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Phosphorylation of Diacylglycerol Kinase-C by Protein Kinase C Regulates its Interaction with the PDZ Domain of Syntrophins

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# Phosphorylation of Diacylglycerol Kinase-ζ by Protein Kinase C Regulates its Interaction with the PDZ Domain of Syntrophins

Elias Daher

Thesis submitted to the Faculty of Graduate
and Postdoctoral Studies in partial fulfillment
of the requirements for the degree of

**Master of Science**

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Department of Cellular and Molecular Medicine
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# Dedication

This work is dedicated to the memory of my mother, Doha, who passed away five years ago. She was the one who always encouraged me to explore the world of science, and nurtured the interest in the field in all of her children. I also dedicate the work to my son Joe, who was born during the course of my studies, as I plan to develop in him a sense of scientific curiosity.

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I wish to express my deepest gratitude to my supervisor, Dr. Stephen Gee, for admitting me into his lab after I was away from the field of biological sciences for more than 10 years. He opened the way for me to be back doing research in the fascinating domain of science. He was also very helpful and understanding along the way, and had a very hands-on involvement with the on-going projects in the lab, which was essential in guiding me through my project.

I also wish to thank our senior student in the lab, the PhD candidate Hanan Abramovici, for providing me with technical and experimental assistance when I first started my work, and for his invaluable comments on my project.

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As for my family, there are no words to describe the sacrifices of my wife, Rima, who has changed her entire world moving into a new country just to be supportive to me. She is sharing with me the risk of shifting my career back into science after a successful experience in business. I wish also to thank my brother, Roni, for his indefinite support, my sister, Gisele, for her strong encouragement, and my father, Youssef, the poor old man who waited three years for us to visit him.

# Abstract

Diacylglycerol kinase-zeta (DGK-ζ) attenuates diacylglycerol (DAG) signaling by converting it into phosphatidic acid (PA). DGK-ζ binds via its C-terminus, which contains a PSD-95/Discs-Large/ZO-1 (PDZ)-binding motif, to a family of PDZ domain-containing scaffold proteins called syntrophins. Previous studies showed that some PDZ-mediated interactions are regulated by phosphorylation of the C-terminal PDZ-binding motif; however, to my knowledge, there are no published reports which demonstrate that a phosphorylation outside of this motif regulates PDZ interactions. Here, I provide evidence that protein kinase C-mediated phosphorylation of the MARCKS domain of DGK-ζ increases its association with syntrophins by a PDZ-dependent mechanism. Compared to wild-type (wt) DGK-ζ, a mutant mimicking phosphorylation of the MARCKS domain (DGK-$\zeta^{M1}$) bound more to recombinant syntrophin PDZ domains in *in vitro* binding assays. Moreover, more endogenous syntrophin co-immunoprecipitated from lysates of COS cells infected with HA-tagged DGK-$\zeta^{M1}$ than with wt HA-DGK-ζ. Protein kinase C (PKC) activation by phorbol myristate acetate enhanced the interaction of wt DGK-ζ and syntrophin PDZ domains, an effect that was blocked by a specific PKC inhibitor. Consistent with the idea that the MARCKS domain regulates binding, deletion of this domain decreased binding to syntrophin PDZ domains. Surprisingly, phosphorylation-mimicking mutants of extracellular signal-regulated kinase phosphorylation sites closer to the C-terminus had no detectable effect on syntrophin binding. Collectively, my findings suggest PKC-mediated phosphorylation of the MARCKS domain regulates the PDZ-dependent interaction between DGK-ζ and syntrophins.

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## List of Abbreviations

| | |
|---|---|
| Anti-HA | : Anti-hemagglutinin |
| DAG | : Diacylglycerol |
| DAPC | : Dystrophin-associated protein complex |
| DGK-$\zeta$ | : Diacylglycerol kinase-zeta |
| DLG | : Drosophila discs-large |
| DMEM | : Dulbecco's modified Eagle's medium |
| DMD | : Duchenne muscular dystrophy |
| ERK | : Extracellular signal-regulated kinase |
| FBS | : Fetal Bovine Serum |
| Grb2 | : Growth factor receptor bound protein 2 |
| GST | : Glutathione S-Transferase |
| ICA512 | : Islet Cell Autoantigen 512 |
| InaD | : Inactivation-no-afterpotential-D |
| IPTG | : Isopropyl-1-thio-$\beta$-D-galactopyranoside |
| LB | : Luria Broth media |
| MARCKS | : Myristoylated alanine-rich C-kinase substrate |
| MOI | : Multiplicity of infection |
| NLS | : Nuclear localization signal |
| nNOS | : Neuronal nitric oxide synthase |
| NO | : Nitric oxide |
| PA | : Phosphatidic acid |
| PBS | : Phosphate buffered saline |
| PCR | : Polymerase chain reaction |
| PDZ | : PSD-95/Discs-Large/ZO-1 |
| PFU | : Plaque forming units |
| PH | : Pleckstrin homology |
| PI | : Phosphatidylinositol |
| PIP | : Phosphatidylinositol 4-phosphate |
| PIP2 | : Phosphatidylinositol 4,5-bisphosphate |

| | |
|---|---|
| PIP5K | : Phosphatidylinositol 4-phosphate 5-kinase |
| PKA | : Protein kinase A |
| PKC | : Protein kinase C |
| PLC | : Phospholipase C |
| PMA | : Phorbol 12-myristate 13-acetate |
| PSD-95 | : Postsynaptic density protein |
| SH | : Src homology |
| SDS-PAGE | : Sodium dodecyl sulfate – polyacrylamide gel electrophoresis |
| SU | : Syntrophin unique |
| TRP | : Transient receptor potential |
| ZO-1 | : Zona Occludens |

# Introduction

A cell is capable of generating biological responses necessary for growth and survival in response to specific stimuli in its environment, such as hormones, neurotransmitters, and growth factors (Barrera et al., 2005). The conversion of the extracellular signals into intracellular responses is mediated through signal transduction pathways that involve the functional coordination of receptors, second messengers, enzymes, and effector molecules (Pawson and Scott, 1997). These signaling components are organized into specific signaling pathways to ensure an adequate control of the enzymatic activity underlying signal transduction (Smith and Scott, 2002). This is achieved through regulatory mechanisms involving the confinement of broad specificity enzymes to distinct sub-cellular sites by the action of scaffold and adaptor proteins (Pawson and Scott, 1997).

### 1. Scaffold Proteins in Signal Transduction

Scaffold proteins are organizing molecules bringing various binding partners in proximity to construct intricate signaling complexes (Jordan et al., 2000). They contain multiple protein interaction domains, such as Src homology (SH), pleckstrin homology (PH), and PSD-95/Discs-Large/ZO-1 (PDZ) domains, responsible for clustering several types of molecules at specific subcellular compartments within the context of a signaling pathway (Fanning and Anderson, 1999a). The presence of different protein-protein interaction modules on a single molecule allows scaffold proteins to assemble macromolecular and large multi-protein complexes (Smith and Scott, 2002). It also

allows scaffold proteins to simultaneously recognize a group of signaling components through a distinct set of interaction modules, and a specific location within the cell through a different set (Jordan et al., 2000). This provides a mechanism for targeting and segregating the signaling proteins into discrete subcellular structures. Once localized to distinct sites, enzymes for example, gain a measure of selectivity in their action due to their controlled and limited access to a specific subset of substrates. As such, scaffold proteins provide a spatial dimension to signaling, thus contributing to the specificity of signal transduction (Burack and Shaw, 2000; Smith and Scott, 2002).

### 1.1 PDZ Domain-Containing Scaffold Proteins

PDZ domain-containing proteins are a major class of scaffold proteins involved in the aggregation of receptors and ion channels at the plasma membrane (Tsunoda et al., 1997; Garner et al., 2000; Nourry et al., 2003; Sierralta and Mendoza, 2004). PDZ domains were first described as a series of Glycine-Leucine-Glycine-Phenylalanine (GLGF) amino acid residue repeats in the postsynaptic density protein (PSD-95) and the cell junction-associated proteins discs-large (DLG) and Zona Occludens (ZO-1) (Kennedy 1995). Most PDZ-containing proteins have multiple PDZ domains, which facilitates the build-up of large macromolecular complexes (Sheng and Sala, 2001). A single PDZ protein could bind several copies of a particular protein in a linear array, thereby clustering them into a single complex (Fanning and Anderson, 1999b). In addition, it can serve as a scaffold for a multitude of molecules, since the individual PDZ domains within a PDZ protein often show distinct specificities (Fanning and Anderson, 1999b).

For example, inactivation-no-afterpotential-D (InaD), a *Drosophila* protein containing five PDZ domains, serves as a scaffold to assemble different components of the phototransduction pathway at the rabdomere (the site for phototransduction in photoreceptor neurons) (Figure 1) (Tsunoda et al., 1997; Ranganathan and Ross, 1997; Tsunoda and Zuker, 1999; Sheng and Sala, 2001). These components include the principal light-activated ion channel (transient receptor potential - TRP), the effector phospholipase C (PLC), and protein kinase C (PKC). In addition, the third and fourth PDZ domains of InaD can form heteromeric and homomeric complexes, allowing InaD to build a higher-order scaffold for the components of the phototransduction pathway at the plasma membrane (Fanning and Anderson, 1999b). Hence, PDZ-containing proteins can organize molecules in large complexes at the plasma membrane due to the presence of several PDZ domains on a single scaffold protein.

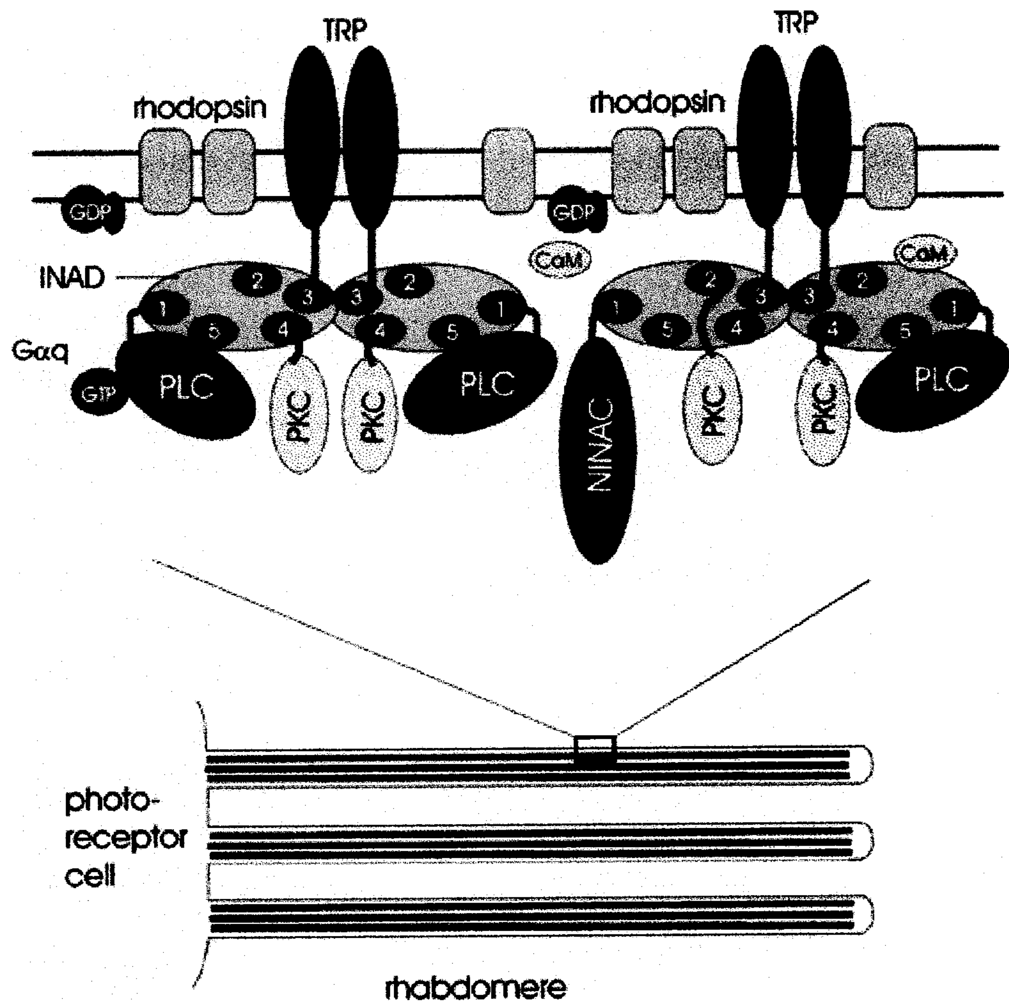
## 2. Syntrophins

### 2.1 Family of PDZ Domain-Containing Scaffold Proteins

PDZ-containing proteins may also assemble signaling complexes around a single PDZ domain, as with syntrophins for example. The syntrophin family is composed of five isoforms ($\alpha 1$, $\beta 1$, $\beta 2$, $\gamma 1$, & $\gamma 2$) that share a common domain structure (Adams et al., 2001). Each has two PH domains, one PDZ domain dividing the first N-terminus PH into two regions, and a C-terminal syntrophin unique (SU) domain (Figure 2) (Adams et al., 1993 and 1995; Ahn et al., 1994 and 1996; Piluso et al., 2000). Syntrophins are members of the dystrophin-associated protein complex (DAPC) that connects the actin cytoskeleton to the extracellular matrix in skeletal muscle (Figure 3) (Campbell and Kahl,

**Fig 1. Organization of the Phototransduction Complex by the Multi PDZ-Containing Protein InaD in *Drosophila* Photoreceptors.** A schematic of the photoreceptor cells and the rhabdomeres is shown at bottom. InaD binds to its major interacting proteins (TRP, PLC, PKC) beneath the plasma membrane, and multimerizes via its PDZ3/PDZ4 domains. The PDZ domains (numbered) are represented by red ovals.

# Figure 1




*From Sheng and Sala, 2001.*

**Fig 2. Domain Structure of Syntrophins.** Syntrophins share a common domain structure characterized by the presence of an N-terminus lipid-binding PH domain, split by a single PDZ domain, a second PH domain, and a C-terminus SU domain. The PDZ domain associates with proteins containing a PDZ-binding motif with the consensus sequence *X*-Serine/Threonine-*X*-Valine. The latter two domains bind dystrophin and also contain actin-binding sites.

