Repositioning of Ritanserin for the Inhibition of DGKα and the Role of the Polybasic Domain in the Phosphoregulation of Lipins 1 and 3

Salome Boroda

Tbilisi, Georgia

St. Paul, Minnesota

MS, Biological and Physical Sciences, University of Virginia, 2014

BS, Biochemistry, University of St. Thomas, 2011

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ABSTRACT

The diversity and multi-functionality of cellular lipids is astounding. At the hub of phospho- and neutral lipid flux are two lipids with many effector proteins: diacylglycerol (DAG) and phosphatidic acid (PA). Enzymes which can simultaneously regulate both of their levels are diacylglycerol kinases (DGKs) and lipins. The former phosphorylates DAG to yield PA and the latter dephosphorylates PA to generate DAG. The work herein approaches the study of DGKs and lipins from two different perspectives.

First, we have built upon previous work, which established DGKα as a therapeutic target in glioblastoma multiforme (GBM), and have repositioned ritanserin, a serotonin receptor (5-HTR) antagonist, as a pharmacological tool for attenuating DGK activity. We have also elucidated the polypharmacology of other DGK inhibitors, and shown that they are potent 5-HTR antagonists.

Second, we hypothesized that the varying PA-binding domains, or polybasic domains (PBDs), of each lipin are involved in the phosphoregulatory differences among these enzymes. We show, for the first time, that insertion of the lipin 3 PBD in the lipin 1 enzyme eliminates the phosphoregulation of lipin 1. Conversely, the insertion of lipin 1 PBD into the lipin 3 protein allows phosphorylated residues on lipin 3 to negatively regulate its activity *in vitro*. These data suggest that the differences in the amino acid sequence between PBDs of these two proteins are sufficient to determine whether or not the enzymes can be controlled by intramolecular phosphoregulation

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APPENDIX OF CONTENTS

APPENDIX OF ACRONYMS

MAG	Monoacylglycerol
DAG	Diacylglycerol
TAG	Triacylglycerol
ATGL	Adipose Triglyceride Lipase
HSL	Hormone-Sensitive Lipase
ATP	Adenosine Triphosphate
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphoinositol-4, 5-Bisphosphate
PDK1	Phosphoinositide-Dependent Kinase 1
GSK3	Glycogen Synthase Kinase 3
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
PKC	Protein Kinase C
PLD	Phospholipase D
WT	Wild Type
SEM	Standard Error of the Mean
LC-MS	Liquid Chromatography-Mass Spectrometry
DMEM	Dulbecco's Modified Eagle's Medium
PMA	*Para*-Methoxyamphetamine
DTT	Dithiothreitol
FBS	Fetal Bovine Serum

NCS	Newborn Calf Serum
IBMX	3 – Isobutyl – 1 – Methylxanthine
cpm	Counts Per Minute
BSA	Bovine Serum Albumin
PAP	Phosphatidic Acid Phosphatase
DGK	Diacylglycerol Kinase
5-HT	Serotonin
5-HTR	Serotonin Receptor
PLC	Protein Lipase C
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PA	Phosphatidic Acid
LPA	Lysophosphatidic Acid
PBD	Polybasic Domain
SDS-PAGE	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis
KO	Knockout
CLIP	C-terminal Domain
NLIP	N-terminal Domain
FRB	FKBP12-Rapamycin Binding Domain
cAMP	Cyclic Adenosine Monophosphate

CHAPTER 1: GENERAL INTRODUCTION

1.1 Membrane Phospholipids

The eukaryotic cell is unique from the prokaryote in its compartmentalization of organelles. Each organelle, as well as the cell itself, is surrounded by dynamic membrane structures that are made of proteins, cholesterol and lipids. Cells contain a large repertoire of lipids and in fact, approximately 5% of their genome is dedicated to coding for proteins responsible for lipid synthesis and modification (1, 2). Lipids maintain membrane structural integrity, but they are also energy storage molecules and second messengers that can modulate the activity of many enzymes (1).

Phospholipids are a major lipid component of membranes and contain diacylglycerol (DAG) as their hydrophobic portion (2, 3). The "building-blocks" of these molecules are: the hydrophilic head-group and the aliphatic chains. The potential variations in these two components are the basis for the cell's ability to generate a vast number of phospholipid species (1). The most abundant are phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and phosphatidylinositols (PI) (1). These are all derivatives of phosphatidic acid (PA), which by itself comprises less than 2% of cellular membrane lipids (1, 4). The structures and basic chemical properties of these compounds are shown in **Fig. 1.1**.

The foci of this dissertation are DAG and PA. These are signaling lipids, which by profoundly influencing the activity of many proteins can have significant effects on cell function. We approach the study of these lipids by investigating the exogenous inhibition, biochemistry and regulation of diacylglycerol kinases (DGKs) and lipins, enzymes that can concurrently impinge on levels of DAG and PA (**Fig. 1.2**). They do so

by catalyzing opposing reactions: DGKs phosphorylate DAG to synthesize PA and lipins generate DAG by the dephosphorylation of PA (**Fig. 1.3**).

The unifying theme in the present study of DGKs and lipins is the fact that they are cytosolic enzymes with substrates that only exist in membranes. Thus, it is important to consider the evolutionary adaptations these proteins developed to efficiently both sense and respond to changes in the levels of these substrates. Although our work with DGKs does not explicitly address this question, it is worth discussing as it underlies its enzymatic inhibition. After briefly summarizing the role of DAG and PA in phospholipid metabolism, this chapter will introduce DAG kinases from a historical perspective followed by a brief discussion of DGK tissue expression and the role of DGKα in cancer and drug discovery. The mechanism of DAG binding and DGK biochemical regulation will then be considered. This will be followed with a historical perspective of lipin discovery and a discussion of lipin expression and relevance to disease. Finally, the biochemical regulation of lipins, the role of electrostatic interactions in PA-lipin association and the polybasic domain (PBD) will be discussed.

1.2 PA and DAG Flux

In addition to being important for membrane structure and having second messenger activities, DAG and PA occupy central nodes in phospholipid and neutral lipid flux (**Fig. 1.3**). Their levels are regulated simultaneously by the action of lipins and DGKs, as discussed above.

DAG is generated by lipin in the *de novo* pathway of fat storage and is a direct precursor of the major energy storage molecule, triacylglycerol (TAG). It is also a precursor of PC and PE, through its reaction with CDP-choline and with CDP-

ethanolamine, respectively. These phospholipids compose a significant portion of cell membranes and they generate PS, another abundant lipid (**Fig. 1.3**). DAG is regenerated by the activity of phospholipase C (PLC), which generates DAG from phosphatidylinositol 4, 5-bisphosphate (PIP_2) in the PI cycle and by sphingomyelin synthase, which synthesized DAG from ceramide and PC.

PA generates phosphatidylglycerol (PG), cardiolipin (CL) and PI through a modified DAG intermediate (**Fig. 1.3**). PI is a precursor of PIP_2, which serves as the substrate for PLC to again synthesize DAG. PA is also a major intermediate in the *de novo* pathway of TAG synthesis. Additionally, it is synthesized by phospholipase D (PLD) from the DAG-derived PC, and of course by DGK (**Fig.1.3**). These pathways are reviewed in (5).

1.3 DGKs – A Historical Perspective

The kinase activity of DGKs against DAG has been extensively investigated, especially *in vitro*. The story begins in the late 1950s and early 1960s when w1orks by Hokin and Hokin described what we now know as the PI pathway. They noted that specific activity in erythrocyte membranes is responsible for phosphate transfer to DAG and generation of PA (6-8). By the 1970s Calls *et al*. had performed biochemical characterizations of the DAG kinase activity in human platelets and demonstrated that the enzyme responsible for the kinase activity had affinity to DAG (K_m = 0.4 mM) and ATP (K_m = 1.3 mM) and was deoxycholate and Mg^{2+} sensitive (9). It is important to note that the latter work measured total DAG kinase activity with no cognizance of the fact that a given cell may express many DGKs. It was not until 1981 and 1983 that the 80 kDa DGK – now known as DGKα – was purified from rat liver and brain (10, 11). In 1990, the

sequences of both the porcine and human 80kDa DGK were reported (12, 13). The analysis identified an EF hand motif found in Ca^{2+} binding proteins, cysteine-rich (C1) domains, known for DAG association, and a putative ATP binding site. Since that time, nine other mammalian DGK isoforms have been cloned – including the human DGKs – characterized and assigned to five classes based on sequence similarity (13).

1.4 DGKs – Tissue Expression

Most cells have DGK activity and several DGK isoforms show tissue-specific expression (14, 15). Almost all of the DGKs are expressed in brain cells as determined by the presence of mRNA and protein, with the exception of DGKδ. This latter enzyme has an important function in skeletal muscle, where it is linked to the development of hyperglycemia (14, 16). DGKα and Type IV DGKζ are additionally very abundant in T lymphocytes and hematopoietic tissues, as seen by mRNA expression. DGKγ, ε and ι are highly expressed in the retina at the protein and mRNA level, and DGKε, η, κ and δ mRNA and proteins are also present in the testis (14, 16-20).

1.5 DGKs –Disease Implications

Diacylglycerol kinases have been slowly emerging as important mediators of the pathogenesis of many diseases (21, 22). The class I DGKα, in particular, has been recently uncovered as a potential therapeutic target for cancers, especially for the very aggressive form of brain cancer, glioblastoma multiforme (GBM) (23, 24). Dominguez *et al*. demonstrated that DGKα positively regulates the transcription of a well-known oncogene, mammalian target of rapamycin complex 1 (mTORC1), a novel mechanism for DGK mediated cancer signaling (21, 23). In addition, DGKα and DGKζ, which are abundantly expressed in T lymphocytes and regulate their development, are proposed to

play an important role in the suppression of T cell activation, making them attractive targets for cancer immunotherapy (15, 17, 25-27).

The inhibitors of DGKs are R59022 (diacylglycerol kinase inhibitor I) and R59949 (diacylglycerol kinase inhibitor II) (28, 29). Since their initial characterization thirty years ago, they have been widely used to attenuate DGK activity *in vitro*. The selectivity profile of these compounds against the 10 DGK isoforms is debated. They were initially believed to only target class I DGKs. However, these findings have subsequently been challenged (30-32). R59022 and R59949 have been used *in vivo* very infrequently and display poor pharmacokinetics in mice (*Olmez et al.* unpublished, and 23, 33)).

In 2006 and 2013, seminal works by several labs showed that DGKα may be involved in multiple hallmarks of oncogenesis and as such, its attenuation could simultaneously target several cancer survival mechanisms (23, 26). Until recently, the only available pharmacological tools for attenuating DGK activity were the above-mentioned compounds. The development of novel inhibitors is a complicated task due to the many mammalian DGKs and the many species of PAs and DAGs that exist in the cell. Drug repositioning may be a viable option for circumventing at least some of these roadblocks. Also known as drug repurposing, this is a process by which a new therapeutic use is found for a preexisting and approved medicine originally meant for a different disease indication (34). My work, which repositions an antipsychotic agent for the attenuation of DGKα, while further characterizing the polypharmacology of the known DGK inhibitors, is discussed in Chapter 3.

1.6 DAG Binding – the Cysteine-Rich Domain of DGKs

The consensus sequence responsible for DAG association is the C1 domain, which coordinates Zn^{2-} ions in its structure (35). Numerous proteins contain C1 domains, including: protein kinas Cs (PKCs), chimaerins, protein kinase D (PKD), RasGRPs and DGKs (35). There are two kinds of C1 domains: typical and atypical. The former is DAG and DAG mimetic (phorbol ester) responsive and the latter is not (35). Diacylglycerol kinases contain two C1 domain repeats (C1A and C1B), with the exception of the class V DGKθ, which contains three (**Fig. 1.4**). The core structures of the C1 domains of DGKβ, γ, δ, η, and θ are very similar to the well-characterized typical C1 domains of PKCγ and PKCε. However, the literature is not in agreement as to the sensitivity of DGK C1 domains to phorbol esters. For example, the class I DGKα, β and γ were initially reported to not bind DAG mimetics, but these findings were later challenged for of DGKβ and γ (35-37). Further, the DGKθ and DGKζ C1 domains are essential for translocation to the membrane, but are unresponsive to DAG mimetics (35, 37-39).

The most C-terminal C1 domain of DGKs is unique in that it contains an extended region of 15 amino acids. Mutations in this region of DGKθ resulted in a very significant reduction in kinase activity (38, 40). Conversely, deletion of both C1 domains of DGKα revealed that they are not absolutely necessary for activity (41). It has been suggested that DGKs very weakly and transiently associate with DAG. Specifically, that the extended region "presents" the substrate to the catalytic site and the C1 domains simply facilitate translocation to the membrane, again with a very weak association to DAG (38).

1.7 DGKs – Biochemical Regulation

i. Calcium

The dependence of some DGKs on Ca^{2+} for activity and translocation to the membrane were demonstrated in parallel to the discovery of their identity (42). Using human erythrocytes labeled with radioactive phosphate (^{32}P), Michell and colleagues showed that incorporation of radioactivity into PA was significantly higher in cells treated with Ca^{2+}, a Ca^{2+} ionophore A23187, or both, compared to control (43). Since this time, Ca^{2+} sensitivity has shown to be unique to the class I DGKs, which contain EF-hand motifs (**Fig. 1.4** and (44)). The N-terminus also contains a region with homology to neuronal Ca^{2+} sensors, termed the recoverin homology (RVH) domain (**Fig. 1.4** and (45)). DGK mutants lacking both of the EF hand and RVH maintain their ability to translocate to the membrane and become constitutively active, indicating that these domains play an auto-inhibitory role on the enzyme function (44). It is now appreciated that Ca^{2+} causes conformation changes in the RVH and EF-hands and disrupts the auto-inhibitory intramolecular association with the C-terminal regions, evidence pointing at a direct interaction with the C1 domains (46, 47).

ii. Phospholipids

The DGKs are divided into different classes based on the presence of specific regulatory domains which may determine their localization and even unique mechanisms of activation (**Fig. 1.4**). The primary focus of this dissertation is the class I DGKα, and as such, the regulation of its activity by lipids will be discussed in further detail. The activities of class I DGKs are PS sensitive, although a distinct PS-binding site has not been elucidated (42, 48). It is also not understood exactly how PS stimulates DGKα

activity and whether it is important for the enzyme's localization (14, 49). In the classical PKCα, PS binding is thought to disrupt an intramolecular association of the C1 and C2 domains to free the C1 domain for insertion into the membrane (50, 51). These data may provide clues for how PS might regulate DGK activity and membrane association. However, PS is able to stimulate activity of the C-terminal DGKα mutant lacking C1 domains, suggesting that it may affect enzymatic activity by mechanisms other than what was observed for the PKC (41). Adding to the complexity of DGKα activity and regulation, is the finding that PE and cholesterol can also stimulate DGKα (and DGKζ) activity.

As mentioned previously, the biological functions of lipid-binding proteins in the cell are dependent on their trafficking from the cytosol to the membranes. The data discussed above, underscores the notion that many complex changes in membrane lipid composition regulate localization of proteins such as DGKs and lipin.

