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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M.Sc. (Neuroscience)

GRADE - DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Syntrophin Regulates the Subcellular Localization of Diacylglyceral Kinase-Zeta

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DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Syntrophin Regulates the Subcellular Localization of Diacylglycerol Kinase-ζ

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This thesis was submitted to the School of Graduate
Studies and Research, University of Ottawa,
in the partial fulfillment of the degree of

Masters of Science

Neuroscience Graduate Program
Department of Cellular and Molecular Medicine
Faculty of Medicine
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Thesis Format

The format of this thesis is that of a collection of manuscripts as permitted by the guidelines set by the School of Graduate Studies at the University of Ottawa. A general introduction is presented in Chapter 1, and following the two manuscripts is an overall discussion. Chapters 2 and 3 each include a manuscript with a summary, acknowledgements, methods, results, discussion, and references. The abbreviations page at the beginning of this thesis covers all abbreviations, while Chapter 5 contains the bibliography, which covers all references except those already listed at the end of each manuscript. All references in Chapters 1 and 4 are listed by author and year within the text and can be found in their complete form in the bibliography where they are listed alphabetically.

The manuscript in Chapter 2 has already been published and is reprinted here with permission from the American Society for Biochemistry and Molecular Biology. This letter of permission is included in the appendices of Chapter 6.

Acknowledgements

Many people have helped me throughout the duration of my graduate studies. One of these people is my supervisor Dr. Stephen Gee, who has guided and encouraged me, been very patient and understanding, and for this I am very grateful. He made me feel proud of what I had accomplished. I would also like to thank Josée Chabot, our indispensable lab technician, who has given me great advice, lots of help, and much laughter, which all made things much easier and more enjoyable. To John Lunde, I would also like to extend my appreciation for his great kindness and his willingness to offer his help whenever he could. My advisory committee members, Dr. Jasmin, Dr. Albert and Dr. Ngsee were very patient, encouraging, insightful, and helped me discover a new appreciation for my research. For this I owe them much gratitude. To my family and the Gougeons, to whom I am so grateful for their generosity, their help and encouragement, and their patience, I would like to say thanks. Without their support life would have been much more difficult and certain events would have even seemed insurmountable. Finally, to the person I am most indebted to, Pierre-Yves Gougeon, who believed in me and my abilities, has helped me overcome so many obstacles, has supported me through even the toughest of times, and who was so proud of me when I reached my goal, I would like to extend the greatest thanks. Without him I would not have made it this far.

Statement of Originality

The format of this thesis is a collection of manuscripts based not only on my own experiments, but also on those of other lab personnel. In the first manuscript, the yeast two-hybrid screening was performed by myself, as were the biochemical assays which gave the results shown in figure 2.2) A, B, C, and F. Stéphane Quenneville, a former graduate student in our lab, performed the overlay assay depicted in figure 2.2) D. All immunoprecipitation experiments and pull-down assays were performed by myself, except for the results shown in figure 2.8) B, which were done by our laboratory technician, Josée Chabot. Immunostaining of transfected cells was also performed by Josée Chabot, except for those seen in figure 2.3) C-F, which I did. Lastly, Lynn Shepherd, a summer student in our lab, tested the γ1-syntrophin and DGK-ζ antibodies, as seen in figure 2.6, and along with Josée Chabot, performed the immunostaining of brain tissue (figure 2.7). All experiments in Chapter 3 were performed by myself and both manuscripts were co-written by both Stephen Gee and myself.

Abstract

Syntrophins are scaffolding proteins that link signaling molecules to the dystrophin protein complex at the plasma membrane. To further understand the roles of syntrophins a yeast two-hybrid screen of a human brain cDNA library was done using the PDZ domain of the recently identified brain-specific γ_1-syntrophin, an isoform for which no signaling ligands had yet been identified. This screen yielded ten overlapping clones coding for the C-terminal portion of diacylglycerol kinase-ζ (DGK-ζ), a kinase that phosphorylates the membrane lipid diacylglycerol (DAG) to phosphatidic acid (PA). Biochemical experiments and binding assays confirmed that this kinase's C-terminus, containing the consensus PDZ-binding motif Q-E-T-A-V-COOH, was both necessary and sufficient for the interaction. This complex can be immunoprecipitated from co-transfected Hela cells, and the C-terminal-PDZ interaction appears to be required for the distribution of this complex between both the cytosol and nucleus. In the brain, DGK-ζ and γ_1-syntrophin colocalize in cerebellar Purkinje fibres and pyramidal neurons of the hippocampus and cortex, areas where dystrophin is expressed, and DGK-ζ can be detected in dystrophin immunoprecipitates from mouse brain extracts.

Furthermore, biochemical experiments also show that DGK-ζ binds to the PDZ domain of α- and β-syntrophins, and, endogenous syntrophin can be detected in DGK-ζ immunoprecipitates from cultured C2C12 skeletal muscle cell extracts. In normal skeletal muscle, DGK-ζ and syntrophin co-localizes at the NMJ, and the sarcolemma of oxidative fibres. In *mdx* mouse skeletal muscle, DGK-ζ's localization at the NMJ appears unaffected, while sarcolemmal levels are decreased in degenerating fibres but not

in regenerating or regenerated ones. Together, our data suggest that syntrophin binds to DGK-ζ and links it to dystrophin-associated protein complexes in neurons and muscle cells, thereby playing a role in regulating the subcellular localization and function of this lipid kinase.

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List of Abbreviations

α-BgTx, α-bungarotoxin

AChR, nicotinic acetylcholine receptor

CaM, calmodulin

CaMKII, Ca^{2+}-calmodulin dependent protein kinase II

CRD, cysteine-rich domain

DAG, diacylglycerol

DAPC, dystrophin-associated protein complex

DGK, diacylglycerol kinase

DMD, Duchenne muscular dystrophy

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

F-actin, filamentous actin

FHA, forkhead-associated

Grb2, growth factor receptor-binding protein-2

GST, glutathione S-transferase

HRP, horse radish peroxidase

ICA-512, islet cell autoantigen 512

InaD, inactivation no-afterpotential

IP_3, inositol-3,4,5 triphosphate

Kir 2.3, inwardly-rectifying potassium channel 2.3

MAb, monoclonal antibody

MAGUK, membrane-associated guanylate kinase

MAPK, mitogen-activated protein kinase

MARCKS, myristolyated alanine-rich C kinase substrate

NLS, nuclear localization sequence

NMDA, *N*-methyl-D-aspartate

NMJ, neuromuscular junction

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

PA, phosphatidic acid

PBS, phosphate buffered saline

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

PH, pleckstrin homology

PI, phosphatidylinositol

PIP, phosphatidylinositol 4-phosphate

PIP_2, phosphatidylinositol-4,5 bisphosphate

PIP-5K, phosphatidylinositol-4-phosphate 5-kinase

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PSD-93, postsynaptic density protein-93

PSD-95, post-synaptic density protein-95

pTyr, phosphotyrosine

RasGAP, Ras GTPase-activating protein

RasGRP, Ras guanyl nucleotide releasing protein

RhoGDI, Rho guanyl nucleotide dissociation inhibitor

RTK, receptor tyrosine kinase

SAPK3, stress-activated protein kinase-3

SAST, syntrophin-associated serine/threonine kinase

SH2, src homology-2

SH3, src homology-3

SOS, son of sevenless

SU, syntrophin unique region

TRP, transient receptor potential channel

TRPL, transient receptor potential-like channel

WW, tryptophan-tryptophan

Chapter 1

Introduction

A cell uses intracellular signaling pathways to accomplish tasks such as growth and differentiation, environmental adaptation, and programmed cell death. These events must be carried out in a timely manner, so the signaling pathways regulating them must be rapid, and specific to the task at hand. Since random diffusion of signaling molecules is inefficient, cells have developed, through the process of evolution, intricate ways of making signaling more rapid, efficient, and precise. This is accomplished by organizing enzymes into different signaling pathways, each giving rise to its own, often distinct, biological response. Interestingly, the same enzymes may be used by different parallel pathways, in various combinations with other signaling molecules, in order to achieve differing effects. This makes signal transduction more efficient because the cell does not have to produce as many different molecules (Smith and Scott, 2002). Cells are able to orchestrate the formation and sorting of proteins into specific signaling cascades by several mechanisms, including targeting and translocation of proteins from one subcellular compartment to another, and the assembly of multiprotein complexes at specific subcellular domains, which act as signaling platforms. It is now known that the basic molecular elements that allow these events to take place are protein-protein interaction modules located within scaffolding molecules.

Intracellular targeting sequences

Upon stimulation of cell-surface receptors specific enzymes translocate from one subcellular area to another, thus ensuring rapid and specific signal transduction. This can bring enzymes closer to their substrates or to molecules that will modify their catalytic

activities, consequently confining them to a particular signaling cascade and accelerating the process of intracellular message transmission. One such mechanism is ligand-induced autophosphorylation of tyrosine-containing motifs (pTyr-X-X-X, where the Xs vary for different SH2 domains) on receptor tyrosine kinases (RTKs), which are recognized by signaling molecules possessing 'Src homology-2' (SH2) protein-protein interaction modules. SH2 domains are named for their homology to a sequence in the Rous sarcoma virus oncogene *src* (Cohen et al, 1995; Pawson and Scott, 1997; Songyang et al., 1993). SH2 targeting sequences can be found in different proteins such as scaffolding/adaptor molecules and enzymes. For example, the SH2 domain in the intracellular scaffolding molecule 'growth factor receptor-binding protein-2' (Grb2) is able to recognize and bind to a specific pTyr-containing motif on the epidermal growth factor receptor (EGFR), an RTK whose cytoplasmic tail becomes autophosphorylated on tyrosine residues upon activation by its ligand EGF. This allows the Grb2-interacting molecule 'son of sevenless' (SOS, a 'Ras small GTP-binding protein' exchange factor) to be localized near its downstream effector molecule Ras, thus linking EGFR activation to the Ras signaling pathway and subsequently to gene expression (Cohen et al.,1995; Pawson and Scott, 1997). One study that demonstrates the importance of protein translocation for effective signal transduction and normal cellular function, showed that mutation of tyrosine residues on the Met receptor tyrosine kinase prevented binding to Grb2's SH2 domain, and led to defects in the proliferation of myoblasts and in muscle formation (Maina et al., 1996).

Thus, effective signaling involves the targeting/translocation of particular enzymes to specific locations within the cell upon receptor activation. This ensures the

rapid local availability of specific signaling molecules that may be common to many cascades, but that consequently become confined to only one. The end result is more rapid, efficient, and specific message transmission, and thus an accelerated and more finely tuned response.

Multimolecular signaling complexes

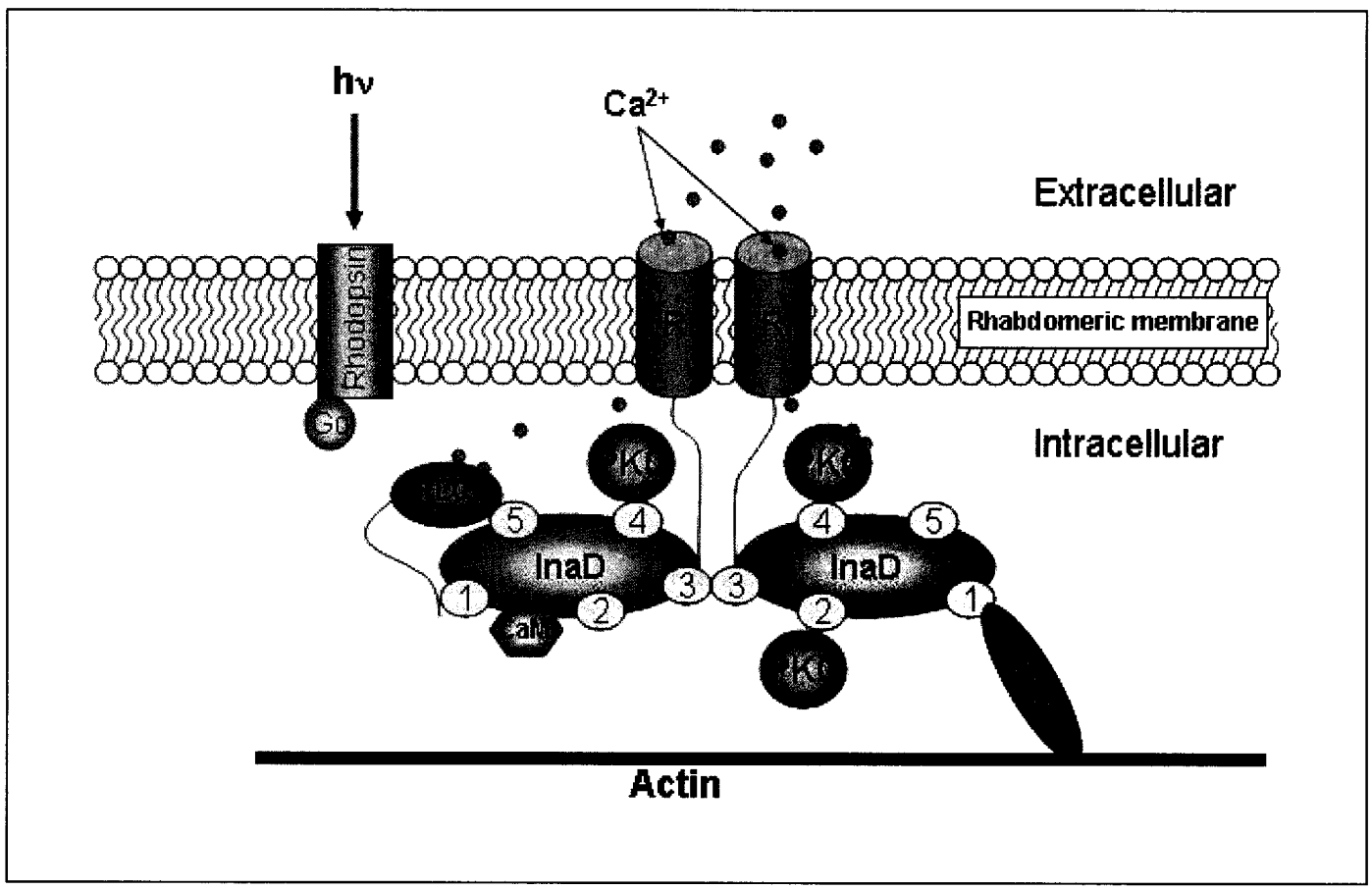
Multiprotein signaling complexes spatially organize distinct transmembrane, membrane-associated, cytosolic and nuclear proteins involved in the same signaling pathways. By doing so, these complexes prevent or minimize molecular "cross-talk", while optimizing the precision, efficiency, and speed of signal transduction. It is thought that signaling molecules do not diffuse randomly within a cell, but are instead grouped with other molecules involved in the same signaling pathway, forming architecturally defined and functionally distinct molecular signaling complexes (Fanning and Anderson, 1999).

The pillars of these multiprotein signaling complexes are scaffolding/adaptor molecules which contain varying combinations of protein-protein interaction modules, which can also be used for targeting/translocation. Examples of such modules include SH2 domains, as well as Src homology-3 (SH3), pleckstrin homology (PH), tryptophan-tryptophan (WW), forkhead-associated domains (FHA), and **P**SD-95 / **D**iscs Large / **Z**O-1 (PDZ) domains (Barinaga, 1999; Cohen et al., 1995; Pawson and Scott, 1997).

One of the best examples of a scaffolding/adaptor molecule able to organize a functional signaling complex is the 'inactivation no-afterpotential' (InaD) protein in the *Drosophila* eye, which contains five PDZ protein-protein interaction modules (Figure

1.1). InaD serves as a scaffold for phototransduction proteins that bind to its various PDZ domains, which together are postulated to mediate unitary visual responses in the rhabdomere (a collection of microvillar projections on the surface of the photoreceptor cell) (Ranganathan and Ross, 1997; Sheng and Sala, 2001). This complex includes three core PDZ-interacting components: eye-specific phospholipase C-β (PLC_β) and protein kinase C (PKC), and the transient receptor potential channel (TRP), all of which interact with InaD at a 1:1 stoichiometry; as well as other components: rhodopsin, the unconventional myosin NINAC, calmodulin (CaM), and the transient receptor potential-like (TRPL) channel (Chevesich et al., 1997; Huber et al., 1996; Fanning and Anderson, 1999; Ranganathan and Ross, 1997; Sheng and Sala, 2001; Shieh and Zhu, 1996; Shieh et al., 1997; Tsunoda et al., 1997; Tsunoda et al., 1998; Wes et al., 1999; Xu et al., 1998). When light stimulates rhodopsin this causes the translocation and binding of $G\alpha_q$ to PLCβ (Bähner et al., 2000), which becomes activated and cleaves the membrane phospholipid phosphatidylinositol-4,5 bisphosphate (PIP_2) to produce the second messengers inositol-3,4,5 triphosphate (IP_3) and diacylglycerol (DAG) (Berridge 1986; Hirasawa and Nishizuka 1985). DAG in turn stimulates the inward Ca^{2+} flux through TRP channels (Raghu et al., 2000), and both DAG and Ca^{2+} are thought to activate downstream effectors of the light response. Furthermore, calcium is thought to allow for negative feedback regulation of the TRP channel through its stimulatory effects on PKC, CaM, and NINAC (Sheng and Sala, 2001). InaD therefore serves the purpose of coupling the activities of transmembrane receptors, ion channels, and downstream effector molecules, involved in the regulation of phototransduction, thus conferring speed and specificity to the highly time-dependent process of vision (Sheng and Sala, 2001).

Figure 1.1 Model of the InaD phototransduction complex in the rhabdomeres of the *Drosophila* photoreceptor cells. Rhabdomeres are the microvillar projections of the *Drosophila* photoreceptor cells. The InaD complex at the membrane of these structures is thought to play a major role in the mediation of the phototransduction process. InaD is a scaffolding molecule containing five PDZ domains (which are represented by numbers here) which interact with several ligands. For instance, rhodopsin, a light-activated G_q-protein coupled receptor, binds to the fourth PDZ domain of InaD. When light stimulates rhodopsin, the $G\alpha_q$ subunit transiently binds to and activates phospholipase C-β (PLCβ), which is bound to the first and fifth PDZ domains of InaD. Activated PLCβ then cleaves the membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP_2) to produce inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). DAG is thought to cause transient release potential (TRP) channels to open, allowing calcium (Ca^{2+}) to flow into the rhabdomeres. These TRP channels interact with the third PDZ of InaD. Although the binding of PKC, PLC and TRP channels to InaD constitute the core complex, InaD may interact with other molecules such as the unconventional myosin NINAC, which binds to its first PDZ domain, and calmodulin, which binds to a region between the first and the second PDZs. Furthermore, core complexes may associate with each other via heteromeric or homomeric interactions between InaD's PDZ3 and PDZ4 domains. Figure adapted from: Bähner et al., 2000; Fanning and Anderson 1999; Ranganathan and Ross, 1997; Sheng and Sala, 2001).


hν
Ca2+
Extracellular
Rhodopsin
Rhabdomeric membrane
Intracellular
InaD
InaD
Actin

Multimolecular signaling complexes locally concentrate signaling molecules. This prevents them from freely diffusing away and becoming less concentrated with distance, thus the cell needs to produce less of them in order to generate a detectable signal (Tsunoda et al., 1998). Scaffolding complexes may also regulate molecular cross-talk between pathways that share some of the same signaling components, ensuring added precision and efficiency to the process of intracellular message transmission.

Aside from grouping together a small number of enzymes, scaffolding proteins appear, in some cases, to self-associate and form large multimers. Because each scaffolding protein may have its own unique set of different molecular interactors, they can "co-cluster" a variety of different signaling molecules. One group of intracellular scaffolding molecules that appear to play such a role is the family of membrane-associated guanylate kinases (MAGUKs), which contain, among other domains, three N-terminal PDZ domains (Dimitratos et al. 1999). PSD-95 is a MAGUK that is expressed in central neurons, and *in vitro,* it can form either homomultimers, or heteromultimers with other MAGUKs (Hsueh et al., 1997; Kim et al., 1996). In heterologous cells, heteromultimerization appears to play a central role in the formation of large heterogeneous membrane clusters of these MAGUKs, and at least two of their PDZ ligands: NMDA receptors (*N*-methyl-D-aspartate) and Shaker type potassium channels (Kim et al., 1996). Clustering together a large number of different signaling molecules may have an important functional significance. For instance, PSD-95 has been shown to form a ternary complex with at least two of its PDZ ligands, namely the NMDA receptor and neuronal nitric oxide synthase (nNOS). Furthermore, this MAGUK has been shown

to specifically couple calcium influx through activated NMDA receptor-channels to the production of NO by Ca^{2+}-dependent activation of nNOS (Christopherson et al. 1999; Sattler et al., 1999). By increasing the number and variety of molecules which can come into close contact, this may allow for coupling of their activities, and may help to diversify and strengthen signaling events (Smith and Scott, 2002; Dumont et al., 2001; Tsunoda et al., 1998).

Interactions between scaffolding molecules and their ligands may be dynamically regulated by one of several possible mechanisms. For instance, protein kinase A (PKA)-phosphorylation of the inwardly-rectifying potassium channel 2.3 (Kir 2.3) inhibits its binding to the second PDZ domain in PSD-95 (Cohen et al., 1996). Additionally, because scaffolding proteins may have several potential binding partners for each of their protein-protein interaction modules, ligands may compete for binding to these domains (Fanning and Anderson, 1999). Overall, dynamic regulation of protein-protein interactions by these or other mechanisms may play a role in the temporal regulation of signaling events.

Specialized Membrane Domains

A major challenge in cell biology is to understand how proteins are localized to different parts of the cell. Multimolecular signaling complexes are located at biochemically and functionally distinct subcellular domains, enabling the cell to respond precisely and efficiently to extracellular signals, such as those transmitted by hormones and neurotransmitters. These domains can be large specialized plasma membrane surfaces such as the basolateral or apical membranes of polarized cells, or the dendritic,

somatic, and axonal membranes of neurons. However, more focused membrane areas such as intercellular junctions, nodes of Ranvier, and the pre- and post-synaptic membranes of synapses, can also be considered domains. The unique combinations of multimolecular signaling complexes located in these areas help to define their special properties and functions (Fanning and Anderson, 1999). In *Drosophila* for instance, InaD serves not only to form the phototransduction complex but also to target it to the rhabdomeres in the eye. As a result, absence of InaD leads to the loss of its core interacting proteins (PKC, PLC, and TRP) from rhabdomeres, while mutants containing mutations of any one of the PDZ domains cause the selective loss of the corresponding PDZ ligand (Adamski et al., 1998; Sheng and Sala, 2001; Shieh and Zhu, 1996; Shieh et al., 1997; Tsunoda et al., 1997). These changes in the properties of this rhabdomeric complex lead to functional abnormalities, namely defects in the eye's light responsiveness. In *Caenorhabditis elegans*, the LET23 receptor tyrosine kinase is localized to the basolateral surface of polarized vulval epithelial cells due to its interaction with a complex of three PDZ proteins: LIN2, LIN7, and LIN10 (Kaech et al., 1998). It has been shown that mutations in any one of these three PDZ proteins leads to mislocalization of the LET23 to the apical surface, and consequently to a vulvaless phenotype (Kaech et al., 1998).

Syntrophins as scaffolding/adaptor molecules involved in signaling

Syntrophin is a scaffolding molecule that was originally discovered in the electric organ of the *Torpedo* ray as a 58 kDa protein associated with the postsynaptic membrane, and also with the mammalian postsynaptic neuromuscular junction (NMJ) (Froehner, et

al., 1984; Froehner, et al., 1987). To date, there are five known syntrophin isoforms (α_1, β_1, β_2, γ_1, and γ_2), each localized to precise areas of the plasma membrane via their interaction with dystrophin and dystrophin-related proteins, major components of dystrophin-associated protein complexes (DAPCs), which will be described in detail later (Adams et al., 2001; Ahn et al., 1996; Butler et al., 1992; Kramarcy et al., 1994; Kramarcy and Sealock, 2000; Peters et al., 1997; Piluso et al., 2000; Yang et al., 1995). These isoforms are encoded by separate genes, expressed at different times during development, and possess distinct subcellular, cellular, and tissular localizations (Table 6.1). (Adams et al., 1993; Adams et al., 1995; Ahn et al., 1996; Froehner et al., 1997; Górecki et al., 1997; Kramarcy and Sealock, 2000; Peters et al., 1997; Piluso et al., 2000). In skeletal muscle for instance, α1-, β2- and γ2-syntrophin are expressed in all fast and slow fibres, while the β1 isoform is restricted to type IIB fast glycolytic fibres (Kramarcy and Sealock, 2000). Furthermore, all of these isoforms are present on the sarcolemma and at the NMJ, except for β2-syntrophin, which is localized almost exclusively at the NMJ. In fact, even within the NMJ, syntrophins do not appear to share the same localization. For example, the α_1 isoform localizes to the crests of junctional folds while the β-syntrophins localize mainly to the troughs (Kramarcy and Sealock, 2000; Peters et al., 1997; Piluso et al., 2000). The different distribution patterns of the individual syntrophin isoforms suggest that each has a unique, but related function.