# Figure 2

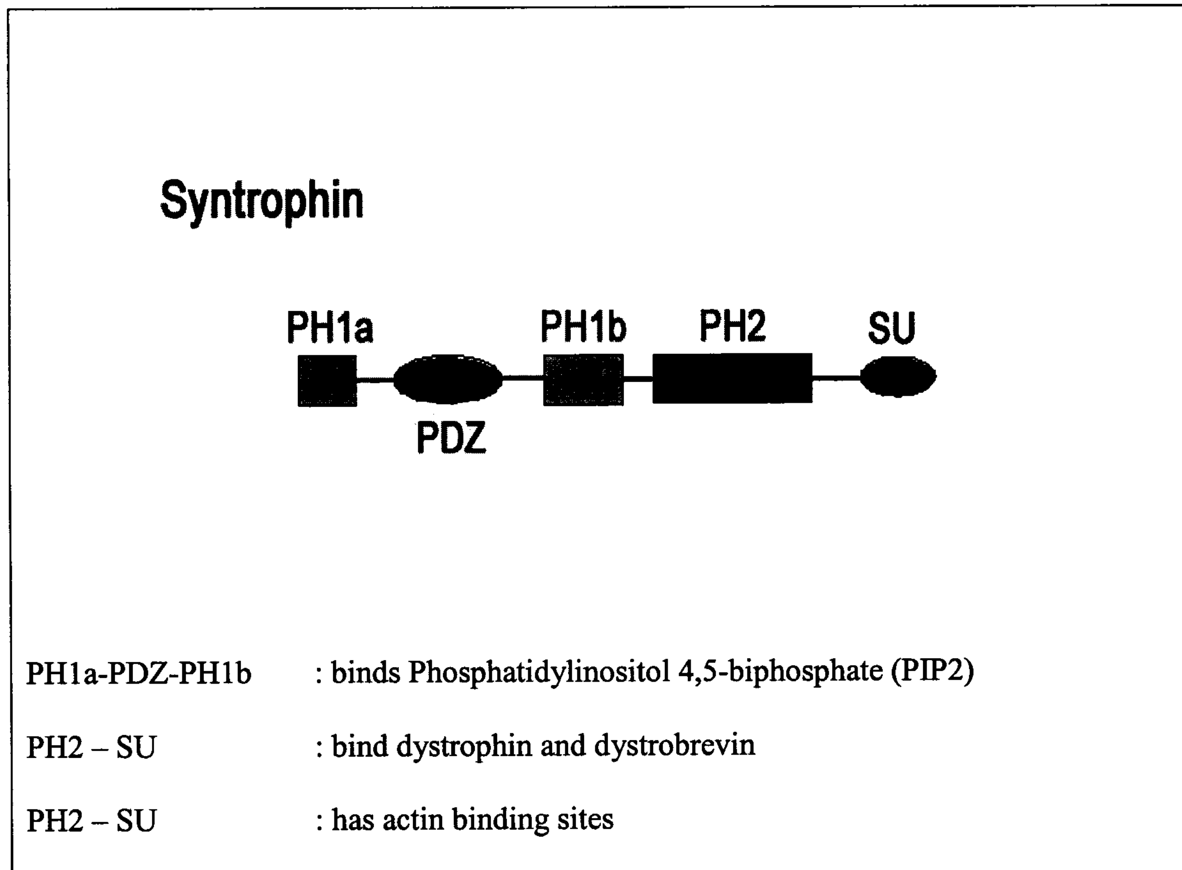
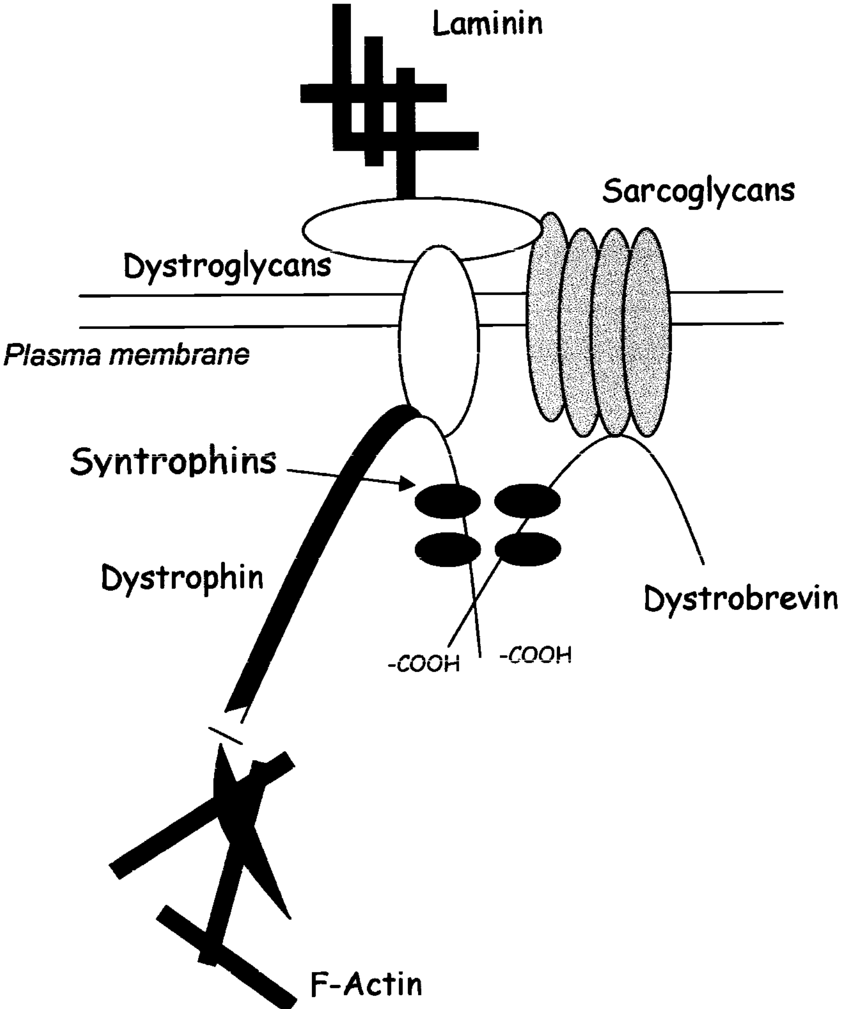


**Fig 3. Dystrophin-Associated Protein Complex.** A schematic of the DAPC linking the internal cytoskeleton to the extracellular matrix in skeletal muscle. Dystrophin binds F-actin beneath the plasma membrane through its N-terminus, and associates with other components of the DAPC through its C-terminus. The dystroglycans connect dystrophin to the extracellular matrix protein laminin across the sarcolemma. A dystrophin C-terminal homologue, dystrobrevin, binds to the C-terminus of dystrophin, and four syntrophin molecules bind to both dystrophin and dystrobrevin. Also shown are other DAPC molecules, such as the sarcoglycans.

# Figure 3



1989; Yoshida and Ozawa, 1990; Rybakova et al., 1996; Adams et al., 2001). The dystrophin protein and other members of the DAPC are also implicated in various forms of muscular dystrophies, when their genes are mutated (Hoffman et al., 1987; Koenig et al., 1988; Dalkilic and Kunkel, 2003; Nowak and Davies, 2004). Up to four syntrophins can bind to the DAPC: two can directly attach to one dystrophin, and two can associate with dystrobrevin, a protein that shows a significant protein sequence homology to the carboxyl terminus of dystrophin (Wagner et al., 1993; Yang et al., 1995a; Peters et al., 1997; Newey et al., 2000). Each syntrophin molecule interacts with dystrophin through its PH2 and SU domains, leaving the N-terminus PH containing the PDZ domain free to interact with other ligands. Hence, the interaction of four syntrophin molecules with the DAPC may allow the single PDZ-containing protein syntrophin to participate in the formation of macromolecular complexes near the plasma membrane (Ahn and Kunkel, 1995; Ahn et al., 1996; Adams et al., 2001).

## 2.2 The Dystrophin-Associated Protein Complex is Involved in Signaling

In addition to the role of the DAPC in protecting the plasma membrane against stresses developed during muscle contraction, by providing a mechanical support (Petrof et al., 1993), the complex is increasingly implicated in signaling functions through the interactions of its members with various signaling molecules (Rando 2001; Lapidos et al., 2004). The integral DAPC components include the dystroglycans, the sarcoglycans, the dystrobrevins, and the syntrophins (Figure 3) (Yoshida et al., 1994; Rando et al., 2001; Blake et al., 2002). The transmembranous proteins sarcoglycans were found to bind to the focal adhesion proteins integrins in myocytes. The complete disappearance of the

sarcoglycans resulted in a significant decrease in integrin levels, causing a reduction of cell adhesion, which suggests sarcoglycans play a role in intracellular signaling through the integrins (Yoshida et al., 1998). The sarcoglycans were also identified as interacting partners to a skeletal muscle-specific form of filamin (Thompson et al., 2000). Filamin family members regulate actin cytoskeletal assembly and are involved in signal transduction cascades associated with cell migration, adhesion, differentiation, and survival. This suggests sarcoglycans may be involved in downstream filamin signaling (Thompson et al., 2000; Rando 2001; Stossel et al., 2001; Blake et al., 2002). Other members of the DAPC, namely the dystroglycans, which bridge dystrophin to laminin across the plasma membrane (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Jung et al., 1995), bind another signaling protein, the growth factor receptor bound protein 2 (Grb2). Grb2 is involved in growth factor-induced cytoskeleton organization (Yang et al., 1995b). Thus, the DAPC components may have a signaling role through their associations with various signaling proteins in skeletal muscle.

Moreover, the expression of some of the DAPC molecules in other tissues, such as smooth muscle, brain, and liver, suggest they might be involved in signaling in those tissues rather than serving a structural role (Rando 2001). In addition to skeletal muscle, the dystroglycans are expressed in brain tissue, where they also associate with the signaling protein Grb2. They possibly play a role in Grb2-mediated signaling in the brain (Yang et al., 1995b; Rando 2001; Lapidos et al., 2004). The sarcoglycans are detected in smooth muscle. Sarcoglycan-deficient mice exhibited a disorder of vascular function resulting in abnormal blood flow in coronary arteries, correlating with the alteration of the sarcoglycan composition in the smooth muscle cells of coronary arteries (Coral-

Vazquez et al., 1999). The vascular dysfunction due to sarcoglycan deficiency may be related to a disruption of one of the signaling pathways regulating vascular tone in smooth muscle, implicating the sarcoglycans in signaling in this tissue (Rando 2001). Hence, the DAPC proteins may serve as structural platforms for signaling molecules in various tissues.

The syntrophins are expressed in a variety of tissues. $\alpha$1-syntrophin is most abundant in skeletal and cardiac muscle, while β-syntrophins are more widely distributed (Adams et al., 1993; Ahn et al., 1996; Peters et al., 1997; Jones et al., 2003). The $\gamma$2-isoform is found in skeletal muscle, while $\gamma$1-syntrophin is brain-specific (Piluso et al., 2000). The existence of syntrophins in muscle and non-muscle tissue suggests they may play structural as well as signaling roles in the different tissues (Adams et al., 2000 and 2004; Rando 2001).

### 2.3 Syntrophins Recruit Signaling Molecules to the Plasma Membrane

Syntrophins function as modular adaptors forming a scaffolding platform for the proper localization and positioning of their binding partners. They target signaling proteins to the plasma membrane, or link them to the DAPC, by PDZ-mediated interactions (Brenman et al., 1996; Gee et al., 1998; Adams et al., 2001). A well studied example is featured here, describing the role of syntrophins in mediating the membrane localization of neuronal nitric oxide synthase (nNOS) in skeletal muscle. nNOS catalyzes the production of nitric oxide (NO), which is a signaling molecule believed to be important for the maintenance of adequate blood flow to exercising muscles through its vasoregulatory function (Thomas et al., 1998 and 2003; Stamler and Meissner, 2001).

nNOS is abundantly expressed in skeletal muscle where it associates with the DAPC at the sarcolemma by binding to the PDZ domain of α1-syntrophin (Brenman et al., 1995 and 1996; Thomas et al., 2003). Kameya *et al.* (1999) have shown that nNOS was absent from the plasma membrane in α1-syntrophin knock-out mice, and Brenman *et al.* (1996) have demonstrated that nNOS isoforms lacking the PDZ domain, which normally heterodimerizes with the PDZ domain of α1-syntrophin (Hillier et al., 1999), do not localize to the sarcolemma in skeletal muscle. These observations suggest syntrophins are vital for the localization of nNOS to the sarcolemma.

A functional consequence was attributed to the sarcolemmal localization of nNOS by Thomas *et al.* (2003), as they revealed that the NO-dependent modulation of α-adrenergic vasoconstriction was greatly impaired in the contracting muscles of α1-syntrophin null mice compared with wild-type mice. This highlights the importance of the role of α1-syntrophin in properly localizing nNOS to initiate the NO-dependent signaling cascade at the plasma membrane in muscle.

Other studies suggest the interactions of syntrophins with signaling molecules through PDZ domains are also fundamental to membrane signaling in non-muscle cell types. α1-syntrophin associates with the brain water channel aquaporin-4 (AQP4) in perivascular membranes of astrocyte endfeet through a PDZ-mediated interaction (Neely et al., 2001). AQP4 contributes to brain water homeostasis and the formation of brain edema (Amiry-Moghaddam et al., 2003 and 2004). AQP4 expression has been reported to be markedly reduced in astrocyte endfeet membranes of α1-syntrophin knockout mice,

suggesting the PDZ domain of $\alpha$1-syntrophin is required for the sarcolemmal localization of the brain water channel (Neely et al., 2001).

### 2.4 Syntrophin Mutations do not Cause Muscular Dystrophy

As syntrophins are part of the DAPC, mutations in genes encoding the different syntrophin isoforms might contribute to the pathogenesis of muscular dystrophies. The most common form of muscular dystrophy is the X-linked recessive and lethal Duchenne muscular dystrophy (DMD) (Nowak and Davies, 2004; Nowak et al., 2005). The DAPC is destabilized in DMD, leading to secondary loss and reduced levels of its components at the sarcolemma (Ozawa et al., 1995; Straub et al., 1997; Blake et al., 2002). In addition, deficiencies in some of the DAPC components could constitute a primary cause for other forms of muscular dystrophies. For example, the disruption of sarcoglycans was associated with limb-girdle muscular dystrophies (Vainzof et al., 1996; Lim and Campbell, 1998), and the deficiency of $\alpha$-dystrobrevin has been linked to a mild form of the disease (Grady et al., 1999). Studies performed to date on syntrophin null-mice have not directly connected syntrophins to the development of any muscular dystrophy however (Kameya et al., 1999; Adams et al., 2000 and 2004). But mice deficient in $\alpha$1-syntrophin present abnormalities of the neuromuscular junction with secondary reductions or mislocalization of acetylcholine receptors (Adams et al., 2000; Hosaka et al., 2002). These findings suggest $\alpha$1-syntrophin may be important for receptor stabilization at synapses to ensure proper receptor signaling (Adams et al., 2000; Rando 2001). The syntrophins are therefore thought to be primarily involved in signal

transduction at the plasma membrane, and their absence was not shown to correlate with any structural dysfunction causing muscular dystrophies.