1.9 Lipins – Discovery and Characterization

That PAP activity was an important part of the pathway of lipid – in particular phospholipid and neutral lipid– synthesis was first indicated in the 1950s by Kennedy and colleagues who discovered the steps of PC and TAG formation and termed it the Kennedy pathway (52-54). These findings were seminal but did not elucidate the specific enzymes responsible for the conversion of glycerol to PC. In the subsequent years, it was demonstrated that PAP activity exists in both the soluble and microsomal cell fractions, is Mg^{2+}-dependent and N-ethylmaleimide (NEM) sensitive (7, 55, 56). This activity was soon distinguished from that of the Mg^{2+} - independent PA phosphatases (LPP) (57). It was not until 2006 that *Pah1*, a gene encoding for a PAP enzyme, was first discovered in

Saccharomyces cerevisiae (58). Pah1 is critical for yeast phospholipid and TAG synthesis, is specific for PA, and contains characteristics of the haloacid dehalogenase (HAD) protein family of phosphatases (58, 59).

The discovery of mammalian lipin genes parallels, and in certain way, precedes that of their yeast counterpart. In the late 1980s, a spontaneous autosomal recessive mutation arose in the mouse colonies of Jackson laboratories. These rodents, called the fatty liver dystrophy (*fld*) mice displayed a range of abnormalities including distinct features of human lipodystrophy, which is a degeneration of fat tissue (60-62). In addition, the mice had enlarged livers and hypertriglyceridemia in early stages of development, peripheral neuropathies due to defects in axon myelination and irregularities in their whole-body metabolism including the development of insulin resistance (62, 63). In the early 2000s, the murine gene mutant responsible for the *fld* phenotype was identified and called *Lpin1*. This was soon followed by the discovery of *Lpins* 2 and 3 in mice and then *LPIN* homologs in humans (60, 64). The identification of Pah1 as the enzyme responsible for *Saccharomyces cerevisiae* PAP activity, and the elucidation of its homology to the mammalian lipins, led to the finding that latter enzymes also possess PAP activity (65).

1.8 Lipins – Tissue Expression

Lipins are found in many tissues with distinct but often shared expression patterns (65, 66). In mice and humans, lipin 1 mRNA predominates in fat, muscle and liver tissues. Lipin 2 is abundant in the mouse brain as well as liver, and the human homolog of lipin 2 is highly expressed in the liver and adipose. Finally, lipin 3 mRNA expression appears most abundantly in both mouse and human digestive tissues (65).

1.10 Lipins – Disease Implications

Deleterious human mutations in the *LPIN1* gene have been reported but are not associated with patients who present with lipodystrophies or with insulin resistant patients (67, 68). Rather, the mutations are a cause of reoccurring rhabdomyolysis in children and may predispose for statin-induced myopathies (69, 70). Rhabdomyolysis is a clinical syndrome which results from skeletal muscle injury. It leads to the breakdown of muscle fibers and the release of the tissue's proteins into the blood stream. An important factor in the pathogenesis of this disease is the release, from the muscles, of the iron containing myoglobin. Dissociation of the iron causes an accumulation of free radicals and oxidative kidney damage (71).

The link between lipin 1 function and statin-induced myopathies has been reported, and it appears that a reduction in DAG levels, resulting from loss of lipin 1 PAP activity, inhibits autophagy clearance (72). That lack of PAP activity does not phenocopy from murine models to humans, suggests that in the latter organisms, lipin 1 plays an important role in muscle cell metabolism, while in mice its primary function is in the adipose tissue. However, a 50% reduction of lipin 1 protein expression (to mimic heterozygous missense mutation in humans) causes statin-induced muscle damage (72). These data implicate the relative levels of the active enzyme as being the predominating factor in the phenotypic differences between human and mouse lipin 1. However, there is still no definite answer for the discrepancy.

Lipin 2 knockout mice do not have an immediately noticeable phenotype, however, they develop tremors and generalized ataxia, associated with an age-dependent loss of lipin 1 in the cerebellum (73). Mutations that result in gene truncations and

inactivity of human *LPIN2* have been linked to an inflammatory disease, Majeed syndrome, which so far has only been identified in three families (74). Lipin 3 is relatively understudied. There is no overt phenotype in KO mice and no mutations have been associated with human diseases (75).

1.11 PA Binding – The Role of Electrostatic Interactions

An electrostatic interaction is the attractive or repulsive force which occurs between two objects of opposing charge. The binding of PA to its effector proteins does not occur through a clearly defined domain, suggesting that attractive electrostatic interactions play an important role in the lipid-enzyme association (76-78).

With this line of thinking, Kooijman *et al*. investigated the biophysical mechanism whereby PA binds target proteins (4, 79). Using ^{31}P-NMR, they studied the ionization states of lysophosphatidic acid (LPA) and PA. LPA contains a hydroxyl at the *sn-2* position instead of an acyl chain. They showed that due to the hydrogen bonding between the phosphomonoester and the hydroxyl, LPA has a lower pKa than PA and at the physiological pH, it carries a greater negative charge (4)

Unlike LPA, PA contains two dissociable protons and pKa's. The pKa_1 is 3 while the pKa_2 is approximately 7.9 (**Fig. 1.5** and (80)). As such, at the physiological pH, PA has a charge of at least -1 (80). Fluctuations in the pKa_2 above or below the membrane pH can cause PA to remain mono-anionic or become di-anionic (**Fig. 1.5** and (4)). Primary amines of lipids such as PE, and lysine and arginine residues in proteins, are a source of hydrogen bond donors and can lower the pKa_2 (4, 77). Kooijman proposed that this occurs via the model of the electrostatic hydrogen bond switch mechanism (77, 80).

According to the model, PA effector proteins are first generally attracted to the

negatively charged membrane surface by nonspecific electrostatic interactions. The proteins sample the bilayer until they encounter a mono-anionic PA. Amino acids with positively charged side-groups, within the proteins hydrogen bond with the PA, cause a decrease in pKa_2, dissociation of the remaining proton and stabilization of the minus charge. This results in the switch of PA from mono- to di-anionic (-2 charge), which enhances the electrostatic interaction between the proteins and the lipid (77, 79, 80).

The critical role of basic residues for PA-protein association has been experimentally demonstrated in several effector enzymes (76). For example, PA activates mTORC1 by binding to the FKBP12-Rapamycin Binding Domain (FRB) and displacing the inhibitors: FK506 binding protein 12 (KBP12) and rapamycin (81, 82). Fang *et al*. demonstrated that three basic residues are critical for the PA-FRB interaction (83). Mutation of all the residues to alanines reduced the association, and mutation of just one of the arginines (R^{2109}) to alanine even further diminished binding (83). Another well-studied PA-effector protein is Raf-1. It contains a cluster of 35 basic residues important for maintaining the integrity of PA binding. However, mutation of just three specific amino acids within this region is enough to reduce binding by 50 – 95% (84). In lipins, the PA binding site is called the polybasic domain (PBD) and contains nine lysines and arginines. Mutation of the entire domain in lipin 1 to alanines renders the enzyme unable to recognize PA electrostatic charge (85). This domain and its function in lipin substrate recognition are discussed in **Section. 1.13.**

PA has also been proposed to act as a pH sensor, again because of the unique structure of its head-group. pKa is the negative logarithm of the ionization constant. As such, even a one-unit pH change in either direction of pKa_2, will cause 90% of PA to

become mono- or di-anionic (4, 79). A decrease in intracellular pH below the pKa_2 will result in protonation of the PA head-group and weakening of the electrostatic interactions with effector proteins (and vice versa). Thus, PA effectors have the ability to respond to changes in the pH-dependent PA charge.

Young *et al*. perhaps best demonstrated the biological implications of this pH sensing in *S. cerevisiae* (86). In yeast, PA regulates Opi1, a transcriptional repressor of phospholipid synthesis genes, by sequestering it to the cytoplasmic leaflet of the ER. Inability of PA to bind Opi1 allows it to localize to the nucleus where it attenuates the expression of over 30 genes, most notably *INO1*, a gene responsible for the synthesis of inositol (87, 88). Constitutive repression of inositol synthesis has been used for inositol auxotrophy screens, and Young *et al*. noted that mutations in proteins involved in the pH regulation of yeast cytosol were elevated in this screen (86). These included plasma membrane proton ATPase (Pma1) and many subunits the vacuolar adenosine triphosphatase (V-ATPase) complex (86, 89). Yeast expressing mutant Pma1 with reduced activity were unable to maintain physiological pH under extracellular acid stress and showed repression of lipid metabolism genes. This was correlated with the inability of Opi1 to properly associate with PA and with its nuclear localization (86). In addition, the direct binding of Opi1 to PA was pH dependent *in vitro*, with decreased binding under acidic conditions due to the protonation of PA (86). Finally, mutation of three basic residues in Opi1 abolished its pH-dependent PA binding, underscoring the dynamic nature of PA ionization and its intimate association with specific protein residues.

1.12 Lipins – Biochemical Regulation

i. Insulin and Phosphorylation

Concurrent with the theme of this dissertation, lipins are the only soluble enzymes in the pathway of TAG synthesis and have been shown to move between the cytosol and the ER membrane (55, 90). As such, the regulation of lipin sub-cellular localization is critical for access to substrate and enzymatic function. Levels of PA and phosphorylation are thought to control the movement of mammalian lipin 1 within the cell in a complex manner that is still under investigation (91, 92).

Prior to the identification of lipin as a PAP enzyme, it was described as being phosphorylated *in vitro*, downstream of mTOR, in response to insulin stimulation (93). Soon after, its specific phosphorylation sites were identified, including that of the amino acid, serine 106 (S106), which is at least one residue directly downstream of mTORC1 and sensitive to Torin 1 and rapamycin treatment (66). A wealth of data now supports our understanding of lipin phosphorylation and its ability to regulate localization and activity (5, 66, 87, 94-97). Other lipin kinases remain to be identified and the direct effect of phosphorylation on enzymatic activity needs to be further elucidated (95). The current consensus is that phosphorylation downstream of insulin signaling negatively regulates the activity and cellular localization of lipin 1, but not lipin 2, by sequestering some of the protein in the cytosol (66, 94, 96, 97).

ii. Electrostatic Charge of PA

Lipin enzymatic activity is also regulated by the electrostatic charge of the PA head-group, as mentioned briefly in **Section 1.11**. Our laboratory was the first to show that lipin 1 senses the change in charge and prefers to bind di-anionic PA, demonstrated

by an increase in PAP activity in the presence of the hydrogen-bond donor, PE (85). As such, lipin associates with its substrate via the electrostatic hydrogen bond switch mechanism, eloquently described by Kooijman *et al*. and discussed in detail in **Section 1.11** (4, 77, 79, 80). We additionally noted that dephosphorylation of lipin 1 caused a significantly greater augmentation in activity in the presence of PE, suggesting that phosphorylation negatively regulates the enzyme's ability to sense and bind -2 charged PA (85). Interestingly, lipin 2 is also regulated by PA charge, but independent of phosphorylation (94). The different regulation of activity and substrate interaction among lipins is unclear but may be due to factors such as protein phosphorylation, variations in intrinsic enzymatic activity or their distinct PBDs. In chapter 5, I address this question and investigate the activity of lipin 3 in the context of phosphorylation and PA charge.

The negative regulation of lipin 1 localization by insulin is paradoxical but can be explained by two hypotheses. First, the phenomena may be in place to prevent lipin 1 from localizing to the plasma membrane (PM) where PA is important for vesicle fusion and glucose uptake. The exclusion of lipin from the PM may be a result of the enrichment of the anionic PS there. Due to its charge, this lipid may interfere with the hydrogen bonding between PE and PA and cause PA to become more mono-anionic. Lipin prefers to bind di-anionic versus mono-anionic PA. Perhaps phosphorylation of lipin 1, which lowers its affinity for substrate, eliminates the recognition of the -1-charged PA predominating in the PM, but still allows for lipin movement to the ER and binding to the -2-charged substrate, albeit in a reduced manner. Another hypothesis, which is not a focus of the present dissertation, is that there are two pools of lipin enzymes. One which responds to the fasting state and transcriptionally up-regulates the genes of beta-oxidation

and another, which responds to the fed state and contributes to TAG accumulation, obesity and the diabetic phenotype. It is plausible that studying lipins in an isolated system, make it difficult to understand their complex function in whole body metabolism.

1.13 The Lipin Polybasic Domains

Lipins belong to an ancient family of HAD proteins, recognized as such by the presence of a DxDx(V/T) catalytic site (66, 91, 98). All lipins also contain the C-terminal CLIP domain (where the catalytic site is found) and the N-terminal NLIP domain. Many lipins also have a PBD, located between the NLIP and CLIP domains in the low homology region (LHR) (91). In addition to being the site of charge sensing and PA binding, the PBD is also a nuclear localization sequence (NLS) (60, 85, 96, 99). The utilization of basic residues to reversibly target proteins to the membrane is a common mechanism employed by PA binding proteins and the PBD is evolutionarily conserved in lipins (76). The lipin PBD is composed of nine basic residues (lysines and arginines) with some evidence that the entire domain is critical for charge sensing (85). The necessity for such a relatively large PA-binding domain is unclear, but suggests that this region is multi-functional.

The lipins of a vast number of animals contain PBDs, with the exclusion of organisms such as bacteria, yeast and worms (**Fig. 1.6**). The domains may have evolved sometime around the evolution of organismal symmetry and the dorsal nerve cord, in animals like the purple sea urchin and the sea squirt (**Fig. 1.6**). The latter is the oldest surviving relative of vertebrates and contains a PBD that is notably different from both its symmetrical ancestor and its vertebrate decedents. In fact, insects, arthropods and all other non-vertebrate organisms I analyzed contain PBDs that are significantly variable

among themselves and in comparison to the PBDs of vertebrate animals (**Fig. 1.6**).

I was unable to identify genomic information on the PBD of very early vertebrates (i.e. hagfish and lampreys). Following the evolution of the vertebra is the appearance of jaws in animals such as sharks and rays. It is with the shark that I first noted the presence of the highly conserved PBD, which contains the recognizable 9 basic residues and is conserved from the ghostshark to the humans, with high sequence similarity (**Fig. 1.6**). In lipin 1, the only exception to this conservation is in birds. The PBDs of lipins 2 and 3 maintain the nine basic residue domains but both have distinct arrangement of lysines and arginines (**Fig. 1.6**). From sharks to humans, the lipin 2 PBD is highly conserved, including 100% similarity between the PBDs of mammals and birds, with only minor changes in some organisms that are inconsistently conserved (**Fig. 1.6**). The PBD of lipin 3, however, shows substantial divergence both within and between clades with 100% amino acid similarity only in the mammalian domains (**Fig. 1.6**).

The presence of these unique PBDs can perhaps be explained by understanding the reason for the evolution of two additional proteins that catalyze the same reaction. It is possible and there is some evidence that these enzymes have non-redundant roles in lipid metabolism, and even in other cell functions (75, 90, 95). In addition to dephosphorylating PA, lipin 1 localizes to the nucleus to regulate transcription, with the PBD acting as the NLS (97, 99). The localization of lipins 2 and 3 is relatively understudied, but it is possible that their PBDs do not function as nuclear localization sequences. Further, in lipin 1 the PBD is the site of PA charge sensing. Our laboratory hypothesized that phosphorylation of lipin 1 negatively regulates its function by interfering with the ability of the PBD to sense this charge. While the PBDs of lipins 2

and 3 have not been directly shown to associate with PA, they are probably also the PA binding domains. Perhaps on lipins 2 and 3, charge sensing is not affected by phosphorylation due to their different PBDs. The studies discussed in chapter 5 will address this hypothesis in more detail.