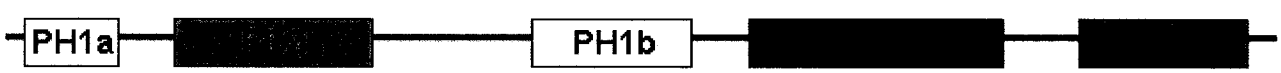
Syntrophins are hypothesized to play a role in cellular signaling. This contention is based in their conserved domain structure, which is composed almost exclusively of protein-protein interaction modules, consistent with the idea that they interact with signaling molecules (Adams et al., 1995; Ahn et al., 1994; Piluso et al., 2000). At their

N-termini, syntrophins contain two PH domains (which are protein-protein and protein-lipid interaction modules), with a PDZ domain inserted into the first PH domain, followed by a C-terminal syntrophin-unique (SU) domain whose specific function remains unknown (Figure 1.2) (Adams et al., 1993; Adams et al., 1995; Piluso et al., 2000; Yang et al., 1994). Of these different modules, the one that is the best characterized is the PDZ domain.

PDZ domains were first discovered in membrane-associated guanylate kinases (Cho et al., 1992; Willott et al., 1993; Woods et al., 1991), and the proteins that contain them are most often found at areas of membrane specialization such as tight junctions, septate junctions, and synapses (Sheng and Sala, 2001). PDZ domains can interact with protein ligands via two principle mechanisms. They can either recognize consensus C-terminal motifs, or internal sequences. For example, syntrophin PDZ domains typically bind to the C-terminus of proteins containing the consensus sequence **Q/R/K-E-S/T**-X-**V/L/I**-COOH (Gee et al., 2000; Songyang et al., 1997; Sheng and Sala, 2001). Alternatively, they can recognize internal sequences in cyclic peptides, or form PDZ-β-finger-PDZ interactions, such as with neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996; Gee et al., 1998a; Hillier et al., 1999).

Examples of syntrophin PDZ-interacting proteins include the cytosolic enzyme neuronal nitric oxide synthase (nNOS), which interacts with α_1-syntrophin in brain and skeletal muscle cells (Adams et al., 2001; Brenman et al., 1996; Hashida-Okumura et al., 1999; Hillier et al., 1999), and the islet cell autoantigen-512 (ICA512) receptor tyrosine phosphatase-like protein associated with secretory granules, whose C-terminus binds to β_2-syntrophin's PDZ domain in pancreatic β-cells (Ort et al., 2000). Furthermore, the C

Figure 1.2 Modular domain organization of syntrophins. All syntrophin isoforms share the same modular domain organization. At their N-termini they contain two pleckstrin-homology domains (PH1 and PH2). A PDZ (**P**SD-95, **D**iscs large, **Z**O-1) domain, whose N- and C-termini are closely apposed, is inserted into the first PH domain in a way that is not thought to disrupt the function of the latter. At their C-termini is a syntrophin-unique (SU) domain (Reviewed in Froehner et al., 1997).



PH = Pleckstrin homology

PDZ = PSD-95/Discs-large/ZO-1

SU = Syntrophin-unique

termini of membrane sodium channels mediate their interaction with syntrophin PDZ domains in skeletal muscle (Gee et al., 1998b; Schultz et al., 1998).

The interaction between nNOS and the α_1-syntrophin PDZ domain has been the most well-studied and may provide some insight into syntrophin PDZ interactions in general. nNOS is a Ca^{2+}-calmodulin-activated enzyme that produces nitric oxide (NO) gas from L-arginine, and thus plays a role in reactive free radical homeostasis (Christopherson and Bredt, 1997). nNOS is highly abundant in brain where it appears to play many roles, such as in the regulation of cerebral blood flow, long-term potentiation (LTP) and long-term depression (LTD) (Christopherson and Bredt, 1997). nNOS is also expressed in skeletal muscle where it is concentrated beneath the sarcolemma of fast twitch (type II) fibres (Kobzik et al., 1994), owing to its association with dystrophin, a protein located at the cytokeleton-plasma membrane interface (Brenman et al., 1995). In this tissue it appears to play many roles, such as in the regulation of muscle contractility, relaxation, metabolism, and even myotube formation and maturation (Christopherson and Bredt, 1997; Kobzik et al., 1994; Thomas et al., 1998). Data from several studies strongly suggest that α_1-syntrophin is the protein directly responsible for linking nNOS to the dystrophin-associated protein complex beneath the sarcolemma. For instance, the sarcolemmal localization of nNOS is lost in the α_1-syntrophin knockout mouse, and is reestablished when this mouse is bred with a transgenic one expressing the full-length α_1-syntrophin gene (Adams et al., 2000; Adams et al., 2001; Kameya et al., 1999). Interestingly, there are no gross histological changes in the α_1-syntrophin knockout mouse, its muscle contractile properties appear to be unaffected, and furthermore these mice are indistinguishable from wild-type mice (Adams et al. 2000; Kameya et al. 1999).

Biochemical experiments demonstrate that α_1-syntrophin and nNOS bind through PDZ-β-finger-PDZ interactions, and in transgenic mice that express a mutated form of α1-syntrophin lacking a functional PDZ domain, nNOS is absent from the sarcolemma (Adams et al., 2001; Brenman et al., 1996). Together these experiments demonstrate a role for syntrophin PDZ domains in localizing nNOS to the plasma membrane, which likely affects its signaling functions.

Aside from PDZ domains, other syntrophin modules have been shown to interact with ligands. For instance, the PH2 and SU domains in tandem are responsible for binding to the C-termini of dystrophin, utrophin, or dystrobrevin, thereby leaving syntrophin's N-terminus free to interact with other molecules (Ahn and Kunkel, 1995; Ahn et al., 1996; Kachinski et al., 1999; Kramarcy et al., 1994). The N-terminal PH1 domain in α_1-syntrophin binds to the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) *in vitro*. Regions in this syntrophin isoform's N-terminus and C-terminus bind *in vitro* to calmodulin and to Ca^{2+}-calmodulin, respectively (Madhavan et al., 1992; Newbell et al., 1997). Thus, due to their modular domain organization, their multiple interactors, and their differing subcellular localizations, syntrophins are hypothesized to play an important role in organizing multimolecular signaling complexes at precise areas within the cell.

Syntrophins, like other scaffolding molecules, may undergo post-translational modifications. For instance, biochemical experiments demonstrate that the plasma membrane-associated stress-activated protein kinase-3 (SAPK3), a mitogen-activated protein kinase (MAPK) family member, binds to and phosphorylates α_1-syntrophin, however the purpose of this is unknown (Hasegawa et al., 1999). Results from another

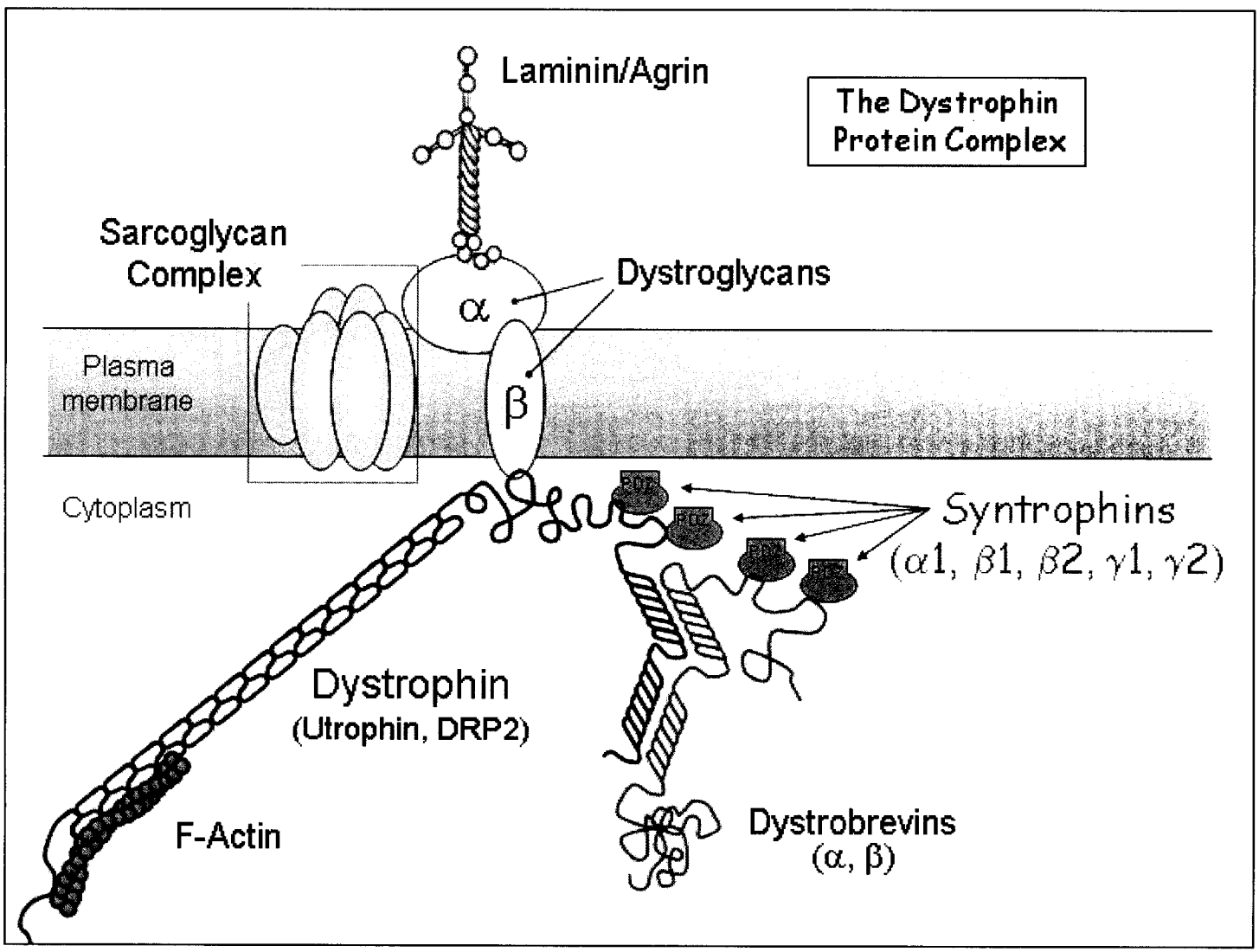
group suggest that Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) can phosphorylate dystrophin and syntrophin, thus inhibiting the binding of these two proteins *in vitro*, raising the possibility that this mechanism may regulate syntrophin docking to the dystrophin-associated protein complex (Madhavan and Jarrett, 1999). Another example is the interaction between the β_2-syntrophin PDZ domain and the C-terminus of islet cell autoantigen 512 (ICA-512), a receptor tyrosine phosphatase-like protein bound to fusion vesicles in pancreatic β-cells (Ort et al., 2001). Ort and colleagues (2001) demonstrated that stimulation of insulin secretion in these cells leads to syntrophin dephosphorylation, its release of ICA512, and ICA512 cleavage by Ca^{2+}/μ-calpain. β_2-syntrophin could in this case play a role in localizing secretory granules to specific areas within the cell. Furthermore, stimulation of insulin secretion, followed by dephosphorylation of β_2-syntrophin by some yet unidentified phosphatase, may serve to mobilize secretory granules to the plasma membrane. Thus, the phosphorylation state of syntrophin could potentially play a role in the functional and/or spatial regulation of this protein and its associated ligands.

The dystrophin-associated protein complex

Syntrophins are localized to precise plasma membrane areas within the cell, and this is thought to be due to its association with the actin cytoskeleton via dystrophin, the product of the gene mutated in Duchenne muscular dystrophy. Dystrophin is a rod-like cytoskeletal protein expressed in skeletal muscle, retina, and brain. Its N-terminus binds to filamentous actin (F-actin), while its C-terminus binds to β-dystroglycan, an integral membrane glycoprotein that links dystrophin to the extracellular matrix through α-

dystroglycan (Figure 1.3) (Davison et al., 1989; Hammonds et al., 1987; Koenig et al., 1988; Roberts, 2001). The latter is a receptor for extracellular matrix proteins like

Figure 1.3 Model of the dystrophin-associated protein complex (DAPC). The best charaterized dystrophin-associated protein complex is the one in skeletal muscle, where it is localized to the sarcolemma. At the heart of this complex is the dystrophin protein, a rod-shaped cytoskeletal molecule whose N-terminus binds to filamentous actin. The C-terminus of dystrophin binds to β-dystroglycan, a transmembrane glycoprotein that links dystrophin to the extracellular matrix via α-dystroglycan. Other transmembrane glycoproteins forming part of the DAPC are the sarcoglycans (α, β, γ, δ, and ε) and sarcospan. The C-terminus of dystrophin also binds to cytosolic DAPC components, syntrophins and dystrobrevin. Dystrobrevin and dystrophin have the potential to bind to two syntrophin molecules each, therefore one DAPC may theoretically comprise a total of four syntrophins. Syntrophin's role is thought to be in organizing multiprotein signaling complexes to the DAPC by interacting with ligands via its multiple protein-protein interaction modules. At the sarcolemma, the dystrophin-associated protein complex localizes to the areas overlying costameres, where it is thought to form a mechanically strong link between the contractile apparatus and the extracellular matrix. Furthermore, the DAPC localizes to the troughs of the folds at the neuromuscular junction, where it appears to associate with voltage-gated sodium channels via syntrophin. At the crests of the junctional folds, and associated with acetylcholine receptor clusters, is a DAPC where dystrophin is replaced by its autosomal homolog utrophin. (Reviewed in; Roberts, 2001; Sanes and Litchtman, 1999).


Laminin/Agrin
The Dystrophin Protein Complex
Sarcoglycan Complex
Dystroglycans
α
β
Plasma membrane
Cytoplasm
Syntrophins
(α1, β1, β2, γ1, γ2)
Dystrophin
(Utrophin, DRP2)
F-Actin
Dystrobrevins
(α, β)

laminin, and proteoglycans such as agrin, perlecan, and biglycan (Bowe et al., 2000; Gee et al., 1993; Gee et al., 1994; Peng et al., 1998; Winder et al., 2001). Dystrophin also binds to transmembrane proteins, such as the sarcoglycans and sarcospan, as well as cytosolic molecules, represented by dystrobrevins and syntrophins. Collectively, dystrophin and its associated proteins are known as the dystrophin-associated protein complex (DAPC). Specific DAPCs are localized to distinct plasma membrane areas. For instance, while DAPCs are thought to be localized to central synapses and areas of the muscle sarcolemma overlying costameres, dystrophin is replaced in this complex by its autosomal homolog, utrophin, at acetylcholine receptor-rich areas of the NMJ's postsynaptic membrane (crests of the junctional folds), and at the basolateral membrane of epithelial cells (Bewick et al., 1996; Kachinski et al., 1999; Lidov et al., 1990; Montanaro et al., 1995; Ohlendieck et al., 1991a; Porter et al., 1992; Rybakova et al., 2000; Schmitz et al., 1993). The role of the dystrophin-associated protein complex in skeletal muscle is believed to be in maintaining the mechanical stability of the sarcolemma during repeated muscle contractions. However, this complex may have additional roles in both excitable and non-excitable cells by virtue of its interaction with syntrophin.

Recent evidence suggests that the DAPC may contain up to four syntrophins, because dystrophin, utrophin, and dystrobrevin, each contain two potential syntrophin-binding sites (Ahn and Kunkel, 1995; Feener et al., 1989; Newey et al., 2000; Susuki et al., 1995). The regions of the messages coding for these sites can be alternatively spliced in tissues such as the brain, heart, and skeletal muscle, a mechanism that is likely used to control the stoichiometry of syntrophins in the dystrophin complex. By binding multiple

syntrophins and their associated signaling molecules, the DAPC could potentially serve to spatially regulate multiple signaling pathways.

Duchenne Muscular Dystrophy

At the molecular level, certain mutations or deletions in the dystrophin gene lead to the loss of dystrophin protein and are the cause of Duchenne muscular dystrophy (DMD). DMD is an X-linked lethal disease that affects 1 in 3500 boys (and girls in some rare cases) and is characterized by progressive proximal muscle weakness beginning in early childhood, wheelchair dependence in their early teens years, and eventually death in their second or third decade due to diaphragm or cardiac muscle failure (Brown, 1997; Duchenne, 1868; Hoffman et al., 1987; Hoffman and Kunkel, 1989; Koenig et al., 1987).

The loss of dystrophin in DMD and in the *mdx* mouse (model of DMD) leads to a deficiency of dystrophin-associated proteins at the sarcolemma of skeletal muscle cells (Brown et al., 1997; Bulfield et al., 1984; Ervasti et al., 1990; Ibraghimov-Beskrovnaya et al., 1992; Matsumura et al., 1993; Ohlendieck et al., 1993a; Ohlendieck and Campbell, 1991b). Since the DAPC links the cytoskeleton to the extracellular matrix, its loss in DMD (and *mdx*) is thought to lead to sarcolemmal instability, thereby rendering the muscle cells susceptible to damage from repeated muscle contractions (Ohlendieck et al., 1993a). Pathological findings in dystrophic muscle that support this idea include sarcolemmal lesions, increased serum creatine kinase levels, elevated intracellular calcium levels, and increased protein turnover (MacLennan and Edwards, 1990; Matsuda et al., 1995; McArdle et al., 1994; Mokri and Engel, 1975; Moser et al., 1984; Ozawa et al., 1999; Turner et al., 1988).

Duchenne muscular dystrophy is a disease that has a primary affect on skeletal muscle, however some DMD patients appear to have cognitive defects, reflected by a leftward shift of average IQ scores from the normal population mean (Blake and Kroger, 2000). The overall brain morphology in these patients appears normal but there have been several accounts of structural changes in the brains of some DMD patients such as mild cerebral atrophy, neuronal loss, and gliosis (Jagadha and Becker, 1988; Yoshioka et al., 1980; Górecki et al., 1992; Górecki and Barnard, 1995). In comparison with skeletal muscle, much less is known about the composition, localization, and function of dystrophin networks in the brain, however they likely have differing functions in these two tissues. For example, in neurons the DAPC is not likely to be necessary for maintaining the integrity of the plasma membrane, as it does in contracting skeletal muscle cells. Furthermore, the expression, localization, and association, of dystrophin-associated proteins in the brain of *mdx* mice, do not appear to be affected, suggesting that they may be anchored together at specific areas by some means other than dystrophin (Blake et al., 1999).

To date, no diseases have been associated with mutations in any of the syntrophin genes, and furthermore, its absence at the sarcolemma of dystrophic muscle does not appear to be directly responsible for the pathogenesis of DMD, as demonstrated by the apparent lack of myopathy in α_1-syntrophin knockout mice (Adams et al., 2000; Kameya et al., 1999). However, syntrophin does appear to play an important role in the formation or maintenance of the NMJ, since α_1-syntrophin knockout mice have abnormal postjunctional membrane folding, absence of utrophin, decreased expression of the nicotinic acetylcholine receptor (AChR), as well as a decline in the levels of

acetylcholinesterase (AChE) (Adams et al., 2000; Kameya et al., 1999). In both this mouse and the α_1-syntrophin-ΔPDZ transgenic mouse, nNOS is mislocalised to the cytosol, clearly indicating that to be properly localized at the sarcolemma, nNOS's C-terminus must interact selectively with α_1-syntrophin's PDZ domain beneath the plasma membrane (Adams et al., 2001). It has previously been shown that the loss of syntrophin from the sarcolemma in *mdx* mice, and the subsequent downregulation and mislocalization of nNOS to the cytosol, are not directly responsible for the pathological features seen in dystrophic mice (Brenman et al., 1995; Chang et al., 1996; Crosbie et al., 1998), however this does not exclude the possibility that these events contribute to them. For instance, in dystrophin-deficient mice, the mislocalization and downregulation of nNOS does appear to be linked to the inability of active skeletal muscle to metabolically inhibit the local α-adrenergic vasoconstriction mediated by NO, an observation supported by similar findings in the nNOS knockout mouse (Thomas et al., 1998). The unopposed sympathetic vasoconstriction in exercising skeletal muscle of Duchenne patients appears to cause muscle ischemia, which may contribute to muscle damage in DMD (Sander et al., 2000). Additionally, normalizing nNOS levels in *mdx* skeletal muscle has been shown to ameliorate muscular dystrophy by reducing macrophage-mediated inflammation, sarcolemmal damage, and degeneration (Wehling et al.,2001).

Together these data suggest that the loss of syntrophin at the sarcolemma in dystrophic muscle, and the subsequent downregulation and loss of nNOS from the plasma membrane, affects the function of nNOS in a way that may contribute to the pathogenesis of Duchenne muscular dystrophy. Furthermore, the possibility remains that

the mislocalization of other syntrophin ligands may also contribute to the development of this or another type of myopathy.

Research Objectives

To more fully understand the role of syntrophins we sought to identify additional syntrophin-interacting proteins. We focused our attention on the PDZ domain of γ_1-syntrophin because no PDZ-interacting proteins had been identified for this isoform. In Chapter 2 I describe how I identified a diacylglycerol kinase (for a description see p.49) as a γ_1-syntrophin-interacting protein, using the yeast two-hybrid screening technique.

Furthermore, we later found that this lipid kinase could interact *in vitro* with the PDZ domains of α- and β-syntrophins, which are expressed in skeletal muscle. Our interest then shifted towards determining if this complex exists in skeletal muscle, and determining its localization. In Chapter 3 I also describe how diacylglycerol kinase does appear to interact with syntrophin in skeletal muscle cells, and that it co-localizes with syntrophin at the sarcolemma and NMJ. We then wanted to determine if, like nNOS, diacylglycerol kinase is mislocalized from the sarcolemma in the absence of dystrophin. In Chapter 3 I describe how sarcolemmal levels of this enzyme are noticeably decreased in degenerating fibres of *mdx* skeletal muscle. Together our results suggest that syntrophin regulates the localization, and consequently the function of DGK-ζ, in both neurons and skeletal muscle fibres.

Chapter 2

Interaction of γ1-Syntrophin with Diacylglycerol Kinase-ζ: Regulation of Nuclear Localization by PDZ Interactions

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Running Title: Diacylglycerol kinase-ζ interaction with γ1-syntrophin

ACKNOWLEDGEMENTS

This work was supported by grant MT-15505 to S.H.G from the Canadian Institutes of Health Research (CIHR). S.H.G. is supported by a CIHR New Investigator Award. The authors would like to thank Drs. Gerry Cox and Lou Kunkel for the γ1-syntrophin cDNA, Dr. Morgan Sheng for PDZ domain constructs and Dr. Stanley C. Froehner for monoclonal antibody 2101 to syntrophins. We would particularly like to thank Dr. Johnny Ngsee and Pierre- Yves Gougeon for assistance with yeast two-hybrid screening and for providing the brain cDNA library.

SUMMARY

Syntrophins are modular adapter proteins that link ion channels and signaling proteins to dystrophin and its homologues. A yeast two-hybrid screen of a human brain cDNA library using the PDZ domain of γ1-syntrophin, a recently identified brain-specific isoform, yielded overlapping clones encoding the C terminus of diacylglycerol kinase-ζ (DGK-ζ), an enzyme that converts diacylglycerol into phosphatidic acid. In biochemical assays, the C terminus of DGK-ζ, which contains a consensus PDZ-binding motif, was found to be necessary and sufficient for association with γ1-syntrophin. When coexpressed in HeLa cells, DGK-ζ and γ1-syntrophin formed a stable complex that partitioned between the cytoplasm and nucleus. DGK-ζ translocates from the cytosol to the nucleus, a process negatively regulated by protein kinase C phosphorylation. We found that DGK-ζ recruits γ1-syntrophin into the nucleus and that the PDZ-binding motif is required. Disrupting the interaction altered the intracellular localization of both proteins; DGK-ζ accumulated in the nucleus, whereas γ1-syntrophin remained in the cytoplasm. The level of endogenous syntrophins in the nucleus of HeLa cells also reflected the amount of nuclear DGK-ζ. In the brain, DGK-ζ and γ1-syntrophin were colocalized in cell bodies and dendrites of cerebellar Purkinje neurons and other neuronal cell types suggesting their interaction is physiologically relevant. Moreover, coimmunoprecipitation and pull-down experiments from brain extracts and cells suggest DGK-ζ, γ1-syntrophin and dystrophin form a ternary complex. Collectively, our results suggest γ1-syntrophin participates in regulating the subcellular localization of DGK-ζ to ensure correct termination of DAG signaling.

INTRODUCTION

Cells respond to extrinsic cues, such as hormone or neurotransmitter binding, by the generation of intracellular second messenger molecules. An efficient response usually requires the precise localization of receptors, ion channels and signal transduction proteins and the coordinated coupling of these proteins into functional signaling pathways. It is becoming increasingly apparent that the clustering and anchoring of these proteins at specific subcellular sites is mediated by scaffold proteins that interact with the cytoskeleton (1). Proteins containing PDZ domains have emerged as key players in the targeting of membrane proteins and the spatial control of intracellular signaling.

The dystrophin-associated protein complex (DAPC) is a group of membrane and cytoplasmic proteins that interacts with dystrophin, the product of the Duchenne muscular dystrophy (DMD) gene (2). In skeletal muscle, the DAPC forms a link between the extracellular matrix and the actin cytoskeleton and is thought to have a role in stabilizing the membrane against the forces of contraction (3). The absence of dystrophin and the concomitant loss of the DAPC from the sarcolemma in Duchenne muscular dystrophy disrupt the cytoskeletal-extracellular matrix link and ultimately lead to the progressive degeneration of muscle fibers (4). Recent evidence suggests the DAPC also organizes functional signaling complexes at the cytoskeleton-plasma membrane interface (2).

Syntrophins are a family of cytoplasmic peripheral membrane proteins that link ion channels and signaling proteins to the DAPC via a direct interaction with the C terminus of dystrophin family proteins (5). The five known isoforms share a common domain organization, consisting of two tandem pleckstrin homology (PH) domains at the N-terminus, a highly conserved C-terminal domain that is unique to syntrophins (SU), and a single PDZ domain (6, 7).