### 2.5 Syntrophins Interact with Diacylglycerol Kinase- zeta

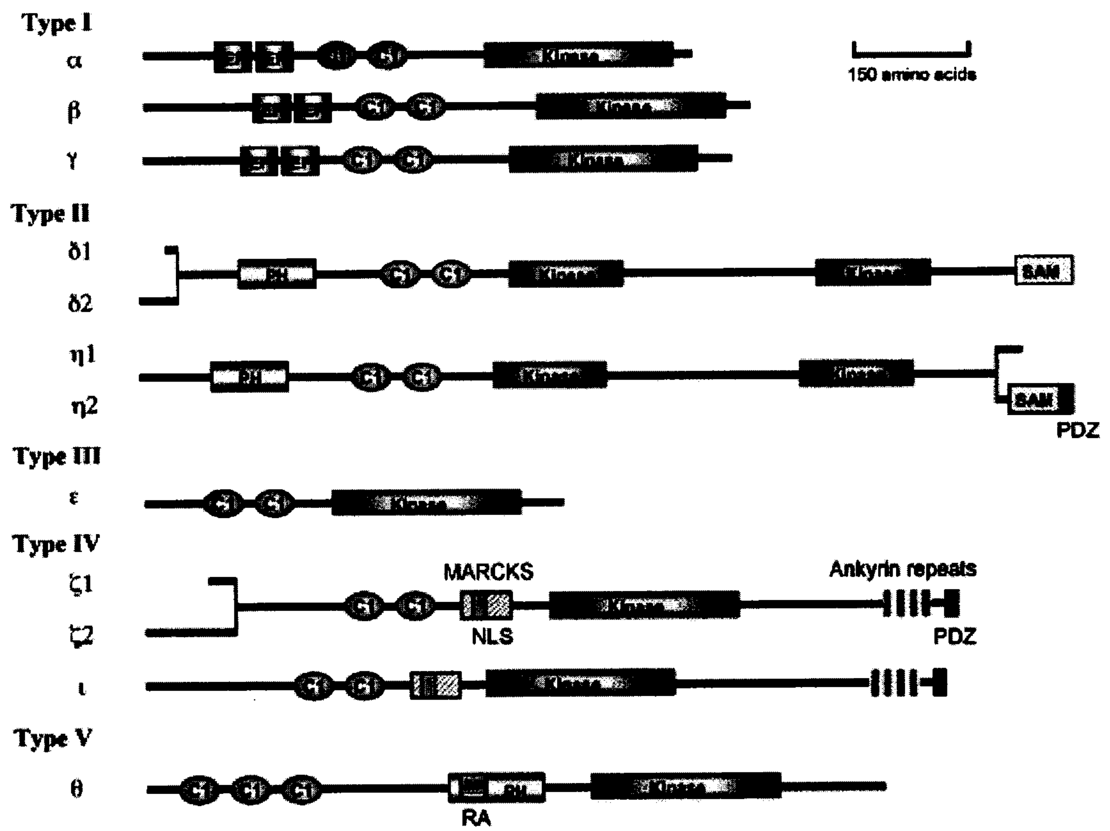
In order to identify new syntrophin binding partners that might be involved in cellular signaling, our lab screened a human brain cDNA library with the PDZ domain of γ1-syntrophin in a yeast two-hybrid assay. A specific interaction was found between the C-terminus of the lipid kinase diacylglycerol kinase-zeta (DGK-ζ), containing a consensus PDZ-binding motif, and the PDZ domain (Hogan et al., 2001).

DGK-ζ belongs to a family of nine mammalian isoforms that catalyze the phosphorylation of the second messenger diacylglycerol (DAG) to generate phosphatidic acid (PA) (Kanoh et al., 1990; Sakane et al., 1990; Bunting et al., 1996; Goto and Kondo, 2004; Luo et al., 2004b). All DGK isoforms contain a conserved catalytic domain in addition to zinc finger motifs rich in cysteine residues, similar to lipid-binding C1 domains found in PKC (Figure 4) (Luo et al., 2004b; Hodgkin et al., 1998). The C1 domains are thought to bind DAG but no conclusive evidence has been reported so far (Luo et al., 2004b).

DAG is one of the products of the hydrolysis of phosphatidylinositol 4,5-biphosphate ($PIP_2$) by PLC (Topham and Prescott, 1999; Parker 2004; Suh and Hille, 2005). DAG binds to and activates signaling proteins that contain cysteine-rich C1 domains, such as PKC (Hodgkin et al., 1998). PKCs are implicated in the regulation of a multitude of cellular functions, such as cell growth and proliferation, actin cytoskeleton

**Fig 4. Diacylglycerol Kinase Family.** Mammalian DGKs share the same catalytic domain in addition to zinc finger motifs similar to lipid-binding C1 domains found in PKC, but are classified under 5 types according to their different regulatory and binding motifs, shown here. DGK-ζ belongs to type IV characterized by the presence of a domain homologous to the phosphorylation site domain of the MARCKS protein (containing an NLS), ankyrin repeats, and a C-terminal PDZ-binding motif.

# Figure 4



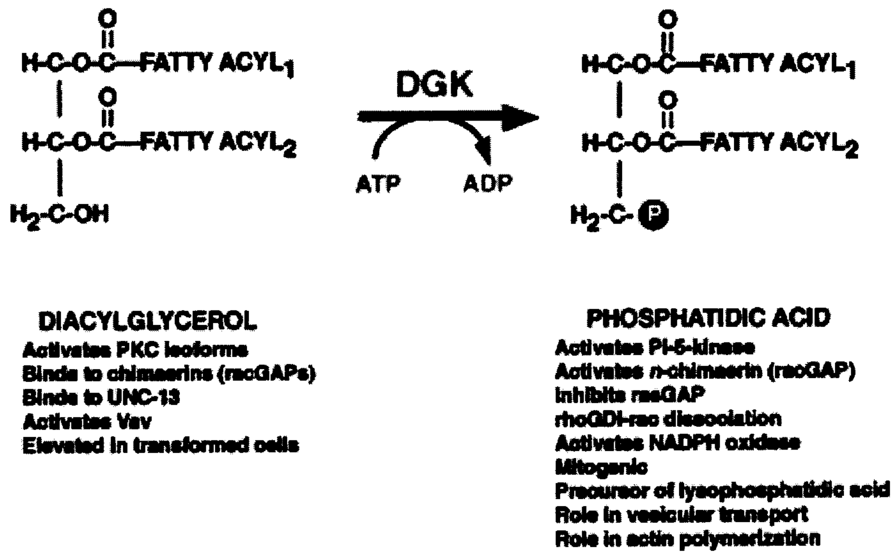

*From Luo et al., 2004b.*

remodelling, and apoptosis (Toker 1998; Topham et al., 1998; Luo et al., 2004b). Hence, the DAG – PKC signaling pathway is a fundamental mechanism for transducing extracellular signals (Newton 2004). Intracellular DAG levels must therefore be strictly regulated to ensure proper cellular responses. This is partly accomplished by DGKs, which attenuate the activity of DAG and consequently affect downstream PKC signaling. This was demonstrated by work of Luo *et al.* (2003a) showing that DGK-$\zeta$ inhibits PKC-$\alpha$ activity in cells in kinase assays.

DGKs are also involved in signal transduction through the production of PA, which regulates the activity of a variety of signaling molecules including phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Figure 5) (Jenkins et al., 1994; Topham and Prescott, 1999; Goto and Kondo, 2004). PIP5K catalyzes the conversion of phosphatidylinositol 4-phosphate (PIP) to yield PIP2 (Tolias et al., 1998; Luo et al., 2004a; Parker 2004). $PIP_2$ acts as a lipid switch that regulates local cellular activities at the plasma membrane, playing an important role in actin polymerization (Luo et al., 2004a; Suh and Hille, 2005). By generating PA, DGK-$\zeta$ stimulates PIP5K activity to increase the levels of PIP2 at the plasma membrane (Luo et al., 2004a). In summary, DGKs play a role in lipid-mediated cellular signaling through their catalytic activity. Therefore, they must be properly localized at the membrane close to DAG sources for adequate control of lipid signaling.

**Fig 5. Downstream Effectors of DAG and PA.** DGKs catalyze the phosphorylation of DAG to produce PA. DGKs may be involved in signal transduction through the reduction of DAG levels or the production of PA, which both regulate the activity of a variety of signaling molecules shown here.

# Figure 5




*From Topham and Prescott, 1999*

## 2.6 Syntrophins are Important for the Subcellular Localization of DGK-ζ

Syntrophins contribute to the recruitment of signaling molecules to specific subcellular domains through PDZ-mediated interactions (Adams et al., 2001). Previous studies by Hogan *et al.* have demonstrated that PDZ interactions regulate the subcellular localization of both DGK-ζ and syntrophin. The disruption of the association between DGK-ζ and γ1-syntrophin, by the introduction of DGK-ζ mutants that cannot bind to the PDZ domain, causes DGK-ζ to accumulate in the nucleus while syntrophin remains extranuclear (Hogan et al., 2001). In the nucleus, DGK-ζ reduces DAG levels and inhibits cell growth, as determined by DAG assays and the increase in the doubling time of cells transfected with DGK-ζ (Topham et al., 1998). These findings suggest the proper localization of DGK-ζ by the PDZ-mediated interaction with syntrophins is one mechanism for the regulation of DGK-ζ activity in the nucleus.

Syntrophin PDZ-mediated interaction with DGK-ζ was also found to have a role in DGK-ζ-induced neurite outgrowth. Overexpression of DGK-ζ in neuronal cells promoted neurite formation through a process dependent on a functional C-terminal PDZ-binding motif (Yakubchyk et al., 2005). DGK-ζ mutants with a blocked C-terminus were unable to induce neurite outgrowth, and also inhibited the process in cells induced for differentiation (Yakubchyk et al., 2005). This suggests the interaction with the PDZ domain of syntrophins may affect the role of DGK-ζ in the process of neurite formation.

Work by Abramovici *et al.* (2003) has also demonstrated that syntrophins seem to promote a more stable association of phosphorylated DGK-ζ with the plasma membrane. DGK-ζ was shown to be phosphorylated by PKC at its myristoylated alanine-rich C-

kinase substrate (MARCKS) domain (KASKKKKRASFKRKSSKK), located upstream of the catalytic domain (Figure 6) (Topham et al., 1998; Luo et al., 2003b). The co-expression of α1-syntrophin with a DGK-ζ mutant that mimics PKC-dependent phosphorylation at the MARCKS domain, led to a substantial increase in the membrane localization of both proteins compared with cells expressing either protein alone (Abramovici et al., 2003). Although DGK-ζ translocation to the plasma membrane is primarily dependent on PKC phosphorylation (Santos et al., 2002), its interaction with syntrophins may stabilize its membrane association (Abramovici et al., 2003).

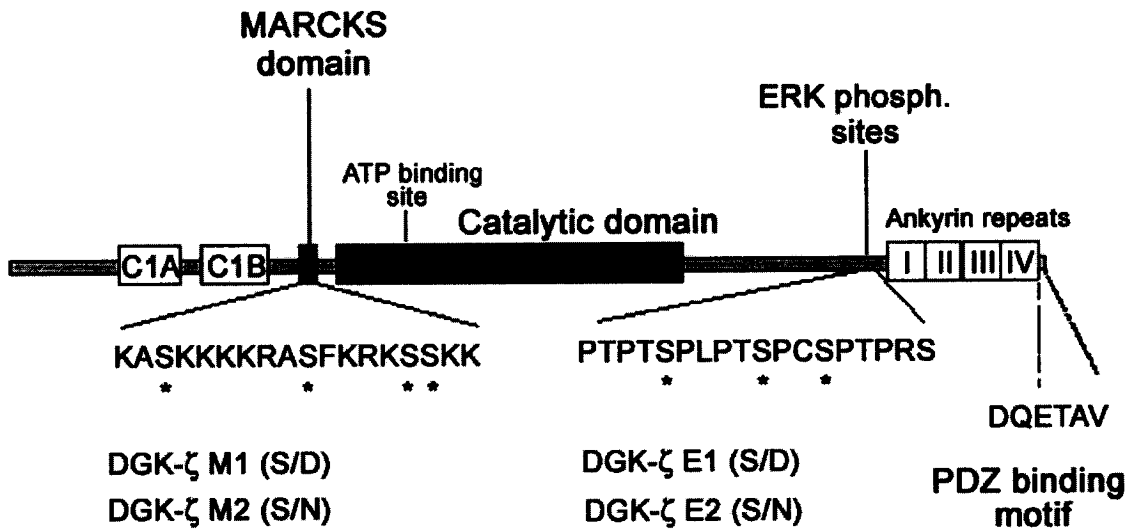
In conclusion, the PDZ-mediated interactions with syntrophins contribute to DGK-ζ localization and function. However, the regulatory mechanisms governing the PDZ-mediated association between DGK-ζ and syntrophins remain to be elucidated.

### 3. DGK-ζ Activity is Regulated by Phosphorylation

Protein phosphorylation is a general mechanism for regulating protein-protein interactions and enzyme activity underlying signal transduction (Pawson and Scott, 1997). Studies by Topham *et al.* (1998) have shown that PKC phosphorylation of DGK-ζ MARCKS domain regulates its intracellular location. The MARCKS motif of DGK-ζ contains a nuclear localization signal (NLS) but the co-expression of DGK-ζ with PKC-α or PKC-γ reduced nuclear DGK-ζ levels, and the down-regulation of PKC isoforms resulted in an increase in nuclear DGK-ζ, suggesting PKC phosphorylation excludes DGK-ζ from the nucleus (Topham et al., 1998). The phosphorylation at the MARCKS domain also causes DGK-ζ to translocate to the plasma membrane (Santos et al., 2002; Abramovici et al., 2003). Santos *et al.* (2002) have shown that DGK-ζ mutants, in which

**Fig 6. Schematic Structure of DGK-ζ Showing the Posphorylation Sites.** The N-terminus contains two domains homologous to the C1A and C1B motifs of PKC, a MARCKS homology domain, and a catalytic region containing an ATP binding site. The C-terminus contains ERK phosphorylation sites upstream of four tandem ankyrin repeats, and a consensus PDZ-binding motif (DQETAV). The schematic shows the sequence of the MARCKS domain, phosphorylated by PKC, and of the proline rich region upstream of the ankyrin repeats, phosphorylated by ERK. The asterisks indicate the serine residues that can be phosphorylated. DGK-ζ mutants that mimic PKC phosphorylation (M1 S/D) or ERK phosphorylation (E1 S/D), by replacing the serines with aspartates, are also shown with their control mutants, DGK-ζ M2 (S/N) and DGK-ζ E2 (S/N) respectively. The controls cannot be phosphorylated due to the replacement of serine with asparagine.

