldolesi,J. (2006). Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events. J.Cell Sci. *119*, 4758-4769.

Faure,J., Vignais,P.V., Dagher,M.C. (1999). Phosphoinositide-dependent activation of Rho A involves partial opening of the RhoA/Rho-GDI complex. Eur.J.Biochem. *262*, 879-889.

Fenteany,G., Janmey,P.A., Stossel,T.P. (2000). Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. Current Biology *10*, 831-838.

Fernandis,A.Z., Wenk,M.R. (2007). Membrane lipids as signaling molecules. Curr.Opin.Lipidol. *18*, 121-128.

Fleming,I.N., Elliott,C.M., Collard,J.G., Exton,J.H. (1997). Lysophosphatidic acid induces threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts via activation of protein kinase C. The Journal of Biological Chemistry *272*, 33105-33110.

Franco,A., Jr., Lansman,J.B. (1990). Calcium entry through stretch-inactivated ion channels in mdx myotubes. Nature. *344*, 670-673.

Franke,T.F., Yang,S.I., Chan,T.O., Datta,K., Kazlauskas,A., Morrison,D.K., Kaplan,D.R., Tsichlis,P.N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell *81*, 727-736.

Froehner,S.C., Adams,M.E., Peters,M.F., Gee,S.H. (1997). Syntrophins: modular adapter proteins at the neuromuscular junction and the sarcolemma. Soc.Gen.Physiol. *52*, 197-207.

Froehner,S.C., Murnane,A.A., Tobler,M., Peng,H.B., Sealock,R. (1987). A postsynaptic Mr 58,000 (58K) protein concentrated at acetylcholine receptor-rich sites in Torpedo electroplaques and skeletal muscle. J Cell Biol. *104*, 1633-1646.

Fulton,A.B., Prives,J., Farmer,S.R., Penman,S. (1981). Developmental reorganization of the skeletal framework and its surface lamina in fusing muscle cells. J Cell Biol. *91*, 103-112.

Futerman,A.H., Banker,G.A. (1996). The economics of neurite outgrowth--the addition of new membrane to growing axons. Trends Neurosci. *19*, 144-149.

Galkin,V.E., Orlova,A., VanLoock,M.S., Rybakova,I.N., Ervasti,J.M., Egelman,E.H. (2002). The utrophin actin-binding domain binds F-actin in two different modes: implications for the spectrin superfamily of proteins. Journal of Cell Biology *157*, 243-251.

Gao,F.B., Brenman,J.E., Jan,L.Y., Jan,Y.N. (1999). Genes regulating dendritic outgrowth, branching, and routing in Drosophila. Genes and Development *13*, 2549-2561.

Garcia,R.A., Vasudevan,K., Buonanno,A. (2000). The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. Proc.Natl.Acad.Sci.U.S.A *97*, 3596-3601.

Gatlin,J.C., Estrada-Bernal,A., Sanford,S.D., Pfenninger,K.H. (2006). Myristoylated, alanine-rich C-kinase substrate phosphorylation regulates growth cone adhesion and pathfinding. Molecular Biology of the Cell *17*, 5115-5130.

Gee,S.H., Madhavan,R., Levinson,S.R., Caldwell,J.H., Sealock,R., Froehner,S.C. (1998). Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins. Journal of Neuroscience *18*, 128-137.

Gee,S.H., Montanaro,F., Lindenbaum,M.H., Carbonetto,S. (1994). Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. Cell *77*, 675-686.

Gee,S.H., Quenneville,S., Lombardo,C.R., Chabot,J. (2000). Single-amino acid substitutions alter the specificity and affinity of PDZ domains for their ligands. Biochemistry *39*, 14638-14646.

Ghosh,A., Greenberg,M.E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron *15*, 89-103.

Gibbs,J.B., Marshall,M.S., Scolnick,E.M., Dixon,R.A., Vogel,U.S. (1990). Modulation of guanine nucleotides bound to Ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). The Journal of Biological Chemistry *265*, 20437-20442.

Gibson,F.S., Lanni,F. (1991). Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy. J.Opt.Soc.Amer.A *8(10)*, 1601-1613.

Godsel,L.M., Hobbs,R.P., Green,K.J. (2008). Intermediate filament assembly: dynamics to disease. Trends Cell Biol. *18*, 28-37.

Goichberg,P., Geiger,B. (1998). Direct involvement of N-cadherin-mediated signaling in muscle differentiation. Mol Biol Cell *9*, 3119-3131.

Goni,F.M., Alonso,A. (1999). Structure and functional properties of diacylglycerols in membranes. Prog.Lipid Res. *38*, 1-48.

Gonzales,M.L., Anderson,R.A. (2006). Nuclear phosphoinositide kinases and inositol phospholipids. J.Cell Biochem. *97*, 252-260.

Gorecki,D.C., Abdulrazzak,H., Lukasiuk,K., Barnard,E.A. (1997). Differential expression of syntrophins and analysis of alternatively spliced dystrophin transcripts in the mouse brain. European Journal of Neuroscience *9*, 965-976.

Gorecki,D.C., Derry,J.M., Barnard,E.A. (1994). Dystroglycan: brain localisation and chromosome mapping in the mouse. Hum.Mol.Genet. *3*, 1589-1597.

Goto,K., Funayama,M., Kondo,H. (1994). Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. Proc.Natl.Acad.Sci.U.S.A *91*, 13042-13046.

Goto,K., Kondo,H. (1996). A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. Proc.Natl.Acad.Sci.U.S.A *93*, 11196-11201.

Govek,E.E., Newey,S.E., Van Aelst,L. (2005). The role of the Rho GTPases in neuronal development. Genes and Development *19*, 1-49.

Grady,R.M., Wozniak,D.F., Ohlemiller,K.K., Sanes,J.R. (2006). Cerebellar synaptic defects and abnormal motor behavior in mice lacking alpha- and beta-dystrobrevin. Journal of Neuroscience *26*, 2841-2851.

Gramolini,A.O., Wu,J., Jasmin,B.J. (2000). Regulation and functional significance of utrophin expression at the mammalian neuromuscular synapse. Microsc.Res.Tech. *49*, 90-100.

Grondin,P., Plantavid,M., Sultan,C., Breton,M., Mauco,G., Chap,H. (1991). Interaction of pp60c-src, phospholipase C, inositol-lipid, and diacyglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. The Journal of Biological Chemistry *266*, 15705-15709.

Guillou,H., Depraz-Depland,A., Planus,E., Vianay,B., Chaussy,J., Grichine,A., Albiges-Rizo,C., Block,M.R. (2008). Lamellipodia nucleation by filopodia depends on integrin occupancy and downstream Rac1 signaling. Exp.Cell Res. *314*, 478-488.

Gulbins,E., Coggeshall,K.M., Baier,G., Telford,D., Langlet,C., Baier-Bitterlich,G., Bonnefoy-Berard,N., Burn,P., Wittinghofer,A., Altman,A. (1994). Direct stimulation of Vav guanine nucleotide exchange activity for Ras by phorbol esters and diglycerides. Mol.Cell Biol. *14*, 4749-4758.

Guo,F., Debidda,M., Yang,L., Williams,D.A., Zheng,Y. (2006). Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. The Journal of Biological Chemistry *281*, 18652-18659.

Hajdo-Milasinovic,A., Ellenbroek,S.I., van Es,S., Van,d., V, Collard,J.G. (2007). Rac1 and Rac3 have opposing functions in cell adhesion and differentiation of neuronal cells. J.Cell Sci. *120*, 555-566.

Hakeda-Suzuki,S., Ng,J., Tzu,J., Dietzl,G., Sun,Y., Harms,M., Nardine,T., Luo,L., Dickson,B.J. (2002). Rac function and regulation during Drosophila development. Nature *416*, 438-442.

Hall,A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu.Rev.Cell Biol. *10*, 31-54.

Hall,A. (1998). Rho GTPases and the Actin Cytoskeleton. Science *279*, 509-514.

Hall,C., Michael,G.J., Cann,N., Ferrari,G., Teo,M., Jacobs,T., Monfries,C., Lim,L. (2001). alpha2-chimaerin, a Cdc42/Rac1 regulator, is selectively expressed in the rat embryonic nervous system and is involved in neuritogenesis in N1E-115 neuroblastoma cells. Journal of Neuroscience *21*, 5191-5202.

Harada,A., Takeuchi,K., Dohmae,N., Takio,K., Uenaka,T., Aoki,J., Inoue,K., Umeda,M. (1999). A monoclonal antibody, 3A10, recognizes a specific amino acid sequence present on a series of developmentally expressed brain proteins. J Biochem.(Tokyo) *125*, 443-448.

Harada,M., Takeishi,Y., Arimoto,T., Niizeki,T., Kitahara,T., Goto,K., Walsh,R.A., Kubota,I. (2007). Diacylglycerol kinase zeta attenuates pressure overload-induced cardiac hypertrophy. Circ.J. *71*, 276-282.

Harden,N., Loh,H.Y., Chia,W., Lim,L. (1995). A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in Drosophila. Development *121*, 903-914.

Harden,N., Ricos,M., Ong,Y.M., Chia,W., Lim,L. (1999). Participation of small GTPases in dorsal closure of the Drosophila embryo: distinct roles for Rho subfamily proteins in epithelial morphogenesis. J Cell Sci. *112 (Pt 3)*, 273-284.

Hardie,R.C., Raghu,P. (2001). Visual transduction in Drosophila. Nature *413*, 186-193.