1.14 The Co-evolution of DGKs and Lipins

Most organisms, all the way back to bacteria contain a DGK enzyme. However, the bacterial DGK is very different from that of its mammalian counterparts. It is difficult to pinpoint the exact time of appearance of all ten DGK isoforms. Interestingly, the purple sea urchin and sea squirt may both have at least four DGK isoforms, and by my analysis these are the earliest organisms to have more than one. All other organisms proceeding symmetry (in the purple sea urchin) and the dorsal nerve cord (sea squirt) have multiple DGKs. The fish, sharks and alligators have at least 8 and mammals have ten. Interestingly, in fruit flies and butterflies we were only able to identify one DGK. However, based on the literature, drosophila probably have multiple DGKs and by 2001, three isoforms had been identified.

Based on the available DNA sequence data, the three distinct lipin isoforms are believed to have appeared with the evolution of the vertebra or jaw. It appears that lipins and multiple DGK isoforms did not evolve together. This is perhaps not surprising as these enzymes are not believed to target the same pools of PA and DAG.

Figure. 1.1 Structures of common cellular phospholipids and diacylglycerol

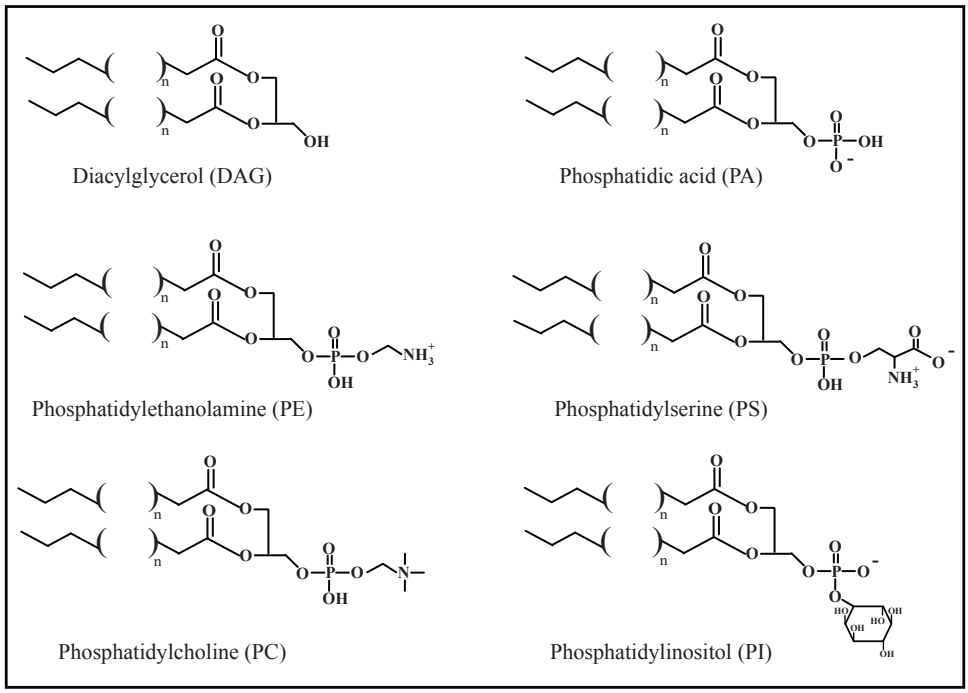

Figure. 1.1 Structures of common cellular phospholipids and diacylglycerol. The cellular lipids, which compose membranes all share the same general architecture: hydrophobic fatty acid chains and a hydrophilic head-group. The structure and chemical properties of the head-group can dictate the lipid's interaction with other lipids, proteins and the membrane environment. The lipids in the above image are shown with the charge that they carry at physiological pH. The parentheses represent the varying chain lengths that compose any given lipid. In addition, the chains can be unsaturated or saturated at various positions. These chains can determine substrate specificity as well as the biophysical properties of the membrane region in which they are enriched.

Figure 1.2 A diagram of the two major foci of the present dissertation.

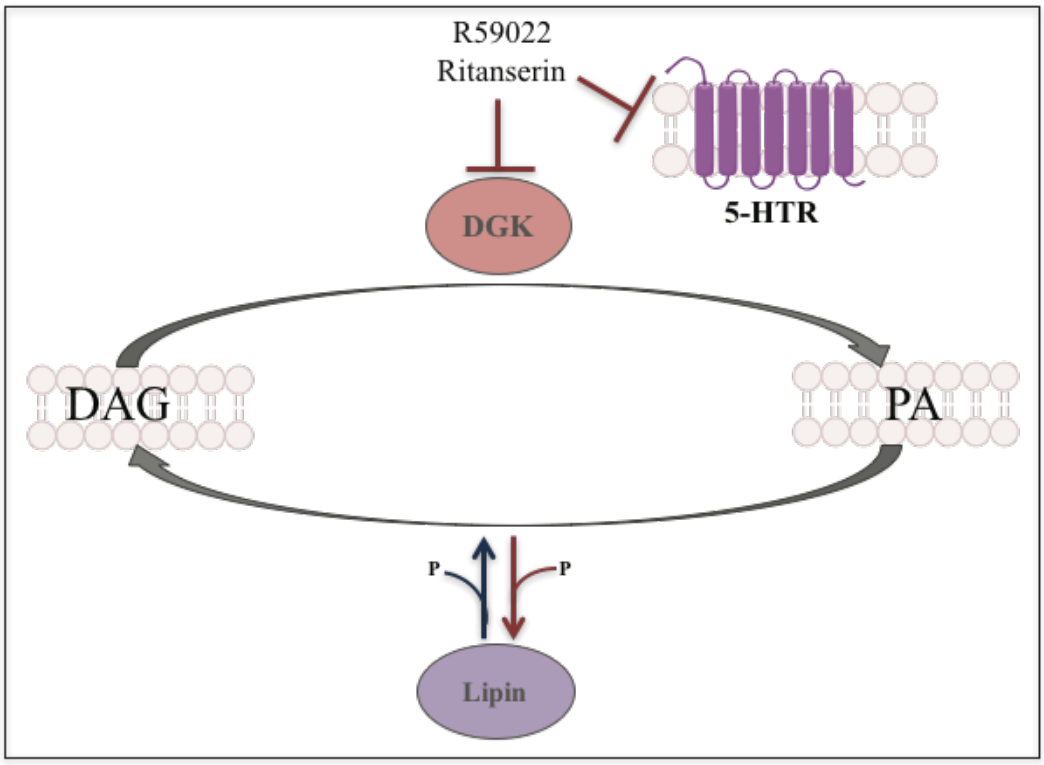

Figure 1.2 A diagram of the two major foci of the present dissertation.

With the work outlined in this dissertation, I investigate two lipid proteins: diacylglycerol kinase (DGK) and lipin. The first enzyme phosphorylates diacylglycerol (DAG) to form phosphatidic acid (PA). The second enzyme dephosphorylates PA to form DAG. In this dissertation, I study the inhibition of DGK and serotonin 5-HT receptors (5-HTRs) by R59022 and ritanserin. I also attempt to answer the fundamental questions of lipin phosphoregulation, indicated in this diagram by the arrows going back and forth from the membrane (gray arrows/pink bilayer).

Figure 1.3 Pathways of phospholipid and neutral lipid synthesis

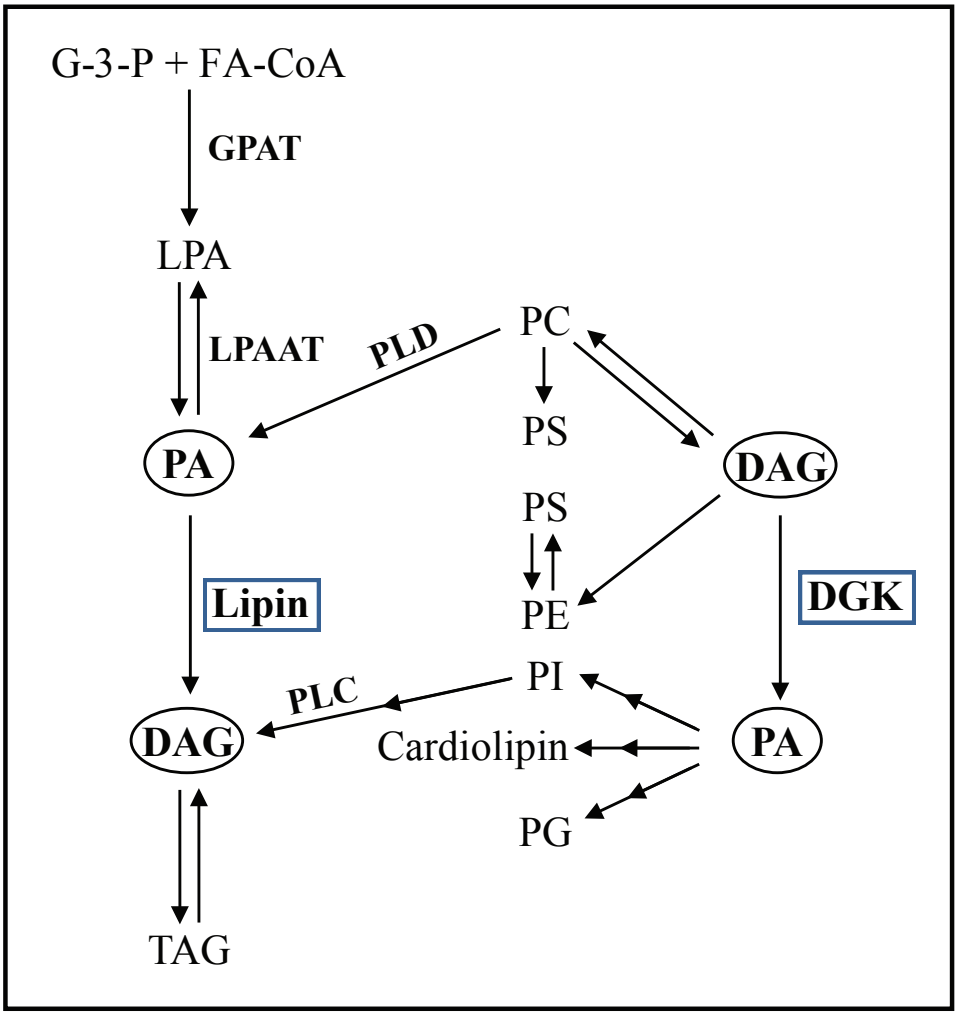

Figure 1.3 Pathways of phospholipid and neutral lipid synthesis. Phosphatidic acid (PA) and diacylglycerol (DAG) are central nodes in the pathways of phospholipid metabolism, as well as intermediates in the *de novo* synthesis of triacylglycerol (TAG). From DAG, TAG, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can all be directly generated. Both PC and PE are precursors of phosphatidylserine (PS). Being the second major node of phospholipid flux, PA is made into cytidine diphosphate (CDP-DAG) and generates phosphatidylglycerol (PG) cardiolipin (CL) and phosphatidylinositol (PI). DAG is regenerated in the TAG synthesis pathway and by lipin, one enzyme at the center point of this dissertation. DAG is also generated during phosphatidylinositol (PI) flux by phospholipase C (PLC) from phosphatidylinositol 4,5-bisphosphate (PIP_2). PA is regenerated via TAG synthesis and also by action of phospholipase D (PLD) which uses PC as a substrate. Finally, DGK, another center point of the present dissertation, phosphorylates DAG to make PA.

Figure 1.4 Ten mammalian DGKs and their regulatory domains

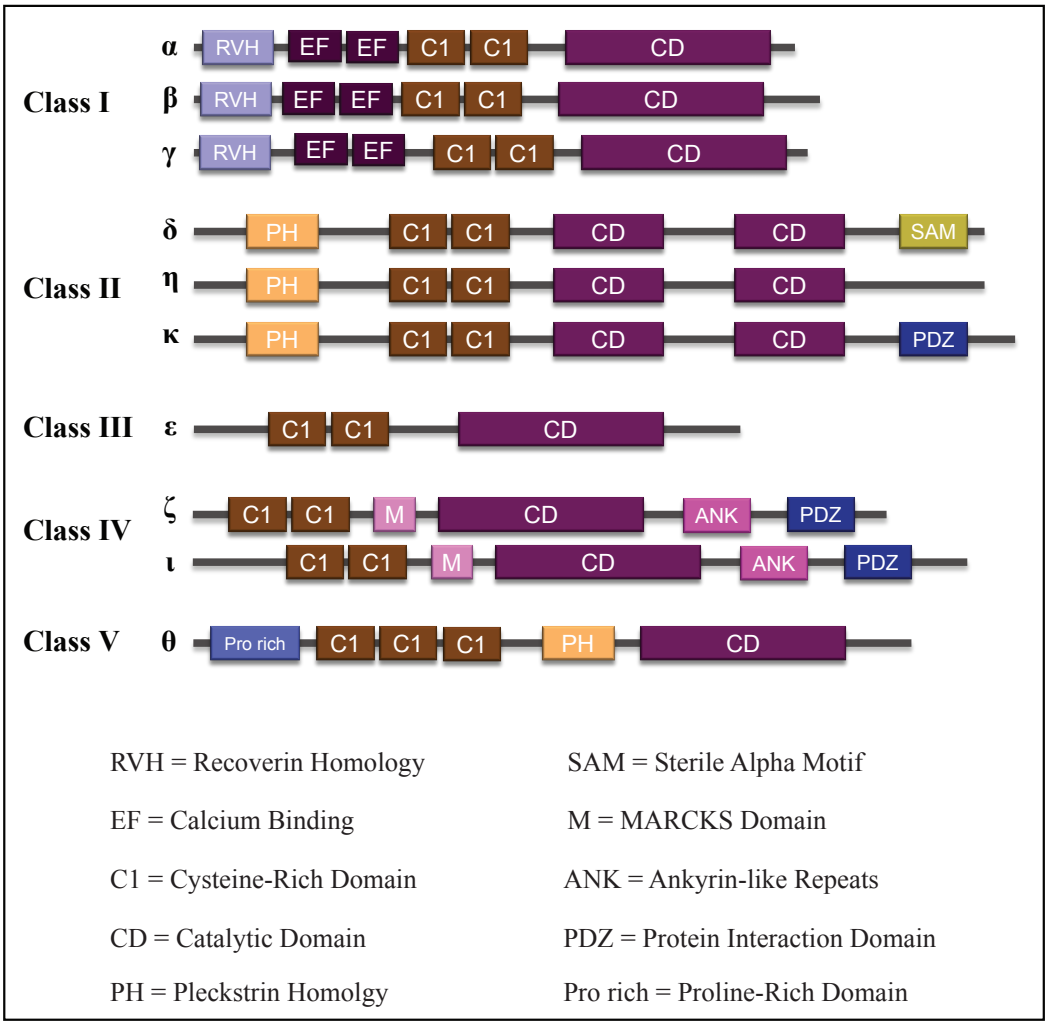

Figure 1.4 Ten mammalian DGKs and their regulatory domains. The mammalian family of diacylglycerol (DAG) kinases is composed of 10 members (100). These are divided into five classes based on sequence similarity and presence of specific regulatory domains. The class I DGKs contain EF-hand motifs and are Ca^{2+} sensitive (101). The class II DGKs have pleckstrin homology (PH) domains, which gives them a distinct mechanism of membrane association (102). These enzymes also have a separated catalytic domain (CD). The class III DGKε is the smallest, contains no regulatory domains other than the C1 domains, and is at least partially an integral membrane protein displaying acyl chain specificity among DAG species (103). The class IV enzymes contain MARCKS domains (M), which may render them subject to unique regulation by phosphorylation, and also contain an NLS (104, 105). Finally, the class V DGKθ also contains a PH, a proline-rich domain and a predicted Ras-association domain, although whether it actually binds Ras has been debated (106, 107).