# Figure 6



phosphorylation of the MARCKS domain was either prevented or mimicked, localized differently in response to receptor stimulation. The mutant that cannot be phosphorylated prevented the translocation of DGK-ζ to the membrane, in contrast to the mutant that mimics the phosphorylation. Abramovici *et al.* (2003) showed that the mutant that mimics phosphorylation at DGK-ζ MARCKS domain was more membrane associated than wild-type DGK-ζ. Hence, the PKC-dependent phosphorylation of the MARCKS domain affects the sub-cellular localization of DGK-ζ.

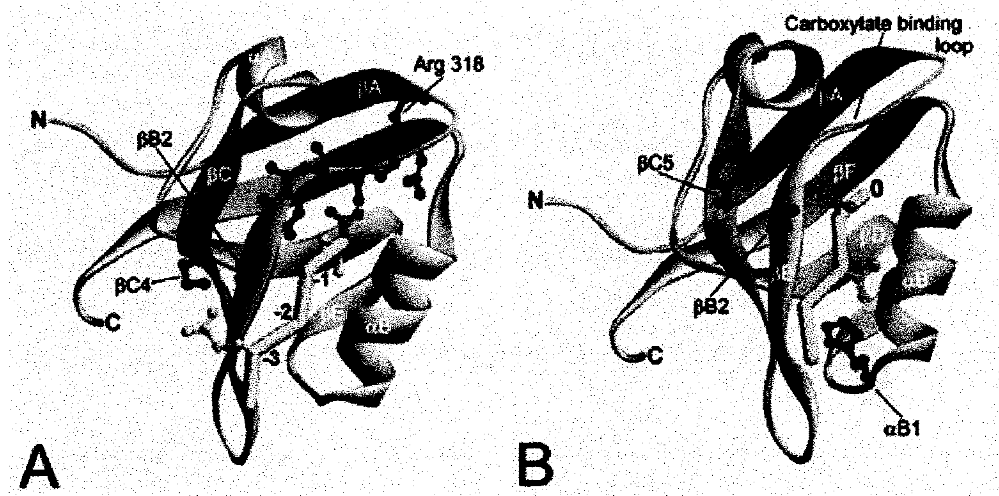
In addition, the phosphorylation of the MARCKS domain causes DGK-ζ to dissociate from PKC-α and inhibits its activity. This was demonstrated through the activation of PKC-α by phorbol 12-myristate 13-acetate (PMA), which impaired its association with DGK-ζ in immunoprecipitation experiments and reduced DGK-ζ activity in kinase assays. These effects were reversed by pre-treatment with a PKC inhibitor (Luo et al., 2003a and 2003b). Also, PKC phosphorylation of the MARCKS motif disrupts the binding between DGK-ζ and active Rac1, a Rho GTPase involved in actin cytoskeletal remodelling (Yakubchyk et al., 2005). An activated Rac1 mutant did not bind a DGK-ζ mutant that mimics phosphorylation at the MARCKS domain, and the association between wild-type DGK-ζ and the Rac1 mutant was abolished following PMA-induced PKC activation in pull-down assays (Yakubchyk et al., 2005). Therefore, it appears that PKC phosphorylation of DGK-ζ MARCKS domain also regulates the association of DGK-ζ with some of its binding partners.

## 4. Phosphorylation Regulates PDZ-Mediated Interactions

PDZ interactions are also regulated by phosphorylation (Kim and Sheng, 2004). The PDZ-mediated interactions involve a sequence-specific binding between a PDZ domain in one protein and a C-terminal PDZ binding motif (*X*-Serine/Threonine-*X*-Valine) in another protein. The specificity of the binding depends on the terminal carboxylate group of the ligand interacting with a hydrophobic pocket on the PDZ domain, formed by the GLGF consensus sequence (Figure 7). In addition, side chain interactions between the PDZ domain and the residue at the -2 position on the ligand (S/T) provide an additional level of binding regulation (Figure 7) (Doyle et al., 1996; Jelen et al., 2003; Zhang and Wang, 2003; Kim and Sheng, 2004). Phosphorylation of this residue usually inhibits the interaction with PDZ domains (Kim and Sheng, 2004). To illustrate, protein kinase A (PKA)-dependent phosphorylation of the C-terminal serine residue at the -2 position of the inwardly rectifying K+ channel Kir2.3 was reported to cause rapid dissociation of the channel from PSD-95 (Cohen et al., 1996). Also, the binding of Kir5.1 with PSD-95 was prevented by PKA-mediated phosphorylation of its carboxyl terminus (Tanemoto et al., 2002), and the phosphorylation of β1-adrenergic receptor by G-protein coupled receptor kinase 5 at the -2 position of its C-terminus attenuated its association with the PDZ domain of PSD-95 (Hu et al., 2002). These examples underline the role of the phosphorylation of a PDZ-binding motif in regulating the PDZ-mediated interactions.

**Fig 7. PDZ Domain Binding Specificity.** Structure of a PDZ domain complexed with a C-terminal peptide ligand, based on PDZ3 of PSD-95 complexed with CRIPT. The ribbon diagram of the PDZ domain (gray) is shown bound to the peptide ligand (main chain represented in yellow). (A) The free carboxylate group (orange) of the C-terminal residue (0 position) of the peptide interacts with the conserved amino acids (Gly-Leu-Gly-Phe) of the carboxylate binding loop (red). (B) The hydroxyl group of the -2 residue (threonine; light blue) interacts with the sidechain of the PDZ domain (histidine; dark blue).

# Figure 7




*From Sheng and Sala, 2001.*

The C–terminal PDZ-binding motif of DGK-ζ contains a threonine residue at the -2 position (DQETAV). According to the NetPhos2.0 server (www.cbs.dtu.dk), which predicts phosphorylation sites, the sequence of DGK-ζ PDZ-binding motif does not predict phosphorylation of the threonine residue, although this does not exclude the possibility that DGK-ζ might be phosphorylated at this location. However, recent studies showed that PDZ–mediated interactions can also be regulated by phosphorylations occurring outside the PDZ-binding motif (shown below); therefore, investigating this possibility for the DGK-ζ / syntrophin interaction is worth considering.

PDZ–mediated interactions can be regulated by phosphorylation of the PDZ domain itself, although this is less common. For instance, the phosphorylation of a PDZ domain on synapse-associated protein 97 by $Ca^{2+}$/calmodulin-dependent protein kinase II disrupted its interaction with the NR2A subunit of NMDA receptors (Gardoni et al., 2003). Another example demonstrates how phosphorylation of a PDZ-containing protein, namely β2-syntrophin, modulates the PDZ-mediated interaction with its binding partner, islet cell autoantigen (ICA) 512. ICA512 is a receptor tyrosine phosphatase-like protein associated with the secretory granules of endocrine cells, including insulin-secreting beta-cells of the pancreas. ICA512 was shown to bind to *β*2-syntrophin through a PDZ-mediated interaction (Ort et al., 2000). This interaction is thought to link the secretory granules with the actin cytoskeleton near the plasma membrane. Additional work by Ort *et al.* (2001) demonstrated that binding of ICA512 to *β*2-syntrophin protects ICA512 from cleavage, while their dissociation may constitute a mechanism for the mobilization of the secretory granules. They also showed that ICA512 preferentially binds a phosphorylated form of β2-syntrophin; however, the exact phosphorylation site was not

identified (Ort et al., 2001). The above studies report about regulatory mechanisms governed by the phosphorylation of either the PDZ domain or the PDZ-containing protein. No evidence was presented to date showing that a phosphorylation of the ligand outside its PDZ-binding motif can regulate a PDZ-mediated interaction. As DGK-ζ can be phosphorylated at two sites (Figure 7), one at the MARCKS domain by PKC and one near the C–terminus by extracellular signal-regulated kinase (ERK) (Abramovici et al., 2003), the examination of whether these phosphorylations affect DGK-ζ binding to syntrophin PDZ domains may help to identify a novel mechanism for the regulation of PDZ-mediated interactions.

# Hypothesis and Rationale

The PDZ-mediated interaction between syntrophins and DGK-ζ plays an important role in properly localizing DGK-ζ and regulating its activity (Hogan et al., 2001; Abramovici et al., 2003; Yakubchyk et al., 2005). One of the best-characterized functions of DGK-ζ is its involvement in lipid signaling through the attenuation of the levels of the second messenger DAG (Goto and Kondo, 2004). The identification of the regulatory mechanisms governing the PDZ-mediated association of DGK-ζ with syntrophins may shed some light on the possible signaling pathways involved in DGK-ζ activity. PDZ-mediated interactions are usually regulated by phosphorylation of the PDZ-binding motif of the ligand (Kim and Sheng, 2004), but no phosphorylation site was identified on the C-terminal PDZ-binding motif of DGK-ζ. How DGK-ζ interaction with syntrophins is regulated still remain to be identified. The PKC-dependent phosphorylation of the MARCKS domain, located outside the C-terminus of DGK-ζ, has been shown to regulate the interaction of the lipid kinase with some of its binding partners, such as PKC and Rac1 (Luo et al., 2003; Yakubchyk et al., 2005). I hypothesize that this phosphorylation also affects the interaction between DGK-ζ and syntrophins by regulating the binding of the C-terminus of DGK-ζ to the PDZ domain.

# Materials & Methods

## Reagents

FuGENE™ 6 transfection reagent was purchased from Roche Diagnostics Corp. (Indianapolis, IN). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Fetal Bovine Serum (FBS) was obtained from Wisent Canadian Laboratories (St-Bruno, QC). Glutathione-Sepharose 4B beads were from Amersham Biosciences AB (Uppsala, Sweden). Protein G-Agarose beads were from Upstate (Lake Placid, NY). Protease inhibitors were from BioShop Canada (Burlington, ON). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich (St-Louis, MO). Super Signal West Pico Chemiluminescent Substrate was from Pierce Laboratories (Rockford, IL). Restriction endonucleases were from New England Biolabs (Pickering, ON).

## Antibodies

Rabbit polyclonal anti-hemagglutinin (HA) was from Zymed Laboratories (San Francisco, CA), and mouse monoclonal anti-HA was from Sigma. Monoclonal antibodies against syntrophins (1351 and 2101) were gifts from Dr Stanley Froehner (University of Washington, Seattle). Horseradish Peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). AlexaFluor 488- and 594-conjugated goat anti-mouse and anti-rabbit secondary antibodies and AlexaFluor 488-conjugated phalloidin were purchased from Molecular Probes/Invitrogen (Carlsbad,

CA). Fluorescein isothiocyanate (FITC)-conjugated phalloidin was from Sigma-Aldrich (St. Louis, MO).

**Plasmids**

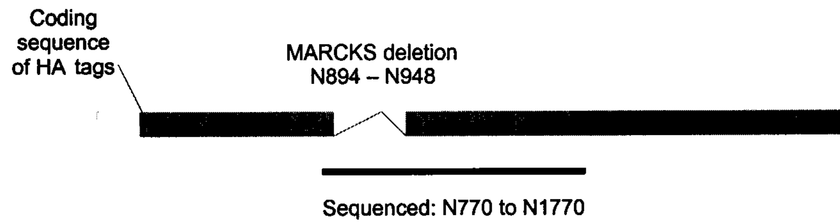
To delete the MARCKS domain from DGK-ζ, a pCMV-HA vector containing DGK-ζ was digested with EcoRI and AgeI. A 5.9kb fragment was isolated and band purified. This fragment contains the vector, three HA-tags, and a DGK-ζ C-terminal fragment downstream of AgeI (1.5kb). Two other DGK-ζ fragments were amplified by polymerase chain reaction (PCR). One fragment contains DGK-ζ N-terminus upstream of the MARCKS domain (0.8kb), and the other spans DGK-ζ downstream of MARCKS up to AgeI (0.5kb). The following primers were used for the PCR: sense (5'AGCTGCGGAATTGTACCCG) and antisense (5'ATATGGTACCCAGAGTATTCTGGGG) for the fragment upstream of MARCKS, and sense (5'ATATGGTACCGGGCCTGAGGAGG) and antisense (5'GCCTCTCGAGACTCGTGG) for the fragment downstream. A KpnI site was introduced in the primers (underlined) to ligate both PCRs. A triple ligation involved the vector fragment, including DGK-ζ C-term downstream of AgeI, and the two DGK-ζ amplified fragments, upstream and downstream of MARCKS, cut with EcoRI and AgeI respectively. The MARCKS deletion plasmid, HA-DGK-ζ ΔM, was verified by sequencing with specific primers (Figure 8).

To shuttle DGK-ζ E1 (S/D) and E2 (S/N) into an HA-tagged vector, the mutants were amplified by PCR from pcDNA1 plasmids. The primers used for the PCR, sense (5'ATATGAATTCGGATGGAGCCGCGGGACGGT) and antisense

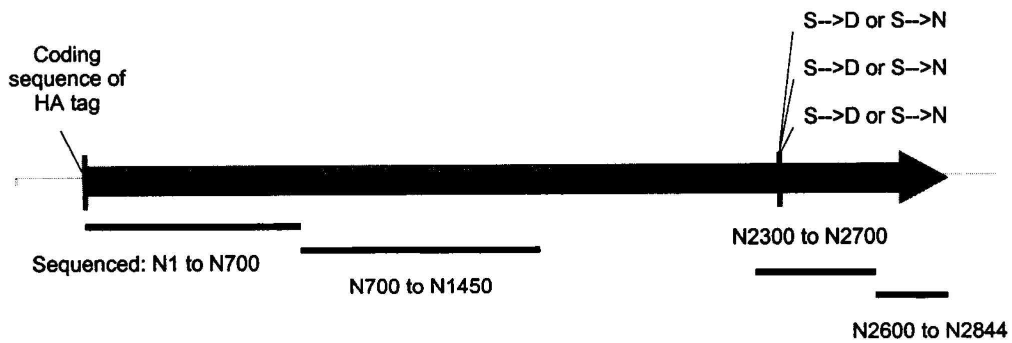
**Fig 8. Sequencing of DGK-ζ ΔM and DGK-ζ E1 (S/D) & E2 (S/N).** As in MATERIALS and METHODS.

# Figure 8

**Sequencing of HA-DGK-ζ ΔM (MARCKS deletion)**




**Sequencing of HA-DGK-ζ E1 (S/D) and HA-DGK-ζ E2 (S/N) - 2844bp**



(5'ATATCGGCCGCCTACACAGCCGTCTC), contain 5' EcoRI and 3' NotI sites (underlined) respectively. The pCMV-HA vector was cut with EcoRI and NotI restriction enzymes, and the digested PCR fragments were ligated into the vector at those sites. The constructed plasmids, HA-DGK-$\zeta$ E1 (S/D) and HA-DGK-$\zeta$ E2 (S/N), were verified by sequencing with specific primers (Figure 8).