Syntrophin PDZ domains (α1, β1, and β2), interact with the C-terminal sequence motif (consensus E(S/T)XV) found in skeletal muscle Na^+ channels (8, 9) and stress-activated protein kinase-3 (SAPK3/ERK6) (10). In addition, α- and β-syntrophins bind to neuronal nitric oxide synthase and microtubule-associated serine/threonine kinases through PDZ-PDZ/β-finger interactions (11-13).

Each syntrophin has a unique tissue and developmental expression pattern and selectively pairs with different dystrophin family proteins *in vivo*, suggesting that complexes containing different isoforms serve distinct functional roles (7, 14-18). For example, α1-syntrophin is necessary for the membrane association of neuronal nitric oxide synthase and for the proper targeting of utrophin, a dystrophin homologue, to the postsynaptic membrane of skeletal muscle (19). In addition, although more than one syntrophin isoform can be expressed in a single cell type, their subcellular distributions can differ dramatically, implying their localization is tightly regulated. For instance, in skeletal muscle, α1-syntrophin is distributed over the entire sarcolemma and is present throughout the folds at neuromuscular junctions, whereas β2-syntrophin is found almost exclusively at junctions and is confined to the lower portion of the folds (20, 21).

Recently, Piluso et al. (22) identified two additional syntrophin isoforms, γ1 and γ2. By Northern analysis, γ1-syntrophin is expressed uniquely in the brain, whereas γ2-syntrophin has a somewhat broader distribution. *In situ* hybridization and immunohistohemistal studies have shown that γ1-syntrophin is expressed exclusively in neurons, and although γ1-syntrophin binds to the C terminus of dystrophin family members like other syntrophins (22), it does not interact with neuronal nitric oxide synthase through its PDZ domain (23). In the present study, we used the yeast two-hybrid system to identify brain proteins that interact with the PDZ domain of γ1-

syntrophin. We found that the enzyme diacylglycerol kinase-ζ (DGK-ζ) interacts with γ1-syntrophin via a PDZ-binding motif at its C terminus. DGK-ζ is one of several known DGK isoforms, all of which convert diacylglycerol (DAG) to phosphatidic acid (PA) and are believed to attenuate the activity of proteins activated by DAG, such as protein kinase C (PKC). Collectively, our results suggest that γ1-syntrophin may participate in regulating the subcellular localization of DGK-ζ to ensure that DAG is rapidly inactivated following receptor activation.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Assay - Yeast two-hybrid screens were performed using the EGY48 yeast strain harboring the reporter genes β-galactosidase (β-gal) on the plasmid EGY48 and *Leu2* on the plasmid pSH18-34. These reporter genes are under the control of six and eight upstream LexA operators, respectively (Invitrogen Corp., Carlsbad, CA). The PDZ domain bait consisted of amino acids 51-149 of human γ1-syntrophin fused in frame with the LexA DNA-binding domain in vector pHybLex/Zeo (Invitrogen Corp.). A human brain cDNA library constructed in the activation domain vector pB42AD (CLONTECH Laboratories, Palo Alto, CA) was screened with the PDZ domain bait.

Several small-scale yeast transformations were performed where 2.5×10^{8} cells containing the bait plasmid were transferred to 50 mL of YPD medium and subsequently incubated until the density reached 2×10^{7} cells/mL, after which the liquid yeast culture was transformed with 5 μg of cDNA library and 0.5 mg of salmon sperm carrier DNA using the lithium acetate method with 50% polyethylene glycol (w/v). After transformation, the yeast were grown 1-2 h in selective ura^{-} trp^{-} medium to allow expression of the *Leu2* reporter gene, before adding Zeocin (Invitrogen) at 200μg/mL. The cultures were grown overnight then plated on ura^{-} trp^{-} leu^{-} plates containing 25 μg/ml Zeocin, 2% galactose, and 1% raffinose.

DNA was isolated from positive yeast colonies and transformed into the KC8 bacterial strain by electroporation. Positives were isolated from the bacteria and retransformed into yeast with either the bait or a negative control (pHybLex/Zeo + Lamin) and plated on selective 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) plates. Positives were sequenced at the University of Ottawa Biotechnology Research Institute. To identify subsequent DGK-ζ clones, the primers 5'-CAGTCAGCACTGGCAGCAAG and 5'-TAGTGCTGCCGGTTCTCCAG were

used to amplify DNA directly from positive yeast colonies. Positive colonies were identified by the presence of a 259-base pair polymerase chain reaction product.

Antibodies - Antigenic peptides 342 (CKILKDSDLLDRRK) and 435 (CKFSQLKGSSDDGKSK) were chosen from the deduced amino acid sequence of human γ1-syntrophin by the method of Hopp and Woods (24). The peptide designation refers to the number of the first amino acid in the sequence. Peptides were synthesized by the University of Waterloo Peptide Synthesis Facility (Waterloo, ON) and contained an additional cysteine residue (underlined) at the N terminus. The peptides were conjugated to keyhole limpet hemocyanin via their N-terminal cysteine residues using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) according to the method described in Harlow and Lane (25). The resultant peptide conjugates were injected into rabbits. The antisera were prepared by Covance, Inc. (Denver, PA) and were affinity purified on immobilized immunogenic peptides as described (8). The peptides were coupled to Ultralink Iodoacetyl (Pierce) according to manufacturer's instructions. The T7 tag monoclonal antibody was purchased from Novagen (Madison, WI). Monoclonal anti-dystrophin, clone MANDRA-1 was purchased from Sigma. Texas Red-, Cy3- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-FLAG M2 monoclonal antibody was purchased from Sigma (F3165), the polyclonal anti-HA antibody was from Zymed Laboratories Inc. (San Francisco, CA), and the monoclonal antibody (B 14) to GST was from Santa Cruz Biotechnology (Santa Cruz, CA). To produce polyclonal antibodies to the N terminus of DGK-ζ, a peptide (CSERDAGPEPDKAPRRLNK) corresponding to human DGK-ζ was synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits. The antibodies were affinity purified from serum on immobilized peptide. The specificity of the antibodies was verified by

Western blotting extracts from cells transfected with DGK-ζ and by preabsorption of antibodies with the immunizing peptide.

DNA Constructs - A cDNA clone (4B1) encoding full-length γ1-syntrophin in the plasmid vector pFHR was a generous gift from Drs. Gerry Cox and Lou Kunkel (7). To construct an N-terminal GFP-γ1-syntrophin fusion, the γ1-syntrophin coding region was amplified by polymerase chain reaction from clone 4B1 and cloned in frame into ApaI and Hind III restriction sites of pQBI25-fC1 (Quantum Biotechnologies, Montréal, Canada) and then subjected to DNA sequencing. To construct T7-tagged γ1-syntrophin, the cassette encoding GFP enzymatically removed using ApaI and NheI restriction sites, and a duplex oligonucleotide encoding the T7 tag was inserted into the same sites. The PDZ domain of γ1-syntrophin was amplified using forward (5'-ATATGAATTCTTCTATTCTGGTGAAAGAACGGTG) and reverse (5'-ATATCTCGAGTG-GGAGTTTGAGGAAAGCAGGTG) primers containing EcoRI and XhoI restriction sites (underlined), respectively, and was cloned into the same sites in pET-32a (Novagen). The γ1-syntrophin PDZ domain was subcloned from pET-32a into pHybLex/Zeo and pGEX-5X1 using the EcoRI and XhoI restriction sites. Constructs encoding the PDZ domains of α- and β-syntrophins have been described previously (8). PSD-95 PDZ constructs were kindly provided by Dr. Morgan Sheng (Harvard Medical School, Boston, MA) and have been described previously (26, 27)

Subcellular Fractionation of Mouse Brain Extracts – Adult C57BL6 mice were sacrificed by CO_2 overdose, and their brains removed, and frozen in liquid nitrogen, and then stored at –80°C until needed. To prepare crude subcellular fractions, 1-2 g of mouse brain were added to 10 volumes of TEE buffer + protease inhibitor mixture (50 mM Tris, pH 7.4, 1mM EDTA, 1mM EGTA, and 10 μg/mL each of leupeptin, antipain, 4-(2-amminoehtyl)-

benzenesulfonyl fluoride HCl, pepstatin A, and benzamidine HCl). The mixture was homogenized in a small sample cup of a Waring blender and centrifuged at 48,000 × *g* for 10 min. An aliquot of the supernatant (cytosolic fraction) was removed, added to 5X concentrated SDS sample buffer, and heated for 5 min at 95°C. The pellet was resuspended in 10 volumes of TEE buffer + protease inhibitors, homogenized as before, and centrifuged at 48,000 × *g* for 10 min. The pellet was resuspended in 10 volumes of TEE buffer containing 0.1 M NaCl, 1% Triton X-100, and protease inhibitors, extracted on an Adams Nutator for 30 min at 4°C, and then centrifuged at 48,000 × *g* for 30 min. The supernatant (membrane fraction) was removed and added to 5× reducing sample buffer as above.

Peptides and Overlay Assays – Synthetic peptides corresponding to the C-terminal 10 amino acids of the adult skeletal muscle Na^+ channel SkM1 (VRPGVKESLV), the embryonic skeletal muscle Na^+ channel SkM2 (SPDRDRESIV), and the Shaker-type K^+ channels Kv1.4 (SNAKAVETDV) and Kv1.5 (CLDTSRETDL) were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO). The DGK-ζ C-terminal peptide (IQREDQETAV) was synthesized by the University of Waterloo Peptide Synthesis Facility (Waterloo, ON). All peptides contained an additional 4-amino acid linker (SGSG) at the N terminus and an N-terminal biotin. Overlay assays were carried out as described previously (8, 28) with the following modifications. Signals generated by enhanced chemiluminescence were captured with a Digital Image Station (Kodak). The amount of fusion protein loaded in each lane was compared by digitally capturing an image of the Ponceau S-stained blot prior to blocking.

Preparation and Purification of Bacterial Fusion Proteins – Fusion proteins were purified as described previously (8) with the following modifications. BL21(λDE3)pLysS cells were transformed and grown in 2 ml LB medium supplemented with 200 μg/ml ampicillin and

34 μg/ml chloramphenicol (LB-Amp-Chlor) overnight at 37°C in a shaking incubator. Bacteria were pelleted by centrifugation and resuspended in 1 ml of fresh LB-Amp-Chlor. This was used to inoculate 1 litre of LB-Amp-Chlor, which was grown until the optical density at 600 nm reached 0.6 and 1.0. Protein expression was then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (BioShop, Canada, Inc., Burlington, Canada), and the culture was incubated overnight at 28°C. All subsequent steps were as described.

Immunofluorescence Localization – C57BL6 mice were anesthetized with sodium pentobarbital (50mg/kg) and transcardially perfused with normal saline, followed by perfusion with a solution of 3% paraformaldehyde, 0.1% glutaraldehyde, 15% picric acid, 0.1M phosphate buffered saline (PBS), pH 7.4. The brains were immediately removed from the cranium, post-fixed for 90 minutes at 4°C in the same buffer, then transferred to PBS, pH 7.2, 10% sucrose, then stored overnight at 4°C. The brains were flash frozen with powdered dry ice, and saggital sections were cut on a cryostat at a thickness of 10 μm at –20 °C. The sections were stored at –80 °C prior to use.

The sections were thawed, ringed with a hydrophobic boundary and rinsed with PBS. The sections were then incubated with affinity-purified antibody diluted in PBS, pH 7.2, containing 0.3% Triton X-100 and 1% normal goat serum in a closed moisture chamber overnight at 4 °C. The sections were subsequently rinsed in PBS for 15 min and then incubated with a 1:200 dilution of FITC- or Texas Red dye-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted in PBS + 0.3% Triton X-100. The sections were then rinsed in PBS for 15 min and coverslipped with Fluoromount G (EMS, Fort Washington, PA). A Zeiss Axioskop microscope was used to visualize the sections, and images

were captured using a Sony CCD camera and Northern Eclipse software. The images were processed using Adobe Photoshop.

Immunoprecipitation – Cells for immunoprecipitation were used 18-24 h post-transfection. All steps were carried out at 4°C or on ice. Cells 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% Triton X-100 and 10 μg/mL each of leupeptin, antipain, 4-(2-amino-ethyl)-benzenesulfonyl fluoride HCl, pepstatin A, and benzamidine HCl)/ 100 mm dish. The cells were incubated on ice for 30 min then scraped from the dish and centrifuged for 10 min at 4°C. The supernatant was removed, and an aliquot of this starting material (Input) was boiled in SDS sample buffer. One to five μg of antibody was added to ~0.5 ml of supernatant and incubated at 4°C with mixing for 1-2 h. To purify immune complexes, 50 μl of a 50% slurry of washed protein A-Sepharose beads (Amersham Pharmacia Biotech) or 20 μl of protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added and incubated an additional 1 h at 4°C with mixing. The immune complexes were collected by centrifugation, and an aliquot of the supernatant was removed and boiled in SDS sample buffer (unbound fraction). The complexes were washed three times for 5 min each with lysis buffer then eluted by boiling in 50 μl of SDS sample buffer. The samples were then subjected to SDS-PAGE.

Cell Culture and Transient Transfections – HeLa cells were grown in Dulbecco's modified Eagle's medium high glucose supplemented with 2 mM glutamine, 5% fetal bovine serum and 100 units/ml penicillin-streptomycin. For transfections, cells were 40-80% confluent. The cells were transfected using the calcium phosphate method as follows. A solution containing 256 mM $CaCl_2$ and 10-30 μg of DNA in sterile water was added dropwise to an equal volume of 2× Hepes-buffered saline, pH 7.05 over a 30-60 s period with constant vortexing. The final

volume of this transfection solution was 250 μl for 35-mm dishes and 1 ml for 100-mm dishes. The Ca^{++}- DNA complexes were allowed to precipitate for 30 min at room temperature. The solution was vortexed and added dropwise onto cells, and the dish was swirled gently to mix. The cells were grown for 8-16 h at 37°C then washed two to three times with nonsupplemented Dulbecco's modified Eagle's medium or sterile PBS.

RESULTS

Identification of DGK-ζ as a γ1-Syntrophin-interacting Protein- A yeast two-hybrid screen of 5.275×10^6 human brain cDNA library clones, using the PDZ domain of γ1-syntrophin as bait, yielded 10 independent overlapping cDNA clones encoding DGK-ζ. The structure of DGK-ζ is shown schematically in Fig. 2.1. All the cDNAs isolated by the yeast two-hybrid screen (four were completely sequenced and are shown here) overlapped in the C-terminal region of DGK-ζ, suggesting that this region mediates the binding to the PDZ domain of γ1-syntrophin (Fig. 2.1). The amino acid sequence (-ETAV) at the C terminus of DGK-ζ is consistent with the class I PDZ domain ligand consensus ((S/T)XV) (29) and with the consensus sequence identified for strong binding to α- and β-syntrophin PDZ domains ((Q/R/K)E(S/T)*X*(V/L/I)) (8, 9, 23). The C-terminal sequence is conserved in human, mouse and rat DGK-ζ (30, 31). The controls for the specificity of the yeast two-hybrid interaction included retesting positive clones by back-transformation into yeast and lack of interaction with a nuclear lamin prey. In addition, γ1-syntrophin PDZ did not self-activate when transformed into yeast with an empty prey vector.

Direct Interaction between the γ1-Syntrophin PDZ Domain and the C Terminus of DGK-ζ – To show direct biochemical association between the PDZ domain of γ1-syntrophin and the C terminus of DGK-ζ, overlay filter-binding assays were performed. A soluble GST fusion protein containing the C terminus of DGK-ζ (clone 8) bound to a His_6 fusion protein of the PDZ domain of γ1-syntrophin but not to any of the PDZ domains of PSD-95 (Fig. 2.2*A*). Interestingly, DGK-ζ appeared to bind more strongly to PDZ domains of α1-, β1-, and β2-syntrophins. Thus, these *in*

vitro binding data indicate that DGK-ζ interacts preferentially with PDZ domains from the syntrophin family of proteins.

We next tested whether this interaction occurs within the context of the full-length γ1-syntrophin protein expressed in mammalian cells. A GST fusion protein containing the C terminus of DGK-ζ efficiently "pulled down" T7-tagged full-length γ1-syntrophin from lysates of transfected HeLa cells (Fig. 2.2*B*), whereas GST alone did not.

To determine whether the C terminus of DGK-ζ is sufficient for interaction with the PDZ domain of γ1-syntrophin, a synthetic peptide corresponding to the C-terminal 10 amino acids of DGK-ζ was assayed for binding to hexahistidine His_6 fusion proteins of various PDZ domains in overlay assays. As shown in Fig. 2.2*C*, the DGK-ζ C terminus bound to all four syntrophin PDZ domains but not to PDZ domains of PSD-95, confirming the results obtained with the GST-DGK-ζ fusion protein. Thus, the C terminus of DGK-ζ is sufficient for binding to γ1-PDZ.

As a further test of the specificity of the interaction between DGK-ζ and γ1-syntrophin, we tested other C-terminal peptide ligands for their ability to bind the γ1-syntrophin PDZ domain. As shown previously, the C terminus of two skeletal muscle sodium channels, SkM1 and SkM2 bound strongly to PDZ domains of α– and β-syntrophins (8, 9) but did not bind to γ1-PDZ (Fig. 2.2*D*). Likewise, the C terminus of the K+ channel Kv1.5 bound strongly to α1-, β1-, and β2-PDZs, but did not bind γ1-PDZ. Of the peptides we tested, only Kv1.4 bound to γ1-PDZ in addition to other syntrophin PDZs. Examination of the C-terminal sequence of Kv1.4 (VETDV) revealed that it is the most similar to that of DGK-ζ (QETAV). These results demonstrate that the interaction of γ1-syntrophin PDZ domain with the C terminus of DGK-ζ is sequence-specific.

We next asked whether the C terminus of DGK-ζ is necessary for interaction with γ1-syntrophin. Most known PDZ domain-mediated interactions occur by the recognition of short C-terminal peptide motifs and require that the peptide ligands have a free carboxylate group (29), but in some cases internal peptide motifs that are well removed from the C terminus can mediate interactions with PDZ domains (12, 28). Therefore, we tested whether the addition of a C-terminal FLAG tag to DGK-ζ would affect its interaction with γ1-syntrophin. The DGK-ζ constructs shown schematically in Fig. 2.2*E* were assayed for binding to γ1-PDZ in pull-down assays. Full-length DGK-ζ containing three tandem N-terminal HA tags (*HA-DGK-ζ*) was efficiently pulled down by GST-γ1-PDZ, but not by GST alone (Fig. 2.2*F*). In contrast, DGK-ζ-FLAG was not pulled down by GST-γ1-PDZ. The specificity of the PDZ-peptide interaction was tested further by replacing the C-terminal sequence ETAV of DGK-ζ with ENSV (Fig. 2.2*F*). This change also completely abolished the interaction with γ1-PDZ, demonstrating that the C-terminal ETAV sequence is a critical determinant of the DGK-γ1-syntrophin interaction. Collectively, our results demonstrate that the C terminus of DGK-ζ is both necessary and sufficient for interaction with γ1-syntrophin.

Interaction of γ1-Syntrophin and DGK-ζ in Mammalian Cells – To further investigate the interaction of γ1-syntrophin and DGK-ζ, we tested whether they associate in mammalian cells. HeLa cells were transiently transfected with T7-tagged γ1-syntrophin and HA-tagged DGK-ζ. After 18-24 h, the cells were solubilized with Triton X-100, and the lysates were subjected to immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted. As shown in Fig. 2.3*A*, an antibody to DGK-ζ coimmunoprecipitated T7-γ1-

syntrophin from HeLa cell lysates. In the reverse experiment, HA-tagged DGK-ζ was coimmunoprecipitated with γ1-syntrophin using a monoclonal T7 tag-specific antibody but was not immunoprecipitated by control mouse IgG (Fig. 2.3*B*). Together, these results indicate that γ1-syntrophin and DGK-ζ form a stable complex in cells.

Proteins containing multiple PDZ domains such as PSD-95/SAP-90 and PSD-93/Chapsyn-110 have been shown to cluster N-methyl-D-aspartate receptors and Shaker-type K^+ channels in heterologous expression systems (26, 32, 33, 33). To determine whether γ1-syntrophin, which contains only a single PDZ domain, could induce clustering of DGK-ζ, we coexpressed HA-tagged DGK-ζ and T7-tagged γ1-syntrophin in HeLa cells and compared their distribution with cells expressing either construct alone. In singly transfected cells, γ1-syntrophin was distributed uniformly throughout the cytoplasm and also appeared to be present at low levels in the nucleus (Fig. 2.3*C*). HA-DGK-ζ, by itself, was similarly distributed (Fig. 2.3*D*). In cotransfected cells, there was no obvious change in the distribution of either protein, but they overlapped throughout the cell (Fig. 2.3*E, F*). Thus, coexpression of γ1-syntrophin and DGK-ζ does not induce clustering of either protein.

PDZ Interactions Regulate the Subcellular Localization of DGK-ζ and γ1-syntrophin–A consistent feature of different DGK isoforms is their ability to shuttle from the cytosol to the plasma membrane in response to cellular signals (34, 35). Some DGK isoforms also translocate to the nucleus, and in the case of DGK-ζ, regulate cell proliferation by reducing nuclear DAG levels (36).

We observed that the partitioning of HA-tagged DGK-ζ between the cytoplasm and nucleus in singly transfected HeLa cells varied considerably from cell to cell. Figure 2.4 (*A, B*) shows single microscopic fields in which the cells have strikingly different levels of nuclear

DGK-ζ. In doubly transfected cells with high levels of HA-DGK-ζ in the nucleus (Fig. 2.4*C*), there were correspondingly high levels of nuclear γ1-syntrophin (Fig. 2.4*D*). Similarly, cells with low levels of nuclear DGK-ζ had low levels γ1-syntrophin in the nucleus (Fig. 2.3*E*, *F*). In all cases, the distributions of γ1-syntrophin and DGK-ζ overlapped exactly, suggesting the two proteins are coordinately regulated and form a stable complex that can translocate into the nucleus.

We hypothesized that the subcellular localization of DGK-ζ may depend on the availability of binding partners in the nucleus and/or cytoplasm. To determine if the subcellular localization of DGK-ζ is influenced by its interaction with γ1-syntrophin, we coexpressed T7-tagged γ1-syntrophin and the C-terminal FLAG-tagged DGK-ζ, which we have shown does not interact with the γ1-syntrophin PDZ domain (see Fig. 2.2*E*, *F*). In virtually all transfected cells, DGK-ζ-FLAG accumulated in the nucleus to high levels (Fig. 2.4*E*). In contrast, γ1-syntrophin remained primarily in the cytoplasm of doubly transfected cells (Fig. 2.4*F*). These results suggest that γ1-syntrophin is recruited to the nucleus via its interaction with the C terminus of DGK-ζ.

Considering that the C terminus of DGK-ζ bound strongly to α- and β-syntrophin PDZ domains in overlay assays (see Fig. 2.2*A*, *C*), we surmised that the variable levels of nuclear DGK-ζ in singly transfected cells (see Fig. 2.4*A*, *B*) might be influenced by the availability of endogenous syntrophins. A Western blot of HeLa cell extracts with a monoclonal antibody (MAntibody 2101) that cross reacts with α- and β-syntrophins confirmed the presence of syntrophins in these cells (not shown). In HeLa cells transfected with HA-DGK-ζ alone, the levels of syntrophins coincided exactly with that of DGK-ζ (compare Figs. 2.5*A* with *B* and C with D). The cells with high levels of nuclear DGK-ζ had correspondingly high levels of

syntrophins in the nucleus (Fig. 2.5 *C*, *D*) suggesting that DGK-ζ can also recruit these syntrophin isoforms to the nucleus.

To confirm that the C-terminal PDZ-binding motif of DGK-ζ is important for controlling its nuclear localization, we examined the subcellular distribution of a mutant in which the consensus PDZ binding motif ETAV was changed to ENSV. This mutant does not bind to the γ1-syntrophin PDZ domain (see Fig. 2.2E). As with DGK-ζ-FLAG, DGK-ζ-ENSV was localized almost exclusively in the nucleus. Moreover, γ1-syntrophin remained predominantly in the cytoplasm in DGK-ζ-ENSV-expressing cells (Fig. 2.4*G*, *H*). Together, these results demonstrate the C-terminal PDZ-binding motif is important for controlling of nuclear/cytoplasmic ratio of DGK-ζ and for recruiting γ1-syntrophin into the nucleus.

Specificity of γ1-Syntrophin and DGK-ζ Antibodies – To study the localization of γ1-syntrophin *in vivo,* we raised polyclonal antibodies against two antigenic peptides (342 and 435) deduced from the primary sequence of the human γ1-syntrophin cDNA. Importantly, the chosen peptide sequences have minimal homology to α– and β-syntrophins. Both antibodies (antibodies 342 and 435) recognize a major band of ~50 kDa in detergent extracts of mouse brain membranes (Fig. 2.6*A*). Preabsorption of the anti-342 and anti-435 antibodies with their respective immunogenic peptides but not with control peptides completely eliminated recognition of the 50 kDa band, confirming the specificity of the antibodies (Fig. 2.6*A*, *lanes marked* + *and* -, respectively). These antibodies also recognize recombinant γ1-syntrophin in Western blots of extracts from transfected cells (not shown).

To examine the tissue distribution of the γ1-syntrophin protein, various tissues were analyzed by Western blotting (for this experiment we used antibody 342 since it had a higher signal-to-noise ratio). γ1-Syntrophin was detected in brain but not in heart, kidney, liver or

skeletal muscle (Fig. 2.6*B*), consistent with the brain-specific expression of the γ1-syntrophin mRNA (22). These results further support the idea that this antibody recognizes authentic γ1-syntrophin protein.