Figure 1.5 Ionization curve of phosphatidic acid [Adopted from Avanti Polar Lipids Webpage].

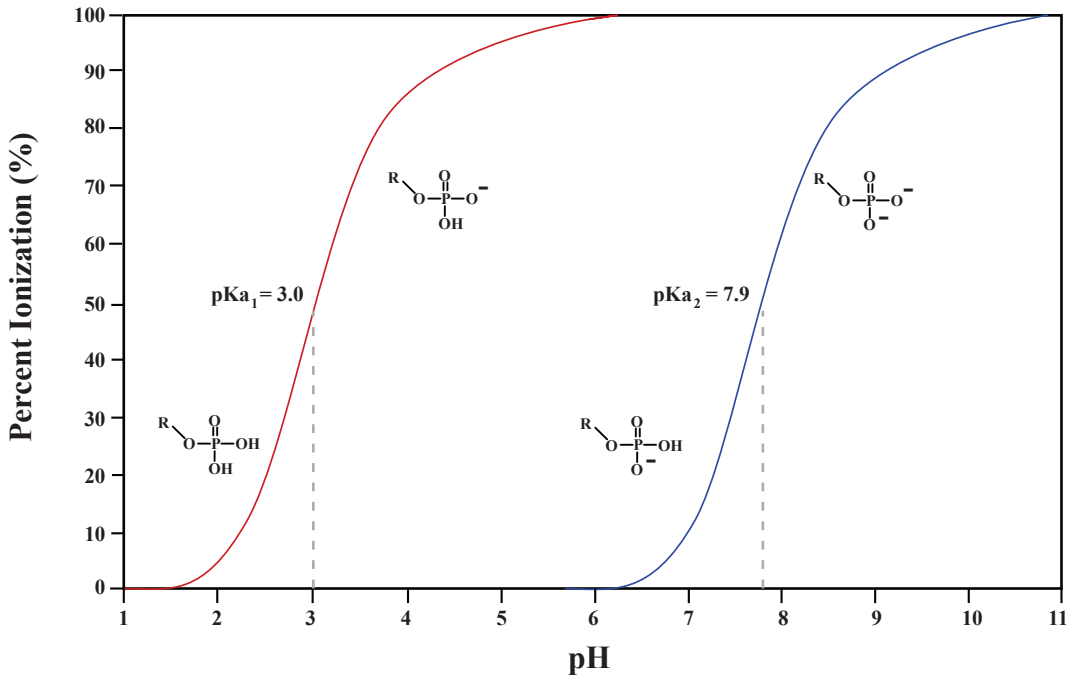

Figure 1.5 Ionization curve of phosphatidic acid [Adopted from Avanti Polar Lipids Webpage]. Phosphatidic acid (PA) is a phosphomonoester, contains two dissociable protons and therefore has two pKa's. The first pKa_1 is far below the physiological pH and he second pKa_2, is slightly below the physiological range. As such, in the cell PA has a charge of at least -1. Fluctuations in the cell membrane pH below the pKa_2 will yield a -1 charged PA, while an increase in the pH above the pKa_2 will cause PA to become di-anionic (4, 79). Similarly, small changes in pKa_2 of PA below or above the pH will cause PA to become di-anionic or remain mono-anionic, respectively.

Figure 1.6 Phylogenetic trees of the PBDs of lipins 1-3 from bacteria to humans

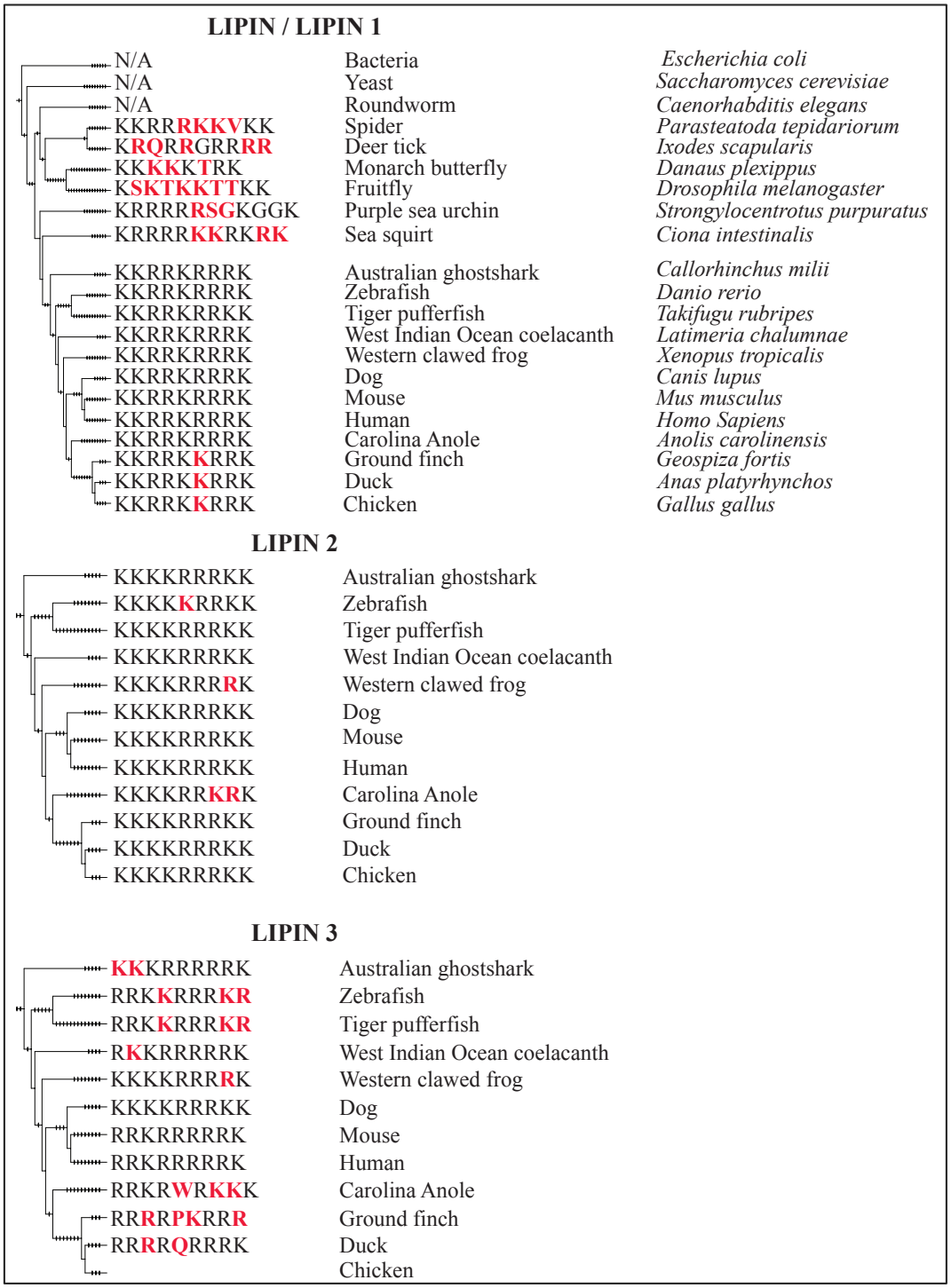

Figure 1.6 Phylogenetic trees of the PBDs of lipins 1-3 from bacteria to humans. Lipin and Lipin 1, 2 and 3 polybasic domains (PBDs) of various organisms were arranged in a phylogenetic tree as indicated. The lipin 1 PBD is highly conserved from the evolution of the vertebra (Australian ghostshark), with the exception of one amino acid difference in birds. The lipin 3 PBD is relatively variable with the only complete conservation of the domain within mammals. We could not find the sequence for chicken lipin 3 protein. N/A marks the absence of the PBD domain in the indicated organisms.

CHAPTER 2: EXPERIMENTAL METHODS

Materials

[γ^{32}P]-ATP was from Perkin Elmer (Boston, MA). The lipid species used in these studies are as follows: 1,2-dioleoyl-sn-glycerol (dioleoyl; 18:1, 18:1), 1,2-octanoyl-sn-glycerol (dioctanoyl; 8:0, 8:0) and 1-stearoyl-2-arachidonoyl-sn-glycerol (stearoyl arachidonoyl; 18:0 20:4). These DAG species as well as 1,2-dioleoyl-*sn*-glycero-phosphate (18:1, 18:1 PA), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), and all materials for the preparation of liposomes were also from Avanti Polar Lipids (Alabaster, AL). M2 FLAG beads, FLAG antibody, rabbit and mouse alkaline-conjugated secondary antibodies, R59949, R59022, and ritanserin were from Sigma-Aldrich (St. Louis, MO). Primary anti-rabbit antibodies against total Akt, total mTOR, phospho-Akt-S473, phospho-Akt T308, phosphor-mTOR S2448, phosphor-mTOR S2481, phosphor-S6K-T389, phosphor-GSK3-S9/S21 and pan-phospho-PKC substrates were from Cell Signaling (Danvers, MA). Ketanserin, bisindolylmaleimide II (bis), PMA, and TCB-2 were from Tocris Bioscience (Avonmouth, Bristol, UK). All other commonly used reagents were from Sigma-Aldrich, unless otherwise indicated. All cell lines were obtained from ATCC (Rockville, MD).

Construction of DGK Expression Plasmids

The expression plasmids, pcDNA3-FLAG-rat-DGKα (108) , pcDNA3-FLAG-rat-DGKβ (19), and pCMV-human-DGKδ1-3xFLAG (109) were gifted to Dr. Kevin Lynch (University of Virginia, School of Medicine) by Dr. Kaoru Goto (Yamagata University, School of Medicine) and Dr. Fumio Sakane (Chiba University) and were kindly shared with us by Dr. Lynch. The expression plasmid, pCMV-HA-human-DGKι, was also gifted

to Dr. Lynch by Dr. Matthew Topham (University of Utah) (106). DGKι cDNA was sub-cloned into the pCMVTag2A vector. The DNA encoding pLenti6-human-DGKθ was from the laboratory of Dr. Daniel Raben (Johns Hopkins University School of Medicine) and was sub-cloned into the pCMVtag2 vector.

Construction of WT Lipin 3 and Venus-WT lipin 3 Expression Plasmids

The *Mus musculus* Lpin 3 cDNA was removed from tripe-HA expression vector using EcoRV and inserted into the pCMV-Tag2C, Strategene (San Diego, CA). This yielded pCMV-Tag2C-Lipin 3. For adenovirus generation, the DNA construct was subcloned into pAdTRACK-CMV shuttle vector and then into the pAdEASY system. High titer virus was purified using CsCl-banding (110). To generate Venus-lipin 3, the triple HA-tagged lipin 3 was inserted downstream of FLAG-tagged Venus in pcDNA3.

Construction of Lipin PBD Mutant Expression Plasmids

The PBD exchange mutants: lipin 1 (3PBD) and lipin 3 (1PBD) were generated by PCR mutagenesis. Venus-tagged lipin 3, lipin 1 (3PBD), and lipin 3 (1PBD) were generated by subcloning the Venus cDNA in frame with the amino-terminus of lipin 3 downstream from the FLAG-tag. Adenoviruses were generated using the pAdEASY system (111). All amino acid numbering conforms to *Mus musculus* phosphatidic acid phosphatase LPIN3 (accession number: NP_075021.1) or when appropriate to LPIN1 isoform b (accession number: NP_056578).

Construction of WT Lipin 1 Expression Plasmids

The FLAG-lipin 1 vector has been described previously (97).

Cell Culture

Human cervical cancer (HeLa) and human embryonic kidney (HEK 293T) cells were cultured in Dulbecco'sModified Eagle's Medium (DMEM) supplemented with 5% Fetal Bovine Serum (FBS) VMR Life Science Seradigm, (Radnor, PA) and 1% penicillin/streptomycin (antibiotics), Fisher Scientific, (Waltham, MA). Human glioblastoma cells, U87 and U251, were cultured in Roswell Park Memorial Institute Medium (RPMI) supplemented with 5% FBS and 1% antibiotics and Minimum Essential Media (MEM) supplemented with 10% FBS and 1% antibiotics, respectively. Mouse fibroblasts were cultured in DMEM containing 10% Newborn Calf Serum (NCS), 1% FBS and 1% antibiotics. Fibroblasts were differentiated to become mouse adipocyte (3T3-L1) cells by addition of 100 U/ml of insulin, Eli Lilly (Indianapolis, IN), 0.5 mM IBMX, and 0.25 μM dexamethasone to DMEM supplemented with 10% FBS and 1% antibiotics. The 3T3-L1 cells were used 7-10 days post-differentiation and were fed DMEM with 10% FBS and 1% antibiotics after differentiation.

Purification of DGKα

HeLa cells were infected with an adenoviral vector expressing rat FLAG-DGKα for 48 h. The cells were fed daily during this period, harvested and lysed using a 22 Ga needle, in 500 μl/plate of kinase lysis buffer (10 mM Na_2HPO_4, pH 7.4, 50 mM Octyl β-D-glucopyranoside, 50 mM NaF (IPBB), 1 mM EDTA, 1 mM EGTA, 0.02% Triton X-100, and the protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin). The cell lysate was cleared by centrifugation at 16,000 *x g* for 10 min. The supernatant was collected and incubated with 15 μl/plate of FLAG (M2) beads for 2 h at 4°C. Following the incubation, the beads were loaded on an affinity screening column,

Fisher Scientific (Waltham, MA) and washed 10 times with kinase lysis buffer. The FLAG-DGKα was eluted with five successive additions of equal volume of 0.5 mg/ml of FLAG peptide. The fractions were collected and dialyzed against kinase lysis buffer without detergent or protease inhibitors. The purified DGKα was visualized on an SDS-PAGE gel stained with Coomassie-blue dye. The protein yield was quantified by comparison to bovine serum albumin (BSA) standards.