**Cell Culture and Transient Transfections**

Cell lines (N1E-115 mouse neuroblastoma cells, NIH-3T3 mouse fibroblasts, Cos-7 monkey kidney cells) were grown on 100-mm dishes in DMEM high-glucose with 10% FBS, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine, to 70-80% confluence. Cells were then transfected with DGK-$\zeta$ WT, DGK-$\zeta$ M1 (S/D), DGK-$\zeta$ M2 (S/N), DGK-$\zeta$ E1 (S/D), DGK-$\zeta$ E2 (S/N), or full-length $\alpha$1-syntrophin using FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions (for 100-mm plates or 10 ml of DMEM: 6 μg of purified DNA was added to 9 μl of FuGENE 6 (3:2 ratio) diluted in serum-free DMEM to a final volume of 300 μl). Transfections proceeded for 18-24 h @ 37 C.

For pull-down assays, cells expressing DGK-$\zeta$ wild type were stimulated with 90nM PMA or vehicle for 30 min. Gö6976 (500 nM) was added 10 min before PMA stimulation, where indicated.

**Recombinant Adenoviral Vectors and Infection**

An adenoviral construct encoding DGK-$\zeta$ wild type or DGK-$\zeta$ M1 (S/D) with three tandem N-terminal HA epitope tags was used for infecting cells. A multiplicity of

infection (MOI) of 100 was used, which indicates the number of plaque forming units (PFU) added per cell. Equivalent amounts of adenoviral constructs were diluted in serum-free DMEM and added to the cells for 1hr @ 37 C. Cells were then incubated in 10% FBS, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine, for 24-48 hr @ 37 C prior to harvesting.

**Immunoprecipitation**

Cell cultures were washed twice with 10 ml of ice-cold phosphate buffered saline (PBS), pH 7.4, and then lysed with 1 ml of a hypotonic solution (50 mM Tris-HCl, pH 7.5, 20 mM NaCl, and protease inhibitors) per 100-mm dish for 15 min, scraped and collected on ice. Cells were then sonicated (power output 1, 50% duty cycle) with a Branson sonifier equipped with a 5-mm tip (Branson, Ultrasonics, Danbury, CT). To solubilize proteins, the detergent Triton X-100 was added to the samples to a final concentration of 1%. Afterwards, NaCl was added back to the cell lysate to a final concentration of 150mM before incubation with the antibody. Cell lysates were then centrifuged at 10,000 rpm for 10 min. The supernatant was collected, protein concentration was measured by the Bradford assay, and an aliquot of this starting material (input) was boiled in reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The appropriate antibody (5μg) was added to 1500μg of supernatant from different samples and incubated at 4°C with mixing for 2 h or overnight. Then, 75 μl of protein G agarose beads was 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 the lysis buffer containing 1% Triton X-100, 150mM NaCl, and 50mM Tris-HCl pH 7.5. Proteins were eluted from the beads by

boiling in reducing SDS-PAGE sample buffer. The samples were then centrifuged at 21,000 x g for 1-2 min and analyzed by SDS-PAGE and Western blotting for bound DGK-ζ or syntrophins.

**Pull-Down Assays**

Cell cultures were lysed with a hypotonic solution (50 mM Tris-HCl, pH 7.5, 20 mM NaCl, and protease inhibitors) for 15 min, scraped and collected on ice. Cells were then sonicated and Triton X-100 was added to the samples to a final concentration of 1%. Sodium chloride was added back to the cell lysate to a final concentration of 150mM, to regain the physiological salt concentration level before incubation with the fusion protein. Cell lysates were then centrifuged at 10,000 rpm for 10 min. The supernatant was collected, protein concentration was measured by the Bradford assay, and an aliquot of this starting material (input) was boiled in reducing SDS-PAGE sample buffer. Equivalent amounts of Glutathione S-Transferase (GST) and GST PDZ fusion proteins conjugated to Glutathione-Sepharose 4B beads were incubated with 1000μg of supernatant for 3 hrs on a rocker at 4°C. The samples were centrifuged and the collected beads were washed 3 x 10 min with the lysis buffer (1% Triton X-100, 150mM NaCl, and 50mM Tris-HCl pH 7.5). The bound proteins were eluted in 75μl of reducing SDS-PAGE sample buffer and boiled for 5 min. The samples were then analyzed by SDS-PAGE and immunoblotting.

**Purification of GST Fusion Proteins**

BL21 competent bacterial cells were transformed with the pGEX-5X-1 vector (Amersham Biosciences), containing syntrophin α1-, γ1-, β1-, & β2- PDZ domains and a

sequence encoding GST, and were grown overnight at 37 °C on LB-ampicillin-chloramphenicol (LB-Amp-Chlor) plates. Luria Broth (LB) medium (0.5 liter) supplemented with 200 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated with a single colony, and the bacterial culture was incubated at 37°C with shaking until the optical density at 600 nm reached between 0.6 and 1.0. Protein expression was then induced with 1mM IPTG (isopropyl-1-thio-$\beta$-D-galactopyranoside) (BioShop, Canada, Inc., Burlington, Canada), and the culture was incubated overnight at 28 °C or for 3-4 hours at 37°C. The cells were harvested by centrifugation at 6000 x g for 10 minutes and subsequently resuspended in 20ml of ice-cold PBS. Protease inhibitors were then added (1mM Benzamidine, 10$\mu$m Leupeptin, 1mM AEBSF, 1$\mu$M Pepstatin A), and bacterial cells were lysed with 100μl lysozyme followed by sonication (power output 5-6, duty cycle at constant; 15 sec blasts), and the addition of 1% Triton X-100. The homogenate was then centrifuged at 16,500 rpm for 30 minutes at 4°C. Glutathione-Sepharose beads (2ml of 50% slurry) were then incubated with the supernatant for 1-2 hr on a rocker at 4°C. Beads were centrifuged, washed with PBS, and resuspended in PBS containing 20% glycerol. The purified GST fusion proteins were run on SDS-PAGE and their purities were determined by Coomassie blue staining.

**Subcellular Fractionation**

NIH-3T3 fibroblasts cultured in 100-mm dishes were washed three times with ice-cold PBS, scraped into 1 ml of PBS, and then centrifuged (650 x g for 5 min). The cells were resuspended in 200 μl of lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and protease inhibitors) and sonicated by 2 x 15 sec blasts (power

output 1, 50% duty cycle). An aliquot, which represents the total fraction, was removed and boiled in reducing SDS-PAGE sample buffer. The lysates were then centrifuged at 14,000 x g for 10 min at 4°C to remove nuclei and cell debris. Afterwards, the supernatant was collected and centrifuged at 100,000 x g for 1 h at 4°C. The cytosol fraction was removed and the pellet was resuspended in the lysis buffer containing 1% Triton X-100, and then centrifuged at 100,000 x g for 1 h at 4°C. The membrane fraction was collected and the pellet (cytoskeleton fraction) was resuspended. All fractions were boiled in reducing SDS-PAGE sample buffer for 5 min, centrifuged at 21,000 x g for 1-2 min, and analyzed by SDS-PAGE and Western blotting.

**Quantification of Western Blots by Densitometry**

Western blots were analyzed by enhanced chemiluminescent (ECL) detection using the Kodak Digital Science Image Station 440. Membranes from western blots were submerged in the ECL substrate (Luminol) solution to allow the HRP-conjugated antibodies to catalyse the chemiluminescent reaction. Light signals emitted were captured by the digital camera and the image was analysed by densitometry. Densitometry evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Net intensities of the different immunoreactive protein bands were measured by densitometry, and specific ratios were computed against the net intensities of their respective input bands. The net intensity represents the sum of the background-subtracted pixel values in the protein band rectangle. The ratios of the different samples were then ploted using SigmaPlot (mean values and standard error). One-tail or two-tail Student t-tests were used for statistical analysis.

# RESULTS

## 1. Altering the Serine Residues in DGK-ζ MARCKS Domain Affects Binding to the PDZ Domain of α1-Syntrophin

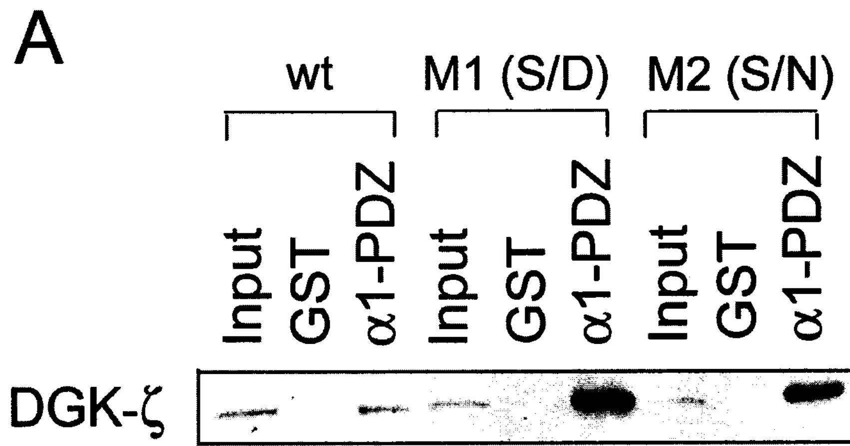
DGK-ζ is phosphorylated at the MARCKS domain by PKC, which causes the dissociation of the two proteins and a reduction of DGK-ζ activity (Luo et al., 2003a and 2003b). To determine whether phosphorylation at this domain affects the interaction between DGK-ζ and syntrophins, the ability of wild-type DGK-ζ (WT) and DGK-ζ M1 (S/D) to bind to the PDZ domain of α1-syntrophin was compared in pull-down assays. DGK-ζ M1 (S/D) is a mutant in which the four serines within the MARCKS domain were changed to aspartates. These negatively charged residues mimic phosphorylation. Lysates of N1E-115 neuroblastoma cells transiently transfected with either wild-type DGK-ζ or DGK-ζ M1 (S/D) were incubated with a GST fusion protein of the α1-syntrophin PDZ domain (α1-PDZ), and the amount of bound DGK-ζ was detected by immunoblotting (Figure 9A). The signals from the immunoreactive bands were quantified by densitometry and the results from three separate experiments were averaged. The data show that DGK-ζ M1 (S/D) binds 2.5-fold more strongly to α1-PDZ GST fusion protein than wild-type DGK-ζ (Figure 9B). These results suggest mimicking phosphorylation of the MARCKS domain increases the association of DGK-ζ and the PDZ domain of α1-syntrophin.

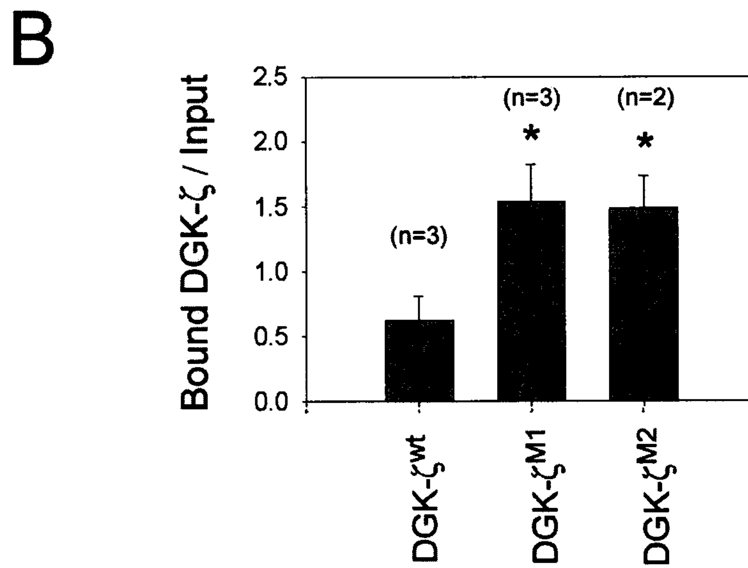
As a control, a DGK-ζ mutant in which the serines at the MARCKS domain were replaced by asparagines, to prevent phosphorylation (DGK-ζ M2 (S/N)), was tested in the

pull-down assay. Asparagine is similar in structure to aspartate, but has no negative charge. Surprisingly, this mutant bound to GST-$\alpha$1-PDZ as much as DGK-$\zeta$ M1 (S/D) (Figure 9A and 9B). These results raise the possibility that a slight modification of the serine residues in the MARCKS domain affects DGK-$\zeta$ binding to the PDZ domain of $\alpha$1-syntrophin. However, only a modification that is caused by a biochemical signal, such as phosphorylation, is relevant in a physiological setting. The idea that phosphorylation at the MARCKS domain does indeed influence the interaction between DGK-$\zeta$ and syntrophin PDZ domains is further explored by inducing phosphorylation and testing for an increase in the strength of binding in in-vitro binding assays.

**Fig 9. Replacing the Serine Residues in DGK-ζ MARCKS Domain with Aspartates or Asparagines Affects Binding to α1-Syntrophin PDZ Domain.** (A) Pull-down of HA-tagged wild-type DGK-ζ, DGK-ζ M1 (S/D), or DGK-ζ M2 (S/N) with the PDZ domain of α1-syntrophin. Beads charged with GST alone or with a GST fusion protein of the α1-syntrophin PDZ domain (α1-PDZ) were incubated with detergent extracts of N1E-115 cells transiently transfected with wild-type DGK-ζ, DGK-ζ M1 (S/D), or DGK-ζ M2 (S/N). Equivalent amounts of bound proteins were loaded on SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting with an anti-HA antibody. The input lane was loaded with 2% of the extract used for the pull-down. (B) Quantification of bands from western blotting by densitometry: Bars represent the ratio of the amount of DGK-ζ bound to α1-PDZ relative to the amount of DGK-ζ expressed in each sample, in arbitrary units. The values are the average of either two or three independent experiments, as specified. An asterisk indicates a statistically significant difference from the wild type ($P < 0.05$, two-tailed $t$ test).