An antibody to a peptide derived from an N-terminal sequence of human DGK-ζ specifically recognizes a major band of ~120 kDa and a minor band of ~100 kDa on immunoblots of mouse brain extracts (Fig. 2.6*C*), consistent with a previous report (31). Recognition of both bands was blocked by preincubation of the antibody with excess immunogenic peptide. The apparently lower molecular weight species may be an incompletely processed form or a proteolytic fragment of DGK-ζ (30, 31).

Colocalization of γ1-syntrophin and DGK-ζ in brain – We used affinity-purified antibodies 342 and 435 for immunocytochemical localization of γ1-syntrophin in mouse brain. These two independent antibodies gave essentially the same staining patterns, strongly suggesting they recognize native γ1-syntrophin in brain sections. Immunofluorescence staining revealed widespread expression of γ1-syntrophin protein in forebrain and cerebellum. At a regional level, γ1-syntrophin staining was prominent in the hippocampal dentate gyrus, the CA1 – CA3 fields of Ammon's horn, the cerebellum, and the cerebral cortex. At the cellular level, γ1-syntrophin immunoreactivity was associated with several types of neurons in the brain.

In the cerebellum, immunoreactivity for γ1-syntrophin was concentrated within cell bodies and dendrites of Purkinje cells (Fig. 2.7*F-H*). There was intense immunoreactivity associated with the plasma membrane of Purkinje neurons as well as diffuse cytoplasmic staining (Fig. 2.7*G*). The labeling of Purkinje dendrites was not as strong and consistent as that for DGK-ζ (see below), but γ1-syntrophin immunoreactivity was detectable in finer dendritic processes (Fig. 2.7*G*, *H*, *arrowheads*). In the hippocampus, pyramidal cells in the CA1, CA2, and CA3

regions were strongly immunoreactive, as were granule cells in the dentate gyrus (Fig. 2.7*I, CA3 region shown*). As in Purkinje neurons, there was diffuse labeling of pyramidal cell bodies that extended into proximal dendrites. The cell bodies of neurons in all layers of the cortex were also positive for γ1-syntrophin, including scattered neurons in the molecular layer (Fig. 2.7*J*).

At the regional level, the distribution of DGK-ζ shows much in common with γ1-syntrophin. Staining was prominent in Purkinje neurons of the cerebellum (Fig. 2.7*A-C*), pyramidal neurons in the hippocampal CA regions (Fig. 2.7*D*), and cell bodies throughout the cortex (Fig. 2.7*E*). DGK-ζ immunoreactivity was particularly strong in the molecular and Purkinje cell layers of the cerebellum (Fig. 2.7*B*). Purkinje cell bodies and dendrites were brightly labeled, with little or no staining of axons (Fig. 2.7*C*). Thus γ1-syntrophin and DGK-ζ show significant overlap in several brain areas and are found within the same neuronal compartments. This colocalization suggests γ1-syntrophin and DGK-ζ are closely associated in neurons.

To verify the biochemical association of DGK-ζ and γ1-syntrophin, we performed pull-down experiments from mouse brain extracts. Beads charged with a GST fusion protein of the γ1-syntrophin PDZ domain (amino acids 55-149) brought down a large fraction of DGK-ζ in the offered extract (Fig. 2.8*A*). GST alone did not precipitate any DGK-ζ, suggesting that the pull-downs are specific. These data demonstrate that native DGK-ζ from brain can interact with γ1-syntrophin. Unfortunately, antibodies to γ1-syntrophin were not useful for immunoprecipitations. Moreover, we were unable to detect γ1-syntrophin in DGK-ζ immunoprecipitates because it comigrated with the immunoglobulin heavy chain.

Interaction of DGK-ζ with Dystrophin in the Brain – Piluso *et al.* (22) recently demonstrated that γ1-syntrophin can associate with dystrophin and other dystrophin family

proteins in yeast two-hybrid assays. In the brain, dystrophin is particularly abundant in cortical and hippocampal pyramidal neurons and in cerebellar Purkinje cells (37), making it a likely candidate for interaction with γ1-syntrophin and DGK-ζ (38). In addition to full-length dystrophin, there are at least two N-terminally truncated isoforms of dystrophin, Dp71 and Dp140 (2). Both isoforms are identical throughout their C termini to full-length dystrophin.

To further examine the interaction between γ1-syntrophin and dystrophin, we tested whether a Dp71-blue fluorescent protein fusion protein formed a complex with T7-tagged γ1-syntrophin in cotransfected HeLa cells. Twenty-four hours after transfection, the cells were solubilized with Triton X-100 and then immunoprecipitated with an antibody to the T7 tag or with an antibody to Dp71. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the same antibodies. As shown in Fig. 2.8*B*, Dp71 and γ1-syntrophin were coimmunoprecipitated from cotransfected cells, indicating they were associated (*left panels*). In contrast, when cells were transfected with γ1-syntrophin alone, γ1-syntrophin was not isolated with the Dp71 antibody (*right panels*), demonstrating the specificity of the coimmunoprecipitation. These results demonstrate that γ1-syntrophin forms stable complexes *in situ* with dystrophin.

To determine if DGK-ζ exists in a complex with dystrophin in the brain, we immunopurified dystrophin complexes and blotted the immunoprecipitates for DGK-ζ (Fig. 2.8C). Although only a relatively small fraction of the total DGK-ζ in the starting material copurified with dystrophin, no DGK-ζ was captured using control rabbit IgG, suggesting that the copurification was specific. Thus, at least some DGK-ζ is associated with dystrophin in the brain.

DISCUSSION

Intracellular levels of the second messenger DAG transiently increase following receptor activation by hormones, trophic factors, and neurotransmitters. DAG produced in response to these external stimuli activates PKC and the guanine nucleotide exchange factors *vav* and RasGRP (39, 40). Prolonged DAG signaling through PKC and RasGRP promotes malignant transformation (40, 41). Therefore, the levels of DAG and its cellular fate must be strictly regulated. To complicate matters, a distinct pool of DAG is an intermediate in the synthesis of phospholipids and triglycerides (34). How these two different pools of DAG are segregated remains an important unanswered question.

DGKs, of which there are nine mammalian isoforms, catalyze the phosphorylation of DAG to phosphatidic acid. This enzymatic conversion is thought to be a key mechanism controlling the cellular levels not only of DAG but also of phosphatidic acid, which has its own biological activities. For this reason, DGKs are postulated to attenuate the activity of certain proteins activated by DAG (42-44).

To efficiently phosphorylate DAG following receptor activation, one or more DGK isozymes must be localized at the plasma membrane where DAG is produced. Indeed, distinct DGK isoforms have differing subcellular distributions and may therefore locally control levels of DAG (34, 45). Regulation of DGK localization may be an important mechanism to segregate different DAG functions within cells. Here, we report that DGK-ζ associates with γ1-syntrophin through a PDZ domain interaction. The C terminus of DGK-ζ, which contains a consensus PDZ-binding motif, was necessary and sufficient for interaction with the PDZ domain of γ1-syntrophin. Because γ1-syntrophin binds to dystrophin ((22) and Fig. 2.8B), DGK-ζ can be linked to submembranous dystrophin complexes and to both the extracellular matrix and the

cytoskeleton. Collectively, these interactions represent a potential mechanism to sequester DGK-ζ at the plasma membrane at sites of receptor-mediated DAG signals.

In the brain, DGK-ζ and γ1-syntrophin are colocalized in cell bodies and dendrites of several distinct neuronal populations, supporting the idea that their interaction is physiologically significant. The expression of both DGK-ζ and γ1-syntrophin was strongest in cerebellar Purkinje neurons, which express a unique isoform of dystrophin (P-type) and do not express any other syntrophin isoforms (46). Dystrophin immunoreactivity in Purkinje cells is restricted to the soma and dendrites and is localized primarily at the plasma membrane (37, 38). Similarly, γ1-syntrophin immunoreactivity is associated with the plasma membrane of Purkinje neurons (Fig. 2.6*G*). Thus, γ1-syntrophin and dystrophin may form a specific complex at the plasma membrane in Purkinje neurons, which may serve to sequester DGK-ζ at the membrane in response to some extracellular signal. In support of this idea, we have shown that a fraction of DGK-ζ exists in a complex with dystrophin in the brain.

DGK-ζ is also found in the nucleus, where it regulates the amount of nuclear DAG. Nuclear DGK-ζ functions to lower DAG levels and attenuate cell growth (36). Translocation of DGK-ζ to the nucleus is negatively regulated by PKC-mediated phosphorylation of the MARCKS phosphorylation site domain (see Fig. 2.1), which acts as a nuclear localization signal (36). In addition to regulation by PKC, Goto and Kondo (47) have shown that the C-terminal region of DGK-ζ influences the nuclear localization of the enzyme; however the molecular basis for this effect was unclear. Our results suggest that retention of DGK-ζ in the cytoplasm depends on interactions between the C-terminal PDZ-binding motif and cytoplasmic PDZ domain-containing proteins. In cotransfected HeLa cells, the PDZ-binding motif mutants of DGK-ζ amassed in the nucleus and were unable to recruit γ1-syntrophin there. Variable nuclear levels

were observed in cells transfected with DGK-ζ alone; therefore, γ1-syntrophin is not the only PDZ-protein that functions to retain DGK-ζ in the cytoplasm. However, HeLa cells express one or more endogenous syntrophin isoforms that redistribute into the nucleus with DGK-ζ. It is likely that these isoforms function similarly.

Because only a subpopulation of cells had elevated levels of DGK-ζ and γ1-syntrophin in the nucleus, the trafficking of the complex between the cytoplasm and nucleus must be regulated somehow. Phosphorylation of the MARCKS domain of DGK-ζ by PKC is one obvious possibility (36), but there may be additional mechanisms to regulate the subcellular distribution of one or both proteins.

To our knowledge, this is the first report that syntrophins, which are usually associated with plasma membrane specializations (5, 21, 48), are found in the nucleus. Their presence there suggests a role for syntrophins in nuclear processes; however, additional studies will be required to determine what that role is. Interestingly, several other PDZ domain-containing proteins have been found to localize in the nucleus (49-51). For example, CASK/LIN-2, a membrane-associated guanylate kinase (MAGUK) required for epidermal growth factor receptor localization in *Caenorhabditis elegans*, interacts with Tbr-1, a transcription factor involved in forebrain development (50). CASK enters the nucleus, and in complex with Tbr-1, activates the transcription of T-element-containing genes. In this case, however, the guanylate kinase domain rather than the PDZ domain of CASK mediates the interaction with Tbr-1.

The sequence of the γ1-syntrophin PDZ domain diverges significantly from α- and β-syntrophins, especially at residues critical for ligand recognition (29). Accordingly, the extended PDZ domain of neuronal nitric oxide synthase interacts with α- and β-syntrophins but does not bind to γ1-PDZ (8, 23). In addition, C-terminal peptide ligands that interact strongly with α– and

β-syntrophins do not bind to γ1-syntrophin (see Fig. 2.2*D*). One exception is the C terminus of DGK-ζ, which interacts with the PDZ domain of γ1-syntrophin and with the PDZ domains of α- and β-syntrophins. Indeed, the binding of the DGK-ζ C terminus to α- and β-syntrophin PDZ domains appeared to be stronger than to γ1-PDZ. Because syntrophins are expressed in multiple tissue and cell types, the interaction of syntrophins with DGK-ζ may be a common mechanism for targeting the enzyme to specialized subcellular sites.

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FIG. 2.1. Schematic structure of DGK-ζ and position of cDNA clones. At its N terminus DGK-ζ contains two domains (*C1*) homologous to the C1A and C1B motifs of protein kinase C: a region homologous to the phosphorylation site domain of the MARCKS protein and a catalytic region containing an ATP binding site. The C terminus of DGK-ζ contains four tandem ankyrin repeats followed by a consensus class I PDZ ligand motif (*QETAV*). The *thick black lines* indicate the extent of overlapping clones isolated in a yeast two-hybrid screen with the γ1-syntrophin PDZ domain. The *number* at the *left* of each *black line* indicates the identity of the clone.

Figure 2.1

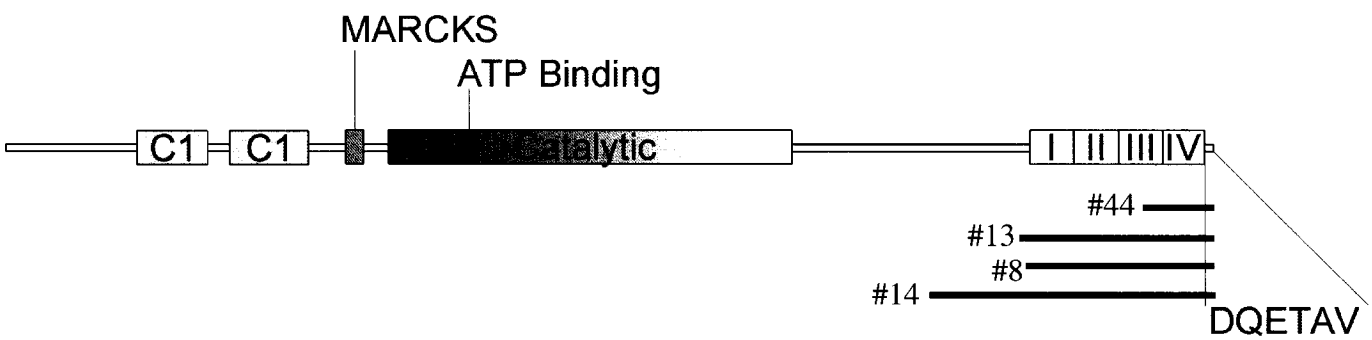


FIG. 2.2. Specific *in vitro* interaction of DGK-ζ with γ1-syntrophin. *A*, filter overlay assay showing binding of DGK-ζ specifically to syntrophin PDZ domains. Approximately equal amounts of His_6-tagged thioredoxin fusion proteins of PDZ domains from α1-, β1-, β2-, and γ1-syntrophin and from PSD-95 (PDZs 1-3) were separated by SDS-PAGE and transferred to a nitrocellulose filter. The position and amount of each protein loaded was determined by staining the filter with Ponceau S (*Protein*). The filter was probed with a purified GST-fusion protein of the C-terminal region of DGK-ζ (*DGK-ζ*). Bound DGK-ζ was visualized with a horseradish peroxidase-conjugated anti-GST antibody followed by enhanced chemiluminescence. *B*, Beads charged with GST alone or with a GST-fusion protein of the C-terminal region of DGK-ζ were incubated with extracts of HeLa cells transfected with T7-tagged γ1-syntrophin. Bound proteins were immunoblotted with an anti-T7 tag antibody. The *Input lane* was loaded with 2.5% of the extract used for the pull-down. *C*, The C-terminal 10 amino acids of DGK-ζ are sufficient to bind to syntrophin PDZ domains. The overlay assay was done as in *A*, except that the filter was probed with a synthetic peptide corresponding to the last 10 amino acids of DGK-ζ. Bound peptide was visualized with streptavidin-horseradish peroxidase. *D*, specificity of the γ1-syntrophin PDZ domain for different C-terminal peptides. In contrast to α- and β-syntrophin PDZs, γ1-PDZ did not bind C-terminal peptides of the SkM1 and SkM2 sodium channels or the K^+ channel subunit Kv1.5. Of the peptides tested, only the C terminus of Kv1.4 bound to γ1-PDZ. *E*, schematic diagram showing the constructs used in the experiments in *F*. Shown are DGK-ζ with an N-terminal HA epitope tag (*HA-DGK-ζ*), a C-terminal mutant in which the last 4 amino acids were changed to ENSV (*DGK-ζ-ENSV*) and DGK-ζ with a C-terminal FLAG epitope tag (*DGK-ζ-FLAG*). *F*, pull-down assays with either GST or GST-γ1-PDZ were carried

out using extracts of HeLa cells transfected with the constructs shown in *E.* DGK-ζ proteins were visualized using anti-HA, anti-FLAG or anti-DGKζ antibodies. Mutation of the C terminus or addition of a FLAG tag blocks the interaction with γ1-PDZ. The *Input lane* was loaded with 5% of the extract used for the pull-down.

Figure 2.2

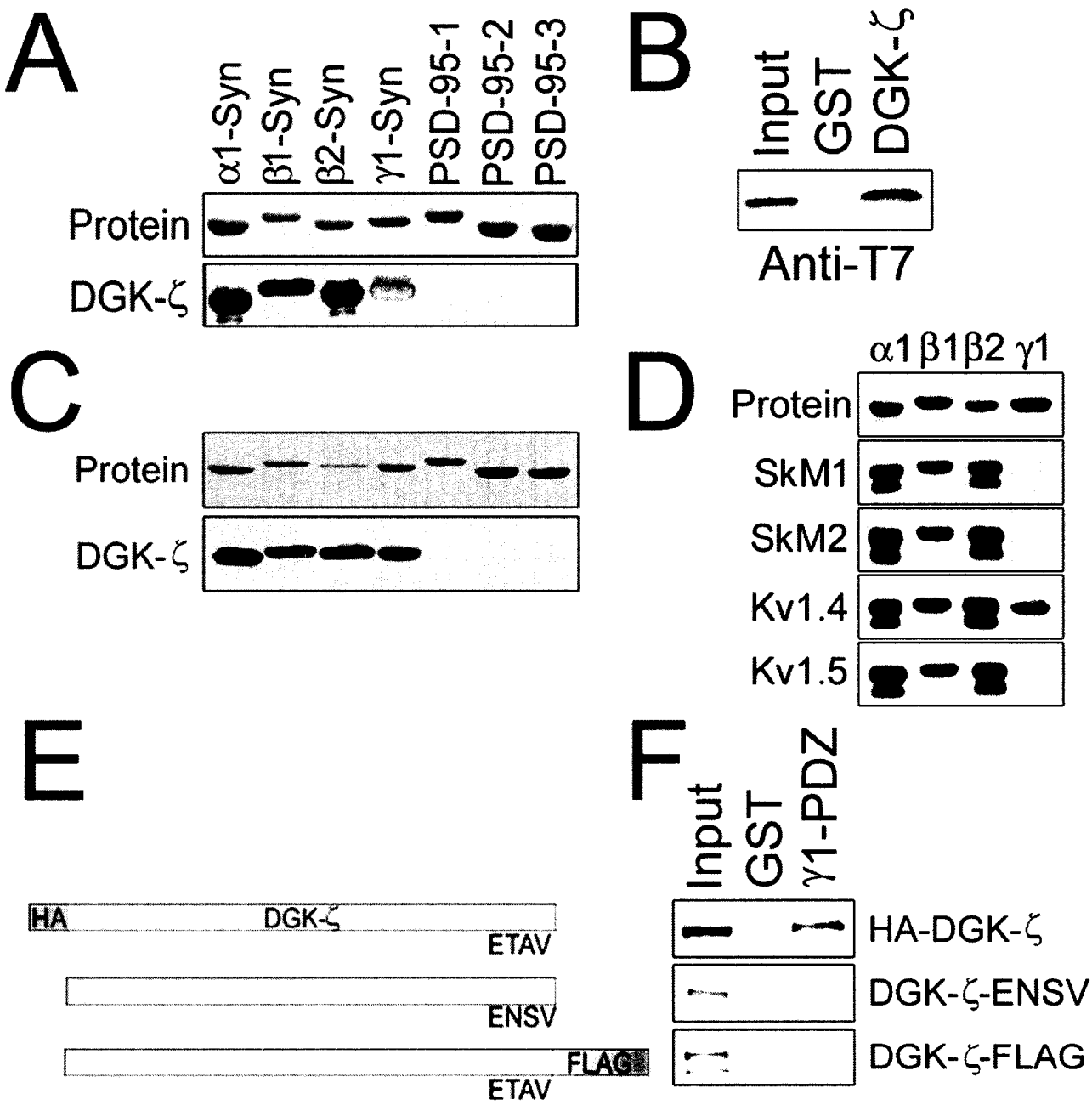

A
α1-Syn
β1-Syn
β2-Syn
γ1-Syn
PSD-95-1
PSD-95-2
PSD-95-3
Protein
DGK-ζ
B
Input
GST
DGK-ζ
Anti-T7
C
Protein
DGK-ζ
D
α1 β1 β2 γ1
Protein
SkM1
SkM2
Kv1.4
Kv1.5
E
HA
DGK-ζ
ETAV
ENSV
FLAG
ETAV
F
Input
GST
γ1-PDZ
HA-DGK-ζ
DGK-ζ-ENSV
DGK-ζ-FLAG

FIG. 2.3. Interaction of γ1-syntrophin with DGK-ζ in Mammalian Cells. *A* and *B*, reciprocal coimmunoprecipitation of DGK-ζ and γ1-syntrophin from transfected cells. HeLa cells cotransfected with T7-tagged γ1-syntrophin and HA-tagged DGK-ζ were lysed with detergent and immunoprecipitated (*IP*) with either an anti-DGK-ζ antibody (*A*) or with an anti-T7 antibody (*B*). The immunoprecipitates were analyzed by Western blotting with anti-T7 (*A*) or anti-HA antibodies (*B*). In singly transfected cells, γ1-syntrophin (*C*) and DGK-ζ (*D*) are distributed uniformly throughout the cytoplasm and nucleus. *E*, γ1-syntrophin and DGK-ζ overlapped exactly in doubly transfected cells, but their subcellular distribution was not noticeably different from that in singly transfected cells.

Figure 2.3

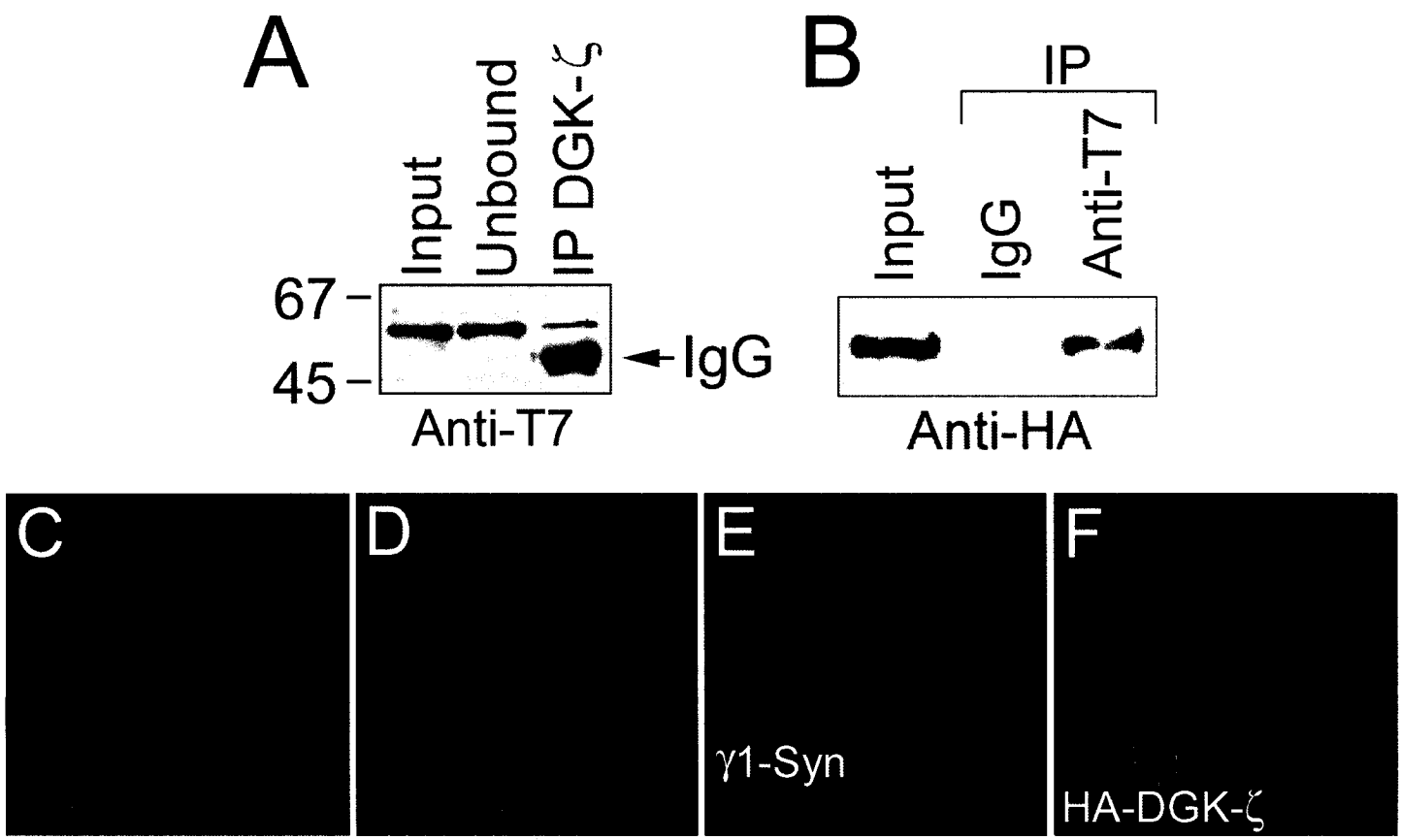

A
Input
Unbound
IP DGK-ζ
67
45
IgG
Anti-T7
B
IP
Input
IgG
Anti-T7
Anti-HA
C
D
E
γ1-Syn
F
HA-DGK-ζ

FIG. 2.4. **The subcellular localization of γ1-syntrophin and DGK-ζ depend on PDZ interactions.** HeLa cells were either transfected with single cDNAs (*A* and B) or co-transfected with two different cDNAs (*C-H*) as indicated in each *panel.* The subcellular distribution of transfected proteins was visualized by indirect immunofluorescence using antibodies specific for each protein followed by FITC- or Texas Red-conjugated secondary antibodies. The cells transfected with HA-DGK-ζ had variable levels of transfected protein in the nucleus. *A* and *B,* two examples of cells in the same microscopic field with dramatically different amounts of nuclear DGK-ζ. *C* and *D,* cells doubly transfected with HA-DGK-ζ and T7-γ1-syntrophin also had variable but matching levels of the two proteins in the nucleus. Shown are two cells with correspondingly high levels of nuclear DGK-ζ (C) and γ1-syntrophin (D). *E* and *F,* in cells cotransfected with DGK-ζ-FLAG and T7-γ1-syntrophin, DGK-ζ-FLAG was mainly in the nucleus (*E*), whereas γ1-syntrophin was primarily in the cytosol (*F*). *G* and *H,* similarly, in cells cotransfected with DGK-ζ-ENSV and γ1-syntrophin, DGK-ζ-ENSV was present at high levels in the nucleus (*G*), whereas γ1-syntrophin remained predominantly in the cytosol and endoplasmic reticulum (*H*).