Hartwig,J.H., Thelen,M., Rosen,A., Janmey,P.A., Nairn,A.C., Aderem,A. (1992). MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature *356*, 618-622.

Hasegawa,M., Cuenda,A., Spillantini,M.G., Thomas,G.M., Buee-Scherrer,V., Cohen,P., Goedert,M. (1999). Stress-activated protein kinase-3 interacts with the PDZ domain of alpha1-syntrophin. A mechanism for specific substrate recognition. The Journal of Biological Chemistry *274*, 12626-12631.

Hasegawa-Sasaki,H. (1985). Early changes in inositol lipids and their metabolites induced by platelet-derived growth factor in quiescent Swiss mouse 3T3 cells. Biochem.J. *232*, 99-109.

He,T.C., Zhou,S., da Costa,L.T., Yu,J., Kinzler,K.W., Vogelstein,B. (1998). A simplified system for generating recombinant adenoviruses. Proc.Natl.Acad.Sci.U.S.A *95*, 2509-2514.

Higgins,P.J., Ryan,M.P. (1989). Cytoarchitecture of ras oncogene-expressing tumor cells: butyrate modulation of substrate adhesion, cytoskeletal actin content and subcellular microfilament distribution. Int.J.Biochem. *21*, 1143-1151.

Hill,M.A., Gunning,P. (1993). Beta and gamma actin mRNAs are differentially located within myoblasts. Journal of Cell Biology *122*, 825-832.

Hillier,B.J., Christopherson,K.S., Prehoda,K.E., Bredt,D.S., Lim,W.A. (1999). Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. Science *284*, 812-815.

Hodgkin,M.N., Pettitt,T.R., Martin,A., Michell,R.H., Pemberton,A.J., Wakelam,M.J. (1998). Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? Trends Biochem.Sci. *23*, 200-204.

Hogan,A., Shepherd,L., Chabot,J., Quenneville,S., Prescott,S.M., Topham,M.K., Gee,S.H. (2001). Interaction of gamma 1-syntrophin with diacylglycerol kinase-zeta. Regulation of nuclear localization by PDZ interactions. The Journal of Biological Chemistry *276*, 26526-26533.

Holinstat,M., Mehta,D., Kozasa,T., Minshall,R.D., Malik,A.B. (2003). Protein kinase Calpha-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. The Journal of Biological Chemistry *278*, 28793-28798.

Honda,A., Nogami,M., Yokozeki,T., Yamazaki,M., Nakamura,H., Watanabe,H., Kawamoto,K., Nakayama,K., Morris,A.J., Frohman,M.A., Kanaho,Y. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521-532.

Hoock,T.C., Newcomb,P.M., Herman,I.M. (1991). Beta actin and its mRNA are localized at the plasma membrane and the regions of moving cytoplasm during the cellular response to injury. Journal of Cell Biology *112*, 653-664.

Houssa,B., de Widt,J., Kranenburg,O., Moolenaar,W.H., van Blitterswijk,W.J. (1999). Diacylglycerol kinase theta binds to and is negatively regulated by active RhoA. The Journal of Biological Chemistry *274*, 6820-6822.

Howe,A.K., Aplin,A.E., Juliano,R.L. (2002). Anchorage-dependent ERK signaling - mechanisms and consequences. Current Opinion in Genetics & Development *12*, 30-35.

Hozumi,Y., Ito,T., Nakano,T., Nakagawa,T., Aoyagi,M., Kondo,H., Goto,K. (2003). Nuclear localization of diacylglycerol kinase zeta in neurons. European Journal of Neuroscience *18*, 1448-1457.

Hunter,T. (2000). Signaling--2000 and beyond. Cell *100*, 113-127.

Hurley,J.H., Newton,A.C., Parker,P.J., Blumberg,P.M., Nishizuka,Y. (1997). Taxonomy and function of C1 protein kinase C homology domains. Protein Sci. *6*, 477-480.

Ile,K.E., Schaaf,G., Bankaitis,V.A. (2006). Phosphatidylinositol transfer proteins and cellular nanoreactors for lipid signaling. Nat.Chem.Biol. *2*, 576-583.

Imai,S., Kai,M., Yasuda,S., Kanoh,H., Sakane,F. (2005). Identification and characterization of a novel human type II diacylglycerol kinase, DGK kappa. The Journal of Biological Chemistry *280*, 39870-39881.

Imai,S., Sakane,F., Kanoh,H. (2002). Phorbol ester-regulated oligomerization of diacylglycerol kinase delta linked to its phosphorylation and translocation. The Journal of Biological Chemistry *277*, 35323-35332.

Ishihara,H., Shibasaki,Y., Kizuki,N., Katagiri,H., Yazaki,Y., Asano,T., Oka,Y. (1996). Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase. The Journal of Biological Chemistry *271*, 23611-23614.

Ishihara,H., Shibasaki,Y., Kizuki,N., Wada,T., Yazaki,Y., Asano,T., Oka,Y. (1998). Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. The Journal of Biological Chemistry *273*, 8741-8748.

Iwata,Y., Pan,Y., Yoshida,T., Hanada,H., Shigekawa,M. (1998). Alpha1-syntrophin has distinct binding sites for actin and calmodulin. FEBS Lett. *423*, 173-177.

Iwata,Y., Sampaolesi,M., Shigekawa,M., Wakabayashi,S. (2004). Syntrophin is an actin-binding protein the cellular localization of which is regulated through cytoskeletal reorganization in skeletal muscle cells. Eur.J.Cell Biol. *83*, 555-565.

Jacinto,A., Wolpert,L. (2001). Filopodia. Curr.Biol *11*, R634.

Jacinto,A., Wood,W., Balayo,T., Turmaine,M., Martinez-Arias,A., Martin,P. (2000). Dynamic actin-based epithelial adhesion and cell matching during Drosophila dorsal closure. Curr.Biol *10*, 1420-1426.

Jacinto,A., Wood,W., Woolner,S., Hiley,C., Turner,L., Wilson,C., Martinez-Arias,A., Martin,P. (2002). Dynamic analysis of actin cable function during Drosophila dorsal closure. Curr.Biol *12*, 1245-1250.

Jaffe,A.B., Hall,A. (2003). Cell biology. Smurfing at the leading edge. Science *302*, 1690-1691.

Jaffe,A.B., Hall,A. (2005). RHO GTPASES: Biochemistry and Biology. Annual Review of Cell and Developmental Biology *21*, 247-269.

Janmey,P.A. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu.Rev.Physiol *56*, 169-191.

Janmey,P.A. (1995). Protein regulation by phosphatidylinositol lipids. Chem.Biol. *2*, 61-65.

Janmey,P.A. (1998). The cytoskeleton and cell signaling: component localization and mechanical coupling. Physiol Rev. *78*, 763-781.

Janmey,P.A., Lindberg,U. (2004). Cytoskeletal regulation: rich in lipids. Nat.Rev.Mol.Cell Biol. *5*, 658-666.

Jemth,P., Gianni,S. (2007). PDZ domains: folding and binding. Biochemistry *46*, 8701-8708.

Jenkins,G.H., Fisette,P.L., Anderson,R.A. (1994). Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. The Journal of Biological Chemistry *269*, 11547-11554.

Johnson,J.E., Goulding,R.E., Ding,Z., Partovi,A., Anthony,K.V., Beaulieu,N., Tazmini,G., Cornell,R.B., Kay,R.J. (2007). Differential membrane binding and diacylglycerol recognition by C1 domains of RasGRPs. Biochem.J. *406*, 223-236.

Joseph,G., Gorzalczany,Y., Koshkin,V., Pick,E. (1994). Inhibition of NADPH oxidase activation by synthetic peptides mapping within the carboxyl-terminal domain of small GTP-binding proteins. Lack of amino acid sequence specificity and importance of polybasic motif. The Journal of Biological Chemistry *269*, 29024-29031.

Judge,L.M., Haraguchiln,M., Chamberlain,J.S. (2006). Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex. J.Cell Sci. *119*, 1537-1546.

Kabir,N., Schaefer,A.W., Nakhost,A., Sossin,W.S., Forscher,P. (2001). Protein kinase C activation promotes microtubule advance in neuronal growth cones by increasing average microtubule growth lifetimes. Journal of Cell Biology *152*, 1033-1044.

Kachinsky,A.M., Froehner,S.C., Milgram,S.L. (1999). A PDZ-containing scaffold related to the dystrophin complex at the basolateral membrane of epithelial cells. Journal of Cell Biology *145*, 391-402.

Kai,M., Sakane,F., Imai,S., Wada,I., Kanoh,H. (1994). Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. J Biol Chem. *269*, 18492-18498.

Kalderon,N., Gilula,N.B. (1979). Membrane events involved in myoblast fusion. J Cell Biol. *81*, 411-425.

Kalil,K., Dent,E.W. (2005). Touch and go: guidance cues signal to the growth cone cytoskeleton. Current Opinion in Neurobiology *15*, 521-526.

Kameya,S., Miyagoe,Y., Nonaka,I., Ikemoto,T., Endo,M., Hanaoka,K., Nabeshima,Y., Takeda,S. (1999). alpha1-syntrophin gene disruption results in the absence of neuronal- type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration. The Journal of Biological Chemistry *274*, 2193-2200.

Katoh,K., Kano,Y., Masuda,M., Onishi,H., Fujiwara,K. (1998). Isolation and contraction of the stress fiber. Molecular Biology of the Cell *9*, 1919-1938.