**Figure 9**



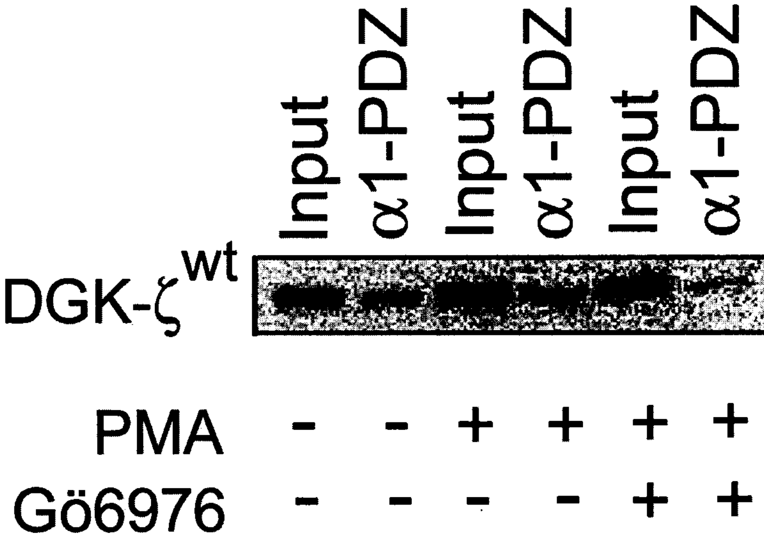



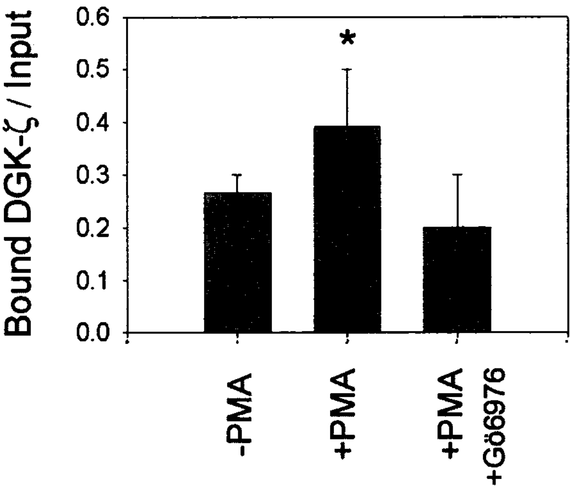
**2. PKC-Dependent Phosphorylation of DGK-ζ MARCKS Domain Increases DGK-ζ Binding to α1-Syntrophin PDZ Domain**

Since the four serine residues of the MARCKS domain of DGK-ζ that were modified in the previous experiment are the predominant sites for PKC-α phosphorylation (Figure 6) (Topham et al., 1998; Luo et al., 2003a and 2003b), pull-down assays were performed to determine whether the binding between syntrophins and DGK-ζ is regulated by this phosphorylation. N1E-115 cells infected with an adenovirus bearing HA-tagged wild-type DGK-ζ were stimulated with PMA, a potent activator of PKC, or vehicle for 30 min prior to harvesting. Detergent-solubilized DGK-ζ from stimulated and unstimulated cells was used in pull-down assays with GST-α1-PDZ. Western blots of bound proteins using an anti-HA antibody revealed that DGK-ζ binding to the PDZ domain of α1-syntrophin increases following PMA stimulation of cells, as measured by densitometry (Figure 10A and 10B). A PKC inhibitor (Gö 6976) applied prior to PMA stimulation reduced the interaction, demonstrating that PKC activity is required (Figure 10A and 10B). These results suggest the PDZ-mediated interaction of DGK-ζ with α1-syntrophin is regulated by PKC-dependent phosphorylation of DGK-ζ MARCKS domain.

**Fig 10. PKC Activation Increases the Association of DGK-ζ with Syntrophin PDZ Domains.** (A) Pull-down of HA-tagged wild-type DGK-ζ with α1-syntrophin PDZ domain. N1E-115 cells infected with an adenoviral vector encoding wild-type DGK-ζ were stimulated with 90nM PMA (a potent PKC activator) for 30 min prior to harvesting. Beads charged with GST alone or with GST-α1-PDZ were incubated with detergent extracts of PMA-stimulated or unstimulated cells. The PKC inhibitor Gö 6976 was added 10 min before PMA stimulation to one of the cultures as a control. Bound proteins were immunoblotted with an anti-HA antibody. Input represents 2% of the extract used for the pull-down. (B) Quantification of bands from western blotting by densitometry: Bars represent the ratio of DGK-ζ bound to α1-PDZ relative to the amount of DGK-ζ expressed in each sample, in arbitrary units. The values are the average of three independent experiments. An asterisk indicates a statistically significant difference from the PMA-unstimulated sample ($P < 0.05$, one-tailed $t$ test).

**Figure 10**



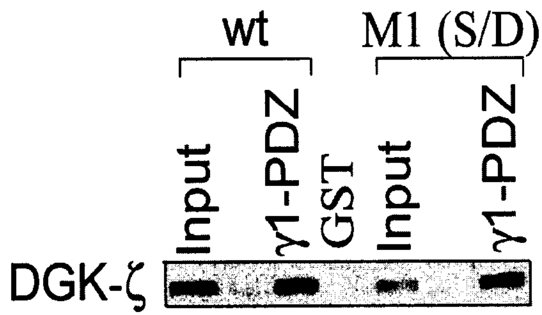
### 3. The Stronger Association between MARCKS-Phosphorylated DGK-ζ and Syntrophin PDZ Domains is not Isoform-Specific

The PDZ domains of the syntrophin isoforms differ slightly in their amino acid composition, which suggests their binding to DGK-ζ may be regulated differently. The PDZ domain of the γ1-isoform is most distantly related to α1-syntrophin PDZ, especially in the sequence of the carboxylate binding groove that is vital for the interaction with PDZ binding motifs. A GST fusion protein of the PDZ domain of γ1-syntrophin (γ1-PDZ) was tested in pull-down assays to determine whether phosphorylation of DGK-ζ MARCKS domain also increases DGK-ζ binding to γ1-syntrophin. Densitometric analysis of western blots showed that DGK-ζ M1 (S/D) bound more strongly than wild-type DGK-ζ to γ1-PDZ (Figure 11A and 11B). In addition, PMA stimulation of cells infected with wild-type DGK-ζ, to induce PKC phosphorylation, also increased DGK-ζ binding to γ1-PDZ (Figure 11C). These findings suggest the stronger association between MARCKS-phosphorylated DGK-ζ and syntrophin PDZ domains does not depend on the syntrophin isoform.

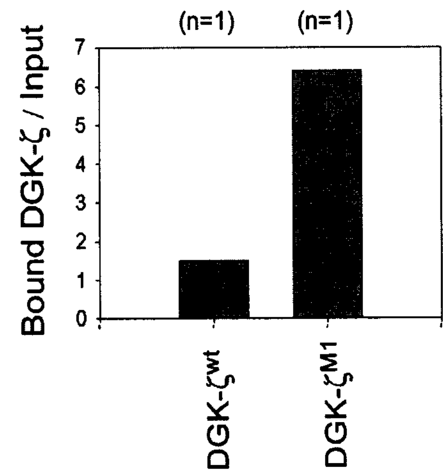
**Fig 11. MARCKS-Phosphorylated DGK-ζ Binds Stronger than the Wild-Type Control to the PDZ Domain of γ1-Syntrophin.** (A) Pull-down of HA-tagged wild-type DGK-ζ or DGK-ζ M1 (S/D) with the PDZ domain of γ1-syntrophin. Beads charged with GST alone or with a GST fusion protein of the γ1-syntrophin PDZ (γ1-PDZ) were incubated with detergent extracts of N1E-115 cells infected with wild-type DGK-ζ or DGK-ζ M1 (S/D). Bound proteins were analyzed by immunoblotting with an anti-HA antibody. Input represents 2% of the extract used for the pull-down. (B) Quantification of bands from western blotting by densitometry: Bars represent the ratio of the amount of DGK-ζ bound to γ1-PDZ relative to the amount of DGK-ζ expressed in each sample, in arbitrary units. The values are taken from a single experiment. (C) Pull-down of HA-tagged wild-type DGK-ζ with γ1-syntrophin or α1-syntrophin PDZ domain. N1E-115 cells infected with wild-type DGK-ζ were treated with 90nM PMA (a potent PKC activator) for 30 min prior to harvesting. Beads charged with GST alone, with GST-γ1-PDZ, or with GST-α1-PDZ (as an additional control) were incubated with detergent extracts of PMA-treated or untreated cells. The PKC inhibitor Gö 6976 was added to one of the cultures as a control. Bound proteins were immunoblotted with an anti-HA antibody. Input represents 2% of the starting material.

**Figure 11**

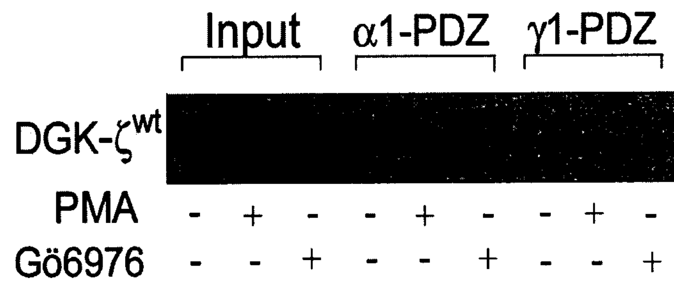
A




B




C

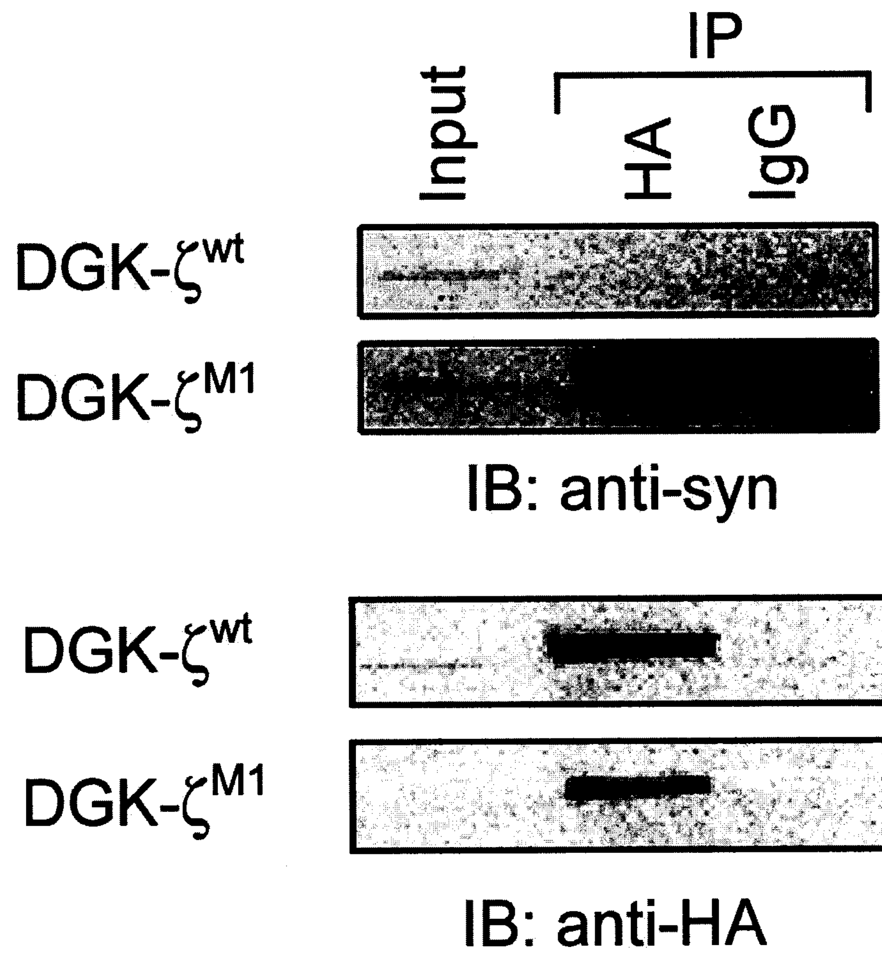


### 4. MARCKS-Phosphorylated DGK-ζ also Binds more Strongly than the Wild-Type Control to Full-Length Syntrophins

To investigate whether the tighter association of MARCKS-phosphorylated DGK-ζ with syntrophin PDZ domains is relevant within the context of the full-length protein, lysates of N1E-115 cells infected with HA-tagged wild-type DGK-ζ or DGK-ζ M1 (S/D), were immunoprecipitated with an anti-HA antibody. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-syntrophin monoclonal antibody (1351). Figure 12 shows that both HA-tagged DGK-ζ species were efficiently immunoprecipitated by the anti-HA antibody but not by normal mouse IgG. Endogenous syntrophins were also immunoprecipitated with HA-tagged DGK-ζ M1 (S/D) using the anti-HA antibody, and no signal was detected from the immunoprecipitation of syntrophins with wild-type DGK-ζ on the western blot. This result suggests the stronger interaction of MARCKS-phosphorylated DGK-ζ with the fusion proteins of syntrophin PDZ domains also applies to endogenous syntrophins.

**Fig 12. Stronger Interaction of Endogenous Syntrophins with MARCKS-Phosphorylated DGK-ζ than with Wild-Type DGK-ζ.** N1E-115 cells infected with HA-tagged wild-type DGK-ζ or DGK-ζ M1 (S/D) were lysed and the extracts were incubated with an anti-HA antibody or with control IgG. The immunoprecipitates were then subjected to immunoblot analysis with a monoclonal antibody against syntrophins (1351) or with anti-HA. Expression of syntrophin, wild-type DGK-ζ, or DGK-ζ M1 (S/D) in the lysate is also shown. Input represents 2% of the starting material.

**Figure 12**



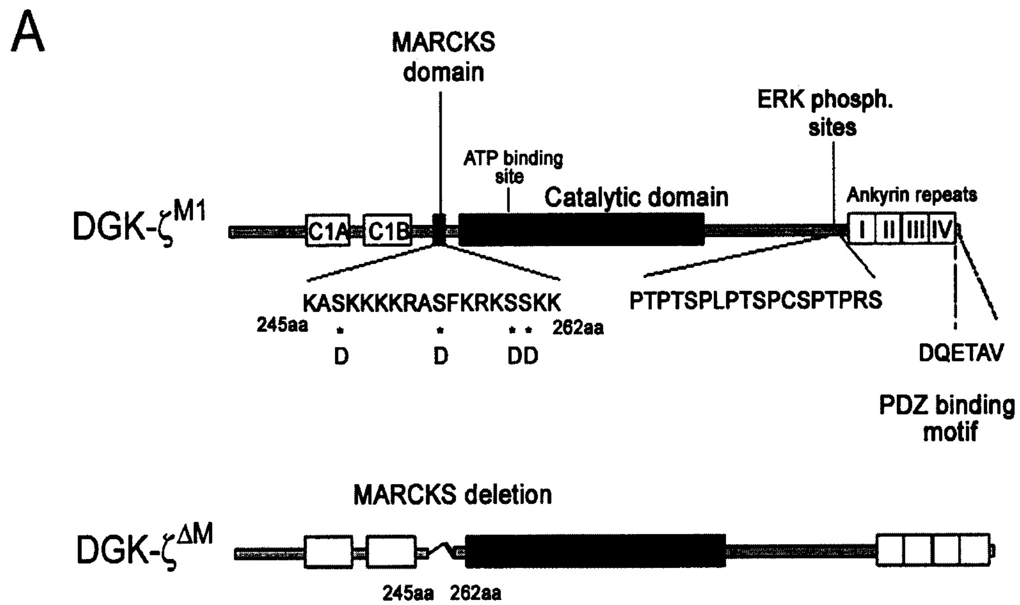
**5. Deleting the MARCKS Domain Reduces the Association of DGK-ζ with the PDZ Domain of α1-Syntrophin**

To further study the role of DGK-ζ MARCKS domain in regulating DGK-ζ binding to syntrophins, the MARCKS domain was deleted (Figure 13A) and the ability of the DGK-ζ MARCKS-deletion mutant (DGK-ζ ΔM) to bind to syntrophin PDZ domains was tested in pull-down assays. Beads charged with GST-α1-PDZ were used to capture DGK-ζ from N1E-115 cell lysates transiently transfected with either DGK-ζ ΔM or DGK-ζ M1 (S/D). Bound proteins were then analyzed by western blotting, revealing that DGK-ζ ΔM bound less strongly to the PDZ domain of α1-syntrophin than MARCKS-phosphorylated DGK-ζ (Figure 13B and 13C). In addition, figure 13 D shows that the amount of DGK-ζ ΔM bound to the PDZ domain of α1-syntrophin is less than the amount of wild-type DGK-ζ bound, taken from cells transiently transfected with either DGK-ζ ΔM or wild-type DGK-ζ. Together, the data suggests the MARCKS domain contributes to the regulation of the interaction of DGK-ζ with syntrophins.