Figure 2.4

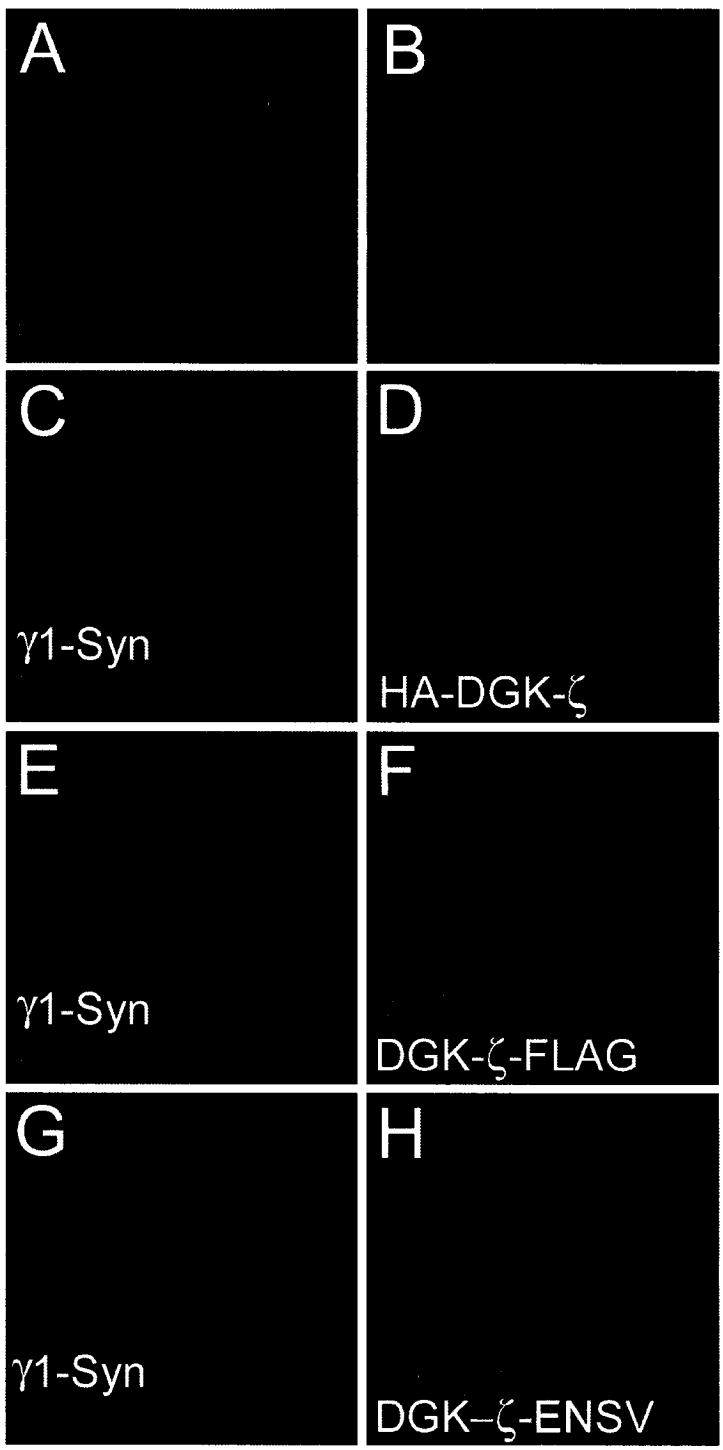


FIG. 2.5. Codistribution of HA-DGK-ζ and α- and β-syntrophins in HeLa cells. HeLa cells transfected with HA-DGK-ζ were fixed and treated for double-label immunofluorescence microscopy. HA-DGK-ζ was visualized with an anti-HA polyclonal antibody followed by a FITC-conjugated secondary antibody (A and C). Endogenous syntrophins were detected with a pan-specific monoclonal antibody (2101) that recognizes α- and β-syntrophins followed by Texas Red dye-conjugated secondary antibody (B and D). The levels of α- and β-syntrophins in nuclei of HeLa cells corresponded exactly with the amount of nuclear HA-DGK-ζ.

Figure 2.5

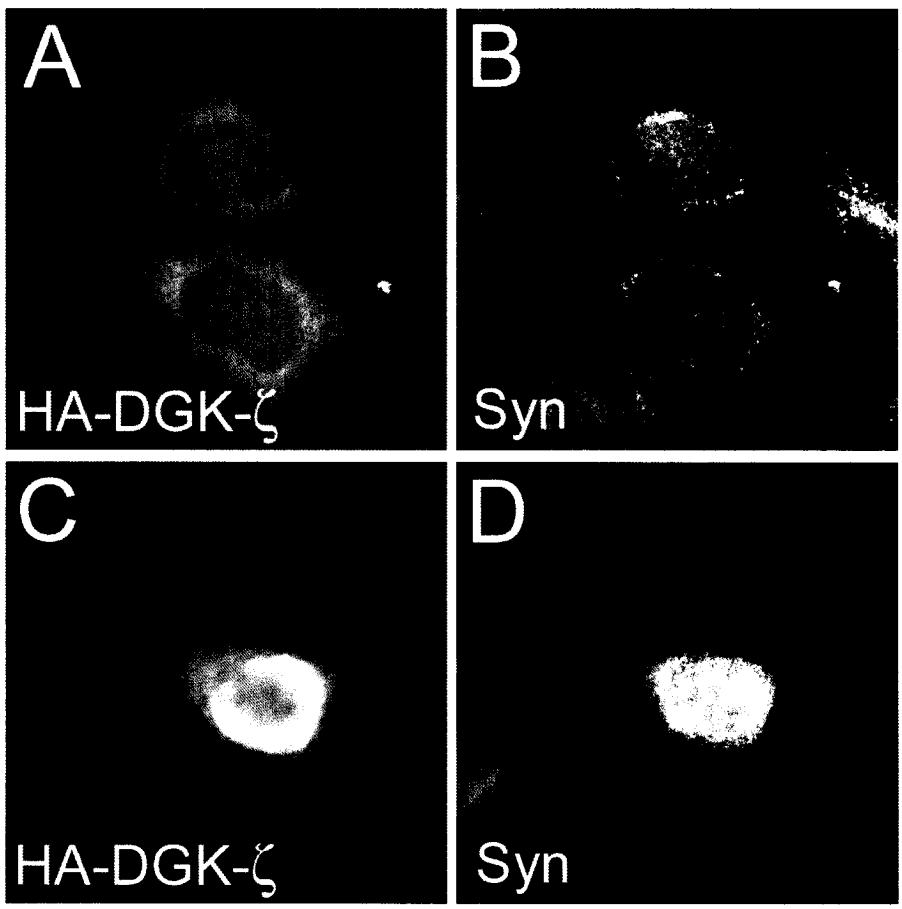


FIG. 2.6. Specificity of γ1-syntrophin and DGK-ζ antibodies. *A*, detergent extracts of mouse brain membranes were analyzed by SDS-PAGE and immunoblotting with affinity-purified antibodies 435 (*Ab 435*) and 342 (*Ab 342*) against γ1-syntrophin. Both antibodies recognize a major band of ~50 kDa that is competed by preincubation of the antibodies with an excess of their respective immunizing peptide (*lanes marked with +, respectively). B*, tissue specificity of γ1-syntrophin. Detergent extracts of membrane preparations of the indicated mouse tissues were immunoblotted with affinity purified antibody 342 to γ1-syntrophin. A major band of ~ 50 kDa is present in adult rat brain but not in any of the other tissues tested. A faint non-specific band of ~ 55 kDa was present in all tissues, indicating that approximately equal amounts of protein were loaded in each lane. C, specificity of DGK-ζ antibody. The soluble fraction of mouse brain homogenates was blotted with an antibody to the N terminus of DGK-ζ. The antibody recognizes a major band at ~120 kDa and a band of ~100 kDa that may be a proteolytic fragment. Recognition of both bands was completely blocked by preincubation of the antibody with the immunizing peptide (+ *pept. lane*).

Figure 2.6

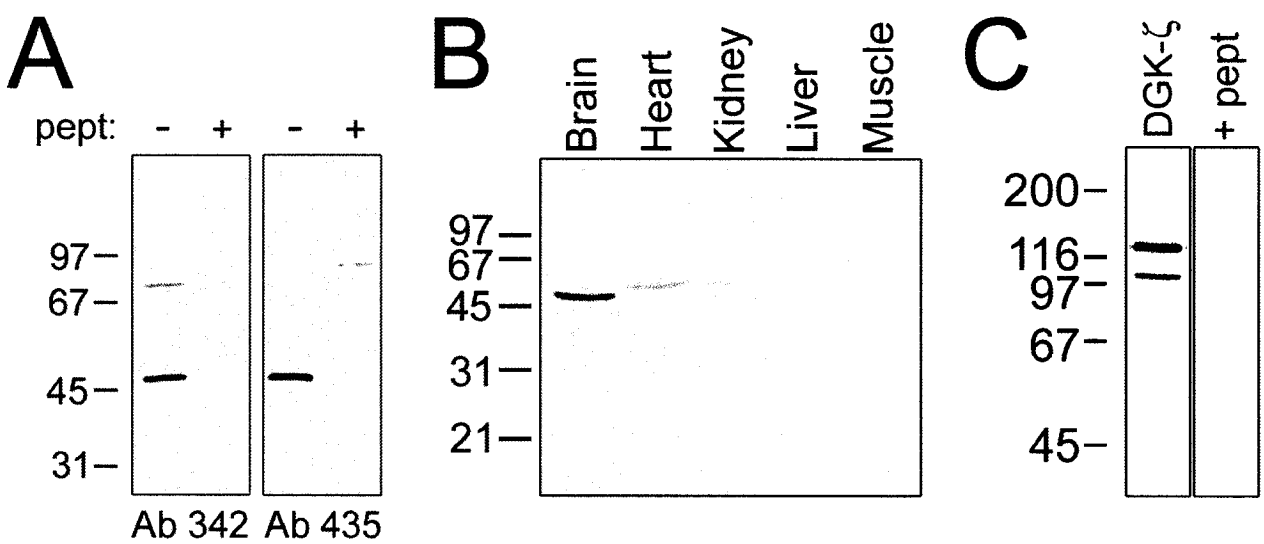

A
pept: - + - +
97
67
45
31
Ab 342
Ab 435
B
Brain
Heart
Kidney
Liver
Muscle
97
67
45
31
21
C
DGK-ζ
+ pept
200
116
97
67
45

FIG. 2.7. Immunofluorescence localization of DGK-ζ and γ1-syntrophin in mouse brain. Saggital mouse brain sections were labeled with affinity purified antibodies to DGK-ζ (*left panels)* and γ1-syntrophin (*right panels*) followed by FITC-conjugated secondary antibodies. A, low magnification saggital section of cerebellum showing strong DGK-ζ immunoreactivity in the molecular and Purkinje cell layers. B and C, higher magnification views of the cerebellar cortex showing prominent labeling of cell bodies and dendrites of Purkinje neurons. F-H, γ1-syntrophin immunoreactivity was also associated with cell bodies and dendrites (*arrowheads* in *G* and *H*) of Purkinje cells but appears to be localized primarily at the plasma membrane. D, and I, somatodendritic localization of DGK-ζ and γ1-syntrophin in pyramidal cells of the hippocampal (*Hc*) CA3 region. The cell bodies in all layers of the cortex (*Ctx*) were also labeled (E and J). Scale Bars, 1000 μm (*A*), 100 μm (*B, D, E, F, I*), 50 μm (*C, G, H*) and 250 μm (*J*).

Figure 2.7

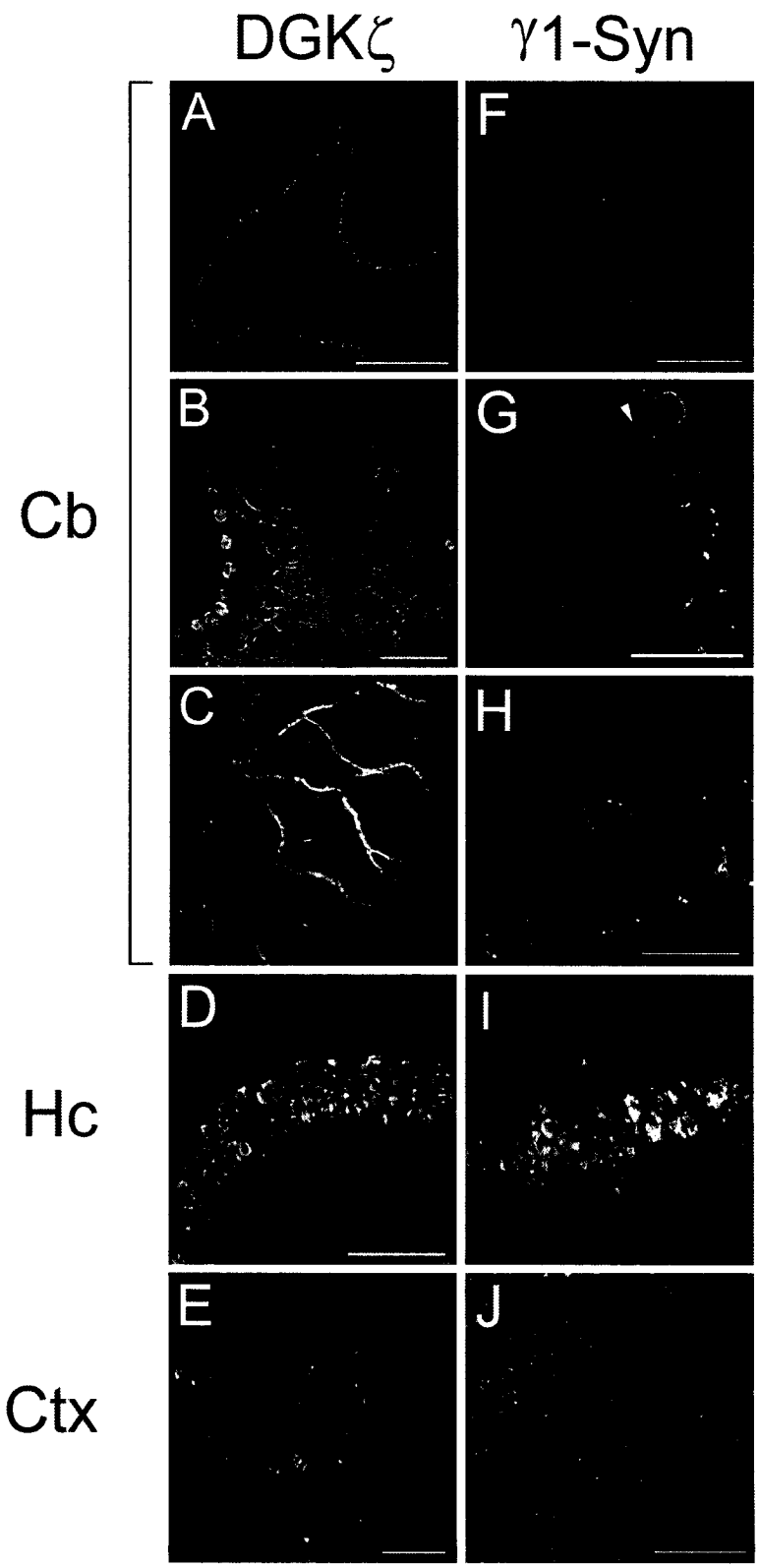
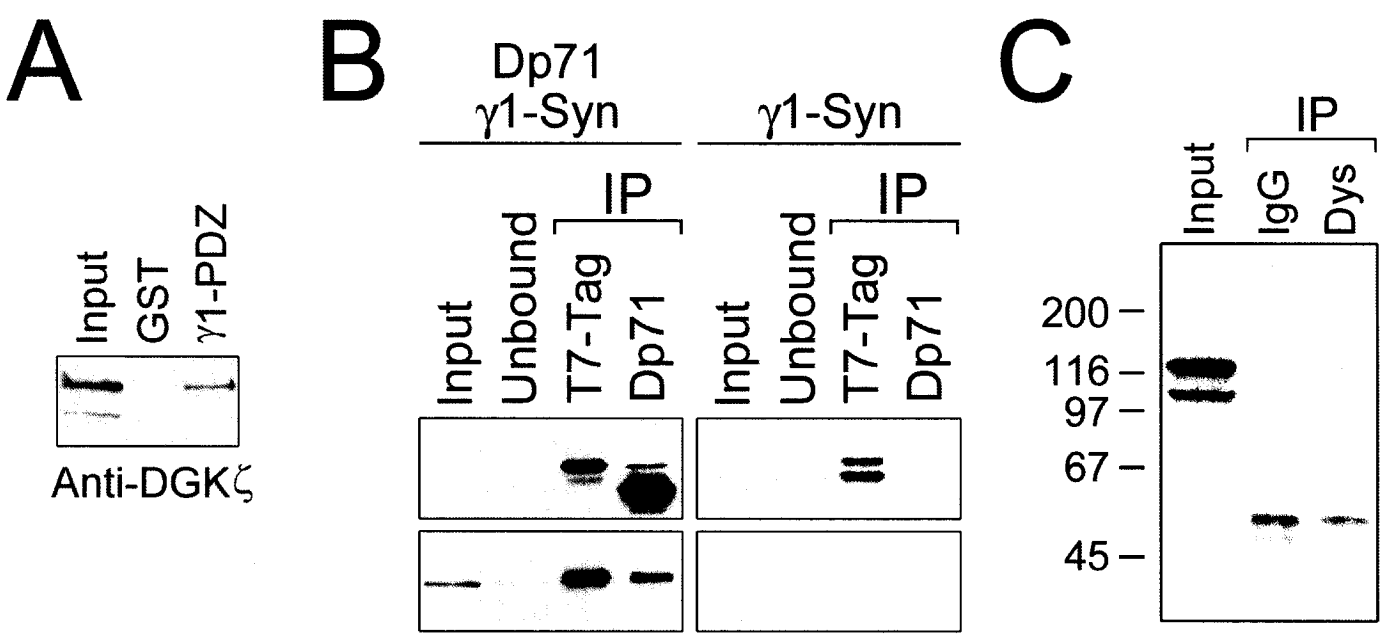


FIG. 2.8. Association of DGK-ζ, γ1-syntrophin, and dystrophin. *A*, pull-down of native DGK-ζ with the PDZ domain of γ1-syntrophin. Mouse brain homogenates solubilized in 1% Triton X-100 were incubated with GST or GST-γ1-PDZ coupled to agarose beads. DGK-ζ was specifically captured by γ1-PDZ. *B*, γ1-syntrophin forms a stable complex with Dp71 in cotransfected cells. Detergent extracts of C2 cells cotransfected with T7-tagged γ1-syntrophin and Dp71-blue fluorescent protein were immunoprecipitated (*IP*) with an anti-T7 tag antibody or with an anti-Dp71 antibody as indicated. The immunoprecipitates were then subjected to Western blotting with the anti-T7 and anti-Dp71 antibodies. *Lanes* marked *Input* were loaded with 2% of the cell extract used for immunoprecipitation and lanes marked *Unbound* were loaded with the same volume of extract after immunoprecipitation. The *lower band* in each of the anti-T7 tag blots is IgG heavy chain. *C*, copurification of DGK-ζ with dystrophin from brain. Mouse brain extracts were incubated with an affinity-purified antibody to the C terminus of dystrophin (*Dys*) or with normal rabbit IgG (*IgG*) conjugated to agarose beads.

Figure 2.8


A
Input
GST
γ1-PDZ
Anti-DGKζ
B
Dp71
γ1-Syn
γ1-Syn
IP
IP
Input
Unbound
T7-Tag
Dp71
Input
Unbound
T7-Tag
Dp71
C
IP
Input
IgG
Dys
200
116
97
67
45

Chapter 3

Fibre Type Specific Expression of Diacylglycerol Kinase-ζ in Skeletal Muscle and its Mislocalization in the *mdx* Mouse

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Running Title: Syntrophin:DGK-ζ Interaction in Muscle

ACKNOWLEDGEMENTS

This work was supported by grant MT-15505 to S.H.G from the Canadian Institutes of Health Research (CIHR). S.H.G. is supported by a CIHR New Investigator Award. The authors would like to thank Dr. Stanley C. Froehner for monoclonal antibody 2101 to syntrophins, John Lunde for his assistance with the isolation of mouse muscles, Dr. Bernard Jasmin for critically evaluating the manuscript, Drs. David Parry and Stephen Prescott for their assistance and helpful discussions, and Josée Chabot for expert technical assistance.

ABSTRACT

Syntrophins are a family of structurally related proteins with multiple protein interaction motifs, which allow them to bind simultaneously to dystrophin family members and to signaling proteins. We recently reported that γ1-syntrophin interacts with, and regulates the subcellular localization of diacylglycerol kinase-ζ (DGK-ζ), an enzyme that converts the lipid second messenger diacylglycerol (DAG) to phosphatidic acid (Hogan et al., *J. Biol. Chem.*, 2001, 276:26526). By lowering DAG levels, DGKs are thought to attenuate the activity of DAG-activated proteins like protein kinase C. To function optimally, DGKs must be targeted to regions of the membrane where DAG is produced. Here, we provide evidence that syntrophins mediate the membrane localization of DGK-ζ in skeletal muscle. We found that DGK-ζ and syntrophins associate in muscle and are colocalized at the sarcolemma of normal muscle fibres. DGK-ζ was present on the sarcolemma and in the cytoplasm of all fibres types, except IIB. Moreover, it was concentrated at neuromuscular junctions (NMJs) and was localized exclusively at synaptic sites in type IIB fibres. In dystrophin-deficient *mdx* muscle, sarcolemmal DGK-ζ was dramatically decreased in most fibres, but was present at near-normal levels in groups of small caliber, newly regenerating fibres, which also showed increased syntrophin expression. DGK-ζ was retained at NMJs in *mdx* muscle, suggesting it is present in complexes containing utrophin and was also found associated with central myonuclei. Collectively, our findings suggest syntrophins participate in the assembly and organization of DGK-ζ signaling complexes at specialized domains of the sarcolemma. Abnormal localization of DGK-ζ may disrupt DAG signaling pathways and contribute to the pathogenesis of muscular dystrophies.

INTRODUCTION

Hormones, growth factors, and other stimuli elicit cellular responses by binding to and activating cell surface receptors. Signals from activated receptors are relayed by intracellular second messenger molecules, which in turn, activate a cascade of events that alter effector molecules such as enzymes and ion channels, and induce the expression of target genes in the nucleus. An efficient biological response requires the coordinated coupling of receptors and their associated signal transduction proteins into functional signaling pathways. Many signaling complexes are assembled around one or more scaffold proteins that contain multiple domains for protein-protein interactions, such as PDZ and SH3 domains (39).

The syntrophin family of scaffold proteins consists of five isoforms (α1, β1, β2, γ1 and γ2) with the same modular domain organization: two pleckstrin homology (PH) domains, a single PDZ domain, and a syntrophin-unique (SU) carboxy terminal domain (2, 6, 37). Syntrophins bind via their PH2 and SU domains to the carboxy (C) terminus of proteins in the dystrophin family of membrane-associated cytoskeletal proteins (5, 25, 32). In humans, defects in the gene encoding dystrophin are the cause of Duchenne muscular dystrophy (DMD), a disorder characterized by skeletal muscle wasting and cognitive impairment (21). Through PDZ interactions, syntrophins recruit ion channels and signaling proteins to specialized dystrophin complexes at the plasma membrane of muscle and other cell types (8, 15, 25, 30, 33, 34).

Each syntrophin isoform has a unique pattern of expression during development and among different tissues (1, 4, 28). More than one syntrophin isoform can be expressed in a single cell type, however, their subcellular distributions can differ dramatically, implying their localization is tightly regulated. For instance, in skeletal muscle, α1-syntrophin is distributed over the entire sarcolemma and is present throughout the folds at neuromuscular junctions,

whereas β2-syntrophin is found almost exclusively at junctions and is confined to the lower portion of the folds (28, 36).

Recently, we demonstrated that the PDZ domain of γ1-syntrophin, an isoform expressed uniquely in the brain, interacts with the C-terminus of diacylglycerol kinase-ζ (DGK-ζ) (23). DGK-ζ is one of nine mammalian DGK isoforms, all of which catalyze the conversion of diacylglycerol (DAG) to phosphatidic acid (PA) (41, 43). DAG is a lipid second messenger generated by phospholipase C (PLC)-mediated breakdown of phosphoinositol-4,5-bisphosphate (PIP_2) in the plasma membrane. DAG exerts its signaling functions mainly by activating lipid-regulated proteins like protein kinase C (PKC), *vav* (a Ras GEF), and RasGRP (41). These, in turn, regulate many cellular responses, including growth and differentiation (14, 26). Inappropriate accumulation of DAG results in prolonged activation of these proteins, which can lead to cellular transformation.