Kawasaki,H., Springett,G.M., Toki,S., Canales,J.J., Harlan,P., Blumenstiel,J.P., Chen,E.J., Bany,I.A., Mochizuki,N., Ashbacher,A., Matsuda,M., Housman,D.E., Graybiel,A.M. (1998). A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc.Natl.Acad.Sci.U.S.A *95*, 13278-13283.

Kazanietz,M.G. (2000). Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. Mol Carcinog. *28*, 5-11.

Keep,N.H. (2000). Structural comparison of actin binding in utrophin and dystrophin. Neurol.Sci. *21*, S929-S937.

Kennedy,T.E., Serafini,T., de,l.T., Jr., Tessier-Lavigne,M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-435.

Kent,C. (1979). Stimulation of phospholipid metabolism in embryonic muscle cells treated with phospholipase C. Proc.Natl.Acad.Sci.U.S.A *76*, 4474-4478.

Kholodenko,B.N., Hoek,J.B., Westerhoff,H.V. (2000). Why cytoplasmic signalling proteins should be recruited to cell membranes. Trends Cell Biol. *10*, 173-178.

Kim,E., Sheng,M. (2004). PDZ domain proteins of synapses. Nat.Rev.Neurosci. *5*, 771-781.

Kim,O., Yang,J., Qiu,Y. (2002). Selective activation of small GTPase RhoA by tyrosine kinase Etk through its pleckstrin homology domain. J Biol Chem. *277*, 30066-30071.

Kim,S., Shilagardi,K., Zhang,S., Hong,S.N., Sens,K.L., Bo,J., Gonzalez,G.A., Chen,E.H. (2007). A critical function for the actin cytoskeleton in targeted exocytosis of prefusion vesicles during myoblast fusion. Dev.Cell *12*, 571-586.

King,C.C., Gardiner,E.M., Zenke,F.T., Bohl,B.P., Newton,A.C., Hemmings,B.A., Bokoch,G.M. (2000). p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1). The Journal of Biological Chemistry *275*, 41201-41209.

Kisseleva,M., Feng,Y., Ward,M., Song,C., Anderson,R.A., Longmore,G.D. (2005). The LIM protein Ajuba regulates phosphatidylinositol 4,5-bisphosphate levels in migrating cells through an interaction with and activation of PIPKI alpha. Mol.Cell Biol. *25*, 3956-3966.

Kitano,Y., Okada,N., Adachi,J. (1986). TPA-induced alteration of actin organization in cultured human keratinocytes. Exp.Cell Res. *167*, 369-375.

Knaus,U.G., Bokoch,G.M. (1998). The p21Rac/Cdc42-activated kinases (PAKs). Int.J.Biochem.Cell Biol. *30*, 857-862.

Knezevic,N., Roy,A., Timblin,B., Konstantoulaki,M., Sharma,T., Malik,A.B., Mehta,D. (2007). GDI-1 phosphorylation switch at serine 96 induces RhoA activation and increased endothelial permeability. Mol.Cell Biol. *27*, 6323-6333.

Knight,B., Laukaitis,C., Akhtar,N., Hotchin,N.A., Edlund,M., Horwitz,A.R. (2000). Visualizing muscle cell migration in situ. Curr.Biol. *10*, 576-585.

Knudsen,K.A. (1990). Cell adhesion molecules in myogenesis. Curr Opin Cell Biol. *2*, 902-906.

Knudsen,K.A. (1992). Fusion of Myoblasts. In: Membrane Fusion, ed. J.Wilschut, D.Hoekstra Marcel Dekker, Inc., 601-626.

Knudsen,K.A., Horwitz,A.F. (1977). Tandem events in myoblast fusion. Dev Biol. *58*, 328-338.

Knudsen,K.A., Horwitz,A.F. (1978). Toward a mechanism of myoblast fusion. Prog.Clin.Biol.Res. *23*, 563-568.

Kobayashi,N., Hozumi,Y., Ito,T., Hosoya,T., Kondo,H., Goto,K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. Eur.J.Cell Biol. *86*, 433-444.

Kohrmann,M., Haubensak,W., Hemraj,I., Kaether,C., Lessmann,V.J., Kiebler,M.A. (1999). Fast, convenient, and effective method to transiently transfect primary hippocampal neurons. J.Neurosci.Res. *58*, 831-835.

Kolkova,K., Stensman,H., Berezin,V., Bock,E., Larsson,C. (2005). Distinct roles of PKC isoforms in NCAM-mediated neurite outgrowth. Journal of Neurochemistry *92*, 886-894.

Komati,H., Naro,F., Mebarek,S., De,A., V, Adamo,S., Lagarde,M., Prigent,A.F., Nemoz,G. (2005). Phospholipase D is involved in myogenic differentiation through remodeling of actin cytoskeleton. Molecular Biology of the Cell *16*, 1232-1244.

Kooijman,E.E., Chupin,V., de Kruijff,B., Burger,K.N. (2003). Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. Traffic. *4*, 162-174.

Kooijman,E.E., Chupin,V., Fuller,N.L., Kozlov,M.M., de Kruijff,B., Burger,K.N., Rand,P.R. (2005). Spontaneous curvature of phosphatidic acid and lysophosphatidic acid. Biochemistry *44*, 2097-2102.

Kozma,R., Ahmed,S., Best,A., Lim,L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol.Cell Biol. *15*, 1942-1952.

Kozma,R., Sarner,S., Ahmed,S., Lim,L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol.Cell Biol. *17*, 1201-1211.

Kranenburg,O., Poland,M., van Horck,F.P., Drechsel,D., Hall,A., Moolenaar,W.H. (1999). Activation of RhoA by lysophosphatidic acid and Galpha12/13 subunits in neuronal cells: induction of neurite retraction. Molecular Biology of the Cell *10*, 1851-1857.

Kranenburg,O., Scharnhorst,V., Van der Eb,A.J., Zantema,A. (1995). Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. Journal of Cell Biology *131*, 227-234.

Krauss,R.S., Cole,F., Gaio,U., Takaesu,G., Zhang,W., Kang,J.S. (2005). Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. J Cell Sci. *118*, 2355-2362.

Kreck,M.L., Freeman,J.L., Abo,A., Lambeth,J.D. (1996). Membrane association of Rac is required for high activity of the respiratory burst oxidase. Biochemistry *35*, 15683-15692.

Kumar,R., Gururaj,A.E., Barnes,C.J. (2006). p21-activated kinases in cancer. Nat.Rev.Cancer *6*, 459-471.

Landreth,G.E., Williams,L.K., Rieser,G.D. (1985). Association of the epidermal growth factor receptor kinase with the detergent-insoluble cytoskeleton of A431 cells. Journal of Cell Biology *101*, 1341-1350.

Lanzetti,L., Palamidessi,A., Areces,L., Scita,G., Di Fiore,P.P. (2004). Rab5 is a signalling GTPase involved in actin remodelling by receptor tyrosine kinases. Nature *429*, 309-314.

Lapidos,K.A., Kakkar,R., McNally,E.M. (2004). The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. Circ.Res. *94*, 1023-1031.

Larsson,C. (2006). Protein kinase C and the regulation of the actin cytoskeleton. Cell Signal. *18*, 276-284.

Lauffenburger,D.A., Horwitz,A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359-369.

Lei,M., Lu,W., Meng,W., Parrini,M.C., Eck,M.J., Mayer,B.J., Harrison,S.C. (2000). Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell *102*, 387-397.

Lemmon,M.A., Ferguson,K.M. (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem.J. *350 Pt 1*, 1-18.

Levchenko,A., Bruck,J., Sternberg,P.W. (2000). Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. Proc.Natl.Acad.Sci.U.S.A *97*, 5818-5823.

Lewis,J.M., Cheresh,D.A., Schwartz,M.A. (1996). Protein kinase C regulates alpha v beta 5-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. Journal of Cell Biology *134*, 1323-1332.

Li,H.S., Montell,C. (2000). TRP and the PDZ protein, INAD, form the core complex required for retention of the signalplex in Drosophila photoreceptor cells. Journal of Cell Biology *150*, 1411-1422.

Li,J., Wurtman,R.J. (1998). Nerve growth factor stimulates diacylglycerol de novo synthesis and phosphatidylinositol hydrolysis in pheochromocytoma cells. Brain Res. *803*, 44-53.

Lidov,H.G.W., Byers,T.J., Kunkel,L.M. (1993). The distribution of dystrophin in the murine central nervous system: An immunocytochemical study. Neuroscience *54*, 167-187.

Lidov,H.G.W., Byers,T.J., Watkins,S.C., Kunkel,L.M. (1990). Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature *348*, 725-728.

Lipton,B.H., Konigsberg,I.R. (1972). A fine-structural analysis of the fusion of myogenic cells. J Cell Biol *53*, 348-364.

Lombardi,L., Ballinari,D., Bongarzone,I., Migliari,M., Mondellini,P., Traversari,C., Modina,S. (1990). Ultrastructural cytoskeleton alterations and modification of actin expression in the NIH/3T3 cell line after transformation with Ha-ras-activated oncogene. Cell Motility and the Cytoskeleton *15*, 220-229.

Loo,T.H., Ng,Y.W., Lim,L., Manser,E. (2004). GIT1 activates p21-activated kinase through a mechanism independent of p21 binding. Mol.Cell Biol. *24*, 3849-3859.

Lorenzo,P.S., Beheshti,M., Pettit,G.R., Stone,J.C., Blumberg,P.M. (2000). The guanine nucleotide exchange factor RasGRP is a high -affinity target for diacylglycerol and phorbol esters. Mol Pharmacol. *57*, 840-846.