Purification of WT lipin 3 and Lipin PBD Mutants

HeLa cells were infected with an adenoviral vector expressing indicated FLAG-mouse-lipin constructs for 48 h. The cells were fed daily during this time, harvested and lysed using a 22 G needle in 500 µl/plate of phosphatase lysis buffer (20 mM HEPES, 150 mM NaCl, 0.1% Brij-35, pH 7.2). The cell homogenates were centrifuged at 16, 000 *x g* for 10 min, and the cleared lysates were incubated with 15 µl/plate of FLAG (M2) beads for 2 h at 4°C. Following the incubation, beads were separated into two fractions and incubated in 50 mM HEPES, 100 mM NaCl, 1 mM $MnCl_2$, 2 mM DTT, pH 7.0 with (+λ) and without (-λ) 2000 units of lambda protein phosphatase for 30 min at 30°C. The breads were then loaded on an affinity-screening column and washed 10 times phosphatase lysis buffer. The lipin proteins were eluted with five successive additions of an equal volume of 0.5 mg/ml FLAG peptide, and dialyzed against phosphatase lysis buffer without detergent. The purified lipins were visualized on an SDS-PAGE gel stained with Coomassie-blue dye. The protein yield was quantified by comparison to bovine serum albumin (BSA) standards.

Analysis of Phosphate Removal by Lambda Phosphatase

HeLa cells were infected with adenovirus overexpressing FLAG-lipin 3 for 48 h. Following the infection period, cells were incubated in low phosphate buffer (145 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.2 mM sodium phosphate, 5 mM glucose, 10 mM HEPES, 0.1% insulin free BSA, pH 7.4), radiolabeled with 0.04 mCi/ml [^{32}P] ATP and incubated at 30ºC for 2 h. The radiochemical specific activities were always between 4000-6000 Ci/mmol. Following the incubation, the cells were harvested, homogenized as described above then incubated with 15 µl/plate of anti-FLAG beads. Following the incubation, the beads were separated into two factions and were treated with (+λ) or without (-λ) 400 units of λ protein phosphatase. The lipin 3 was detached from the beads by addition of 1X Laemmli buffer containing β-ME. The proteins were separated by 8.75% SDS-PAGE gel under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane, Immobilon (Darmstadt, Germany). The radiolabeled protein and removal of phosphate was visualized using autoradiography.

Overexpression of DGK Isoforms

To study the activity and inhibition of various DGK isoform (α, β, δ, ι, θ), HEK 293T cells (10 cm plates) were cultured and transiently transfected with 15 µg of FLAG-DGK plasmid DNA using Lipofectamine 2000, Invitrogen (Carlsbad, CA). Forty-eight hours following the transfection, the cells were harvested and homogenized with a 22 G needle using 250 µl/plate of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitors. To solubilize DGKι, kinase lysis buffer was used. The cell homogenates were cleared by centrifugation at 16,000 *x g* for 10 min. The supernatant

was collected and used immediately or stored at -80°C. We chose to use HEK 293T cells because they are amenable to liposome-mediated transformations.

Preparation of Liposomes for Kinase Assays

The preparation of liposomes generally followed a previously reported protocol from MacDonald *et al.* (112). Briefly, PC, DAG, and PS were dissolved in $CHCl_3$, combined, and dried *in vacuo* to remove all solvent. All liposomes contained dioleoyl DAG unless otherwise indicated. For assays using purified enzyme, the liposomal concentration of lipids was as follows: 55 mol% PC, 40 mol% PS and 5 mol% DAG. For measurement of purified DGKα inhibition and changes in kinetics in the presence of R59022 and ritanserin, the compounds were incorporated into the liposomes. They were first dissolved in $CHCl_3$ then added to and dried down with the lipids at 0.5 and 2.0 mol%. The lipids were hydrated to 19 mM in 50 mM (3-(*N*-morpholino)propanesulfonic acid) (MOPS), pH 7.5, 100 mM NaCl and 5 mM $MgCl_2$. For assays using cell homogenate, 50 mol% PC, 40 mol% PS and 10 mol% DAG were used. The lipids were hydrated to 10 mM in the above-mentioned buffer. In both cases, the lipids were subjected to five freeze-thaw cycles in liquid nitrogen, followed by extrusion through a 100 nm polycarbonate filter 11 times.

Preparation of Liposomes for Phosphatase Assays

Lipids and [^{32}P]-PA (10,000 cpm/nmol) were dissolved in chloroform, combined and dried *in vacuo*. The liposomal concentration of lipids was as follows: 90 mol% PC and 10 mol% PA or 60 mol% PC, 30 mol% PE and 10 mol% PA. For investigating the effect of PS on lipin activity, liposomes were composed of: 10, 30 and 40 mol% PS at the expense of PC, 30 mol% PE and 10 mol% PA. The dried lipids were hydrated in 50 mM Tris-

HCl, 0.5 mM $MgCl_2$, and 10 mM βME, pH 7.4, and subjected to five freeze-thaw cycles, followed by extrusion 11 times with a mini extruder (Avanti), through a 100 nm polycarbonate filter.

Preparation of Mixed Micelles

The preparation of Triton X-100/PA mixed micelles followed a previously described protocol (113). Briefly, PA and [^{32}P]-PA were combined in chloroform and dried *in vacuo*. Triton X-100 was added to solubilize the lipids to the indicated concentrations. The micelles were generated with 10 mol% surface concentration of PA, which was calculated using the following formula: mol % = 100 x [lipid(M)/([lipid(M)+[Triton X-100 (M)]).

Preparation of [^{32}P]-PA

The protocol for radiolabeling of PA has been described previously (114). Briefly, using [γ-^{32}P]-ATP and *E.coli* DGK were used to phosphorylate dioleoyl DAG and generate radiolabeled PA. The [^{32}P]-PA was purified by thin layer chromatography.

Kinase assays

The protocol for measurement of purified DGKα activity was modified from Epand *et al*. (115). Briefly, the reactions contained 50 mM MOPS, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), purified enzyme, and 4.75 mM lipids, with and without indicated total liposomal concentrations of R59022 and ritanserin. For reactions testing the activity of DGKα in the absence of $CaCl_2$, 1 mM EGTA was also used. The reactions were initiated by the addition of 10 µl of 10 mM [γ^{32}P]-ATP to a final volume of 100 µl and were allowed to proceed for 15 min at 25°C. The kinase assays

using cell homogenate contained 50 mM MOPS, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 2 mM lipids and 5 μg of protein from homogenate. For assays with DGKα and DGKβ, 1 mM $CaCl_2$ was also added. The reactions were initiated as described above and were allowed to proceed for 20 min at 30°C. For measuring DGK isoform inhibition in cell homogenates, indicated compounds were dissolved in DMSO, serially diluted and added directly to kinase assays to the desired concentration. To solubilize the compounds, water bath sonication was used. The final concentration of DMSO in the reactions was less than 1% (v/v) and did not affect enzyme activity. All reactions were terminated with the addition of 0.5 ml of methanol with 0.1 N HCl, followed by 1 ml of $CHCl_3$. The organic phase was washed two times with 1 M $MgCl_2$ and thoroughly vortexed. To measure the incorporation of [^{32}P] into DAG, 0.5 ml of the organic phase was removed and the radioactivity was measured using a scintillation counter.

The activity of purified protein was normalized to background radioactivity measured from assays without enzyme. The activities of lysates overexpressing DGKs (signal) were normalized to activities of lysates expressing only GFP (background). The signal to background ratio was calculated as follows: S/B = mean signal / mean background.

Phosphatase Assays

Purified lipin proteins and radioactive liposomes, and buffer were combined to a final volume of 100 μl. The final concentrations were as follows: 50 mM Tris-HCl, 0.5 mM $MgCl_2$, 10 mM β-ME, and indicated concentrations of PA. The reactions were allowed to proceed for 20 min at 30°C with gentle agitation and were terminated with the addition of 500 μl acidic methanol (methanol + 0.1 N HCl). To this was added 1 ml of chloroform followed by 1 ml of 1 M NaCl. The organic extraction was vortexed and 500 μl of the

aqueous phase was placed in scintillation vials to measure the removal of ^{32}P from PA by a scintillation counter. The activity from assays containing lipins was normalized to activity in assays without enzyme.

Measurement of WT lipin 3 and Lipin PBD Mutant Membrane Binding

Membrane binding of lipins to large unilamellar vesicles was measured following a slightly modified version of Hofer *et al* (116). In short, lipids and PC-Pyrene were dissolved in chloroform and dried *in vacuo*. The liposomal surface concentration of lipids were as follows: 80 mol% PC, 20 mol% PA and 0.1 mol% PC-Pyrene or 50 mol% PC, 30 mol% PE, 20 mol% PA and 0.1 mol% PC-Pyrene. The lipids were hydrated in binding buffer, 20 mM Tris-HCl, 1 mM EDTA, pH 7.2. Liposomes were prepared as described above. The reaction volume was 100 µl and contained Venus-FLAG-lipins, liposomes and binding buffer. The final bulk concentration of PA in the reactions was 2 mM. The reactions were allowed to proceed for 30 min at 30 ºC with gentle agitation, and terminated by addition of equal volume of 80% sucrose (w/v). The mixtures was layered over 270 µl of 80% sucrose (w/v) in 5 x 41 –mm Beckman centrifuge tubes and were overlaid with 150 µl of 20% sucrose (w/v) and 100 µl of binding buffer. The sucrose gradients were centrifuged in a SW 55Ti swinging bucket rotor containing nylon inserts at 240,000 *x g* for 1 h. Following the centrifugation, the top 100 µl of the gradients were collected and PC-Pyrene and Venus absorption were measured. The Venus absorption from samples was normalized to input Venus read out from protein and buffer only and PC-Pyrene from samples was normalized to input PC-Pyrene measurement from liposomes and buffer only. To determine the fraction of lipins bound to liposomes, Venus

absorption values were normalized to that of PC-Pyrene. Binding was also performed for liposomes containing PC only, as a background control.

Western immunoblot analysis

To verify the expression of the DGK isotypes, lipins, enzymes of indicated insulin signaling and their phosphorylation and detect PKC substrates, 50 μg of proteins were separated by 8.75% SDS-PAGE gel under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes, Immobilon (Darmstadt, Germany). The membranes were blocked by incubation in Tris-buffered saline with detergent Tween 20 (TBST) containing 10% (w/v) dried milk for 1 h at 25°C. The TBST contained the following: 50 mM Tris, 150 mM NaCl, and 0.05% (w:v) Tween 20, pH 7.4. For detection of DGKs and lipins, the membranes were incubated with monoclonal anti-FLAG antibody (1:1000) in TBST, at 25°C for 1 h with gentle agitation. For the detection of PKC substrates, the PVDF membranes were incubated with Phospho-PKC substrate antibody, (1:1000) in TBST overnight at 4°C with gentle agitation. The membranes were then incubated with alkaline phosphatase-conjugated mouse or rabbit secondary antibody (1:10,000) diluted in TBST with 2% (w/v) dried milk, for 1 h at 25°C. After three 15 min washes with TBST, the membranes were briefly incubated in chemiluminescent alkaline phosphatase substrate, Applied Biosystems (Poster City, CA). The immunoreactivity was detected using a Fuji LAS 4000.

Generation of lipin 1 S106 Antibody

The generation of anti-rabbit poly-clonal antibody against phospho-serine 106 of lipin 1 is described in Harris *et al*. (66).

Protein kinase C (PKC) Activation

HeLa, U87 and U251 cells were treated as follows: 4 and 40 μM of R59022 and ritanserin, 40 μM ketanserin, and 100 nM PMA for 30 min with and without pre-treatment with 500 nM bis for 1 h. For the detection of PMA and Torin 1 time induced changes in insulin signaling proteins, HeLa cells were treated with 100 nM PMA and 250 nM Torin 1 for the indicated periods of time. For additional PKC activation experiments, HeLa cells were treated with 10 μM TCB-2 for 6 h with and without co-treatment with 40 μM ketanserin, 40 μM ketanserin alone, and with 500 nM bis for 1 h. The cells were lysed in IPBB, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors and the cell lysate was cleared by centrifugation at 16,000 *x g* for 10 min. The cleared homogenate was loaded on an 8.75% SDS-PAGE gel and PKC substrate phosphorylation was detected using Western immunoblot analysis.

Radiolabeling of WT Lipin 1 and WT Lipin 3 in 3T3-L1 cells

Differentiated 3T3-L1 cells were cultured in 10 cm plates (2 plates/condition) and infected with an adenoviral vector expressing FLAG-lipin 3 or FLAG-lipin 1 for 48 h. In the last 2 h of the infection, cells were serum starved in low phosphate buffer supplemented with 0.2 mC/ml of [^{32}P]-orthophosphate. During this time, the cells were treated as follows: vehicle, Torin 1 for the last 45 min followed by 10 mU/ml of insulin the last 15 min or 10 mU/ml of insulin only for the last 15 min. The cells were harvested and homogenized in 20 mM HEPES, 150 mM NaCl, pH 7.2 and 0.1% Brij-35 with protease inhibitors and cleared by centrifugation at 16,000 *x g* for 10 min. The supernatant was incubated with 15 μl/plate of anti-FLAG (M2) beads for 4 h at 4°C. Proteins were displaced from the beads with the addition of SDS and were separated on

an 8.75% SDS-PAGE gel. Phosphate incorporation into lipins 1 and 3 was visualized using autoradiography and total protein expression was detected using Western immunoblot analysis. The incorporation of ^{32}P was normalized to total protein.

Mass Spectrometry Analysis of Purified Lipin 3

Protein was precipitated from affinity-purified lipin aliquots using 20% TCA, solubilized in 8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2, reduced using 5 mM dithiothreitol at 55ºC for 30 min, alkylated with 10 mM iodoacetamide at room temperature, diluted in 4 equal volumes of 50 mM Tris, pH 8.2, and digested overnight using trypsin (Promega), Lys-C (Wako), or Glu-C (Worthington). The resulting peptides were desalted and analyzed in duplicate by LC-MS/MS using both Thermo LTQ-Velos-Orbitrap and Q-Exactive mass spectrometers using a data-dependent acquisition strategy. Raw mass spectra was searched using Comet (version 2014.01) against UniProt human protein sequence database (v11.29.2012) and concatenated to the 3 overexpressed mouse lipin sequences allowing for phosphorylated serine, threonine, or tyrosine and oxidized methionine. Peptide false discovery rate was filtered to less than 1% using Percolator (117, 118). Phosphorylated peptides were scored for site localization using the Ascore algorithm (119). An Ascore value of greater than 13 corresponds to phosphorylation sites localized to 95% confidence.

qPCR

Quantitative qPCR was carried out as per the MIQE guidelines. Total RNA was isolated with TRIzol reagent, Ambion (Carlsbad, CA), according to the manufacturer's instructions. Briefly, cells were harvested and suspended in 1 ml TRIzol reagent. Isolated RNA was treated with DNase I, NEB (Ipswich MA) in a 1:10 ratio of DNase and DNase

reaction buffer (10 mM Tris-HCl, pH 7.6, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) and incubated at 37°C for 30 min. cDNA was synthesized from 1 μg of RNA with the SensiFAST cDNA Synthesis Kit, Bioline (Taunton, MA). Real-time qPCR was performed using SensiMix SYBR and Fluorescein Kit, Bioline (Taunton, MA) according to manufacturer's instruction. All the samples were assayed in duplicates and analyzed using a CFX96 Real-Time PCR Detection System, Bio-Rad (Hercules, CA). Relative expression was calculated using 2^-ddCt method (120). For the internal control, 18S rRNA was used.