DGK-mediated conversion of DAG to PA is thought to be a key mechanism by which the activities of DAG-activated proteins are attenuated. Thus, DGKs are hypothesized to serve as an "off" signal for DAG signaling at the plasma membrane (43). Recent studies suggest that individual DGK isoforms modulate the activity of one, or a few, DAG-activated proteins. For example, the activity of RasGRP, a guanine nucleotide exchange factor for Ras, is selectively attenuated by DGK-ζ, and not by other DGK isoforms, most likely because DGK-ζ and RasGRP are organized into a regulated signaling complex (41). These findings support the idea that local regulation of DAG signaling by DGK isozymes may be a general mechanism to regulate the activity of DAG-activated proteins.

Other studies have shown that regulation of DGK subcellular localization is an important mechanism by which DGK activity is controlled (38). Here, we present evidence that

syntrophins mediate the association of DGK-ζ with the plasma membrane of skeletal muscle fibres. In dystrophin-deficient *mdx* muscle, the membrane localization of DGK-ζ was dramatically decreased; raising the possibility that mislocalization of DGK-ζ in skeletal muscle contributes to the pathogenesis of muscular dystrophies.

METHODS

Antibodies – Alternative splicing generates two different sized DGK-ζ transcripts in muscle, one of 3.5 kb, which is similar in size to the transcript found in other non-muscle tissues, and a second major transcript of 4.1 kb, which encodes an alternatively-spliced muscle-specific isoform (11). The latter transcript encodes a ~130 kDa protein with a unique N-terminal domain that is identical throughout its remainder to the shorter isoform. This report focuses uniquely on the shorter isoform, which migrates with an apparent molecular mass of ~116 kDa (12), because no antibodies were produced that specifically recognized the longer isoform. To produce polyclonal antisera to the shorter DGK-ζ isoform, a peptide (CSERDAGPEPDKAPRRLNK) corresponding to the unique N-terminal domain of human DGK-ζ was synthesized, conjugated to KLH, and injected into rabbits. The antibodies were affinity purified from serum on immobilized peptide. The specificity of the affinity-purified antibodies was verified by western blotting extracts from cells transfected with DGK-ζ (9) and by preabsorption of antibodies with the immunizing peptide.

The pan-specific syntrophin monoclonal antibody 2101 was a generous gift from Dr. Stanley Froehner (University of Washington at Seattle, WA). The BF-F3 monoclonal antibody against the type IIB myosin heavy chain isoform was purchased from the German Collection of Microorganisms and Cell cultures (Braunschweig, Germany). Texas Red-conjugated α-Bungarotoxin was purchased from Molecular Probes. Texas Red-, and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, and peroxidase-conjugated secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA).

Subcellular Fractionation of Mouse Skeletal Muscle Extracts – Adult male C57BL6, and C56BL/10ScSn-Dmd^{MDX}/J (*mdx*) mutant mice aged 8-10 weeks were sacrificed by CO_2 overdose.

Skeletal muscle was harvested, flash-frozen in liquid nitrogen and then stored at –80°C. To prepare crude subcellular fractions, 1-2 grams of mouse thigh muscle were added to 10 volumes of modified TEE buffer cocktail (25 mM Tris, pH 7.4, 1mM EDTA, 1mM EGTA) + 100mM NaCl + protease inhibitor cocktail [10 ug/mL each of leupeptin, antipain, 4-(2-Aminoethyl)-benzenesulfonyl-fluoride HCl (AEBSF), pepstatin A, and benzamidine 10 HCl (Bioshop Canada Inc., Burlington, ON)]. The mixture was homogenized in a small sample cup of a Waring blender, followed by 5-10 sec with a PT-3100 Polytron (Kinematica, Luzern, Switzerland). Nuclei were pelleted for 10 min at 1,000 x g and the supernatant was centrifuged at 48,000 x g for 10 min. The supernatant (cytosolic fraction) was removed and protein was quantified using the Bio-Rad Protein Assay kit (Hercules, CA). A fraction of the sample was added to a 5X concentrated SDS reducing sample buffer and heated for 5 minutes at 95°C.

Cell Culture – C2C12 cells were grown on plates coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in DMEM high-glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine until they were 80-100% confluent. To induce differentiation, fetal bovine serum was replaced with 5% horse serum. C2C12 myoblast cells were allowed to fuse and form mature myotubes over a five-day period.

Immunofluorescence Localization – C57BL6 and C56BL/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), and frozen in liquid nitrogen-cooled isopentane, and stored at –80°C. Transverse sections were cut on a cryostat at a thickness of 8 μm at –20 °C. Sections were stored at –80 °C prior to use. Sections were thawed, ringed with a hydrophobic boundary and rinsed with buffer A (0.5% BSA and

0.15% glycine in phosphate buffered saline, pH 7.4), fixed in 4% paraformaldehyde, and washed again before being blocked in buffer A + 5% normal goat serum. Sections were then incubated with affinity-purified antibody diluted in buffer A, containing 0.3% Triton X-100 in a closed moisture chamber either overnight or for one hr at 4°C. Muscle sections were subsequently rinsed in buffer A and then incubated with a 1:200 to 1:300 dilution of FITC- or Texas Red dye-conjugated secondary antibody, or with a 1:1000 dilution of Cy3-conjugated secondary, in buffer A + 0.3% Triton X-100, for 45 min to 1 hr at room temperature. Sections were then 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.

C2C12 myotubes that were grown for five days on coverslips were washed twice in warm PBS, then fixed with 4% paraformaldehyde for 20 min and washed again. Cells were permeabilized in 0.1% Triton X-100 in PBS, then blocked for 40 min in PBS containing 1% BSA. Anti-DGK-ζ polyclonal antibody and FITC-conjugated donkey anti-rabbit secondary antibody were used at a concentration of 1:100 and 1:300 respectively in blocking buffer for 1 hour at room temperature, followed by 3 x 5 min washes in blocking buffer. Coverslips were mounted on slides using Fluoromount G (EMS, Ft. Washington, PA).

Immunoprecipitation – Five-day old C2C12 myotubes were used for immunoprecipitation experiments. All steps were carried out at 4°C or on ice. Cells 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% Triton X-100 and 10 ug/mL each of leupeptin, antipain, AEBSF, pepstatin A, and benzamidine HCl) per 100 mm dish. Cells were lysed on ice for 30 min, then scraped from the dish and centrifuged for 10 min at 4°C. The supernatant was collected and an

aliquot of this starting material (Input) was boiled in concentrated SDS reducing sample buffer. Samples were pre-cleared for 1-2 hours using 50 ul 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 hours. To purify immune complexes, 50 ul of a 50% slurry of washed Protein A/G Plus agarose beads was added and incubated an additional 2-3 hrs 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 reducing sample buffer. The samples were then centrifuged at 21,000 x g for 1-2 min and subjected to SDS-PAGE and Western analysis.

Pull-Down Assays – GST and GST-DGK-ζ bound to Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) were prepared by incubating 2.5 mg of GST or GST-fusion protein with 500 μl of a 50% slurry of PBS-washed beads for 20 min at 4°C, followed by washing in PBS. Mouse skeletal muscle cytosolic fractions were added to 50-100μL of a 50% slurry of Glutathione Sepharose 4B beads bound to GST or GST-DGK-ζ (N-terminally truncated, see Positive # 8; Hogan et al., 2001). A fraction of the starting material was boiled in SDS reducing sample buffer. Samples were incubated with the beads for 2-3 hours with mixing at 4°C. Beads were then washed twice with cold PBS, then once with 1% Triton X-100 in PBS. Proteins were eluted in an equal volume of SDS reducing sample buffer and boiled for 5 minutes. Samples were then analyzed by SDS-PAGE and Western blot analysis.

Western Blotting – Protein samples were separated on 7.5% SDS-PAGE, transferred onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.), then incubated with either monoclonal antibody 2101 (1:1000 dilution), or anti-DGKζ (N-terminal antibody, 1:500) in a total volume of 2-4 mL for one hour at room temperature. Membranes were incubated with

peroxidase-conjugated secondary antibodies for one hour at room temperature at a 1:4000 dilution. Detection of bands was carried out as described previously (16, 23).

RESULTS

Association of DGK-ζ with Syntrophins in Muscle – To identify DGK-ζ in skeletal muscle, an antibody directed against a peptide sequence within the N-terminal domain was produced in rabbits and affinity purified (see Methods). This antibody specifically recognized a doublet of ~116 kDa in western blots of mouse skeletal muscle extracts (Fig. 3.1*A*). The lower band of the doublet is less abundant and represents an unphosphorylated form of DGK-ζ (Abramovici et al., in press). To identify syntrophins in skeletal muscle, we used a monoclonal antibody (2101) that recognizes an epitope in the PDZ domain of α- and β-syntrophins (3, 15). In western blots of skeletal muscle extracts, MAb 2101 recognized a band of ~59 kDa as expected (Fig. 3.1*B*).

We previously demonstrated that the C-terminus of DGK-ζ binds to purified recombinant α1-, β1-, and β2-syntrophin PDZ domains in overlay assays (23). To verify the biochemical association of DGK-ζ and syntrophins in muscle, we performed pull-down experiments from mouse skeletal muscle extracts using beads charged with a GST fusion protein of DGK-ζ (amino acids 787-928). The bound proteins were analyzed by immunoblotting with MAb 2101. GST-DGK-ζ, but not GST alone captured a significant fraction of syntrophins in the offered extract (Fig. 3.1*B*).

For coimmunoprecipitation experiments, C2C12 myotubes were used as the starting material, since more DGK-ζ could be solubilized from these cells than from adult muscle tissue. As shown in Fig. 3.1*C*, DGK-ζ was efficiently precipitated from C2 myotube extracts by its specific antibody, but not by control rabbit IgG. Moreover, syntrophins were specifically coimmunoprecipitated by the DGK-ζ antibody. We were unable to carry out reverse coimmunoprecipitation experiments because MAb 2101 interferes with ligand binding to the

PDZ domain of syntrophins (S.G., unpublished observations). Collectively, these results suggest DGK-ζ and syntrophins exist as a stable complex in muscle cells *in vivo*.

Colocalization of DGK-ζ and Syntrophins in Skeletal Muscle – Affinity purified antibodies to DGK-ζ and fluorescein-isothiocyanate (FITC)-conjugated secondary antibodies were used to examine the distribution of DGK-ζ in transverse cryosections of mouse tibialis anterior (TA) muscle by indirect immunofluorescence. Strong staining of the sarcolemma, as well as diffuse intracellular staining was observed (Fig. 3.2*A*). Interestingly, the intensity of labeling of individual muscle fibres varied considerably, suggesting DGK-ζ expression is fibre-type specific (see below). The labeling was completely blocked by preincubation of the antibody with its immunogenic peptide (Fig. 3.2B). Interestingly, at higher magnification, the cytoplasmic DGK-ζ labeling appeared to be punctate (Fig. 3.2C).

To compare the localization of syntrophins and DGK-ζ, sections of mouse TA muscle were double-labeled with MAb 2101 and anti-DGK-ζ antibodies. Primary antibodies were visualized with species-specific secondary antibodies conjugated to either FITC or Texas-Red Dye. In transverse sections, DGK-ζ and syntrophins were colocalized at the sarcolemma (Fig. 3.2*D*-D"). Combined with the biochemical data above, these results suggest DGK-ζ and syntrophins are present in a complex at the plasma membrane of skeletal muscle fibres.

DGK-ζ is Expressed in Specific Muscle Fibre Types – The mouse TA muscle contains a mix of fast twitch fibre types, both oxidative (types IIA and IIX) and glycolytic (type IIB). To determine the fibre-type distribution of DGK-ζ in TA muscle, sections were double-labeled for DGK-ζ (Fig. 3.3*A*) and myosin IIB, a marker of type IIB fibres (Fig. 3.3*B*). Type IIB fibres (*asterisks*) displayed little or no DGK-ζ staining. In some cases, the low level of sarcolemmal staining made it difficult to distinguish the boundary between two individual type IIB fibres (see

arrows in Fig. 3.2*D*). These areas often coincided with a modest decrease in syntrophin staining (*arrows* in Fig. 3.2*D*'), suggesting a reduction or absence of one or more of the syntrophin isoforms recognized by MAb 2101. A mosaic pattern of DGK-ζ labeling was also seen in the plantaris muscle, which contains a mix of fast-twitch fibre types (not shown) . In contrast, all fibres were strongly labeled in the soleus muscle, which contains a mix of both type I and IIA fibres (not shown). Collectively, our results suggest DGK-ζ is expressed on the sarcolemma of all fibres types except IIB.

Specific Loss of Sarcolemmal DGK-ζ in mdx Muscle – In *mdx* mouse skeletal muscle, the absence of dystrophin results in a dramatic reduction of sarcolemmal syntrophins (10, 35, 36, 46). To determine if DGK-ζ is similarly affected, sections of control and *mdx* TA muscle were immunostained for DGK-ζ, then photographed with identical exposure times. Compared to control muscle fibres (Fig. 3.4A), most *mdx* fibres had substantially reduced levels of both sarcolemmal and cytoplasmic DGK-ζ staining, although weak but detectable sarcolemmal staining remained (Fig. 3.4*B*). Interspersed among the DGK-ζ-negative fibres were small groups of fibres in which the sarcolemmal DGK-ζ immunoreactivity was noticeably increased (Fig. 3.4*C* and *D*, *arrows*). In some of these fibres, the punctate cytoplasmic staining characteristic of normal fibres was observed (Fig. 3.4*D*, *arrows*). The small caliber and centrally located nuclei of these fibres suggested they were newly regenerated or regenerating. Consistent with this idea, previous studies have shown that syntrophins are upregulated in regenerating fibres, concomitant with an increase in utrophin expression (35). To determine if the increase in DGK-ζ expression mirrored an increase in syntrophin expression in the same fibres, sections of *mdx* muscle were double labeled with polyclonal anti-DGK-ζ antibodies and MAb 2101. In *mdx* muscle fibres with

increased DGK-ζ expression (Fig. 3.4*D*), there was a parallel increase in syntrophin expression (Fig. 3.4*D'*). Thus, the level of DGK-ζ at the plasma membrane reflects that of syntrophins.

DGK-ζ is Concentrated at NMJs in Normal Muscle and Specifically Retained at NMJs in mdx Muscle – Three syntrophin isoforms are enriched at NMJs in normal skeletal muscle: β2-syntrophin is largely restricted to the postsynaptic membrane, but α1- and β1-syntrophin are additionally present on the extrajunctional sarcolemma (35, 36). To determine if DGK-ζ is also concentrated at NMJs, frozen sections of mouse TA muscle were double-labeled with DGK-ζ antibodies and Texas Red-conjugated α-bungarotoxin (α-BgTx), which specifically labels nicotinic acetylcholine receptors (nAChRs). In fibres with strong sarcolemmal DGK-ζ labeling, there was a noticeable increase in staining that overlapped with AChRs, suggesting DGK-ζ is concentrated at NMJs (Fig. 3.5*A* and *A', arrows*). Junctional DGK-ζ staining was particularly apparent in type IIB fibres (*asterisks* in Fig. 3.5*B*), where there was almost no sarcolemmal staining. In these fibres, DGK-ζ immunoreactivity was localized almost exclusively at the synaptic membrane (Fig. 3.5*B and B', arrows*). Thus, in type IIB fibres, DGK-ζ is specifically expressed at NMJs.

All three syntrophin isoforms are specifically retained at NMJs in *mdx* muscle (10, 35, 36, 46), most likely due to their association with postsynaptic utrophin. We assessed whether DGK-ζ is also retained at NMJs in *mdx* muscle. Although DGK-ζ staining was reduced on the extrajunctional sarcolemma, it remained concentrated at synaptic sites (Fig. 3.5*C-C''*). In a magnified view of the merged image, DGK-ζ immunoreactivity could be seen to extend beyond the AChR-rich synaptic regions into the surrounding perisynaptic membrane (Fig. 3.5*C''*, *boxed*

region). Thus, although DGK-ζ is absent from the sarcolemma of most *mdx* fibres, it is specifically retained at NMJs.

Nuclear Localization of DGK-ζ in mdx Skeletal Muscle – In cross sections of *mdx* TA muscle, we often observed bright central patches of DGK-ζ immunoreactivity (Fig. 3.6*A*; see also Fig. 3.4*B-D*). We speculated that this staining might correspond to central nuclei. Consistent with this idea, a significant fraction of DGK-ζ is found in the nucleus of cells, where it regulates proliferation by reducing nuclear DAG (40). Hoechst stain was used to identify nuclei in sections of *mdx* muscle labeled for DGK-ζ (Fig. 3.6*B*). As expected, most *mdx* muscle fibres had at least one identifiable centrally located nucleus. The merged image reveals that virtually every nucleus was associated with DGK-ζ immunoreactivity (Fig. 3.6*C*). Interestingly, many of the patches had a ring-like appearance, suggesting DGK-ζ is associated with the nuclear membrane. In transverse sections of normal muscle, it was not possible at the light microscope level to differentiate peripheral muscle nuclei from nuclei in epithelial cells of blood vessels. Thus, further studies are needed to determine if DGK-ζ is associated with myonuclei of normal muscle fibres and whether dystrophin deficiency alters nuclear DGK-ζ levels.

DISCUSSION

To be active, DGKs have to be located close to the plasma membrane, in the vicinity of their substrate, DAG. DGK activity is increased in response to the transient accumulation of DAG evoked by receptor activation, but not by global DAG production, consistent with the view that DGKs regulate signaling events through the spatial metabolism of second messenger DAG (44). Supporting this, recent studies suggest that individual DGK isoforms metabolize the local accumulation of DAG and modulate the activity of one, or a few, lipid-activated proteins (42). This localized regulation arises by the association of DGK and its target proteins into organized signaling complexes. To function optimally, these complexes must be targeted to regions of the membrane where receptor activation occurs. Our findings suggest syntrophins participate in the assembly and organization of DGK-ζ signaling complexes at specialized domains of the sarcolemma, which may be critical for the regulation of DGK-ζ activity by receptor-derived signals and for the spatial regulation of DAG signaling pathways.

In the present report, we found that DGK-ζ and syntrophins associate in skeletal muscle cells and are colocalized at the plasma membrane of adult muscle fibres. In a previous study, we showed that, of the four syntrophin isoforms expressed in skeletal muscle (10), at least three (α1, β1, β2) bind directly to the C-terminus of DGK-ζ via their PDZ domains (23). Since syntrophins also bind to the C-terminus of dystrophin and other members of the dystrophin family (4, 10, 13, 29, 35, 37, 46), they likely provide a link between DGK-ζ and sarcolemmal dystrophin complexes. The finding that sarcolemmal DGK-ζ was markedly decreased in dystrophin-deficient *mdx* muscle fibres, in which there is a secondary loss of syntrophins (10), supports the idea that the membrane localization of DGK-ζ depends in part on the formation of a complex containing both syntrophin and dystrophin. (1, 4, 6, 37, 46) Moreover, it raises the possibility

that the mislocalization of DGK-ζ contributes to the pathogenesis of muscular dystrophies. One possibility is that DGK-ζ accumulates in the cytoplasm of dystrophic muscle fibres. This cytosolic DGK-ζ is unlikely to be active, because it is not close to a source of DAG production.

Mislocalized DGK-ζ may also accumulate in nuclei of dystrophin-deficient muscle fibres. In agreement with this idea, we found that DGK-ζ immunoreactivity was associated with central nuclei of *mdx* muscle. Further support for this idea comes from a recent study in which we demonstrated that DGK-ζ recruits γ1-syntrophin to the nucleus. In the absence of a functional PDZ-binding motif, DGK-ζ accumulated in the nucleus and γ1-syntrophin remained in the cytoplasm (23). By extension, the absence of dystrophin and the secondary loss of syntrophins may cause DGK-ζ to accumulate in nuclei of *mdx* muscle fibres. In a previous study, Topham et al. (40) showed that a fraction of DGK-ζ is found in the nucleus. This nuclear pool of DGK-ζ decreases the amount of DAG in the nucleus, which attenuates cell growth. Thus, inappropriate levels of nuclear DGK-ζ could adversely affect the regulation of cell growth. Additional experiments will be necessary to distinguish among these and other possibilities. Nevertheless, in either scenario, the failure of DGK-ζ to associate with the sarcolemma likely affects its activity and may consequently affect the regulation of DAG-activated proteins.

In normal TA muscle, there is substantially less DGK-ζ on the sarcolemma of type IIB fibres than other fibre types. Interestingly, it is this fibre type that is preferentially affected in muscles of patients with Duchenne muscular dystrophy (45), even though dystrophin is expressed at similar levels in all myofibres (22). The fibre-type specificity of DGK-ζ contrasts with that of β1-syntrophin, which is enriched in type IIB fibres (35). For this reason, it seems unlikely that β1-syntrophin mediates the membrane localization of DGK-ζ. Furthermore, since

α1-syntrophin is uniformly distributed among fibre types, it too is unlikely to be the cognate partner for sarcolemmal DGK-ζ. Although β2-syntrophin is found at high levels only at the postsynaptic membrane of NMJs (28, 36), it is present at lower levels on the extrajunctional sarcolemma of some fibres (35). Therefore, β2-syntrophin could conceivably mediate the sarcolemmal localization of DGK-ζ. Consistent with this idea, DGK-ζ is localized exclusively at synaptic sites in type IIB fibres. We cannot rule out the possibility that DGK-ζ also associates with γ2-syntrophin, but MAb 2101, which was used in our studies to isolate syntrophin complexes, does not recognize this isoform (Marvin E. Adams, U. Washington at Seattle, unpublished observations).

Several observations suggest DGK-ζ is also part of distinct utrophin complexes at specialized membrane domains in skeletal muscle. First, DGK-ζ is concentrated at NMJs in normal muscle, where utrophin replaces dystrophin at the AChR-rich crests of the postsynaptic folds (7). Second, DGK-ζ is retained at NMJs in *mdx* muscle, where dystrophin is absent, but utrophin remains (31). Third, DGK-ζ is upregulated in small caliber, newly regenerated *mdx* fibres, in which both syntrophin and utrophin expression are increased (18, 19, 35). These overlapping patterns of localization suggest DGK-ζ might also associate with utrophin on the extrasynaptic sarcolemma, especially in slow muscle fibres where utrophin is expressed at relatively high levels in extrasynaptic regions (17). This could explain why some sarcolemmal DGK-ζ immunoreactivity remained in dystrophin-deficient *mdx* muscle fibres and the lack of sarcolemmal DGK-ζ staining in type IIB fibres, which have comparatively low levels of utrophin outside of NMJs.

The possible presence of DGK-ζ in specialized utrophin complexes at adult NMJs suggests that it may have a role in synaptic function or in the maintenance of synaptic structure.

DAG is the major physiological activator of several PKC isoforms, some of which are specifically concentrated at synaptic sites in skeletal muscle (20). Studies using cultured myotubes have provided evidence that PKC plays a role in the activity-dependent repression of AChR biosynthesis, which eliminates AChRs from extrajunctional areas of developing and adult muscle (24, 27). In one study, down regulation of PKC by prolonged exposure to phorbol ester, or inhibition of PKC with staurosporine, increased AChR α subunit mRNA levels by 8- to 10-fold, suggesting PKC modulates AChR α subunit biosynthesis at the transcriptional level (27). It was later shown that electrical stimulation of muscle greatly increases the activity of PKC in the nucleus (24). Since DGK-ζ regulates cellular DAG levels (40), control of its localization and activity at synaptic sites may be important for activity-dependent changes in AChR subunit gene expression.

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Fig. 3.1. Interaction of DGK-ζ and syntrophins in skeletal muscle. *A*, An antibody to the N-terminus of DGK-ζ specifically recognizes a doublet of ~116 kDa in Western blots of mouse skeletal muscle homogenates. The positions of molecular mass standards are shown at the left. *B*, Beads charged with either 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 the offered extract. *Input* = 5% of starting material. *C*, Coimmunoprecipitation of endogenous DGK-ζ and syntrophins from untransfected cultured muscle cells. Lysates of C2 myotubes were immunoprecipitated with a polyclonal antibody to DGK-ζ or with control rabbit IgG. The immunoprecipitates were then analyzed by western blotting with antibodies to DGK-ζ and syntrophins. DGK-ζ was efficiently precipitated by its specific antibody, but not by control rabbit IgG. Moreover, syntrophins specifically coimmunoprecipitated with DGK-ζ. With longer exposures, DGK-ζ was detectable in the *Input* lane. *Input* = 5% of starting material.