Los,A.P., Vinke,F.P., de Widt,J., Topham,M.K., van Blitterswijk,W.J., Divecha,N. (2006). The retinoblastoma family proteins bind to and activate diacylglycerol kinase zeta. The Journal of Biological Chemistry *281*, 858-866.

Lu,M., Kinchen,J.M., Rossman,K.L., Grimsley,C., deBakker,C., Brugnera,E., Tosello-Trampont,A.C., Haney,L.B., Klingele,D., Sondek,J., Hengartner,M.O., Ravichandran,K.S. (2004). PH domain of ELMO functions in trans to regulate Rac activation via Dock180. Nat Struct.Mol Biol *11*, 756-762.

Lukiw,W.J., Cui,J.G., Musto,A.E., Musto,B.C., Bazan,N.G. (2005). Epileptogenesis in diacylglycerol kinase epsilon deficiency up-regulates COX-2 and tyrosine hydroxylase in hippocampus. Biochemical and Biophysical Research Communications *338*, 77-81.

Lumeng,C., Phelps,S., Crawford,G.E., Walden,P.D., Barald,K., Chamberlain,J.S. (1999). Interactions between beta 2-syntrophin and a family of microtubule- associated serine/threonine kinases. Nat.Neurosci. *2*, 611-617.

Luo,B., Prescott,S.M., Topham,M.K. (2004a). Diacylglycerol kinase zeta regulates phosphatidylinositol 4-phosphate 5-kinase Ialpha by a novel mechanism. Cell Signal. *16*, 891-897.

Luo,B., Regier,D.S., Prescott,S.M., Topham,M.K. (2004b). Diacylglycerol kinases. Cell Signal. *16*, 983-989.

Luo,B., Prescott,S.M., Topham,M.K. (2003a). Association of diacylglycerol kinase {zeta} with protein kinase C {alpha}: spatial regulation of diacylglycerol signaling. The Journal of Cell Biology *160*, 929-937.

Luo,B., Prescott,S.M., Topham,M.K. (2003b). Protein Kinase Cα Phosphorylates and Negatively Regulates Diacylglycerol Kinase ζ. J Biol.Chem. *278*, 39542-39547.

Luo,L. (2000). Rho GTPases in neuronal morphogenesis. Nat.Rev.Neurosci. *1*, 173-180.

Luo,L. (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annu.Rev Cell Dev.Biol *18*, 601-635.

Luo,L., Hensch,T.K., Ackerman,L., Barbel,S., Jan,L.Y., Jan,Y.N. (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature *379*, 837-840.

Luo,L., Liao,Y.J., Jan,L.Y., Jan,Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes and Development *8*, 1787-1802.

Luo,S., Chen,Y., Lai,K.O., Arevalo,J.C., Froehner,S.C., Adams,M.E., Chao,M.V., Ip,N.Y. (2005). {alpha}-Syntrophin regulates ARMS localization at the neuromuscular junction and enhances EphA4 signaling in an ARMS-dependent manner. Journal of Cell Biology *169*, 813-824.

Lykissas,M.G., Batistatou,A.K., Charalabopoulos,K.A., Beris,A.E. (2007). The role of neurotrophins in axonal growth, guidance, and regeneration. Curr.Neurovasc.Res. *4*, 143-151.

Lynch,G.S., Rafael,J.A., Chamberlain,J.S., Faulkner,J.A. (2000). Contraction-induced injury to single permeabilized muscle fibers from mdx, transgenic mdx, and control mice. Am.J.Physiol Cell Physiol *279*, C1290-C1294.

Machesky,L.M., Insall,R.H. (1999). Signaling to actin dynamics. Journal of Cell Biology *146*, 267-272.

Manabe,R., Kovalenko,M., Webb,D.J., Horwitz,A.R. (2002). GIT1 functions in a motile, multi-molecular signaling complex that regulates protrusive activity and cell migration. J.Cell Sci. *115*, 1497-1510.

Manser,E., Chong,C., Zhao,Z.S., Leung,T., Michael,G., Hall,C., Lim,L. (1995). Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family. The Journal of Biological Chemistry *270*, 25070-25078.

Manser,E., Huang,H.Y., Loo,T.H., Chen,X.Q., Dong,J.M., Leung,T., Lim,L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol.Cell Biol. *17*, 1129-1143.

Manser,E., Leung,T., Salihuddin,H., Zhao,Z.S., Lim,L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature *367*, 40-46.

Manser,E., Loo,T.H., Koh,C.G., Zhao,Z.S., Chen,X.Q., Tan,L., Tan,I., Leung,T., Lim,L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol.Cell *1*, 183-192.

Massarwa,R., Carmon,S., Shilo,B.Z., Schejter,E.D. (2007). WIP/WASp-based actin-polymerization machinery is essential for myoblast fusion in Drosophila. Dev.Cell *12*, 557-569.

Matsubara,T., Shirai,Y., Miyasaka,K., Murakami,T., Yamaguchi,Y., Ueyama,T., Kai,M., Sakane,F., Kanoh,H., Hashimoto,T., Kamada,S., Kikkawa,U., Saito,N. (2006). Nuclear transportation of diacylglycerol kinase gamma and its possible function in the nucleus. The Journal of Biological Chemistry *281*, 6152-6164.

McLaughlin,S., Aderem,A. (1995). The myristoyl-electrostatic switch: a modulator of reversible protein- membrane interactions. Trends Biochem.Sci. *20*, 272-276.

McMullan,R., Hiley,E., Morrison,P., Nurrish,S.J. (2006). Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in C. elegans. Genes and Development *20*, 65-76.

McNamee,H.P., Ingber,D.E., Schwartz,M.A. (1993). Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. Journal of Cell Biology *121*, 673-678.

Mege,R.M., Goudou,D., Diaz,C., Nicolet,M., Garcia,L., Geraud,G., Rieger,F. (1992). N-cadherin and N-CAM in myoblast fusion: compared localisation and effect of blockade by peptides and antibodies. J Cell Sci. *103 (Pt 4)*, 897-906.

Mehta,D., Rahman,A., Malik,A.B. (2001). Protein kinase C-alpha signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. The Journal of Biological Chemistry *276*, 22614-22620.

Mehta,K.D., Radominska-Pandya,A., Kapoor,G.S., Dave,B., Atkins,B.A. (2002). Critical role of diacylglycerol- and phospholipid-regulated protein kinase C epsilon in induction of low-density lipoprotein receptor transcription in response to depletion of cholesterol. Mol.Cell Biol. *22*, 3783-3793.

Meigs,J.B., Wang,Y.L. (1986). Reorganization of alpha-actinin and vinculin induced by a phorbol ester in living cells. Journal of Cell Biology *102*, 1430-1438.

Mellstrom,K., Heldin,C.H., Westermark,B. (1988). Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor. Exp.Cell Res. *177*, 347-359.

Menard,R.E., Mattingly,R.R. (2003). Cell surface receptors activate p21-activated kinase 1 via multiple Ras and PI3-kinase-dependent pathways. Cell Signal. *15*, 1099-1109.

Menon,S.D., Chia,W. (2001). Drosophila rolling pebbles: a multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. Dev.Cell *1*, 691-703.

Merida,I., Avila-Flores,A., Merino,E. (2008). Diacylglycerol kinases: at the hub of cell signalling. Biochem.J. *409*, 1-18.

Mesmin,B., Robbe,K., Geny,B., Luton,F., Brandolin,G., Popoff,M.R., Antonny,B. (2004). A phosphatidylserine-binding site in the cytosolic fragment of Clostridium sordellii lethal toxin facilitates glucosylation of membrane-bound Rac and is required for cytotoxicity. The Journal of Biological Chemistry *279*, 49876-49882.

Miller,J.B., Boyce,F.M. (1995). Gene therapy by and for muscle cells. Trends Genet *11*, 163-165.

Misra,A., Lim,R.P., Wu,Z., Thanabalu,T. (2007). N-WASP plays a critical role in fibroblast adhesion and spreading. Biochemical and Biophysical Research Communications *364*, 908-912.

Mitchison,T.J., Cramer,L.P. (1996). Actin-based cell motility and cell locomotion. Cell *84*, 371-379.

Miyashita,M., Ohnishi,H., Okazawa,H., Tomonaga,H., Hayashi,A., Fujimoto,T.T., Furuya,N., Matozaki,T. (2004). Promotion of neurite and filopodium formation by CD47: roles of integrins, Rac, and Cdc42. Molecular Biology of the Cell *15*, 3950-3963.

Miyata,Y., Nishida,E., Koyasu,S., Yahara,I., Sakai,H. (1989). Protein kinase C-dependent and -independent pathways in the growth factor-induced cytoskeletal reorganization. The Journal of Biological Chemistry *264*, 15565-15568.

Montgomery,R.B., Moscatello,D.K., Wong,A.J., Stahl,W.L. (1997). Epidermal growth factor receptor stimulation of diacylglycerol kinase. Biochemical and Biophysical Research Communications *232*, 111-116.

Moore,S.A., Saito,F., Chen,J., Michele,D.E., Henry,M.D., Messing,A., Cohn,R.D., Ross-Barta,S.E., Westra,S., Williamson,R.A., Hoshi,T., Campbell,K.P. (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. Nature *418*, 422-425.