**Fig 13. MARCKS-Deletion DGK-ζ Binds Weaker to the PDZ Domain of α1-Syntrophin than MARCKS-Phosphorylated DGK-ζ.** (A) Schematic structure of DGK-ζ showing the MARCKS deletion. Amino acids 245 to 262 were deleted and KpnI site was introduced to ligate the two fragments of DGK-ζ. (B) Pull-down of HA-tagged DGK-ζ ΔM or DGK-ζ M1 (S/D) with the PDZ domain of α1-syntrophin. Beads charged with GST alone or with GST-α1-PDZ were incubated with detergent extracts of Cos-7 cells transiently transfected with DGK-ζ ΔM or DGK-ζ M1 (S/D). Bound proteins were immunoblotted with an anti-HA antibody. The input lane was loaded with 2% of the extract used for the pull-down. (C) Quantification of bands from western blotting by densitometry: Bars represent the ratio of the amount of DGK-ζ bound to syntrophin α1-PDZ relative to the amount of DGK-ζ expressed in each sample, in arbitrary units. The values are taken from a single experiment. (D) Pull-down of HA-tagged DGK-ζ ΔM or wild-type DGK-ζ with the PDZ domain of α1-syntrophin. Beads charged with GST alone or with GST-α1-PDZ were incubated with detergent extracts of Cos-7 cells transiently transfected with DGK-ζ ΔM or wild-type DGK-ζ. Bound proteins were immunoblotted with an anti-HA antibody. The input lane was loaded with 2% of the extract used for the pull-down.

# Figure 13

A

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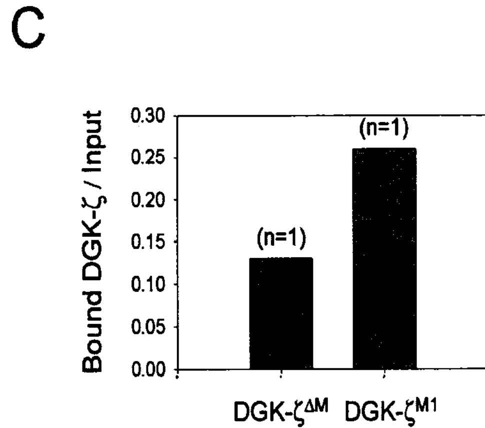
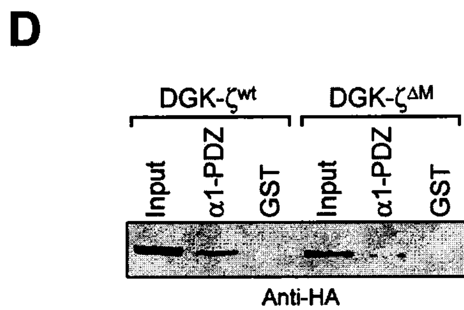



D



**6. A DGK-ζ Mutant Mimicking ERK-Dependent Phosphorylation Binds as much as its Control Mutant to α1-Syntrophin PDZ Domain**

Phosphorylation at the C-terminus of ligands with PDZ binding motifs often disrupts their binding to PDZ-containing proteins. To my knowledge, the PDZ binding motif of DGK-ζ does not contain any known consensus sequence for a phosphorylation site; however, DGK-ζ can be phosphorylated near the C-terminus, in a proline-rich region upstream of the ankyrin repeats (Figure 6). Three serine residues were found to be potential sites for ERK phosphorylation (Abramovici et al., 2003).

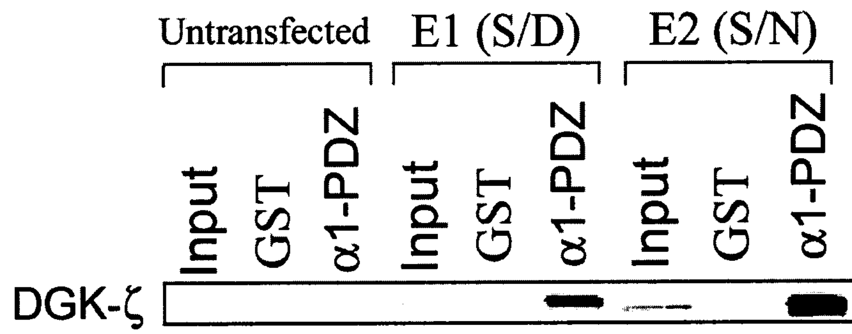
Since the serine residues lie close to the C-terminus, ERK-dependent phosphorylation may affect the PDZ-mediated interaction between DGK-ζ and syntrophins. To study this possibility, the ability of a DGK-ζ mutant that mimics ERK-dependent phosphorylation (DGK-ζ E1 (S/D)) was tested in pull-down assays with the PDZ domain of α1-syntrophin, using a mutant that cannot be phosphorylated as a control (DGK-ζ E2 (S/N)). The mutants either contain aspartates (DGK-ζ E1 (S/D)) or asparagines (DGK-ζ E2 (S/N)) in replacement for serine. Untransfected Cos-7 cells, which express very low levels of endogenous DGK-ζ, were used as a control for the specific detection of the DGK-ζ mutants with a polyclonal antibody against DGK-ζ N-terminus that can bind indiscriminately to the native protein. Both DGK-ζ mutants were captured with GST-α1-PDZ from transfected COS-7 cell lysates, and the densitometry of the western blots showed no difference in the intensities of the immunoreactive bands relative to their respective inputs (Figure 14A and 14B). This suggests ERK-dependent phosphorylation of DGK-ζ does not influence the binding between DGK-ζ and the PDZ domain of α1-syntrophin. However, the ability of DGK-ζ E1 (S/D) to bind to syntrophin

PDZ domains should also be tested in comparison to wild-type DGK-ζ, to have a better understanding of the role of ERK-dependent phosphorylation in regulating DGK-ζ / syntrophin interaction.

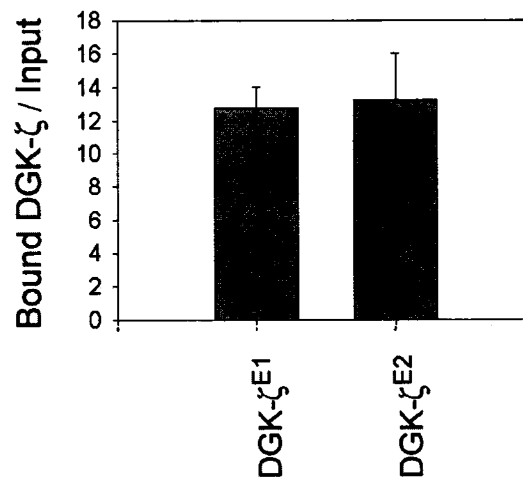
**Fig 14. A DGK-ζ Mutant Mimicking ERK-Dependent Phosphorylation Binds as much as its Control Mutant to α1-syntrophin PDZ Domain.** (A) Pull-down of DGK-ζ E1 (S/D) or DGK-ζ E2 (S/N) with the PDZ domain of α1-syntrophin. Beads charged with GST alone or with GST-α1-PDZ were incubated with detergent extracts of either untransfected Cos-7 cells or transiently transfected cells with DGK-ζ E1 (S/D) or DGK-ζ E2 (S/N). Bound proteins were analyzed by immunoblotting with a polyclonal antibody against DGK-ζ N-terminus. Input represents 2% of the extract used for the pull-down. (B) Quantification of bands from western blotting by densitometry: Bars represent ratios of the amount of DGK-ζ bound to syntrophin α1-PDZ relative to the amount of DGK-ζ expressed in each sample, in arbitrary units. The values represent the mean of two independent experiments.

**Figure 14**

A




B



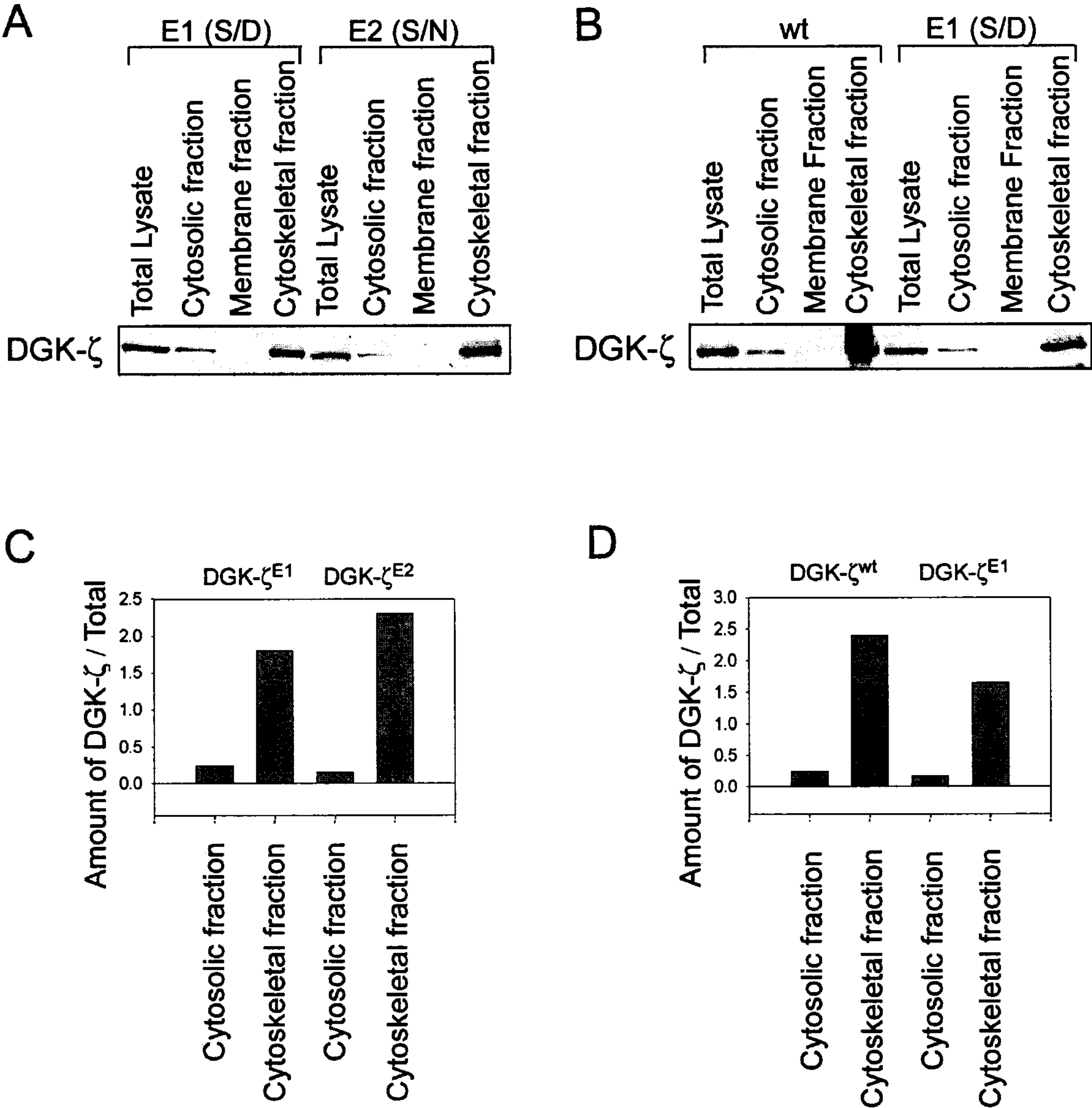
## 7. ERK Phosphorylation Alters the Subcellular Distribution of DGK-ζ

To further explore the role of ERK phosphorylation in regulating DGK-ζ activity, the association of ERK-phosphorylated DGK-ζ with the cytoskeleton was examined. Abramovici *et al.* (2003) showed that DGK-ζ migrates as a doublet and the two species detected in immunoblots have different localizations as determined by subcellular fractionation. The slower migrating (upper) band was present in the cytosolic fraction while the faster migrating (lower) band was recovered in the detergent-insoluble cytoskeleton fraction. Immunoblotting also revealed that DGK-ζ E1 (S/D) comigrates with the upper band whereas DGK-ζ E2 (S/N) comigrates with the lower band, suggesting ERK phosphorylation regulates the association of DGK-ζ with the cytoskeleton (Abramovici et al., 2003). Although the presence of a faster migrating DGK-ζ species remains to be confirmed, the possibility that ERK phosphorylation affects the subcellular distribution of DGK-ζ was tested by subcellular fractionation. NIH-3T3 cells transfected with HA-tagged DGK-ζ E1 (S/D), its control mutant DGK-ζ E2 (S/N), or wild-type DGK-ζ were lysed and fractionated by multiple high-speed centrifugations. Immunoblotting showed that DGK-ζ E1 (S/D) and DGK-ζ E2 (S/N) display similar localization patterns, as they were both found in the cytosolic fraction as well as in association with the cytoskeletal fraction (Figure 15A). However, careful quantification of the immunoreactive protein bands by densitometry revealed that the ERK-phosphorylated mutant is slightly more cytosolic compared to the mutant that cannot be phosphorylated (Figure 15C). The reverse was observed in the cytoskeletal-associated fraction, where DGK-ζ E2 (S/N) was more abundant compared to DGK-ζ E1 (S/D) (Figure 15C). This result constitutes additional evidence that ERK phosphorylation

regulates the association of DGK-ζ with the cytoskeleton. In addition, the examination of the localization of DGK-ζ E1 (S/D) against wild-type DGK-ζ revealed that the ERK-phosphorylated mutant is found less abundantly in the cytoskeletal fraction (Figure 15B and 15D), further supporting that ERK-phosphorylation affects DGK-ζ association with the cytoskeleton. However, DGK-ζ E1 (S/D) was also found less abundantly in the cytoslic fraction compared to wild-type DGK-ζ (Figure 15B and 15D). A possible interpretation for the reduction of DGK-ζ in both fractions could be that DGK-ζ translocates to the nucleus in response to ERK signaling. Collectively, the above findings suggest phosphorylation by ERK alters the subcellular distribution of DGK-ζ.