Measurement of serotonin receptor antagonism

The compounds R59949 and R59022 were tested against 5-HTRs by the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH PDSP) at the University of North Carolina, Chapel Hill. To identify the potency and K_i of each compound against the receptors, radioligand competition binding assays were performed and the Cheng-Prusoff equation was used as described in more detail in Besnard *et al*. (121, 122). To confirm whether the compounds are agonists or antagonists at the receptors of interest, functional assays were performed as described in Kroeze *et al.* (123). The detailed protocols can also be found online (http//pdsp.med.unc.edu/pdspw/binding.php).

Statistical Analysis for DGK Studies

The statistical analysis and determination of all kinetic constants were done using GraphPad Prism software. For calculation of K_m^{app} values, non-linear regression of Michaelis-Menten plots were used. The R59022 and ritanserin dose dependent curves were fitted to linear interpolation and a GraphPad Prism function called log [inhibitor] vs.

normalized response was used to calculate IC_{50} values. For comparisons between and within more than two groups, one-way Analysis of Variance (ANOVA) and two-way ANOVA were used, followed by Dunnett's or Tukey post-hoc analysis (as indicated in the figure legends). All values are reported as the mean of triplicate values ± SEM. Data shown are representative of at least three independent experiments and when appropriate, from separate enzyme preparations. Significance was set to $p < 0.05$.

Statistical Analysis for Lipin Studies

The statistical analysis and determination of all kinetic constants were done using GraphPad Prism software. Statistical significance between two groups was determined using Student t-test. Statistical analysis between more than two groups was determined using one-way ANOVA followed by Tukey's postdoc analysis. The statistical tests used for each figure are indicated. All values are expressed as means ± SEM. Data shown are representative of at least three independent experiments and when appropriate, from separate enzyme preparations. Significance was set to $p < 0.05$.

CHAPTER 3: RITANSERIN IS A DGKα INHIBITOR *IN VITRO* AND DGK INHIBITORS ARE SEROTONIN RECEPTOR ANTAGONISTS

ABSTRACT

Diacylglycerol kinase alpha (DGKα) catalyzes the conversion of diacylglycerol (DAG) to phosphatidic acid (PA). Recently, DGKα was identified as a therapeutic target in various cancers, as well as in immunotherapy. Application of small-molecule DGK inhibitors, R59022 and R59949, induces cancer cell death *in vitro* and *in vivo*. The pharmacokinetics of these compounds in mice, however, are poor. Thus, there is a need to discover additional DGK inhibitors not only to validate these enzymes as targets in oncology, but also to achieve a better understanding of their biology. In the present study, we investigate the activity of ritanserin, a compound structurally similar to R59022, against DGKα. Ritanserin, originally characterized as a serotonin (5-HT) receptor (5-HTR) antagonist, underwent clinical trials as a potential medicine for the treatment of schizophrenia and substance dependence. We document herein that ritanserin attenuates DGKα kinase activity while increasing the enzyme's affinity for ATP *in vitro*. In addition, R59022 and ritanserin function as DGKα inhibitors in cultured cells and activate protein kinase C (PKC). While recognizing that ritanserin attenuates DGK activity, we also find that R59022 and R59949 are 5-HTR antagonists. In conclusion, ritanserin, R59022 and R59949 are combined pharmacological inhibitors of DGKα and 5-HTRs *in vitro*.

INTRODUCTION

Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol (DAG) species to yield the corresponding phosphatidic acid (PA) (100). To date, ten DGK isotypes have been identified, characterized, and divided into 5 classes based on amino acid sequence similarities (11, 21, 22, 124, 125). While much remains to be learned about the function of these enzymes, there is an increasing body of evidence highlighting their critical role in many pathological states (reviewed in (21)). In particular, recent work has implicated DGKα as a positive regulator of carcinogenesis; DGKα was shown to induce T-cell anergy, a hypo-activation of T-cells that suppresses their immunological response (26). Additionally, Dominguez and colleagues demonstrated that pharmacological attenuation of DGKα activity in glioblastoma multiforme (GBM) cells resulted in apoptosis and the genetic knockdown of DGKα caused a reduction in tumor growth (23, 24). This and other work strongly implicated DGKα as a novel therapeutic target in the most common and malignant primary brain tumor and possibly other cancers as well (21, 23, 126).

Despite advances in linking the function of DGKs to the development of cancer and other diseases, the study of their biology is challenging. Genetic and pharmacological manipulations of DGKs have been informative but with significant caveats. This is due to the large number of mammalian DGKs, the diversity of DAG and PA species in cells, and a lack of isotype-selective inhibitors. Currently available chemical tools for understanding the role of DGKs in biology and disease are confined to the two long-known inhibitors, R59022 and R59949 (DGK inhibitor I and II, respectively) (28, 29). Very recently, another small molecule, CU-3, was identified as a novel DGKα inhibitor *in vitro* (127). The selectivity of R59022 and R59949 for the different DGK isotypes has

been debated. Some studies suggest that they are selective for class-I, Ca^{2+}-dependent DGKs, particularly DGKα, while some have reported inhibition of other DGKs (30-32, 41, 44, 47, 101). The *in vivo* application of these compounds has been difficult due to their poor pharmacokinetics and limited ability to cross the blood-brain barrier (23). The emerging role of DGKs in pathological states and the current limitations that exist in the study of these enzymes increase the need for the discovery of novel and perhaps more potent inhibitors, not only for translation to the clinic but also as effective probes for understanding DGK function on a cellular and physiological level.

We recently noted that ritanserin has striking structural similarity to R59022 (128). Ritanserin was first identified as a serotonin (5-HT) receptor (5-HTR) antagonist and was shown to have drug-like properties (129). Its use as a treatment of schizophrenia and substance dependence advanced to clinical trials but development was eventually discontinued (130-132). Despite the obvious structural similarities between R59022 and ritanserin, these compounds, as well as R59949, were to our knowledge never grouped as being functionally similar. In this study, we present evidence that ritanserin is a DGKα inhibitor while both R59022 and R59949 are 5-HTR antagonists (128).

RESULTS

Purification and enzymatic characterization of DGKα.

FLAG-DGKα was affinity-purified from HeLa cells (**Fig. 3.1A**). I confirmed that DGKα was active and that it was linear in a time- and concentration-dependent manner to 15 min and 3.2 μg of protein (**Fig. 3.1B and C**).

Classical biochemical assays for studying DGK activity involve the use of partially purified enzyme or cell homogenate and detergent mixed micelles (11, 30, 133). Few studies have utilized liposomes, which are composed of a lipid bilayer and thus may more closely resemble cellular membranes (115, 134). I investigated the kinetics and catalytic properties of DGKα using purified enzyme and PC:PS:DAG liposomes. The two substrates of DGKs are ATP and DAG. **Fig. 3.2A** shows the ATP-dependent activity of DGKα. The K_m^{app} of DGKα for ATP determined with this assay was 0.05 mM (**Fig. 3.2A and Table 1**). The K_m^{app} against DAG was 0.12 mM (**Fig. 3.2B** and **Table 3.1**). These data are consistent with what has been previously reported for DGKα (30, 31). Furthermore, the activity of DGKα has long been known to depend on Ca^{2+} (12, 44, 101, 135). This is thought to be due to the Ca^{2+}-dependent dissociation of an auto-inhibitory, intra-molecular interaction between the enzyme's C1 domains and the EF-hand motifs (47). In the absence of exogenous Ca^{2+}, the activity of DGKα was reduced by 75%; the addition of 10 μM Ca^{2+} was sufficient to restore the majority of the kinase activity (**Fig. 3.2C**). Some DGKs, including DGKα, are also strongly activated by PS (41, 42, 136). Titration of 10 – 40 mol% PS into PC:DAG liposomes resulted in a PS dose-dependent increase in kinase activity (**Fig. 3.2D**).

Ritanserin is an inhibitor of DGKα enzymatic activity in vitro

The structures of the known small-molecule inhibitors of DGKs as well as two 5-HTRs, ritanserin and ketanserin, are depicted in **Fig. 3.3**. I assessed the extent to which ritanserin inhibits the activity of DGKα as compared to R59022. Since these compounds are lipophilic, they can easily be incorporated into the liposome bilayer. I added 0.5 mol% and 2.0 mol% R59022 and ritanserin into the PC:PS:DAG liposomes. The bulk concentrations of the drugs were 24 μM and 95 μM. The rationale for using high concentrations of inhibitors was based on the reasoning that some of the compounds would incorporate into the inner leaflet of the liposome bilayer and would thus be unable to access enzyme. The activity of DGKα was assayed in the context of the substrates ATP and DAG.

The presence of 0.5 mol% R59022 decreased the V_{max} of purified DGKα by 25% in the context of ATP and 40% in the context of DAG. With 2.0 mol% R59022, about 50% of the specific activity was inhibited for both substrates (**Fig. 3.4A and C**, **Table 3.1**). Interestingly, the apparent K_m of DGKα for ATP decreased in the presence of 2.0 mol% R59022 (**Table 3.1**). Under the same conditions, 0.5 mol% ritanserin decreased V_{max} of DGKα by greater than 50% in the context of both ATP and DAG. At 2.0 mol%, enzymatic activity was attenuated by about 70% (**Fig. 3.4B and D, Table 3.1**). As R59022, ritanserin significantly decreased the apparent K_m of DGKα towards ATP (**Table 3.1**). Together, these data suggest that under these *in vitro* conditions, ritanserin is an inhibitor of DGKα. Further, the increase in affinity of DGKα for ATP but not DAG in the presence of inhibitors, suggests that R59022 and ritanserin may bind an allosteric site on the enzyme and affect its interaction with each substrate differently.

R59022 and ritanserin are more potent inhibitors of DGKα than of other, selected DGKs

The compounds R59022 and R59949 were reported to be specific inhibitors of class I DGKs (30, 124). This claim has been subsequently challenged. For example, R59022 was reported to inhibit DGKε and DGKθ, and it was demonstrated that 30 μM R59022 did not inhibit the two other class I isotypes, DGKβ and DGKγ (31, 32). Motivated by these conflicting results, I investigated the activity of R59022 and ritanserin against various DGKs, first using liposomes containing dioleoyl DAG. The expression of FLAG-tagged-DGKs was forced in HEK 293T cells (**Fig. 3.5A**). R59022 and ritanserin were dissolved in DMSO, diluted, and added directly to the kinase reactions, which contained cell homogenate over-expressing the indicated DGK enzyme. **Fig. 3.5B** and **C** show the percent of activity from control (kinase assay with DMSO only) of a representative DGK from each of the five classes (DGKε was excluded because it is partially an integral membrane protein (103, 137)). The activity of DGKα decreased by about half with 20 μM inhibitors (**Fig. 3.5B and C**). Under these conditions, the activities of the indicated DGKs were not detectably inhibited, including that of DGKβ (another Class I DGK; **Fig. 3.5B and C**). I examined whether this pattern of inhibitor selectivity would be observed in the context of other DAG species. It was found that R59022 and ritanserin were similarly potent against DGKα when assayed using liposomes containing dioctanoyl (**Fig. 3.5D and E**) and stearoyl arachidonoyl (**Fig. 3.5F and G**) DAGs. To expand on these findings, the activities of DGKα and DGKι were tested against a range of R59022 and ritanserin concentrations. Under the given conditions, the IC_{50} values of R59022 and ritanserin for DGKα were approximately 25 and 15 μM, respectively (**Fig. 3.5H and I**).

On the other hand, for DGKι the IC_{50} values were between 55 and 65 μM (**Fig. 3.5J and K**).

In order to verify that the concentrations of ATP and DAG used in the above experiment were saturating for not just DGKα but all of the DGKs used, I performed ATP and DAG dose dependent curves. Under these conditions, I found that DGKα has the highest apparent affinity for ATP, followed by DGKβ, while DGKδ has the lowest affinity for ATP, at 0.13 mM (**Tables 3.1 and Table 3.2**). With respect to 18:1,18:1 DAG, DGKα had the lowest apparent affinity and DGKθ had the highest, with a K_m^{app} of 0.03 mM (**Table 3.1 and Table 3.2**). In summary, the concentrations of DAG and ATP in the kinase experiments in **Fig. 3.5** were saturating for the DGKs tested.

R59022 and R59949 are 5-HTR antagonists

R59022 (structure in **Fig. 3.3A**) is a close structural analog of ritanserin, with the sole difference being the substitution of the hydrogen isostere, fluorine, at a second position to generate ritanserin. Despite the similarities between the DGK inhibitors and some 5-HTR antagonists, including ritanserin, the action of R59022 and R59949 against 5-HTRs has not been reported. To test the potency of these DGKα inhibitors against these receptors, radioligand competition assays were used as described in (121). Briefly, in the primary screen, 10 μM of the compounds were used to investigate % inhibition (of binding) of a radioligand. Both molecules inhibited binding at all receptors except 5-HT_3 (**Table 3.3**). In the secondary assays, K_i values were determined at the receptors where significant inhibition was observed. The lowest K_i values of R59022 and R59949 were against 5-HT_{2A}R. Interestingly, R59022 was also potent against 5-$HT_{2B/2C}$ receptors as well as 5-HT_{1D} and 5-HT_7 (**Table 3.3**).

These assays elucidate the potency of R59022 and R59949 for 5-HTRs but do not describe whether they function as antagonists or agonists. As such, functional assays were used to determine the action of these compounds at 5 receptors where low K_i values were observed for either R59022 or R59949, as described in (123). The agonist assays showed very low to no activity (data not shown). Both compounds showed high antagonist activity at all of the receptors, with the exception of R59949 at 5-HT_{2C} and 5-HT_7 (**Table 3.3**). Together with the low K_i values, these data support the conclusion that R59949 and R59022 are antagonists of 5-HT_2Rs and possibly other serotonin receptors.

R59022 and ritanserin stimulate PKC in HeLa and U87 cells but not in U251 cells

The ability of chemical compounds to inhibit DGKs must ideally be demonstrated by treatment of intact cells and detection of subsequent changes in various DAG and PA species. Probably the most robust method for detecting such changes is by using mass spectrometry. However, this has proven to be difficult without genetic manipulations (23, 127, 138). Using LC-MS, we analyzed changes in DAG, PA and PC in cells expressing endogenous DGKs after treatment with ritanserin and R59022 but could not obtain statistically significant results. Both DAG and PA are signaling lipids and serve as effectors of many proteins (21, 22, 125). For example, DAG activates PKC (139). The phosphorylation of PKC downstream targets was investigated following treatment of cells with ritanserin and R59022, as an indirect measure of DGKα inhibition and DAG accumulation (**Fig. 3.6A**). Treatment with 40 μM of either compound increased the phosphorylation of PKC downstream targets by about 2.5-fold in HeLa cells. The treatment of cells with the known PKC activator, PMA, induced substrate phosphorylation by greater than 3-fold (**Fig. 3.6B and C**). Induction of PKC activity was

reversible by bis, a PKC inhibitor (**Fig. 3.6B and C**). I next tested the activity of ketanserin, another 5-HT_2R antagonist with structural similarities to ritanserin and R59022, against DGKα (140). The compound was dissolved in DMSO and added to kinase assays containing purified DGKα and PC:PS:DAG liposomes. Under the given *in vitro* conditions, the IC_{50} of ketanserin was 264 μM (**Fig. 3.6D**). Importantly, when HeLa cells were treated with ketanserin, no activation of PKC was observed (**Fig. 3.6B and C**).