Moritz,A., De Graan,P.N., Gispen,W.H., Wirtz,K.W. (1992). Phosphatidic acid is a specific activator of phosphatidylinositol-4- phosphate kinase. The Journal of Biological Chemistry *267*, 7207-7210.

Morrison,R.S., Gross,J.L., Moskal,J.R. (1988). Inhibition of protein kinase C activity promotes the neurotrophic action of epidermal and basic fibroblast growth factors. Brain Res. *473*, 141-146.

Moukhles,H., Carbonetto,S. (2001). Dystroglycan contributes to the formation of multiple dystrophin-like complexes in brain. Journal of Neurochemistry *78*, 824-834.

Mulcahy,L.S., Smith,M.R., Stacey,D.W. (1985). Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature *313*, 241-243.

Musto,A., Bazan,N.G. (2006). Diacylglycerol kinase epsilon modulates rapid kindling epileptogenesis. Epilepsia *47*, 267-276.

Myat,M.M., Anderson,S., Allen,L.A., Aderem,A. (1997). MARCKS regulates membrane ruffling and cell spreading. Curr.Biol. *7*, 611-614.

Nagaya,H., Wada,I., Jia,Y.J., Kanoh,H. (2002). Diacylglycerol kinase delta suppresses ER-to-Golgi traffic via its SAM and PH domains. Molecular Biology of the Cell *13*, 302-316.

Nameroff,M., Trotter,J.A., Keller,J.M., Munar,E. (1973). Inhibition of cellular differentiation by phospholipase C. I. Effects of the enzyme on myogenesis and chondrogenesis in vitro. Journal of Cell Biology *58*, 107-118.

Nasuhoglu,C., Feng,S., Mao,J., Yamamoto,M., Yin,H.L., Earnest,S., Barylko,B., Albanesi,J.P., Hilgemann,D.W. (2002). Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. Anal.Biochem. *301*, 243-254.

Newey,S.E., Velamoor,V., Govek,E.E., Van Aelst,L. (2005). Rho GTPases, dendritic structure, and mental retardation. Journal of Neurobiology *64*, 58-74.

Newton,A.C. (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem.Rev. *101*, 2353-2364.

Ng,P., Graham,F.L. (2002). Construction of first-generation adenoviral vectors. Methods Mol Med. *69*, 389-414.

Niizeki,T., Takeishi,Y., Arimoto,T., Takahashi,H., Shishido,T., Koyama,Y., Goto,K., Walsh,R.A., Kubota,I. (2007). Cardiac-specific overexpression of diacylglycerol kinase zeta attenuates left ventricular remodeling and improves survival after myocardial infarction. Am.J.Physiol Heart Circ.Physiol *292*, H1105-H1112.

Nishimura,T., Yamaguchi,T., Kato,K., Yoshizawa,M., Nabeshima,Y., Ohno,S., Hoshino,M., Kaibuchi,K. (2005). PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. Nat.Cell Biol. *7*, 270-277.

Nishizuka,Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. *9*, 484-496.

Nobes,C.D., Hall,A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

Nobes,C.D., Hall,A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. Journal of Cell Biology *144*, 1235-1244.

Olenchock,B.A., Guo,R., Carpenter,J.H., Jordan,M., Topham,M.K., Koretzky,G.A., Zhong,X.P. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. Nat.Immunol. *7*, 1174-1181.

Olofsson,B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cell Signal. *11*, 545-554.

Ort,T., Maksimova,E., Dirkx,R., Kachinsky,A.M., Berghs,S., Froehner,S.C., Solimena,M. (2000). The receptor tyrosine phosphatase-like protein ICA512 binds the PDZ domains of beta2-syntrophin and nNOS in pancreatic beta-cells. Eur.J.Cell Biol. *79*, 621-630.

Ort,T., Voronov,S., Guo,J., Zawalich,K., Froehner,S.C., Zawalich,W., Solimena,M. (2001). Dephosphorylation of beta2-syntrophin and Ca2+/mu-calpain-mediated cleavage of ICA512 upon stimulation of insulin secretion. The EMBO Journal *20*, 4013-4023.

Orth,J.D., McNiven,M.A. (2006). Get off my back! Rapid receptor internalization through circular dorsal ruffles. Cancer Res. *66*, 11094-11096.

Pankov,R., Endo,Y., Even-Ram,S., Araki,M., Clark,K., Cukierman,E., Matsumoto,K., Yamada,K.M. (2005). A Rac switch regulates random versus directionally persistent cell migration. Journal of Cell Biology *170*, 793-802.

Partridge,M.A., Marcantonio,E.E. (2006). Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. Molecular Biology of the Cell *17*, 4237-4248.

Paterson,H.F., Self,A.J., Garrett,M.D., Just,I., Aktories,K., Hall,A. (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. Journal of Cell Biology *111*, 1001-1007.

Paululat,A., Breuer,S., Renkawitz-Pohl,R. (1999). Determination and development of the larval muscle pattern in Drosophila melanogaster. Cell Tissue Res. *296*, 151-160.

Pawson,T., Scott,J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075-2080.

Payrastre,B., Missy,K., Giuriato,S., Bodin,S., Plantavid,M., Gratacap,M. (2001). Phosphoinositides: key players in cell signalling, in time and space. Cell Signal. *13*, 377-387.

Payrastre,B., van Bergen en Henegouwen PM, Breton,M., den Hartigh,J.C., Plantavid,M., Verkleij,A.J., Boonstra,J. (1991). Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. Journal of Cell Biology *115*, 121-128.

Peters,M.F., Adams,M.E., Froehner,S.C. (1997). Differential association of syntrophin pairs with the dystrophin complex. J Cell Biol *138*, 81-93.

Peters,M.F., Kramarcy,N.R., Sealock,R., Froehner,S.C. (1994). β2-Syntrophin: Localization at the neuromuscular junction in skeletal muscle. NeuroReport *5*, 1577-1580.

Petrof,B.J., Shrager,J.B., Stedman,H.H., Kelly,A.M., Sweeney,H.L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc.Natl.Acad.Sci.USA *90*, 3710-3714.

Piluso,G., Mirabella,M., Ricci,E., Belsito,A., Abbondanza,C., Servidei,S., Puca,A.A., Tonali,P., Puca,G.A., Nigro,V. (2000). gamm 1- and gamma 2-Syntrophins, Two Novel Dystrophin-binding Proteins Localized in Neuronal Cells. The Journal of Biological Chemistry *275*, 15851-15860.

Plopper,G.E., McNamee,H.P., Dike,L.E., Bojanowski,K., Ingber,D.E. (1995). Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Molecular Biology of the Cell *6*, 1349-1365.

Pouliot,Y., Holland,P.C., Blaschuk,O.W. (1990). Developmental regulation of a cadherin during the differentiation of skeletal myoblasts. Dev.Biol. *141*, 292-298.

Price,L.S., Leng,J., Schwartz,M.A., Bokoch,G.M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. Molecular Biology of the Cell *9*, 1863-1871.

Prives,J., Shinitzky,M. (1977). Increased membrane fluidity precedes fusion of muscle cells. Nature *268*, 761-763.

Raben,D.M., Tu-Sekine,B. (2008). Nuclear diacylglycerol kinases: regulation and roles. Front Biosci. *13*, 590-597.

Raghu,P., Usher,K., Jonas,S., Chyb,S., Polyanovsky,A., Hardie,R.C. (2000). Constitutive activity of the light-sensitive channels TRP and TRPL in the Drosophila diacylglycerol kinase mutant, rdgA. Neuron *26*, 169-179.

Raich,W.B., Agbunag,C., Hardin,J. (1999). Rapid epithelial-sheet sealing in the Caenorhabditis elegans embryo requires cadherin-dependent filopodial priming. Curr.Biol *9*, 1139-1146.

Ramirez-Weber,F.A., Kornberg,T.B. (1999). Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. Cell *97*, 599-607.

Ramirez-Weber,F.A., Kornberg,T.B. (2000). Signaling reaches to new dimensions in Drosophila imaginal discs. Cell *103*, 189-192.

Rando,T.A. (2001). The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle and Nerve *24*, 1575-1594.

Rash,J.E., Fambrough,D. (1973). Ultrastructural and electrophysiological correlates of cell coupling and cytoplasmic fusion during myogenesis in vitro. Dev Biol. *30*, 166-186.

Ren,X.D., Bokoch,G.M., Traynor-Kaplan,A., Jenkins,G.H., Anderson,R.A., Schwartz,M.A. (1996). Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. Molecular Biology of the Cell *7*, 435-442.

Ren,X.D., Kiosses,W.B., Schwartz,M.A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. The EMBO Journal *18*, 578-585.

Ridley,A.J. (2001). Rho family proteins: coordinating cell responses. Trends Cell Biol *11*, 471-477.

Ridley,A.J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. *16*, 522-529.

Ridley,A.J., Hall,A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell *70*, 389-399.

Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D., Hall,A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell *70*, 401-410.

Rifkin,D.B., Crowe,R.M., Pollack,R. (1979). Tumor promoters induce changes in the chick embryo fibroblast cytoskeleton. Cell *18*, 361-368.

Rincon,E., Santos,T., Avila-Flores,A., Albar,J.P., Lalioti,V., Lei,C., Hong,W., Merida,I. (2007). Proteomics identification of sorting nexin 27 as a diacylglycerol kinase zeta-associated protein: new diacylglycerol kinase roles in endocytic recycling. Mol.Cell Proteomics. *6*, 1073-1087.

Ritzenthaler,S., Suzuki,E., Chiba,A. (2000). Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. Nat Neurosci *3*, 1012-1017.