Figure 3.1

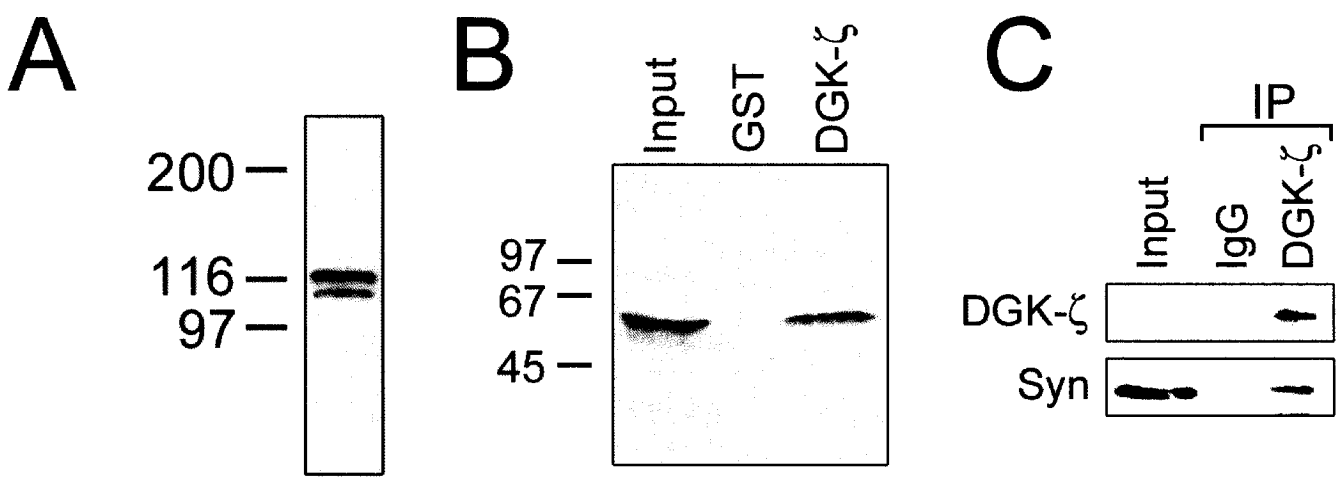

A
200
116
97
B
Input
GST
DGK-ζ
97
67
45
C
IP
Input
IgG
DGK-ζ
DGK-ζ
Syn

Fig. 3.2. Immunofluorescence localization of DGK-ζ in mouse skeletal muscle and colocalization with syntrophins. A-C, Transverse cryosections of mouse tibialis anterior (TA) muscle were fixed and stained with affinity-purified antibodies to DGK-ζ followed by FITC-conjugated secondary antibodies, then were visualized by immunofluorescence microscopy. *A*, At low magnification, DGK-ζ immunoreactivity was observed in a subset of muscle fibres, with diffuse cytoplasmic labeling and strong labeling of the sarcolemma. *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*, At higher magnification, the cytoplasmic labeling appeared as discreet puncta. In D-D", mouse TA muscle was double labeled for DGK-ζ (*D*) and syntrophins (*D'*). Syntrophins were visualized using monoclonal antibody 2101 followed by Texas-Red-conjugated secondary antibodies. The merged image shows that syntrophins and DGK-ζ were colocalized at the sarcolemma (*D"*). The *arrows* indicate regions where DGK-ζ staining is absent and where there is a corresponding reduction in syntrophin staining. Note: In *D-D"*, the brightness levels were adjusted to emphasize the sarcolemmal labeling and to make the reduction in syntrophin staining more apparent. Scale bars = 100 ums (A, B) and 25 ums (C, D-D").

Figure 3.2

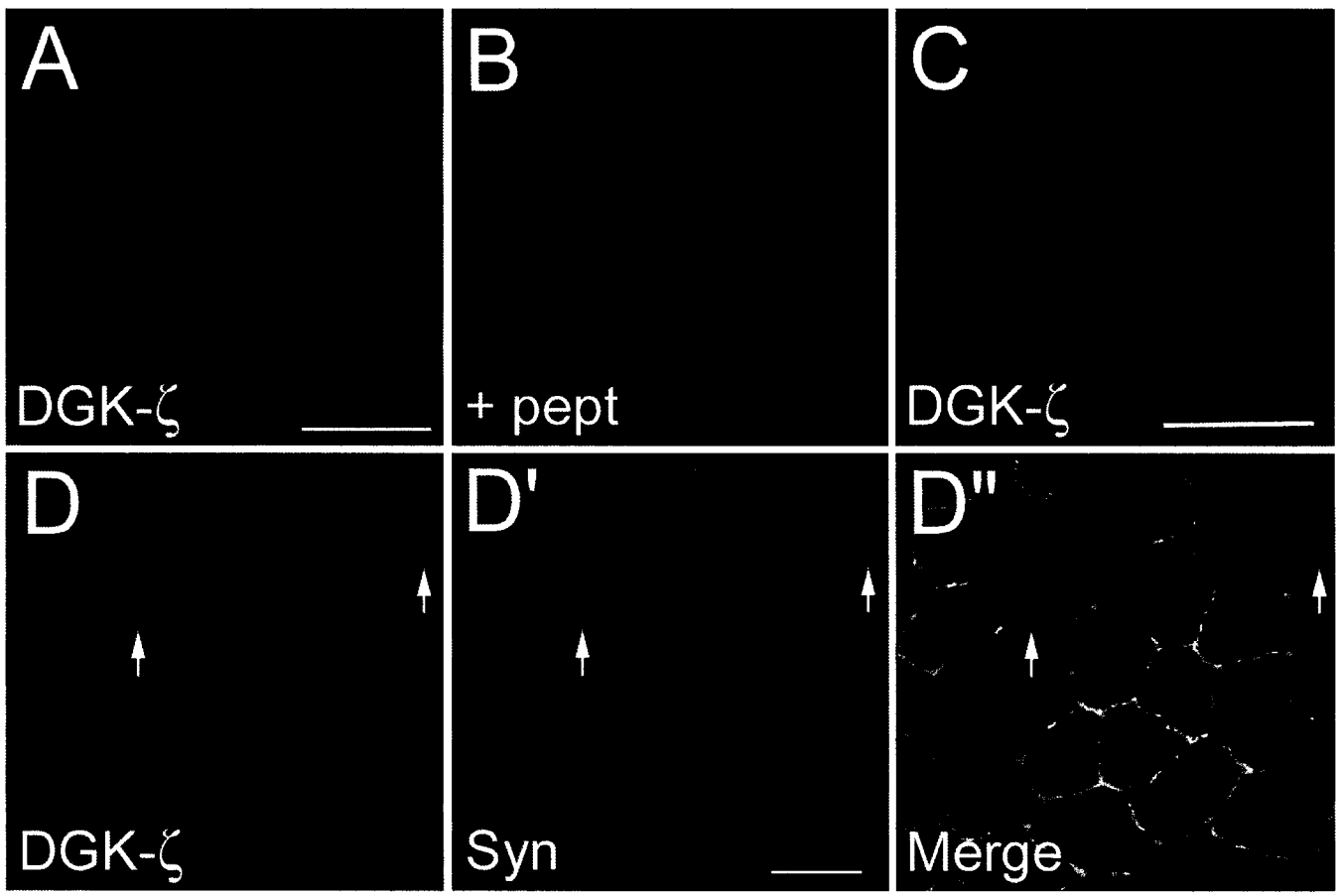


Fig. 3.3. Fibre type specificity of DGK-ζ expression. Double labeling for DGK-ζ (*A*) and myosin IIB (*B*) in mouse TA muscle reveals that DGK-ζ is absent or greatly reduced in type IIB fibres. The *asterisks* show DGK-ζ-negative fibres that are stained for myosin IIB.

Figure 3.3

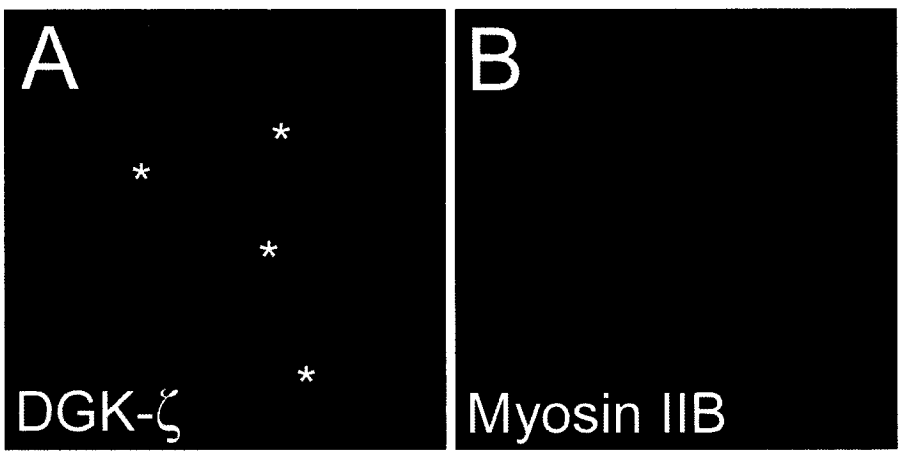


Fig. 3.4. Reduction of sarcolemmal DGK-ζ in *mdx* mouse muscle. Sections of TA muscle from normal (*A*) and *mdx* (*B*) mice were stained for DGK-ζ and photographed with identical exposure times. In *mdx* muscle, the sarcolemmal DGK-ζ staining was dramatically reduced, as was the punctate intracellular staining seen in normal fibres. *C*, Among the fibres with reduced DGK-ζ immunoreactivity in *mdx* muscle, there were occasional groups of small caliber fibres (*arrows*) with increased DGK-ζ expression. *D-D"*, The increased DGK-ζ immunoreactivity in small caliber fibres coincided with increased syntrophin expression in the same fibres. In *D*, the *arrows* indicate fibres with punctate intracellular staining similar to normal muscle fibres. Scale bar = 25 ums.

Figure 3.4

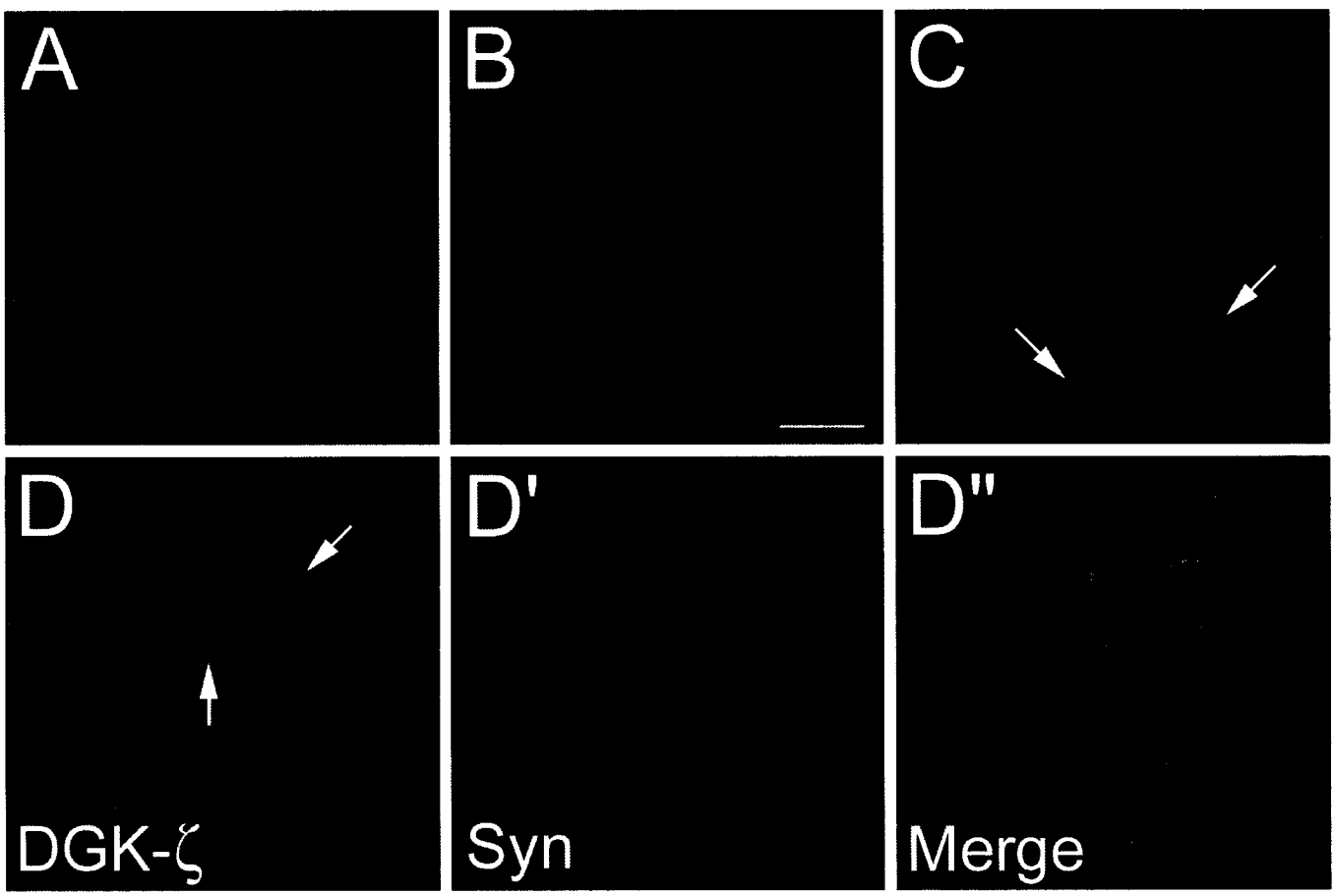


Fig. 3.5. DGK-ζ is specifically expressed at NMJs in type IIB fibres and is retained at NMJs in *mdx* muscle. Transverse sections of mouse TA muscle were double-labeled with an anti-DGK-ζ antibody followed by FITC-conjugated secondary antibody (*A* - *C*) and Texas Red-conjugated α-BgTx, which specifically labels AChRs (*A'* – *C'*). *A, A'*, In Type IIA and IIX fibres, DGK-ζ was present on the extrajunctional sarcolemma and was concentrated at NMJs defined by α-BgTx staining (*arrows*). *B, B'*, In contrast, DGK-ζ immunoreactivity was confined to junctional regions (*arrows*) in type IIB fibres (indicated by *asterisks*), which were otherwise devoid of staining. *C-C''*, Sections of *mdx* TA muscle double-labeled for DGK-ζ (*C*) and α-BgTx (C') illustrate that DGK-ζ is virtually absent from extrajunctional regions, but remains concentrated at synaptic sites. A magnified view of the boxed region in *C''* (*bottom* right) shows that synaptic DGK-ζ immunoreactivity extends slightly beyond the AChR-rich regions. Scale bar = 25 ums.

Figure 3.5

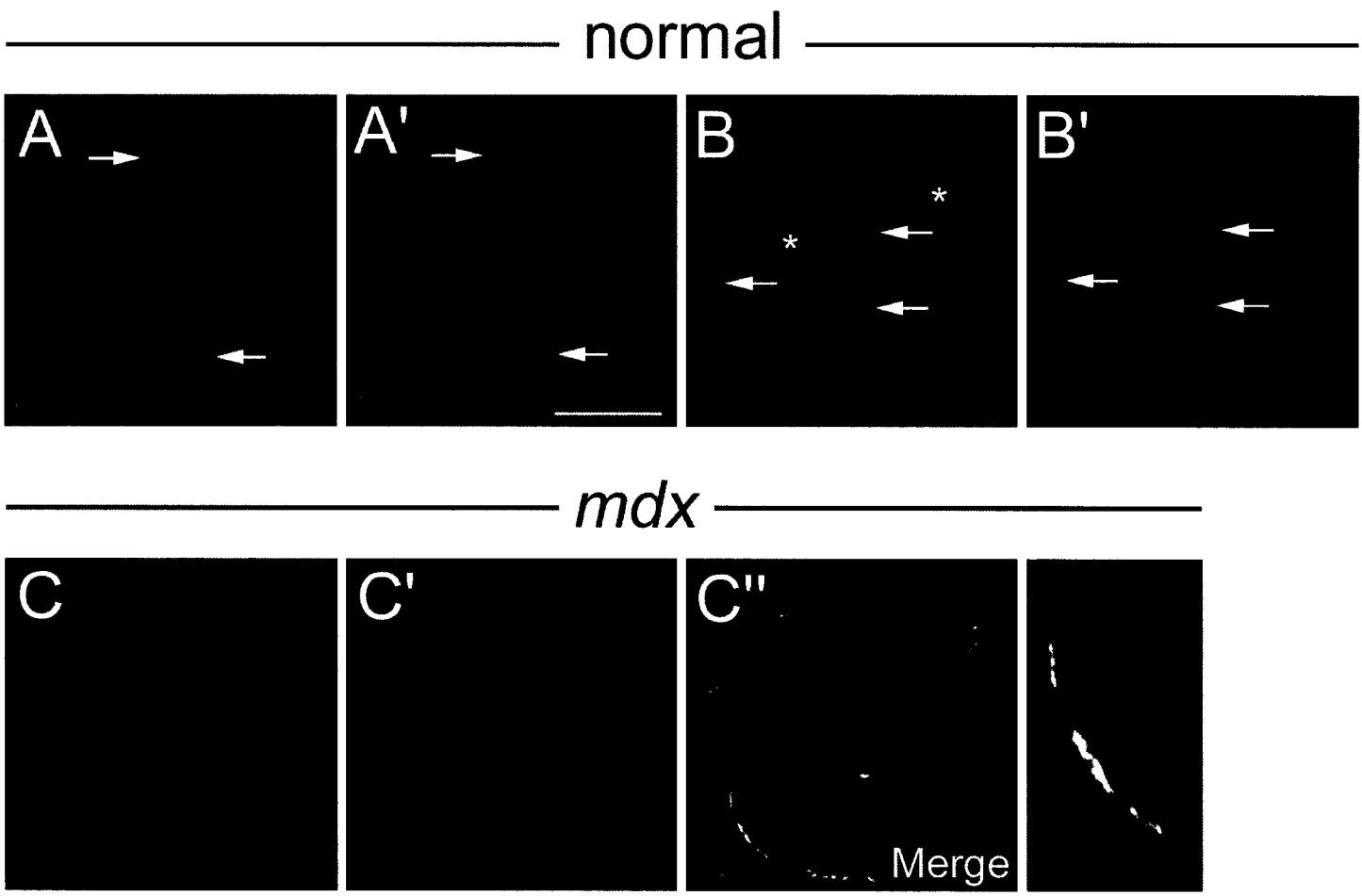
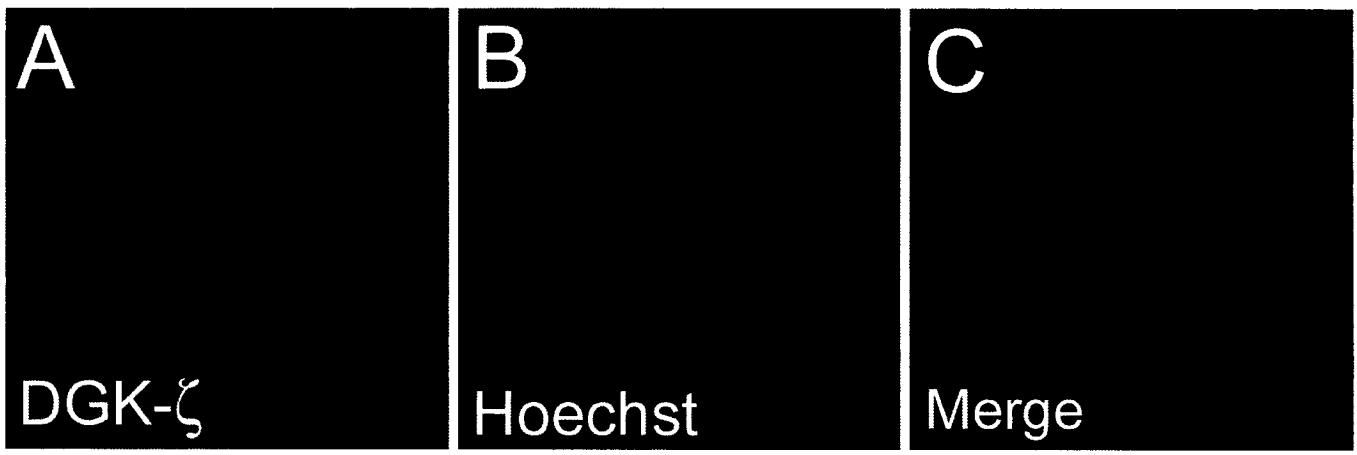


Fig. 3.6. DGK-ζ is associated with central nuclei in *mdx* muscle fibres. Sections of *mdx* mouse TA muscle were double-labeled with anti-DGK-ζ antibodies (*A*) and Hoechst stain (*B*) to visualize nuclei. In the merged image (*C*), virtually every nucleus is associated with DGK-ζ immunoreactivity.

Figure 3.6



Chapter 4

General Discussion

The major finding of this thesis is the identification of DGK-ζ as a novel syntrophin-interacting protein. Furthermore, DGK-ζ is the first protein found to interact with the PDZ domain of the γ_1-syntrophin isoform. We have shown that syntrophin likely plays a role in regulating the subcellular localization of DGK-ζ, and thus may affect the spatial regulation of DAG-activated signaling pathways.

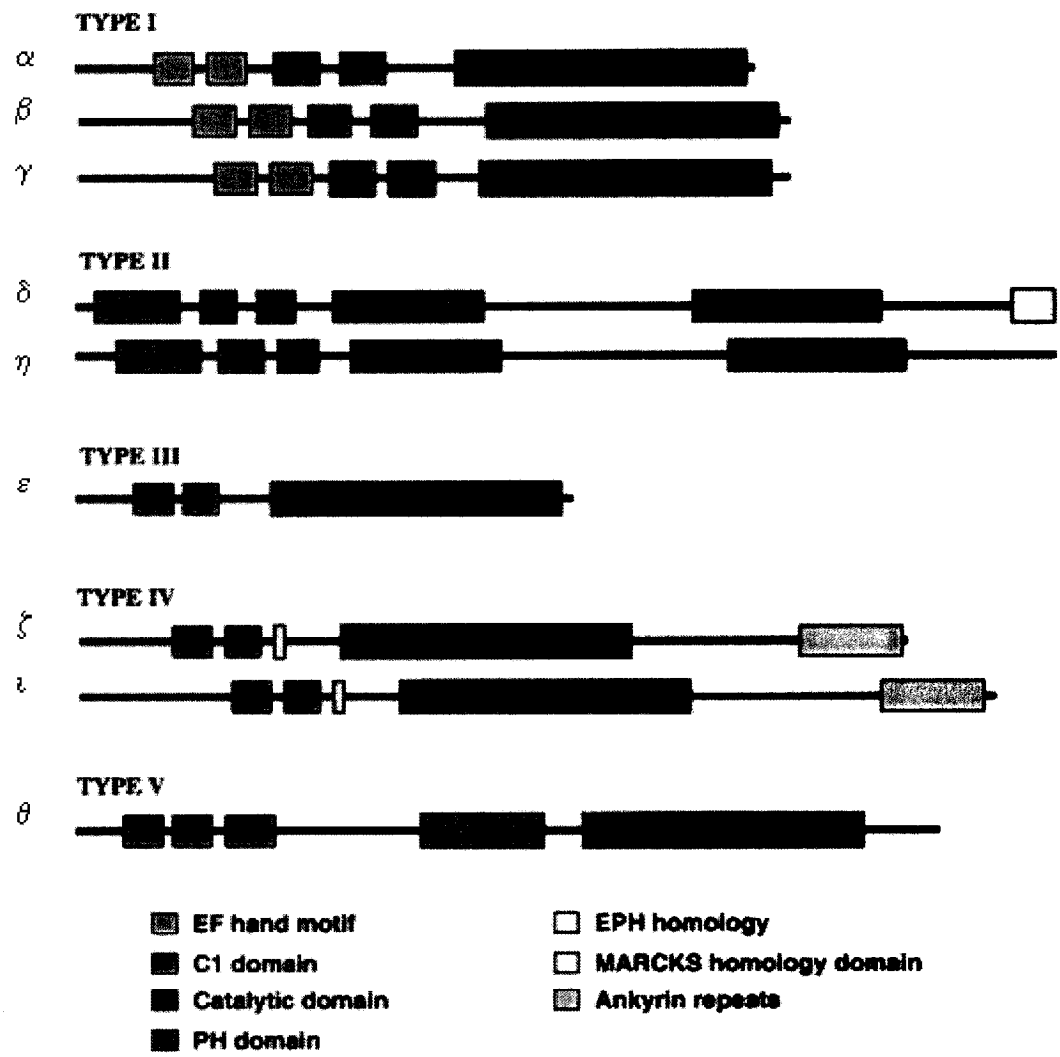
The Importance of DGK Subcellular Localization

Diacylglycerol kinase phosphorylates diacylglycerol (DAG), converting it to phosphatidic acid (PA). DAG is a lipid second messenger that is produced, along with soluble inositol-3, 4, 5-triphosphate (IP_3), upon cleavage of phosphatidylinositol-4, 5-biphosphate (PIP_2) by $G\alpha_q$-activated phospholipase C-β (PLCβ) (Neer, 1995). DAG activates, and in some cases recruits to the membrane, several C1 domain-containing signaling proteins (Hurley et al., 1997) such as RasGRP (Tognon et al., 1998), *vav*, protein kinase-C (PKC) (Ron and Kazanietz, 1999), protein kinase D (PKD), UNC 13 (Nurrish et al., 1999), and β_2-chimaerin (Caloca et al., 1999). Furthermore, PA, like DAG, is a membrane-bound lipid that has its own signaling functions. For instance, PA is mitogenic (Moolenaar et al., 1986) and has been shown to activate several enzymes such as type 1 phosphatidylinositol-4-phosphate 5-kinase (Jenkins et al., 1994), *n*-chimaerin (Ahmed et al., 1993), PKC-ζ (Limatola et al., 1994), and RasGAP (Exton, 1997). Thus, by terminating DAG signaling, DGK is thought to attenuate the activity of DAG-activated proteins (Sanjuán et al., 2001), while promoting the activity of PA-

activated proteins (Aragonés et al., 2001). Because DAG is produced in, and is confined to the plasma membrane, DGK must associate with the plasma membrane to inactivate its substrate (Hodgkin et al., 1998). At the plasma membrane, DGK serves as an "off switch" to ensure that DAG signaling is short-lived. This is of utmost importance since the sustained DAG-dependent activation of PKC or the Ras guanyl nucleotide releasing protein (RasGRP) leads to the malignant transformation of cells (Ebinu et al., 1998; Housey et al., 1988; Topham and Prescott, 1999).