These effects were also observed in the glioblastoma U87 but not U251 cells, perhaps due to low expression of DGKα (**Fig. 3.6E and F**). To test this hypothesis, the relative mRNA expression of DGKs and 5-HTRs were measured in all three cell lines. In both HeLa and U87 cells, DGKα showed the highest relative expression (**Fig. 3.6G and H**). Both cell lines also expressed 5-$HT_{2A/2C}$Rs but not 5-HT_{2B}R (**Fig. 3.6G and H**). On the other hand, although U251 cells had some expression of DGKα, it was low compared to expression of other DGKs such as DGKδ and DGKθ (**Fig. 3.6I**).

Ketanserin is a 5-HTR antagonist but does not inhibit DGKα (**Fig. 3.6** and (129)). Thus, ketanserin should decrease levels of DAG and attenuate PKC. We did not observe a decrease in the phosphorylation of PKC downstream targets when we treated cells with ketanserin alone (**Fig. 3.6B-F**). One explanation is that under control conditions, 5-HTRs (particularly 5-HT_{2A}R and 5-HT_{2C}R) and/or PKCs are not detectably active. As such, we cannot detect the antagonistic effects of ketanserin. To verify this hypothesis, I treated HeLa cells with bis alone. As demonstrated in **Fig. 3.6**, bis reverses PMA, R59022, and ritanserin-stimulated phosphorylation of PKC substrates. I did not see any changes in basal PKC activity with only bis treatment (**Fig. 3.7**). Additionally, we treated HeLa cells with TCB-2, a 5-HT_{2A}R agonist, and stimulated 5-HT_{2A}R and PKC (**Fig. 3.7** and (141)).

Under the conditions of active 5-HT_{2A}R, co-treatment with ketanserin and TCB-2 inhibited the ability of TCB-2 to activate PKC (**Fig. 3.7**).

DISCUSSION

DGKα was recently established as a therapeutic target in GBM and other cancers, and the DGKα inhibitor R59022 was effective in countering cancer cell growth and progression both *in vitro* and *in vivo* (23). In the present study, we hypothesized that the structurally related 5-HT_2R antagonist, ritanserin, would also inhibit DGKα activity. Using purified DGKα in combination with PC:PS:DAG liposomes, I show that ritanserin attenuates kinase activity *in vitro* and establish it as a third small-molecule inhibitor of DGKα. Further, we demonstrate that R59022 and R59949 are 5-HT_2R antagonists.

More specifically, both R59022 and ritanserin inhibit the activity of DGKα with respect to ATP and DAG (**Fig. 3.4**). At 2 mol%, R59022 decreased the V_{max} of DGKα by 50% (**Fig. 3.4A and C, Table 3.1**). The same concentration of ritanserin attenuated DGKα activity by 70% (**Fig. 3.4B and D, Table 3.1**). These data suggest that under the given *in vitro* conditions, ritanserin may be a better inhibitor of DGKα than R59022. Further, the inhibition of enzymatic activity was not surmountable by increasing concentrations of either substrate and a significant decrease in the K_m^{app} for ATP was observed in the presence of both inhibitors (**Fig. 3.4 and Table 3.1**). This complements previous findings using R59949 and suggests that R59022 and ritanserin prefer to bind to an ATP-enzyme complex (30). One plausible explanation is that in the absence of ATP, the drug-binding pocket is less accessible to the inhibitors. A conformational change in response to the binding of ATP may allow the compounds to better interact with the enzyme. At the same time, the apparent affinity of DGKα for DAG was not significantly altered in the presence of the inhibitors (**Table 3.1**). It is possible that this is a case of mixed inhibition, where R59022 and ritanserin have higher affinity for the ATP-bound

DGKα than free enzyme, but do not affect binding of DAG. In-depth structural studies are needed to know for certain where and how these compounds bind to DGKα. Little is known about how DAG and ATP bind mammalian DGKs and how the enzyme catalyzes phosphate transfer. In the case of *E. coli* DAGK, however, ATP and DAG were shown to bind the enzyme independent of each other (142). If this is also the case with mammalian DGKα, then perhaps it is not surprising that the DGK inhibitors display mixed inhibition in the context of ATP and DAG.

Ritanserin is a potent 5-HT_2R antagonist that was in late-stage clinical trials for schizophrenia and substance abuse (129-131). It is orally bioavailable, has a half-life of approximately 40 hours in humans, and was found to have few adverse side effects (143, 144). As such, repurposing ritanserin as a DGKα inhibitor for an oncological indication might be a viable option. However, aspects of its polypharmacology, including its selectivity among the ten mammalian DGKs, need to be elucidated. While some of these studies are outside the scope of our work, it is of interest to investigate whether ritanserin attenuates the activity other mammalian DGKs. To date, there are ten known DGK isotypes divided into 5 classes (21). The R59949 and R59022 compounds were initially thought to be selective for class I, Ca^{2+}-dependent DGKs (α, β, γ), but recent work has indicated that this may not be the case (30-32). Under the given assay conditions, 20 μM ritanserin and R59022 attenuated the activity of DGKα but did not significantly inhibit the other DGK isotypes (**Fig. 3.5B and C**). The liposomes used for these experiments contained dioleoyl DAG. Cellular membranes contain a variety of DAG species but *in vitro*, only DGKε differentiates among them, preferring the *sn*-2 arachidonoyl DAG (145, 146). Using dioctanoyl and stearoyl arachidonoyl DAGs, I found that R59022 and

ritanserin still significantly inhibited the activity of only DGKα (**Fig. 3.5D-G**). This is also reflected in the dose-response curves, which show that the IC_{50}s of R59022 and ritanserin for DGKι are around 60 μM (**Fig. 3.5J and K**)—about 2-3 fold higher than the IC_{50}s observed for DGKα (**Fig. 3.5H and I**). These data suggest that R59022 and ritanserin are most potent towards DGKα but at higher concentrations may attenuate other DGKs as well. The variation between our findings and those of others may be due to differences in assay conditions.

While they share obvious structural similarities to ritanserin, the activities of R59022 and R59949 against 5-HTRs have, to our knowledge, not been reported. In the present study, we show that the two known DGK inhibitors are also potent 5-HTR antagonists, with highest affinity for 5-HT_2Rs, particularity 5-$HT_{2A/2C}$ (**Table 3.3**). Interestingly, although ritanserin has activity against various 5-HT receptors, its K_i is lowest for 5-$HT_{2A/2C}$ receptors as well (121, 129).

The 5-HT_2Rs signal through $G\alpha_q$ GPCR and activate protein lipase C (PLC) to generate DAG and inositol triphosphate (IP_3) (147). Thus, antagonizing these receptors would result in a decrease in DAG (**Fig. 3.6A**; red arrows). Attenuation of DGK activity, on the other hand, would cause elevations in DAG (**Fig. 3.6A**; blue arrows). Since DAG is an activator of PKC, I wanted to elucidate whether R59022 and ritanserin reach DGKα inside the cell by detecting changes in PKC activity. Early work characterizing R59022 suggested that treatment of intact human platelets with R59022 resulted in an increase in the phosphorylation of an unknown, 40 kDa PKC substrate (28). A more recent report studying the role of DGKδ in insulin resistance found that treatment of isolated rat muscle cells caused attenuation of total DGK activity and increased PKC activity (16).

We showed that treatment of HeLa cells with 40 μM R59022 and ritanserin resulted in a significant increase in the phosphorylation of PKC substrates as compared to untreated cells. This was reversible with a known PKC inhibitor (**Fig. 3.6B and C**). Further, these results were recapitulated in U87 but not in U251 glioblastoma cells (**Fig. 3.6E and F**). Congruent with this data, it was found that U251 cells have a low relative mRNA expression of DGKα, compared to many of the other DGKs (**Fig. 3.6I**). On the other hand, HeLa and U87 cells express DGKα most abundantly (**Fig. 3.6G and H**). Additionally, I showed that another 5-HTR antagonist, ketanserin, does not inhibit DGKα *in vitro* and does not activate PKC in intact cells (**Fig. 3.6B-F**). These data increase our confidence that ritanserin and R59022 are functional DGKα inhibitors.

Ketanserin is a 5-HTR antagonist and as such, should attenuate PLC, decrease DAG, and PKC activity (**Fig. 3.6**). However, we did not notice an attenuation of PKC in **Fig. 3.6**. We reasoned that perhaps this is due to a lack of 5-HT_2R and/or PKC activity under basal conditions. This hypothesis was tested by treatment of cells with bis, which resulted in no difference in the phosphorylation of PKC substrates (**Fig. 3.7**). To address the question of basal 5-HTR activity, I treated HeLa cells with the 5-HT_{2A}R agonist, TCB-2, which has been shown to stimulate production of DAG (141). TCB-2 caused a significant elevation of PKC activity and co-treatment with ketanserin reversed this effect (**Fig. 3.7**). Together, these data suggest that in HeLa, as well as perhaps in U87 and U251 cells, PKC and 5-HTRs require exogenous stimulation for detectable activity. Additionally, these data give us further confidence that the increase in phosphorylation of PKC substrates with R59022 and ritanserin treatment is a result of DAG accumulation.

Our study has some limitations. We have demonstrated that ritanserin and R59022 can inhibit the function of more than one enzyme. Furthermore, ritanserin is reported to also have activity against dopamine receptors (129). This data must be considered when investigating the effects of these compounds *in vivo.* Our results summarized in **Fig. 3.6 and 3.7**, however, support the conclusion that at least in cultured cells, ritanserin and R59022 cause DAG accumulation. While showing DGK attenuation, our experiments do not simultaneously demonstrate the effects of ritanserin and R59022 on 5-HTR signaling. These phenomena have been well studied by others and the ability of R59022 to antagonize 5-HTRs has now been shown by us (**Table 3.3** and (129, 130)). The purpose of the experiment in **Fig. 3.6** was to test the hypothesis that ritanserin and R59022 can *also* attenuate DGK activity, which we showed by the increase in PKC activity (**Fig. 3.6 and 3.7)**. We are also limited by *in vitro* assays due to the fact that cells express several DGK isotypes that are dynamically regulated and differentially expressed (100). As such, it is difficult to say whether these compounds are selective for DGKα *in vivo*. Finally, cells contain many DAG and PA species, while we can only study only the small number of the lipid species that are commercially available.

In conclusion, we have demonstrated that the 5-HTR antagonist, ritanserin, is an inhibitor of DGKα and increases the affinity of the enzyme for ATP *in vitro* and that the two known DGK inhibitors, R59022 and R59949, are also 5-HTR antagonists. Further, ritanserin and R59022 are more potent against DGKα than against four other DGKs and have similar selectivity within these DGKs when assayed using various DAG species. Finally, we demonstrate that treatment of cells with ritanserin and R59022, but not ketanserin, activates PKC in cells that have a high relative expression of DGKα. Our data

provide evidence that ritanserin may be a viable option for *in vivo* translation and an additional pharmacological tool for studying DGK biology.

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AUTHOR CONTRIBUTIONS

Dr. Salome Boroda, Dr. Thurl Harris and Dr. Benjamin Purow conceived the study. Dr. Boroda and Dr. Harris designed the study. Dr. Harris also significantly contributed to the editing of the manuscript. Dr. Boroda designed, performed and analyzed the experiments shown in Fig. 1-7, with the exception of Fig. 7 G-I, which were performed by Dr. Vidisha Raje. Maria Niccum contributed to the experimental design, treatment optimization and many of the experiments in Fig. 6B and C. Dr. Boroda performed the experiments in

Tables 1 and 2. The NIH PDSP screening center at UNC Chapel Hill performed the experiments in Table 3. All authors contributed to the editing of the manuscript, especially Maria Niccum. All authors reviewed the results and approved the final version of the manuscript.

Figure 3.1 Purification of recombinant DGKα.

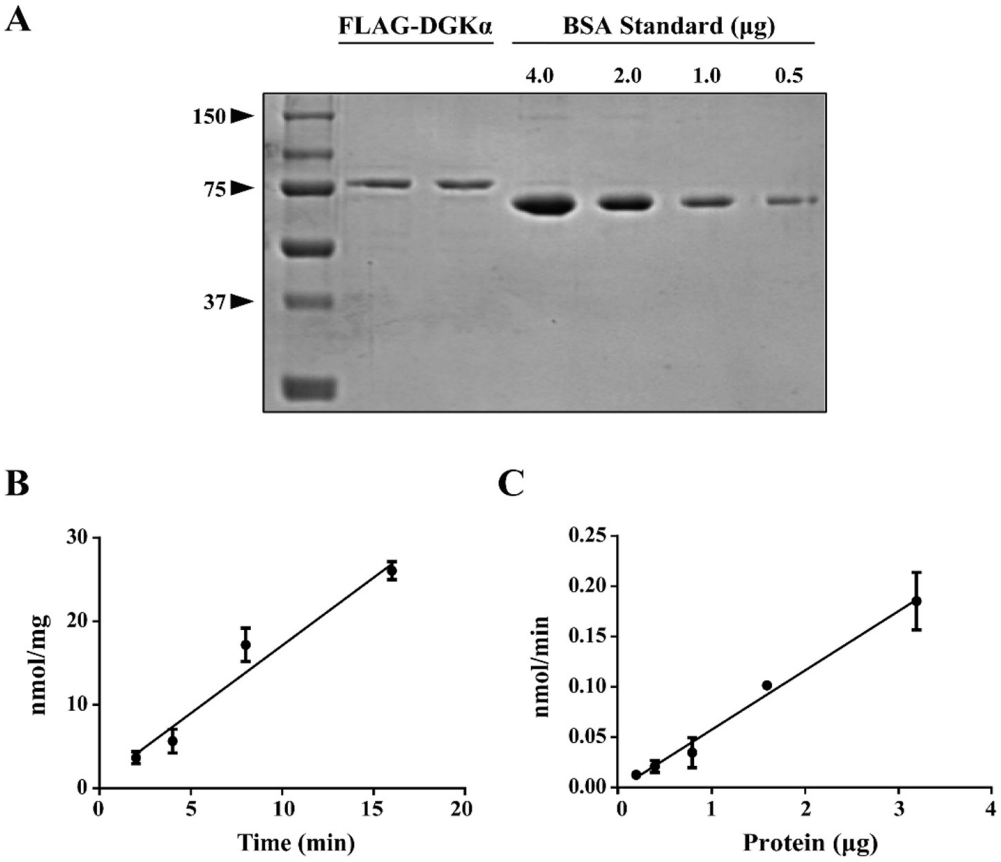

Figure 3.1 Purification of recombinant DGKα. HeLa cells overexpressing FLAG-DGKα were harvested, the DGKα protein was affinity-purified, and activity was tested against DAG-containing liposomes. (A) The enzyme and BSA standards were separated on an SDS-PAGE and stained with Coomassie blue; a representative image is shown. Purified DGKα activity was linear in a (B) Time dependent manner with 0.5 μg protein and (C) Linear in a protein concentration dependent manner by 15 minutes. Each data point represents the mean of triplicate ± SEM of a representative protein preparation.