Robbe,K., Otto-Bruc,A., Chardin,P., Antonny,B. (2003). Dissociation of GDP dissociation inhibitor and membrane translocation are required for efficient activation of Rac by the Dbl

homology-pleckstrin homology region of Tiam. The Journal of Biological Chemistry *278*, 4756-4762.

Robertson,E.J. (1987). Embryo Derived Stem Cell Lines., ed. E.J.RobertsonOxford: IRL Press, 71-112.

Ron,D., Kazanietz,M.G. (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J. *13*, 1658-1676.

Rorth,P. (2003). Communication by touch: role of cellular extensions in complex animals. Cell *112*, 595-598.

Rosner,H., Vacun,G. (1999). 1,2-dioctanoyl-s,n-glycerol-induced activation of protein kinase C results in striking, but reversible growth cone shape changes and an accumulation of f-actin and serine 41-phosphorylated GAP-43 in the axonal process. Eur.J.Cell Biol. *78*, 698-706.

Roy,L.M., Gittinger,C.K., Landreth,G.E. (1989). Characterization of the epidermal growth factor receptor associated with cytoskeletons of A431 cells. J.Cell Physiol *140*, 295-304.

Ruiz-Gomez,M., Coutts,N., Price,A., Taylor,M.V., Bate,M. (2000). Drosophila dumbfounded: a myoblast attractant essential for fusion. Cell *102*, 189-198.

Rushton,E., Drysdale,R., Abmayr,S.M., Michelson,A.M., Bate,M. (1995). Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development. Development *121*, 1979-1988.

Rustom,A., Saffrich,R., Markovic,I., Walther,P., Gerdes,H.H. (2004). Nanotubular highways for intercellular organelle transport. Science *303*, 1007-1010.

Rybakova,I.N., Amann,K.J., Ervasti,J.M. (1996). A new model for the interaction of dystrophin with F-actin. Journal of Cell Biology *135*, 661-672.

Sakane,F., Imai,S., Kai,M., Yasuda,S., Kanoh,H. (2007). Diacylglycerol kinases: why so many of them? Biochim.Biophys.Acta *1771*, 793-806.

Sakane,F., Imai,S., Yamada,K., Murakami,T., Tsushima,S., Kanoh,H. (2002). Alternative splicing of the human diacylglycerol kinase delta gene generates two isoforms differing in their expression patterns and in regulatory functions. The Journal of Biological Chemistry *277*, 43519-43526.

Samuels,M.L., Weber,M.J., Bishop,J.M., McMahon,M. (1993). Conditional transformation of cells and rapid activation of the mitogen- activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Mol.Cell Biol. *13*, 6241-6252.

Sander,E.E., van Delft,S., ten Klooster,J.P., Reid,T., van der Kammen,R.A., Michiels,F., Collard,J.G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. Journal of Cell Biology *143*, 1385-1398.

Sanders,L.C., Matsumura,F., Bokoch,G.M., de Lanerolle,P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. Science *283*, 2083-2085.

Sanes,J.R., Lichtman,J.W. (1999). Development of the vertebrate neuromuscular junction. Annu.Rev.Neurosci. *22*, 389-442.

Sanger,J.W., Holtzer,H. (1972). Cytochalasin B: effects on cell morphology, cell adhesion, and mucopolysaccharide synthesis (cultured cells-contractile microfilaments- glycoproteins-embryonic cells-sorting-out). Proc.Natl.Acad.Sci.U.S.A *69*, 253-257.

Sanger,J.W., Holtzer,S., Holtzer,H. (1971). Effects of cytochalasin B on muscle cells in tissue culture. Nat.New Biol. *229*, 121-123.

Sanjuan,M.A., Jones,D.R., Izquierdo,M., Merida,I. (2001). Role of Diacylglycerol Kinase alpha in the Attenuation of Receptor Signaling. Journal of Cell Biology *153*, 207-220.

Santos,T., Carrasco,S., Jones,D.R., Merida,I., Eguinoa,A. (2002). Dynamics of diacylglycerol kinase zeta translocation in living T-cells. Study of the structural domain requirements for translocation and activity. The Journal of Biological Chemistry *277*, 30300-30309.

Sarner,S., Kozma,R., Ahmed,S., Lim,L. (2000). Phosphatidylinositol 3-kinase, Cdc42, and Rac1 act downstream of Ras in integrin-dependent neurite outgrowth in N1E-115 neuroblastoma cells. Mol.Cell Biol. *20*, 158-172.

Sasaki,T., Takai,Y. (1998). The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control. Biochemical and Biophysical Research Communications *245*, 641-645.

Satoh,A., Huard,J., Labrecque,C., Tremblay,J.P. (1993). Use of fluorescent latex microspheres (FLMs) to follow the fate of transplanted myoblasts. J.Histochem.Cytochem. *41*, 1579-1582.

Satoh,T., Endo,M., Nakafuku,M., Akiyama,T., Yamamoto,T., Kaziro,Y. (1990). Accumulation of p21ras.GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. Proc.Natl.Acad.Sci.U.S.A *87*, 7926-7929.

Schliwa,M., Nakamura,T., Porter,K.R., Euteneuer,U. (1984). A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. Journal of Cell Biology *99*, 1045-1059.

Schultz,J., Hoffmuller,U., Krause,G., Ashurst,J., Macias,M.J., Schmieder,P., Schneider-Mergener,J., Oschkinat,H. (1998). Specific interactions between the syntrophin PDZ domain and voltage- gated sodium channels. Nat.Struct.Biol. *5*, 19-24.

Sechi,A.S., Wehland,J. (2000). The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. J.Cell Sci. *113 Pt 21*, 3685-3695.

Sekine,A., Fujiwara,M., Narumiya,S. (1989). Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. The Journal of Biological Chemistry *264*, 8602-8605.

Sells,M.A., Boyd,J.T., Chernoff,J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. Journal of Cell Biology *145*, 837-849.

Sells,M.A., Chernoff,J. (1997). Emerging from the Pak: the p21-activated protein kinase family. Trends Cell Biol. *7*, 162-167.

Sells,M.A., Knaus,U.G., Bagrodia,S., Ambrose,D.M., Bokoch,G.M., Chernoff,J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Curr.Biol. *7*, 202-210.

Sells,M.A., Pfaff,A., Chernoff,J. (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. Journal of Cell Biology *151*, 1449-1458.

Serafini,T., Kennedy,T.E., Galko,M.J., Mirzayan,C., Jessell,T.M., Tessier-Lavigne,M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell *78*, 409-424.

Seykora,J.T., Myat,M.M., Allen,L.A., Ravetch,J.V., Aderem,A. (1996). Molecular determinants of the myristoyl-electrostatic switch of MARCKS. The Journal of Biological Chemistry *271*, 18797-18802.

Sharma,M., Henderson,B.R. (2007). IQ-domain GTPase-activating protein 1 regulates beta-catenin at membrane ruffles and its role in macropinocytosis of N-cadherin and adenomatous polyposis coli. The Journal of Biological Chemistry *282*, 8545-8556.

Sharrocks,A.D., Yang,S.H., Galanis,A. (2000). Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem.Sci. *25*, 448-453.

Shcherbata,H.R., Yatsenko,A.S., Patterson,L., Sood,V.D., Nudel,U., Yaffe,D., Baker,D., Ruohola-Baker,H. (2007). Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. The EMBO Journal *26*, 481-493.

Sheng,M., Sala,C. (2001). PDZ domains and the organization of supramolecular complexes. Annu.Rev.Neurosci. *24*, 1-29.

Shi,L., Fu,W.Y., Hung,K.W., Porchetta,C., Hall,C., Fu,A.K., Ip,N.Y. (2007). Alpha2-chimaerin interacts with EphA4 and regulates EphA4-dependent growth cone collapse. Proc.Natl.Acad.Sci.U.S.A *104*, 16347-16352.

Shirai,Y., Segawa,S., Kuriyama,M., Goto,K., Sakai,N., Saito,N. (2000). Subtype-specific translocation of diacylglycerol kinase alpha and gamma and its correlation with protein kinase C. The Journal of Biological Chemistry *275*, 24760-24766.

Siddiqui,R.A., English,D. (1997). Phosphatidic acid elicits calcium mobilization and actin polymerization through a tyrosine kinase-dependent process in human neutrophils: a mechanism for induction of chemotaxis. Biochim.Biophys.Acta *1349*, 81-95.

Sistonen,L., Keski-Oja,J., Ulmanen,I., Holtta,E., Wikgren,B.J., Alitalo,K. (1987). Dose effects of transfected c-Ha-rasVal 12 oncogene in transformed cell clones. Exp.Cell Res. *168*, 518-530.

Slack-Davis,J.K., Eblen,S.T., Zecevic,M., Boerner,S.A., Tarcsafalvi,A., Diaz,H.B., Marshall,M.S., Weber,M.J., Parsons,J.T., Catling,A.D. (2003). PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. Journal of Cell Biology *162*, 281-291.

Small,J.V. (1988). The actin cytoskeleton. Electron Microsc.Rev. *1*, 155-174.

Small,J.V., Stradal,T., Vignal,E., Rottner,K. (2002). The lamellipodium: where motility begins. Trends Cell Biol. *12*, 112-120.

Songyang,Z., Fanning,A.S., Fu,C., Xu,J., Marfatia,S.M., Chishti,A.H., Crompton,A., Chan,A.C., Anderson,J.M., Cantley,L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science *275*, 73-77.