There are nine mammalian isoforms of DGK, each having different tissular and subcellular distributions. All members of this family have in common at least two N-terminal zinc-finger-like cysteine-rich (CRD or C1) domains, and a catalytic domain (Topham and Prescott, 1999). They are further divided into five subclasses according to the presence of other regulatory domains (Figure 4.2). Together, these findings suggest that different DGK isoforms have non-redundant roles and that the subcellular localization of each must be tightly regulated (van Blitterswijk and Houssa, 2000). For instance, DGK-ζ belongs to the type IV subclass along with DGK-ι. They are characterized by the presence of a MARCKS homology domain, which contains a nuclear localization sequence, four C-terminal ankyrin repeats, and are the only isoforms known to contain a PDZ-binding consensus sequence at their C-terminus. This PDZ-binding consensus sequence suggests that these enzymes are regulated by PDZ domain-containing proteins, which are typically localized at specialized areas of the plasma membrane.

Figure 4.1 The Structural Domain Organization of the Mammalian Family of Diacylglycerol Kinases. This family is divided into five subtypes according to which structural motifs they comprise. All mammalian isoforms contain at least two cysteine-rich motifs (C1 domains), which are homologous to the C1A and C1B domains of PKC, as well as a catalytic domain comprising a putative ATP binding site. DGK-ζ belongs to the type IV subfamily, along with DGK-ι. Figure from Topham and Prescott, 1999.


TYPE I
α
β
γ
TYPE II
δ
η
TYPE III
ε
TYPE IV
ζ
ι
TYPE V
θ
EF hand motif
C1 domain
Catalytic domain
PH domain
EPH homology
MARCKS homology domain
Ankyrin repeats

Syntrophin Localizes DGK-ζ to the Plasma Membrane

Syntrophins are scaffolding molecules known to associate with the sarcolemma of normal muscle fibres, and data from Adams and colleagues (2001) strongly suggest that dystrophin is both necessary and sufficient for the sarcolemmal localization of syntrophins. In Chapters 2 and 3, I provide evidence suggesting syntrophins and DGK-ζ form a stable complex in cells. Furthermore, I have shown that syntrophins and DGK-ζ co-localize at the plasma membrane of normal skeletal muscle fibres, but are noticeably decreased at the sarcolemma of dystrophic fibres in *mdx* skeletal muscle. Collectively, these results suggest syntrophins play a role in localizing DGK-ζ to the sarcolemma via their association with dystrophin.

As was mentioned above, in larger degenerating fibres DGK-ζ immunostaining was decreased but not absent. Moreover, utrophin is expressed at lower levels at the sarcolemma of large degenerating fibres versus small regenerating ones (Helliwell et al., 1992; Karpati et al., 1993; Mizuno et al., 1994), which raises the possibility that the residual sarcolemmal levels of DGK-ζ in degenerating dystrophic muscle are due to the association of this enzyme with utrophin-associated protein complexes. However, aside from localizing to the plasma membrane via dystrophin or utrophin complexes, DGK-ζ may possess some ability to partially localize to the plasma membrane by itself or via some other complex. For instance, Santos and colleagues (2002) have shown that PKC phosphorylation of DGK-ζ's MARCKs domain signals the translocation of the latter from the cytosol to the plasma membrane, and that the C terminus of DGK-ζ is not

necessary for its membrane localization. However, because our results show that DGK-ζ immunostaining is increased at the sarcolemma of normal versus *mdx* fibres, this suggests that even if syntrophin is not necessary for the membrane localization of DGK-ζ, it serves to increase its presence at the plasma membrane (Chapter 3). Collectively, these data suggest that syntrophin, located at the DAPC at the cytoskeleton-plasma membrane interface, facilitates the plasma membrane association of the phosphorylated form of DGK-ζ.

The absence of dystrophin and the secondary loss of syntrophins and DGK-ζ from the plasma membrane may contribute to the pathology of dystrophic muscle. For instance, DAG levels at the plasma membrane may not be properly regulated, leading to the inappropriate activation of DAG-activated signaling enzymes, such as PKC and RasGRP. For instance, both PKC and the Ras signaling pathway are involved in the regulation of cell growth, and as mentioned previously, the sustained activation of PKC or the Ras guanyl nucleotide releasing protein (RasGRP) by DAG leads to the malignant transformation of cells (Ebinu et al., 1998; Housey et al., 1988; Topham and Prescott, 1999). Therefore, excessive DAG levels may lead to aberrant cell growth.

In chapter 1 I described the INAD phototransduction complex (p.4) in the rhabdomeres of the *Drosophila* eye. In the *Drosophila* fly three different genes coding for separate DGKs have been isolated thus far: DGK1, DGK2, and the *Drosophila* homologue of the mammalian DGKε (van Blitterswijk and Houssa, 2000). DGK2 most closely resembles type IV mammalian DGKs, but without a MARCKS domain and C-terminal PDZ-binding motif. Mutations in the *rdg* A gene that codes for this eye-specific DGK lead to retinal degeneration, and this is thought to be due to the constitutive

activation of transient release potential (TRP) calcium channels by unattenuated DAG signaling (Hofmann et al. 1999; Masai et al., 1993; Raghu et al., 2000). Interestingly, mammalian isoforms of these channels, called TRPCs, have been identified as the main channels responsible for the elevated intracellular calcium levels seen in dystrophic *mdx* muscle fibres, which lead to necrosis of these cells (Vandebrouck et al., 2002). Since some of these mammalian isoforms have been shown to be directly activated by DAG, this suggests a role for DGK-ζ in the regulation of TRPCs in skeletal muscle (Hofmann et al. 1999). Furthermore, in dystrophic muscle, these data suggest the interesting possibility that it's the mislocalization of DGK-ζ that leads to unattenuated DAG signaling at the membrane, leading to the over-activation of TRPC channels, and finally to the increased intracellular calcium levels and subsequent necrosis which are seen in these fibres.

The Localization of DGK-ζ to Specialized Plasma Membrane Domains

Immunohistochemical data in Chapter 3 show that DGK-ζ is concentrated at neuromuscular junctions (NMJs), regions of the plasma membrane opposed to nerve terminals that are specialized for the receipt of postsynaptic signals. In type II glycolytic fibres, DGK-ζ is absent from the general sarcolemma and is restricted to the NMJ. Furthermore, in dystrophic muscle, DGK-ζ remains concentrated at NMJs. Collectively, these findings suggest that DGK-ζ is linked, via syntrophin, to utrophin protein complexes at NMJs. In support of this contention, DGK-ζ levels are increased on the sarcolemma of regenerating dystrophic fibres, where utrophin is known to be present (Helliwell et al., 1992; Karpati et al., 1993; Mizuno et al., 1994).

Although the function of DGK-ζ in skeletal muscle has not been studied, its localization at the NMJ could possibly implicate it in the synaptic clustering of nicotinic acetylcholine receptors (AChRs). During the maturation of the NMJ, the motor neuron orchestrates the redistribution of AChRs from the sarcolemma, where they are uniformly distributed, to the postsynaptic membrane, where they subsequently become clustered and highly concentrated (Sanes and Lichtman, 1999). Weston and colleagues (2000) have recently demonstrated that Rac 1, a member of the Rho family of small GTPases involved in multiple cellular processes such as cytoskeletal rearrangement, cell proliferation and differentiation, plays an essential role in agrin-induced acetylcholine receptor (AChR) clustering at the mammalian neuromuscular junction (Takai et al., 2001). DGK-ζ could potentially play a role in regulating AChR clustering since it can bind to Rac 1 *in vivo* and activate it (Tolias et al., 1998; van Blitterswijk and Houssa, 2000).

Alternatively, DGK-ζ may play a negative-regulatory role in the synapse-specific expression of AChR subunit genes. During the maturation of the NMJ, neurally derived neuregulin stimulates AChR subunit gene expression in synaptic nuclei via the Ras-Raf-Mek-Erk signaling pathway, a cascade regulated in part by DAG-activated RasGRP (Sanes and Lichtman, 1999). DGK-ζ acts as a spatially discrete negative regulator of Ras signaling by binding to and inhibiting RasGRP through the phosphorylation of local pools of DAG (Topham et al., 2001). Thus, the localization of DGK-ζ to the NMJ may regulate the synaptic expression and localization of AChRs at the crests of the junctional folds during the maturation of skeletal muscle cells. Furthermore, the association of DGK-ζ with utrophin-associated protein complexes, which tightly associate with AChRs

(Campanelli et al., 1994; Gee et al., 1994; Pons et al., 1993) provide a strategic way for this enzyme to regulate the synaptic clustering and expression of these receptors.

Syntrophin may play a part in localizing DGK-ζ to plasma membrane areas rich in PIP_2. This stems from the finding that the first PH domain of α_1-syntrophin binds to the phospholipid PIP_2 (Chockalingham et al., 1999). Such a mechanism is particularly attractive for DGK-ζ, since it would target it to areas where its substrate DAG is produced via breakdown of PIP_2 by PLC-β.

In Chapter 2 we show that in the brain DGK-ζ and γ1-syntrophin co-localize in neurons in specific areas of the brain. In cerebellar Purkinje neurons they specifically localize to the soma and dendrites. Previous work has shown that dystrophin is localized to cell bodies and dendrites in Purkinje cells, where it appears to associate with the plasma membrane at post-synaptic areas (Blake and Kröger, 2000; Lidov et al., 1990; Lidov et al., 1993). Furthermore, our immunoprecipitation experiments from co-transfected cells, as well as results from Piluso and colleagues (2000), suggest that γ1-syntrophin can form a complex in cells with dystrophin and its related proteins. Therefore, γ1-syntrophin may serve to link DGK-ζ to dystrophin protein complexes at the plasma membrane, possibly at post-synaptic areas. This line of thinking is supported by our results from binding assays using mouse brain preparations, which suggest that DGK-ζ can form a complex *in vivo* with dystrophin (Chapter 2). Thus, while the lack of reliable γ1-syntrophin antibodies prevented us from performing co-immunoprecipitations of DGK-ζ and γ_1-syntrophin from brain tissue, our data still suggest that DGK-ζ, γ1-syntrophin, and dystrophin, can form a complex in distinct areas of central neurons.

Possible roles for DGK-ζ in neurons include the regulation of neurite outgrowth. For instance, in neurons Rac 1 appears to play a role in the formation of lamellipodia and membrane ruffles, two morphological structures believed to be involved in neurite outgrowth and the formation of new synaptic connections (Gallo and Letourneau, 1998; Kozma et al., 1997; Takai et al., 2001). Rac also plays a role in regulating branch additions and retractions during dendritic arbor growth in central neurons *in vivo* (Li et al., 2000). Recent results from our lab indicate that DGK-ζ localizes to neuronal growth cones, and it co-localizes with actin in lamellipodia and filopodia (unpublished results). Furthermore, over-expression of wild type DGK-ζ causes neurite outgrowth, an effect that is abolished when dominant-negative Rac 1 is overexpressed or when DGK-ζ's C-terminal syntrophin-binding sequence is mutated (unpublished data). Therefore, syntrophin may play a role in localizing DGK-ζ to specific areas in neurons where it can regulate neurite outgrowth through its effects on Rac 1.

Another possibility is that DGK-ζ might play a role in regulating the dendritic morphology of cerebellar Purkinje neurons through its effect on PKC. For instance, PKC is important for normal dendritic development in Purkinje cells (Schrenk et al., 2002), and is also a key enzyme involved the mediation of long-term depression at the parallel fibre-Purkinje cell synapse (Abeliovich et al., 1993; Levenes et al., 1998). This raises the possibility that, due to its localization to Purkinje cell bodies and dendrites, DGK-ζ plays a regulatory role in the formation of synaptic connections by regulating PKC activity.

Syntrophin and DGK-ζ form a Complex that Localizes to the Nucleus

DGK-ζ and at least three other DGK family members, DGK-θ, -ι, and -α, have been shown to localize to the nuclear compartment (for review see: van Blitterswijk and Houssa, 2000). Thus far, DGK-ζ and -ι are the only isoforms that contain a known nuclear localization sequence, and it is located in their MARCKS homology domain. PKC appears to regulate the nuclear translocation of DGK-ζ since phosphorylation of its MARCKS domain by either PKCα or PKCγ prevents its accumulation in the nucleus (Goto and Kondo, 1996; Topham et al., 1998; van Blitterswijk and Houssa, 2000). The nuclear compartment contains inositol lipids and a parallel cycle of DAG conversion to PA (Divecha et al., 1993; D'Santos et al., 1998; Vann et al., 1997). Furthermore, results from Topham and colleagues (1998) show that nuclear DGK-ζ attenuates cell growth by decreasing DAG levels in this compartment. Since DAG is an allosteric activator of PKC, and PKC is mitogenic, it has been suggested that the decrease in cellular proliferation may be the result of a reduction in nuclear PKC activity caused by the DGK-dependent decrease in nuclear DAG levels. Moreover, in the nucleus, DGK-ζ may regulate the expression of AChR subunit genes through its inhibitory effects on nuclear PKC. For instance, in skeletal muscle nuclear PKC couples membrane depolarization and muscle activity to the down-regulation of AChR subunit gene expression in non-synaptic nuclei (Huang et al., 1992; Klarsfeld et al., 1989; Sanes and Lichtman, 1999).

Our results in Chapter 2 indicate that syntrophin participates in the regulation of DGK-ζ nuclear translocation. Syntrophin and DGK-ζ form a complex that partitions between the cytosolic and nuclear compartments, but when this interaction is prevented, DGK-ζ accumulates in the nucleus, while syntrophin remains in the cytoplasm. Thus,

both the interaction of DGK-ζ with syntrophin and its phosphorylation by PKC each serve to regulate nuclear translocation. Syntrophin appears to act as a cytoplasmic anchor, preventing accumulation of DGK-ζ in the nucleus, whereas phosphorylation of DGK-ζ by PKC could act as a switch to regulate nuclear translocation of DGK-ζ/Syntrophin complexes.

On the other hand, DGK-ζ appears to be able to recruit syntrophin to the nucleus. Abolishing the interaction between these two proteins causes syntrophin to remain in the cytoplasm. Since syntrophin does not contain a known nuclear localization sequence (NLS), it likely binds to DGK-ζ to gain access to the nucleus. Futhermore, there likely exists a molecular "switch" that signals the translocation of the syntrophin/DGK-ζ complex into the nucleus. One possibility is that since phosphorylation appears to play a role in the membrane localization of DGK-ζ, dephosphorylation of this enzyme or even of syntrophin, may allow this complex to be recruited to the nucleus. Furthermore, in the case of dystrophic fibres, the absence of dystrophin at the plasma membrane of skeletal muscle fibres could translate to increased nuclear levels of the DGK-ζ/syntrophin complex. This in turn could have physiological consequences, such as the attenuation of cell growth through the downregulation of nuclear DAG levels. Although our data shows that both DGK-ζ and syntrophin are associated with central nuclei in dystrophic fibres, technical limitations prevented us from determining whether their levels are decreased in the peripheral nuclei of normal muscle.

One important open question is what role syntrophin plays in the nucleus. An interesting possibility is that nuclear syntrophin localizes DGK-ζ to specific subdomains of the nucleus. Experiments by Goto and Kondo (1996) suggest that the C terminus of

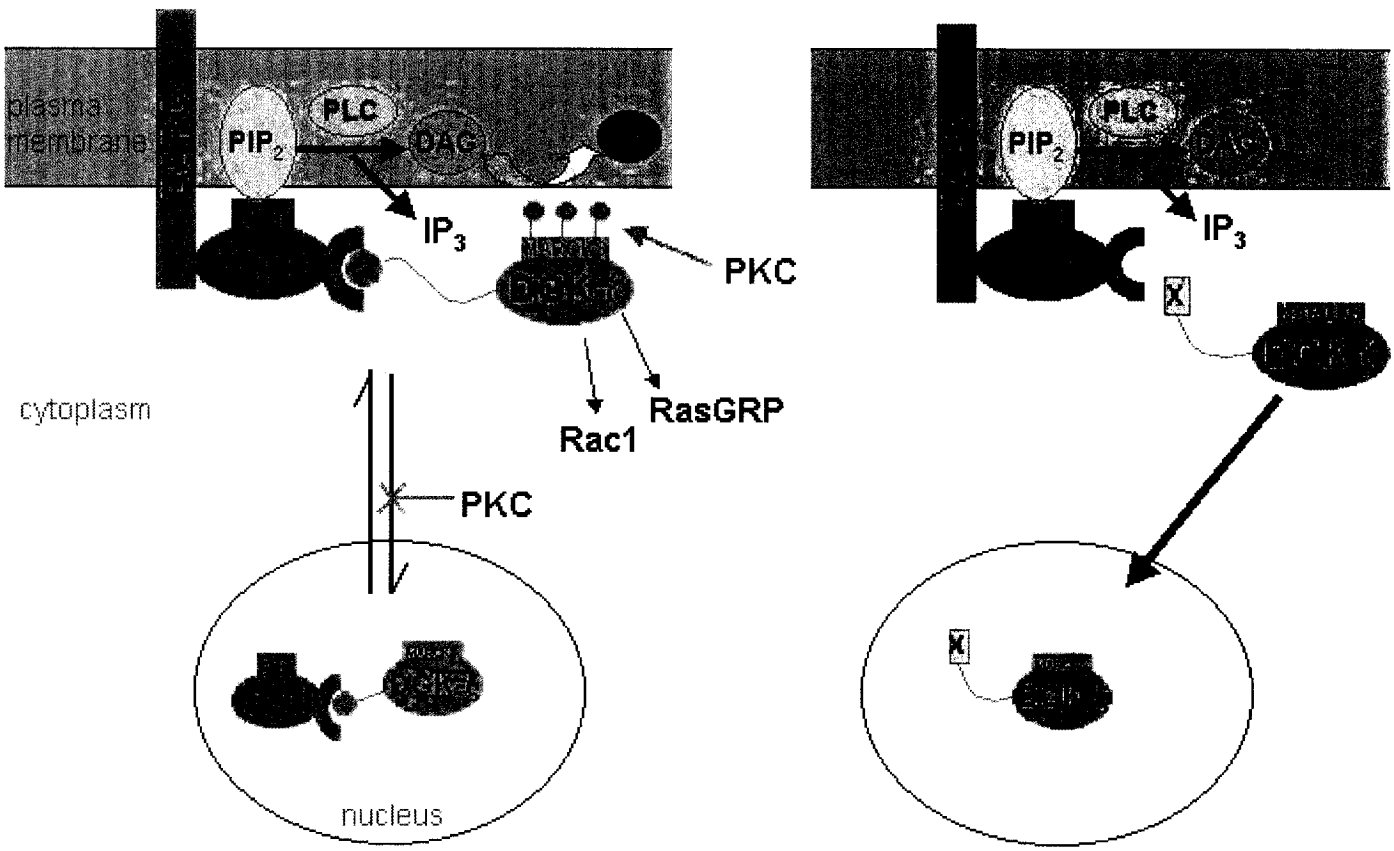
DGK-ζ is important for its localization to detergent-soluble fractions of the nucleus (probably the nuclear envelope). Therefore, syntrophin may link DGK-ζ to specific areas of the nuclear membrane, much like it does at the plasma membrane. However, Goto and Kondo (1996) also suggest that DGK-ζ can localize to detergent-insoluble fractions of the nucleus (nuclear matrix). Here, there exists a smaller pool of DAG, which makes a major contribution to the total amount of PA produced in the nucleus (Vann et al., 1997). Thus, through its subnuclear localization of DGK-ζ, syntrophin may play a part in determining which specific DAG pools this enzyme interacts with, and thus regulate physiological processes such as cellular proliferation (D'Santos et al., 1998).

One interesting possibility is that dystrophin-like complexes are present in the nucleus. Evidence to support this line of thinking comes from data indicating the presence of dystrophin, Dp71 (an N-terminally truncated dystrophin isoform), β-dystrobrevin, as well as 'syntrophin-associated serine/threonine kinase' (SAST), in the nucleus (Blake et al., 1999; González et al., 2000; Lumeng et al., 1999). Therefore, if functional dystrophin-like complexes do exist in the nucleus, syntrophins and DGK-ζ could be components of these.

Our data further strengthens the role of syntrophins in the regulation of the subcellular localization of signaling molecules. Furthermore, our finding that syntrophin can localize to the nucleus opens up new avenues for research into the role of these scaffolding molecules in areas other than the plasma membrane. Based on our results we have proposed a hypothetical model describing the interaction of the syntrophin-DGK-ζ complex and its translocation in cells (Figure 4.2).

Figure 4.2 Proposed Model of the Interaction Between DGK-ζ and γ1-Syntrophin. **A)** In this model syntrophin would play a role in localizing DGK-ζ to the plasma membrane and possibly to specific areas of the nucleus. One way that syntrophin may localize DGK-ζ at the plasma membrane is by linking it to the dystrophin protein complex at the cytoskeleton-plasma membrane interface. The PH1 in syntrophin may also be able to bind to plasma membrane PIP_2 and therefore target DGK-ζ to areas where DAG is produced. PKC-phosphorylation of DGK-ζ's MARCKS domain leads to the translocation of the DGK-ζ/syntrophin complex out of the nucleus and/or to the plasma membrane. Futhermore, dephosphorylation of the DGK-ζ/syntrophin complex may act as a 'switch' that causes this complex to be released from the DAPC at the plasma membrane, and be translocated to the nucleus **B)** If DGK-ζ's PDZ-binding motif is disrupted then it cannot interact with syntrophin, therefore syntrophin stays in the cytosol and DGK-ζ accumulates in the nucleus. DGK-ζ might also accumulate in the nucleus if the levels of syntrophin within the cell are lowered, such as in the case of dystrophic muscle.

Model of the Interaction Between DGK-ζ and Syntrophin



Chapter 5

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Chapter 6

Table 6.1 Differences between syntrophin isoforms

	Species	α1-syntrophin	β1-syntrophin	β2-syntrophin	γ1-syntrophin	γ2-syntrophin
No. of amino acids	mouse human	503 505	537 538	520 540	- 517	- 539
Molecular mass (kDa)	mouse human	53.7 54.0	58.1 58.0	56.4 57.9	- 57.9	- 60.1
Chromozomal localization	mouse human	2 20q11.2	? 8q23-24	8 16q23-24	- 8q11	- 2p25
Exons	mouse human	- 8	- ?	- 7	- 19	- 17
Isoelectric point	mouse human	6.7 6.4	8.3 8.6	8.7 8.7	- 6.24	- 7.59
Tissue distribution		Skeletal and cardiac muscle	Ubiquitous	Ubiquitous	Brain	Brain, testes, skeletal muscle
Localization in skeletal muscle		NMJ and sarcolemma	NMJ of all fibres, and sarcolemma of type IIB fast-twitch fibres	NMJ	-not expressed in this tissue	Sarcolemma
Localization at the NMJ		Crests and troughs of folds	Troughs of folds	Troughs of folds	-N/A	Not determined
Localization in the brain	rodent	Blood vessels, neurons in CA regions, olfactory bulb, cerebral cortex, and dentate gyrus	Blood vessels	Blood vessels, neurons in dentate gyrus pituitary, cerebral cortex, Ammon's horn, and caudate putamen	Cell bodies of Purkinje neurons, hippocampal (CA1-4), dentate gyrus and cortical neurons	Pyramidal and multipolar neurons of the frontal cortex

(Adams et al., 1993; Adams et al., 1995; Ahn et al., 1994; Ahn et al., 1996; Butler et al., 1992; Froehner et al., 1997; Górecki et al., 1997; Hogan et al., 2001; Kramarcy and Sealock, 2000; Peters et al., 1997; Piluso et al., 2000; Yang et al., 1994)

This was adapted from a table published in 1997 by Froehner et al., 1997.

Table 6.2 Additional Positive Preys Retrieved from the Yeast Two-Hybrid Screen of a Brain cDNA Library using the PDZ Domain of γ1-Syntrophin

OTHER YTH POSITIVE PREYS	C-TERMINAL PDZ-BINDING CONSENSUS SEQUENCE	# TIMES RETRIEVED (OUT OF 21)	IN VITRO INTERACTION WITH OTHER SYNTROPHINS	DESCRIPTION	REFEREN-CES
BAS GRIP / ***TAPP1*** (**ta**ndem **P**H-domain-containing **p**rotein-**1**)	PVSDV	2	yes (α_1-, β_1-, and β_2-syn.)	contains two PH domains and binds to PI(3,4)P_2	Dowler et al., 2000
Autotaxin	YESEI	3	undet.	extracellular enzyme & autocrine motility factor	Clair et al., 1997; Stracke et al. 1992.
MARK1 (**M**AP/ **m**icrotubule **a**ffinity-**r**egulating **k**inase)	NELKL	1	undet.	protein kinase that triggers microtubule disruption	Drewes et al. 1997
Bob! / ***SLIP1*** (**s**lit-**l**ike **p**rotein-**1**)	QETQI	2	undet.	still unidentified	none
Human chromoso-me 7q31	QETQI	3	undet.	still unidentified	none

August 14, 2001

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Dear Sir/Ma'am

I am presently preparing to submit my M.Sc. thesis written as a collection of manuscripts, and would like to include our paper entitled "Interaction of γ1-syntrophin with diacylglycerol kinase-ζ. Regulation of nuclear localization by PDZ interactions", published in the Journal of Biological Chemistry, 2001 Jul 13; 276(28) 26526-33 I am therefore requesting permission to reproduce this article as part of my thesis.

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