**Fig 15. ERK-Dependent Phosphorylation Alters the Subcellular Distribution of DGK-ζ.** NIH-3T3 cell lysates transfected with HA-tagged DGK-ζ E1 (S/D), DGK-ζ E2 (S/N), or wild-type DGK-ζ were fractionated as described in MATERIALS and METHODS. (A) Equal amounts of protein from each fraction of cells transfected with DGK-ζ E1 (S/D) or DGK-ζ E2 (S/N) were analyzed by SDS-PAGE and immunoblotting with an anti-HA antibody. (B) Equal amounts of protein from each fraction of cells transfected with wild-type DGK-ζ or DGK-ζ E1 (S/D) were analyzed by SDS-PAGE and immunoblotting with an anti-HA antibody. (C) Quantification of bands from western blotting of A (above) by densitometry: Bars represent ratios of the amount of DGK-ζ in each fraction relative to the amount of DGK-ζ expressed in the total lysate of each sample, in arbitrary units. The total lysate lane represents 2% of the starting material. The values are taken from a single experiment. (D) Quantification of bands from western blotting of B (above) by densitometry: Bars represent ratios of the amount of DGK-ζ in each fraction relative to the amount of DGK-ζ expressed in the total lysate of each sample, in arbitrary units. The values are taken from a single experiment.

# Figure 15



# Discussion

This study suggests PKC-dependent phosphorylation of DGK-ζ MARCKS domain increases its association with syntrophin PDZ domains. PDZ interactions are usually implicated in the recruitment of signaling protein complexes to specified locations in the cell through a sequence-specific interaction between a PDZ domain in a scaffold protein and a C-terminal PDZ-binding motif of a ligand (Kim and Sheng, 2004). Previous reports showed that PDZ-mediated interactions are regulated by phosphorylation, either on the C-terminus of the ligand (Hegedus et al., 2003; Zhang and Wang, 2003) or on the PDZ domain (Gardoni et al., 2003). In addition, a study by Ort *et al.* (2001) demonstrated that the PDZ-mediated association between ICA512 and β2–syntrophin is modulated by phosphorylation of the latter protein. However, the phosphorylation site was not identified, suggesting it could be either within or outside the PDZ domain of β2–syntrophin. To my knowledge, my findings are the first evidence that a PDZ-mediated interaction is regulated by phosphorylation at a site located outside the PDZ-binding motif or the PDZ domain.

It was previously demonstrated that DGK-ζ can be phosphorylated by PKC at its MARCKS domain (Figure 6) (Topham et al., 1998; Luo et al., 2003a and 2003b). The present study shows that mimicking phosphorylation at this domain, by replacing the four serine residues with aspartates, caused DGK-ζ to bind more strongly to recombinant PDZ domains of syntrophins. The phosphorylation-mimicking DGK-ζ mutant also enhanced DGK-ζ binding to endogenous syntrophins. However, a DGK-ζ mutant where the serine residues are substituted with asparagines, to prevent phosphorylation, bound as much as

the MARCKS phosphorylated DGK-$\zeta$ mutant to a GST fusion protein of the PDZ domain of $\alpha$1-syntrophin. A possible explanation for this finding is that even a subtle modification of the serine residues at the MARCKS domain may influence binding to sytnrophin PDZ domains, perhaps due to a conformational change in DGK-$\zeta$ caused by the modification. Another possibility is that the hydroxyl group of the serine residue might be binding to a third protein, negatively regulating the association with syntrophin. A modification of the serine residue releases the inhibition. However, an additional control, in which the serines at the MARCKS domain would be substituted with alanines, could also be tested. Since alanine has a closer structure to serine than asparagine does, the effect of introducing this amino acid residue might be minimal on the conformation of the protein, which would then serve as a better control for the phosphorylation-mimicking DGK-$\zeta$ mutant in binding assays.

Physiologically, a conformational change in an enzyme can be induced by phosphorylation (Stock et al., 2000; Pawson and Scott, 2005). For example, the phosphorylation of Vav proteins, which are GDP/GTP exchange factors for Rho/Rac molecules, on a specific tyrosine residue led to a conformational change that allowed the activation of the catalytic activity of Vav proteins (Bustelo 2002). Also, the phosphorylation of the actin filament-associated protein AFAP-110 by PKC affected its conformation, which correlated with an increase in the capability of AFAP-110 to bind and cross-link actin filaments (Qian et al., 2002). These examples highlight the role of phosphorylation in inducing a conformational change in a protein, which might influence its activity and binding capabilities.

This study demonstrates that the proposed conformational change leading to a modification in the binding between DGK-$\zeta$ and syntrophins is a mechanism caused by phosphorylation. *In-vitro* binding experiments conducted using a PKC activator (PMA) resulted in an increase in the strength of binding of DGK-$\zeta$ to recombinant PDZ domains of syntrophins. In support of this idea, the association between DGK-$\zeta$ and the fusion proteins was reduced following the application of a PKC inhibitor, suggesting the stronger association between DGK-$\zeta$ and syntrophin PDZ domains is due to PKC phosphorylation. Although, the amount of HA-tagged wild-type DGK-$\zeta$ bound to the PDZ domain of $\alpha$1-syntrophin is much less than its expressed amount in the cell, this does not underestimate the result. This might be caused by a low amount of fusion protein incubated with the cell lysates, or by the interaction of endogenous DGK-$\zeta$ with the fusion protein. The western blots however, clearly show an increase in the amount of wild-type DGK-$\zeta$ bound to the PDZ domain following PKC activation, in respect to unstimulated cells. Hence, PKC phosphorylation increases the association of DGK-$\zeta$ to the PDZ domain of $\alpha$1-syntrophin.

The PDZ domains of the various syntrophin isoforms differ slightly in their amino acid sequences, especially in the carboxylate-binding loop consensus sequence motif (GLGI for $\alpha$1, $\beta$1, & $\beta$2, and GFGL for $\gamma$1). This motif is a critical determinant of the binding specificity for ligands containing PDZ-binding motifs (Doyle et al., 1996; Gee et al., 2000), suggesting the strength of the binding between DGK-$\zeta$ and syntrophin PDZ domains may depend on the syntrophin isoform. However, it was observed that MARCKS-phosphorylated DGK-$\zeta$ bound stronger than wild-type DGK-$\zeta$ to fusion proteins of both $\alpha$1- and $\gamma$1-PDZ, and the association of wild-type DGK-$\zeta$ was enhanced

in PKC activation experiments with either α1- or γ1-PDZ. Therefore, the stronger binding between DGK-ζ and the PDZ domain of syntrophins caused by PKC phosphorylation is not limited to the α1-syntrophin isoform.

The role of the MARCKS domain in regulating PDZ binding was also examined by constructing a deletion mutant (DGK-ζ ΔM). This construct bound less to the PDZ domain of α1-syntrophin than either wild-type DGK-ζ or MARCKS-phosphorylated DGK-ζ. The absence of the MARCKS domain causes a reduction in the phosphorylation state of DGK-ζ, as it is the predominant site of PKC phosphorylation (Luo et al., 2003b). Hence, it would be expected that a less phosphorylated DGK-ζ exhibits a weaker association with syntrophins. The behaviour of the MARCKS-deletion mutant is in line with the evidence presented thus far in support of the findings of this study.

The phosphorylation at the MARCKS domain was reported to cause the dissociation of PKC-α from DGK-ζ (Luo et al., 2003) and to inhibit the interaction between DGK-ζ and an activated Rac1 mutant (Yakubchyk et al., 2005). Here, we show that this phosphorylation regulates the interaction of DGK-ζ with syntrophins differently, working to enhance their association. This suggests the effect of the PKC phosphorylation on the interaction of DGK-ζ with its binding partners is specific and not merely an artefact.

In addition, PKC phosphorylation may serve to anchor DGK-ζ at specific subcellular domains through its stronger association with syntrophins. Previous studies have shown that DGK-ζ accumulates in the nucleus when its PDZ-mediated interaction with γ1-syntrophin is disrupted (Hogan et al., 2001), while its nuclear levels are reduced

following PKC phosphorylation (Topham et al., 1998). Hence, PKC phosphorylation may add another level of regulation to the localization of DGK-ζ, whereby phosphorylated DGK-ζ that is excluded from the nucleus binds more tightly to syntrophin PDZ domains in the cytoplasm to help retain DGK-ζ away from sources of nuclear DAG. This possible mechanism may reduce the down regulation of DAG by DGK-ζ, which could subsequently affect cell growth and proliferation.

A recent study by Abramovici *et al.* (2003) has shown that MARCKS-phosphorylated DGK-ζ and syntrophins were more membrane associated when co-expressed than when either protein is expressed alone. The stronger binding between the two proteins, shown in my work, may explain this observation. It may induce a conformational change in syntrophin causing it to bind more strongly to dystrophin or its homologues, thus anchoring DGK-ζ more tightly to the DAPC near the plasma membrane. Syntrophins are also known to contain PH lipid-binding modules, and α1-syntrophin was shown to bind to the membrane-associated lipid PIP2 through its first PH domain, which is split by a PDZ insertion (Chockalingam et al., 1999). More recent work by Yan *et al.* (2005) has demonstrated that although the presence of the PDZ insertion does not have significant impact on the structure of the split PH1 domain of α1-syntrophin, binding of the PH1a–PDZ–PH1b module to PIP2 is much stronger than binding of the joined PH1a–PH1b domain, suggesting the PDZ domain contributes to the creation of a PH–PDZ supramodule with unique membrane lipid-binding properties (Yan et al., 2005). Since the lipid-binding site does not overlap with the PDZ ligand-binding site on the α1-syntrophin PDZ domain (Yan et al., 2005), increasing the strength of the PDZ-interaction with DGK-ζ could also strengthen the association of syntrophin with

PIP2, thus leading to a rise in DGK-ζ levels directly at the membrane. The amount of DGK-ζ at the plasma membrane may be important for adequate control of DAG signaling.

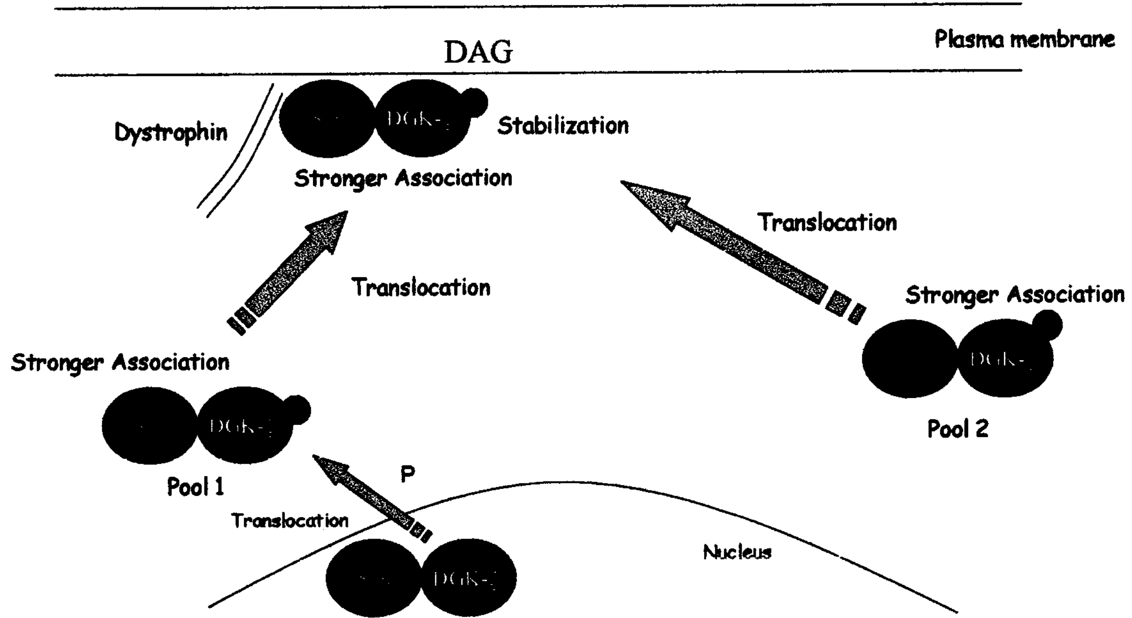
Furthermore, the increase in the strength of DGK-ζ binding to syntrophins may modulate the association of syntrophin with the actin cytoskeleton. Iwata *et al.* (1998 and 2004) have identified high-affinity actin binding sites located on the PH2 and SU domains of α1-syntrophin, and have shown that syntrophins were able to bind to F-actin. A MARCKS-phosphorylated DGK-ζ mutant that cannot bind to syntrophins induced changes in the actin cytoskeleton in myoblasts, suggesting the interaction between DGK-ζ and syntrophins is important for proper control of actin cytoskeletal remodelling (Abramovici et al., 2003). A modulation of this interaction, caused by PKC phosphorylation in this case, could in turn change the strength of binding of syntrophin to actin, and consequently influence the role of DGK-ζ in actin organization.

In summary, this work shows that the phosphorylation of a domain distant from the C-terminus of the ligand regulates the interaction between DGK-ζ and syntrophin PDZ domains. In addition, this study shows that interactions involving syntrophins can be regulated by phosphorylation of their binding partners. Although another study has shown that syntrophin-ligand interactions are regulated by phosphorylation, the phosphorylation sites are located on the syntrophin molecule rather than on the ligand (Ort et al., 2001). Therefore, this study represents a first in showing that PKC-dependent phosphorylation of DGK-ζ MARCKS domain, which is located at a significant distance from the C-terminal PDZ-binding motif, increases the strength of the binding between

DGK-ζ and the PDZ domains of syntrophins, and that syntrophin PDZ-mediated interactions can be regulated by phosphorylation of DGK-ζ. The enhanced binding between DGK-ζ and syntrophins may sequester DGK-ζ in the cytoplasm, away from DAG sources in the nucleus, as it may account for the stabilization of the association of DGK-ζ with the DAPC or with PIP2 near DAG sources at the plasma membrane.

**Fig 16. Model.** PKC phosphorylation excludes DGK-$\zeta$ from the nucleus. In the cytoplasm, PKC phosphorylation increases the association of DGK-$\zeta$ with syntrophin. The complex of phosphorylated DGK-$\zeta$ and syntrophin (pool 1) translocates to the plasma membrane, where syntrophin binds dystrophin. The stronger association between DGK-$\zeta$ and syntrophin might enhance the binding of syntrophin to dystrophin, stabilizing the DGK-$\zeta$ / syntrophin complex at the plasma membrane near DAG sources. In pool 2, PKC phosphorylation of DGK-$\zeta$ in the cytoplasm strengthens the interaction with syntrophin and translocates the complex to the plasma membrane, where syntrophin associates with dystrophin.

# Figure 16



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