Straight,S.W., Pieczynski,J.N., Whiteman,E.L., Liu,C.J., Margolis,B. (2006). Mammalian lin-7 stabilizes polarity protein complexes. The Journal of Biological Chemistry *281*, 37738-37747.

Strunkelnberg,M., Bonengel,B., Moda,L.M., Hertenstein,A., de Couet,H.G., Ramos,R.G., Fischbach,K.F. (2001). rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. Development *128*, 4229-4239.

Suetsugu,S., Yamazaki,D., Kurisu,S., Takenawa,T. (2003). Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. Dev.Cell *5*, 595-609.

Swanson,J.A., Watts,C. (1995). Macropinocytosis. Trends Cell Biol. *5*, 424-428.

Tabellini,G., Bortul,R., Santi,S., Riccio,M., Baldini,G., Cappellini,A., Billi,A.M., Berezney,R., Ruggeri,A., Cocco,L., Martelli,A.M. (2003). Diacylglycerol kinase-theta is localized in the speckle domains of the nucleus. Exp.Cell Res. *287*, 143-154.

Takahashi,H., Takeishi,Y., Seidler,T., Arimoto,T., Akiyama,H., Hozumi,Y., Koyama,Y., Shishido,T., Tsunoda,Y., Niizeki,T., Nozaki,N., Abe,J., Hasenfuss,G., Goto,K., Kubota,I. (2005). Adenovirus-mediated overexpression of diacylglycerol kinase-zeta inhibits endothelin-1-induced cardiomyocyte hypertrophy. Circulation *111*, 1510-1516.

Takahashi,Y., Nakayama,T., Soma,M., Izumi,Y., Kanmatsuse,K. (1997). CA repeat polymorphism of the neuronal nitric oxide synthase gene. Human Heredity *47*, 58-59.

Takeda,M., Kagaya,Y., Takahashi,J., Sugie,T., Ohta,J., Watanabe,J., Shirato,K., Kondo,H., Goto,K. (2001). Gene expression and in situ localization of diacylglycerol kinase isozymes in normal and infarcted rat hearts: effects of captopril treatment. Circ.Res. *89*, 265-272.

Takeishi,Y., Goto,K., Kubota,I. (2007). Role of diacylglycerol kinase in cellular regulatory processes: a new regulator for cardiomyocyte hypertrophy. Pharmacol.Ther. *115*, 352-359.

Tang,Y., Zhou,H., Chen,A., Pittman,R.N., Field,J. (2000). The Akt proto-oncogene links Ras to Pak and cell survival signals. The Journal of Biological Chemistry *275*, 9106-9109.

Tatin,F., Varon,C., Genot,E., Moreau,V. (2006). A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. J.Cell Sci. *119*, 769-781.

ten Klooster,J.P., Hordijk,P.L. (2007). Targeting and localized signalling by small GTPases. Biology of the Cell *99*, 1-12.

Teruel,M.N., Meyer,T. (2000). Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. Cell *103*, 181-184.

Tognon,C.E., Kirk,H.E., Passmore,L.A., Whitehead,I.P., Der,C.J., Kay,R.J. (1998). Regulation of RasGRP via a phorbol ester-responsive C1 domain. Mol.Cell Biol. *18*, 6995-7008.

Tolias,K., Carpenter,C.L. (2000). In vitro interaction of phosphoinositide-4-phosphate 5-kinases with Rac. Methods Enzymol. *325*, 190-200.

Tolias,K.F., Couvillon,A.D., Cantley,L.C., Carpenter,C.L. (1998). Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. Mol.Cell Biol. *18*, 762-770.

Topham,M.K., Bunting,M., Zimmerman,G.A., McIntyre,T.M., Blackshear,P.J., Prescott,S.M. (1998). Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. Nature *394*, 697-700.

Topham,M.K., Prescott,S.M. (1999). Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. The Journal of Biological Chemistry *274*, 11447-11450.

Topham,M.K., Prescott,S.M. (2001). Diacylglycerol kinase zeta regulates Ras activation by a novel mechanism. Journal of Cell Biology *152*, 1135-1143.

Traynor,A.E. (1984). The relationship between neurite extension and phospholipid metabolism in PC12 cells. Brain Res. *316*, 205-210.

Troller,U., Raghunath,A., Larsson,C. (2004). A possible role for p190RhoGAP in PKCepsilon-induced morphological effects. Cell Signal. *16*, 245-252.

Tsushima,S., Kai,M., Yamada,K., Imai,S., Houkin,K., Kanoh,H., Sakane,F. (2004). Diacylglycerol kinase gamma serves as an upstream suppressor of Rac1 and lamellipodium formation. J Biol Chem. *279*, 28603-28613.

Tu,H., Wigler,M. (1999). Genetic evidence for Pak1 autoinhibition and its release by Cdc42. Mol.Cell Biol. *19*, 602-611.

Uberall,F., Hellbert,K., Kampfer,S., Maly,K., Villunger,A., Spitaler,M., Mwanjewe,J., Baier-Bitterlich,G., Baier,G., Grunicke,H.H. (1999). Evidence that atypical protein kinase C-lambda and atypical protein kinase C-zeta participate in Ras-mediated reorganization of the F-actin cytoskeleton. Journal of Cell Biology *144*, 413-425.

Ueyama,T., Eto,M., Kami,K., Tatsuno,T., Kobayashi,T., Shirai,Y., Lennartz,M.R., Takeya,R., Sumimoto,H., Saito,N. (2005). Isoform-specific membrane targeting mechanism of Rac during Fc gamma R-mediated phagocytosis: positive charge-dependent and independent targeting mechanism of Rac to the phagosome. J.Immunol. *175*, 2381-2390.

Ugolev,Y., Molshanski-Mor,S., Weinbaum,C., Pick,E. (2006). Liposomes comprising anionic but not neutral phospholipids cause dissociation of Rac(1 or 2) x RhoGDI complexes and support amphiphile-independent NADPH oxidase activation by such complexes. The Journal of Biological Chemistry *281*, 19204-19219.

Uhm,C.S., Neuhuber,B., Lowe,B., Crocker,V., Daniels,M.P. (2001). Synapse-forming axons and recombinant agrin induce microprocess formation on myotubes. J Neurosci *21*, 9678-9689.

Vaidya,T.B., Weyman,C.M., Teegarden,D., Ashendel,C.L., Taparowsky,E.J. (1991). Inhibition of myogenesis by the H-ras oncogene: implication of a role for protein kinase C. Journal of Cell Biology *114*, 809-820.

Van Aelst,L., D'Souza-Schorey,C. (1997). Rho GTPases and signaling networks. Genes and Development *11*, 2295-2322.

van Bergen en Henegouwen PM, Defize,L.H., de Kroon,J., van Damme,H., Verkleij,A.J., Boonstra,J. (1989). Ligand-induced association of epidermal growth factor receptor to the cytoskeleton of A431 cells. J.Cell Biochem. *39*, 455-465.

van Blitterswijk,W.J., Houssa,B. (2000). Properties and functions of diacylglycerol kinases. Cell Signal. *12*, 595-605.

van der Bend,R.L., de Widt,J., Hilkmann,H., van Blitterswijk,W.J. (1994). Diacylglycerol kinase in receptor-stimulated cells converts its substrate in a topologically restricted manner. The Journal of Biological Chemistry *269*, 4098-4102.

van Leeuwen,F.N., Kain,H.E., Kammen,R.A., Michiels,F., Kranenburg,O.W., Collard,J.G. (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. Journal of Cell Biology *139*, 797-807.

van Rheenen,J., Song,X., van Roosmalen,W., Cammer,M., Chen,X., Desmarais,V., Yip,S.C., Backer,J.M., Eddy,R.J., Condeelis,J.S. (2007). EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells. Journal of Cell Biology *179*, 1247-1259.

Vandebrouck,C., Martin,D., Colson-Van Schoor,M., Debaix,H., Gailly,P. (2002). Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers. J Cell Biol. *158*, 1089-1096.

Vasioukhin,V., Bauer,C., Yin,M., Fuchs,E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell *100*, 209-219.

Vidali,L., Chen,F., Cicchetti,G., Ohta,Y., Kwiatkowski,D.J. (2006). Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. Molecular Biology of the Cell *17*, 2377-2390.

Wagner,S., Flood,T.A., O'Reilly,P., Hume,K., Sabourin,L.A. (2002). Association of the Ste20-like kinase (SLK) with the microtubule. Role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading. The Journal of Biological Chemistry *277*, 37685-37692.

Wahrman,M.Z., Gagnier,S.E., Kobrin,D.R., Higgins,P.J., Augenlicht,L.H. (1985). Cellular and molecular changes in 3T3 cells transformed spontaneously or by DNA transfection. Tumour.Biol. *6*, 41-56.

Wakelam,M.J. (1983). Inositol phospholipid metabolism and myoblast fusion. Biochem.J *214*, 77-82.

Wakelam,M.J. (1985). The fusion of myoblasts. Biochem.J *228*, 1-12.

Wakelam,M.J., Pette,D. (1982). The breakdown of phosphatidylinositol in myoblasts stimulated to fuse by the addition of Ca2+. Biochem.J *202*, 723-729.

Walma,T., Aelen,J., Nabuurs,S.B., Oostendorp,M., van den,B.L., Hendriks,W., Vuister,G.W. (2004). A closed binding pocket and global destabilization modify the binding properties of an alternatively spliced form of the second PDZ domain of PTP-BL. Structure. *12*, 11-20.

Wang,J., Guo,K., Wills,K.N., Walsh,K. (1997). Rb functions to inhibit apoptosis during myocyte differentiation. Cancer Res. *57*, 351-354.

Wang,Z., Gluck,S., Zhang,L., Moran,M.F. (1998). Requirement for phospholipase C-gamma1 enzymatic activity in growth factor-induced mitogenesis. Mol.Cell Biol. *18*, 590-597.

Watanabe,H., Yamazaki,M., Miyazaki,H., Arikawa,C., Itoh,K., Sasaki,T., Maehama,T., Frohman,M.A., Kanaho,Y. (2004a). Phospholipase D2 functions as a downstream signaling molecule of MAP kinase pathway in L1-stimulated neurite outgrowth of cerebellar granule neurons. Journal of Neurochemistry *89*, 142-151.

Watanabe,H., Yokozeki,T., Yamazaki,M., Miyazaki,H., Sasaki,T., Maehama,T., Itoh,K., Frohman,M.A., Kanaho,Y. (2004b). Essential role for phospholipase D2 activation downstream of ERK MAP kinase in nerve growth factor-stimulated neurite outgrowth from PC12 cells. The Journal of Biological Chemistry *279*, 37870-37877.

Weeks,B.S., Wilson,P.J., Heffernan,C.C., Ahmad,A., Mahadeo,K. (1999). The role of protein kinase C in laminin-mediated neurite outgrowth. Biochemical and Biophysical Research Communications *256*, 98-103.

Weernink,P.A., Meletiadis,K., Hommeltenberg,S., Hinz,M., Ishihara,H., Schmidt,M., Jakobs,K.H. (2004). Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. The Journal of Biological Chemistry *279*, 7840-7849.

Wegmeyer,H., Egea,J., Rabe,N., Gezelius,H., Filosa,A., Enjin,A., Varoqueaux,F., Deininger,K., Schnutgen,F., Brose,N., Klein,R., Kullander,K., Betz,A. (2007). EphA4-

dependent axon guidance is mediated by the RacGAP alpha2-chimaerin. Neuron *55*, 756-767.

Welch,H., Eguinoa,A., Stephens,L.R., Hawkins,P.T. (1998). Protein kinase B and rac are activated in parallel within a phosphatidylinositide 3OH-kinase-controlled signaling pathway. The Journal of Biological Chemistry *273*, 11248-11256.

Wells,C.D., Fawcett,J.P., Traweger,A., Yamanaka,Y., Goudreault,M., Elder,K., Kulkarni,S., Gish,G., Virag,C., Lim,C., Colwill,K., Starostine,A., Metalnikov,P., Pawson,T. (2006). A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. Cell *125*, 535-548.

Weston,C., Yee,B., Hod,E., Prives,J. (2000). Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. Journal of Cell Biology *150*, 205-212.

Weston,C.A., Teressa,G., Weeks,B.S., Prives,J. (2007). Agrin and laminin induce acetylcholine receptor clustering by convergent, Rho GTPase-dependent signaling pathways. J.Cell Sci. *120*, 868-875.

Wiegant,F.A., Blok,F.J., Defize,L.H., Linnemans,W.A., Verkley,A.J., Boonstra,J. (1986). Epidermal growth factor receptors associated to cytoskeletal elements of epidermoid carcinoma (A431) cells. Journal of Cell Biology *103*, 87-94.

Williams,E.J., Furness,J., Walsh,F.S., Doherty,P. (1994). Characterisation of the second messenger pathway underlying neurite outgrowth stimulated by FGF. Development *120*, 1685-1693.

Williams,M.W., Bloch,R.J. (1999). Extensive but Coordinated Reorganization of the Membrane Skeleton in Myofibers of Dystrophic (mdx) Mice. Journal of Cell Biology *144*, 1259-1270.

Wojciak-Stothard,B., Ridley,A.J. (2003). Rho GTPases and the regulation of endothelial permeability. Vascul.Pharmacol. *39*, 187-199.

Wong,R., Hadjiyanni,I., Wei,H.C., Polevoy,G., McBride,R., Sem,K.P., Brill,J.A. (2005). PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes. Curr.Biol. *15*, 1401-1406.

Wrenn,R.W., Creazzo,T.L., Herman,L.E. (1996). Beta 1 integrin ligation stimulates tyrosine phosphorylation of phospholipase C gamma 1 and elevates intracellular Ca2+ in pancreatic acinar cells. Biochemical and Biophysical Research Communications *226*, 876-882.

Wright,T.M., Hoffman,R.D., Nishijima,J., Jakoi,L., Snyderman,R., Shin,H.S. (1988). Leukocyte chemoattraction by 1,2-diacylglycerol. Proc.Natl.Acad.Sci.U.S.A *85*, 1869-1873.

Wu,H., Feng,W., Chen,J., Chan,L.N., Huang,S., Zhang,M. (2007). PDZ domains of Par-3 as potential phosphoinositide signaling integrators. Mol.Cell *28*, 886-898.

Wymann,M.P., Schneiter,R. (2008). Lipid signalling in disease. Nat.Rev.Mol.Cell Biol. *9*, 162-176.

Xie,Y., Hong,Y., Ma,X.Y., Ren,X.R., Ackerman,S., Mei,L., Xiong,W.C. (2006). DCC-dependent phospholipase C signaling in netrin-1-induced neurite elongation. The Journal of Biological Chemistry *281*, 2605-2611.

Yakubchyk,Y., Abramovici,H., Maillet,J.C., Daher,E., Obagi,C., Parks,R.J., Topham,M.K., Gee,S.H. (2005). Regulation of Neurite Outgrowth in N1E-115 Cells through PDZ-Mediated Recruitment of Diacylglycerol Kinase {zeta}. Mol Cell Biol *25*, 7289-7302.

Yamaguchi,Y., Katoh,H., Yasui,H., Mori,K., Negishi,M. (2001). RhoA inhibits the nerve growth factor-induced Rac1 activation through Rho-associated kinase-dependent pathway. The Journal of Biological Chemistry *276*, 18977-18983.

Yamashita,T., Tohyama,M. (2003). The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat.Neurosci. *6*, 461-467.

Yan,J., Wen,W., Xu,W., Long,J.F., Adams,M.E., Froehner,S.C., Zhang,M. (2005). Structure of the split PH domain and distinct lipid-binding properties of the PH-PDZ supramodule of alpha-syntrophin. The EMBO Journal *24*, 3985-3995.

Yang,B., Jung,D., Rafael,J.A., Chamberlain,J.S., Campbell,K.P. (1995). Identification of alpha-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. The Journal of Biological Chemistry *270*, 4975-4978.

Yasuda,S., Kai,M., Imai,S., Kanoh,H., Sakane,F. (2007). Diacylglycerol kinase gamma interacts with and activates beta2-chimaerin, a Rac-specific GAP, in response to epidermal growth factor. FEBS Lett. *581*, 551-557.

Yasui,H., Katoh,H., Yamaguchi,Y., Aoki,J., Fujita,H., Mori,K., Negishi,M. (2001). Differential responses to nerve growth factor and epidermal growth factor in neurite outgrowth of PC12 cells are determined by Rac1 activation systems. The Journal of Biological Chemistry *276*, 15298-15305.

Yonemura,S., Itoh,M., Nagafuchi,A., Tsukita,S. (1995). Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells. J Cell Sci. *108 (Pt 1)*, 127-142.

Young,P.E., Richman,A.M., Ketchum,A.S., Kiehart,D.P. (1993). Morphogenesis in Drosophila requires nonmuscle myosin heavy chain function. Genes and Development *7*, 29-41.

Zeidman,R., Lofgren,B., Pahlman,S., Larsson,C. (1999). PKCepsilon, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. Journal of Cell Biology *145*, 713-726.

Zhang,M., Wang,W. (2003). Organization of signaling complexes by PDZ-domain scaffold proteins. Acc.Chem.Res. *36*, 530-538.

Zhao,Z.S., Manser,E., Loo,T.H., Lim,L. (2000). Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. Mol.Cell Biol. *20*, 6354-6363.

Zhong,X.P., Hainey,E.A., Olenchock,B.A., Jordan,M.S., Maltzman,J.S., Nichols,K.E., Shen,H., Koretzky,G.A. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. Nat Immunol. *4*, 882-890.

Zhou,B., Wang,Z.X., Zhao,Y., Brautigan,D.L., Zhang,Z.Y. (2002). The specificity of extracellular signal-regulated kinase 2 dephosphorylation by protein phosphatases. The Journal of Biological Chemistry *277*, 31818-31825.

Zhou,D., Luini,W., Bernasconi,S., Diomede,L., Salmona,M., Mantovani,A., Sozzani,S. (1995). Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. The Journal of Biological Chemistry *270*, 25549-25556.

Zicha,D., Dobbie,I.M., Holt,M.R., Monypenny,J., Soong,D.Y., Gray,C., Dunn,G.A. (2003). Rapid actin transport during cell protrusion. Science *300*, 142-145.

Zimmermann,P. (2006). The prevalence and significance of PDZ domain-phosphoinositide interactions. Biochim.Biophys.Acta *1761*, 947-956.

Zimmermann,P., Meerschaert,K., Reekmans,G., Leenaerts,I., Small,J.V., Vandekerckhove,J., David,G., Gettemans,J. (2002). PIP(2)-PDZ domain binding controls the association of syntenin with the plasma membrane. Mol.Cell *9*, 1215-1225.