Figure 3.2 Characterization of purified DGKα enzymatic properties.

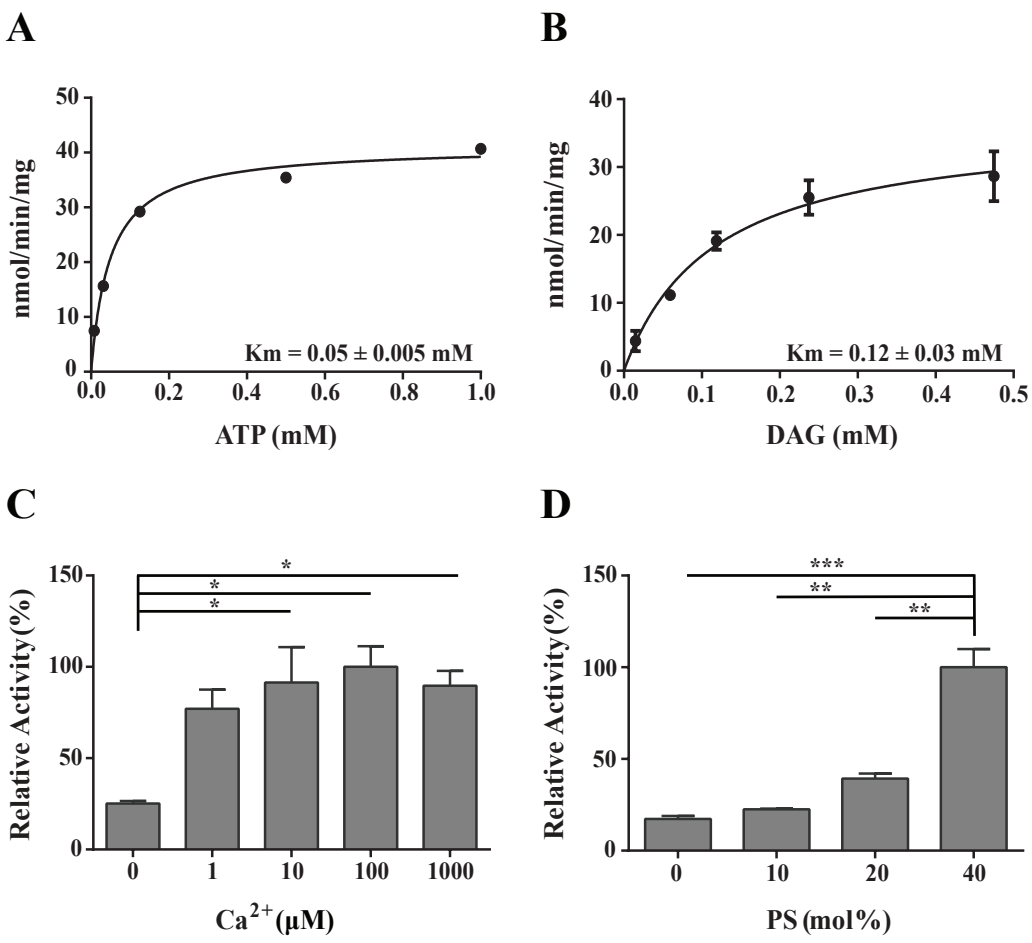

Figure 3.2 Characterization of purified DGKα enzymatic properties. (A) ATP-dependent increase in DGKα activity with DAG at 5 mol%. (B) DAG-dependent increase in DGKα activity with ATP at 1 mM. (C) Effect of indicated concentrations of Ca^{2+} on the activity of DGKα. (D) The change in activity of DGKα with increasing mol% PS titrated into PC:DAG liposomes. Each point represents the mean of triplicate ± SEM of a representative experiment. One-way ANOVA was used to analyze statistical significance, followed by Tukey post-hoc analysis: ***$p<0.0001$, **$p<0.0005$, *$p<0.05$

Figure 3.3 Chemical structures of the compounds used in this study.

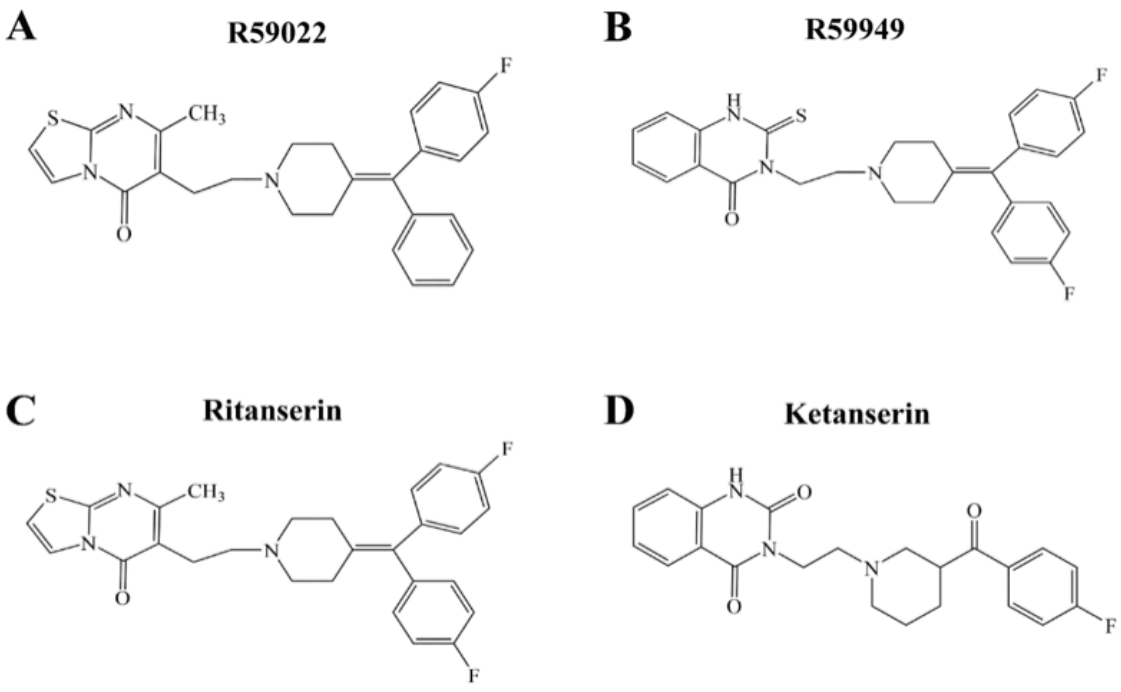

Figure 3.3 Chemical structures of the compounds used in this study. Previous to these studies, the only known DGK inhibitors were (A) R59022 (28) and (B) R59949 (29). These were characterized around the same time as the 5-HTRs, (C) Ritanserin (129) and (D) Ketanserin (140).

Figure 3.4 Ritanserin inhibits the activity of purified DGKα.

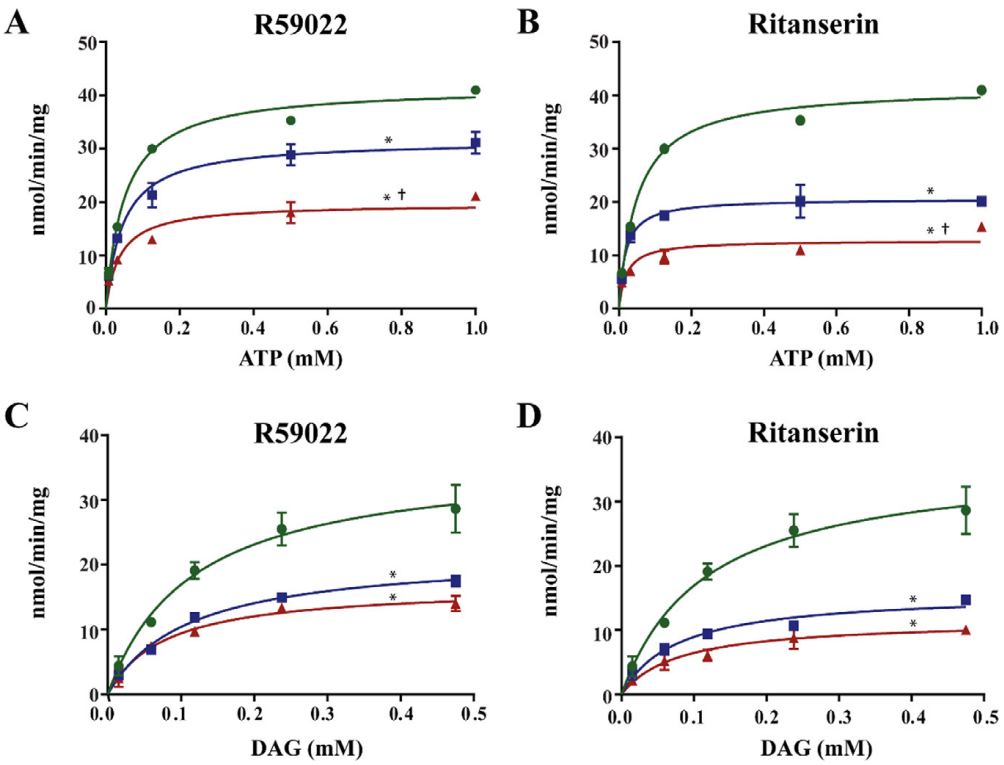

Figure 3.4 Ritanserin inhibits the activity of purified DGKα. Compounds were titrated to 0.5 and 2 mol% into PC:PS:DAG liposomes containing DAG. Change in ATP-dependent DGKα activity in the presence of (A) R59022 and (B) Ritanserin with 5 mol% DAG. Change in DAG-dependent activity of DGKα in the presence of (C) R59022 and (D) Ritanserin with 1 mM ATP. Key: (—●—) No inhibitor, (—■—) 0.5 mol% drug, (—▲—) 2.0 mol% drug. Each point represents a mean of triplicate ± SEM of a representative experiment. Two-way ANOVA was used to analyze statistical significance, followed by Tukey post-hoc analysis: *p <0.0001 differences between V_{max} of control and each drug dose, †p<0.0001 differences between V_{max} with 0.5 mol% and 2.0 mol% drugs.

Table 3.1 Steady state kinetics of DGKα inhibition by R59022 and ritanserin.

		R59022			Ritanserin		
		No Drug	0.5 mol%	2.0 mol%	No Drug	0.5 mol%	2.0 mol%
ATP	V_{max}	43 ± 1	32 ± 1*	20 ± 1*†	41 ± 1*	20 ± 1*	12 ± 0.01*†
	K_m^{app}	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01 #	0.05 ± 0.01	0.03 ± 0.004 #	0.02 ± 0.01 #
DAG	V_{max}	37 ± 4	22 ± 1*	16 ± 1.5*	37 ± 4*	17 ± 2*	11 ± 2*
	K_m^{app}	0.12 ± 0.03	0.11 ± 0.02	0.1 ± 0.02	0.12 ± 0.03	0.12 ± 0.04	0.07 ± 0.04

Table 3.1 Steady state kinetics of DGKα inhibition by R59022 and ritanserin.

R59022 and ritanserin decrease V_{max} and K_m^{app} in the context of ATP and decrease V_{max} in the context of DAG. The units for V_{max} are nmol/min/mg. The K_m^{app} is indicated in mM concentration. Two-Way ANOVA was used to analyze statistical significance: *$p<0.0001$ between control (no drug) and each drug dose, †$p<0.0001$ between the two drug doses, # = significant difference in the K_m^{app} of control (no drug) and each drug dose as determined by 95% confidence interval.

Figure 3.5 R59022 and ritanserin are more potent against DGKα than other DGKs when tested using various DAG species.

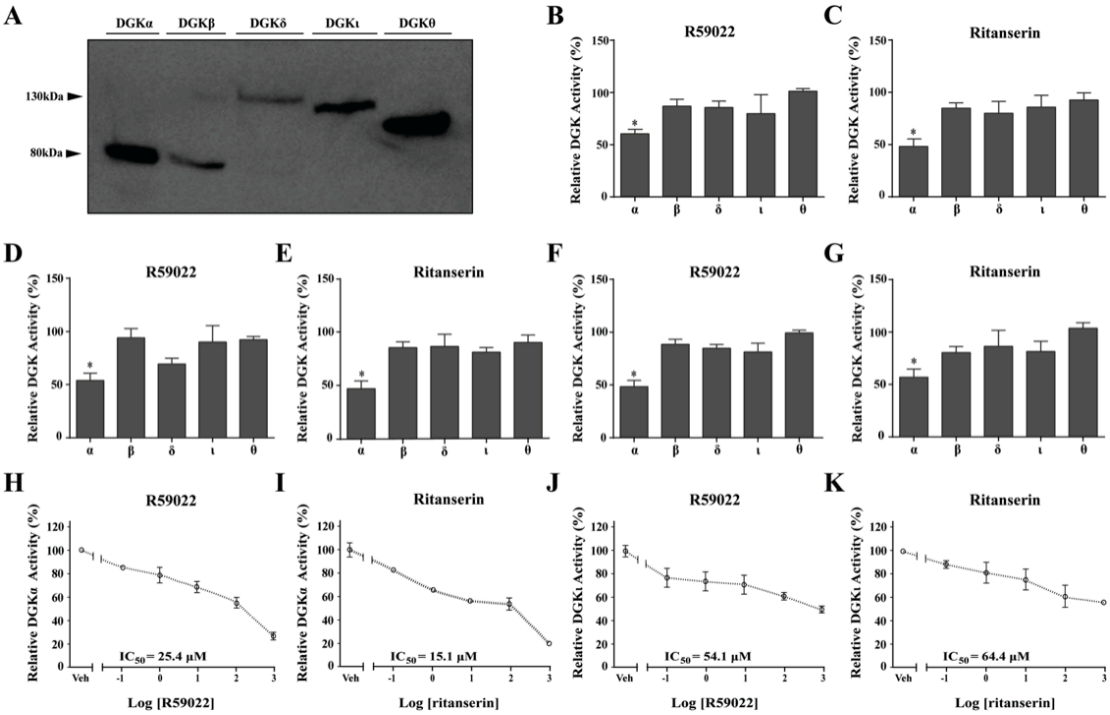

Figure 3.5 R59022 and ritanserin are more potent against DGKα than other DGKs when tested using various DAG species. (A) FLAG-DGKs over-expressed in HEK 293 T cells were separated on an SDS-PAGE gel and probed with FLAG antibody. A representative image is shown. Using PC:PS:DAG liposomes with dioleoyl DAG, DGK activity was tested using cell homogenate with and without (B) 20 μM R59022 and (C) 20 μM ritanserin. Liposomes were prepared with dioctanoyl DAG and DGK activity was tested with and without (D) 20 μM R59022 and (E) 20 μM ritanserin. Liposomes were prepared with stearoyl arachidonoyl DAG and DGK activity was tested with and without (F) 20 μM R59022 and (G) 20 μM ritanserin. The values shown are percent of activity from no inhibitors, which was set to 100. The activity of lysates over-expressing only GFP was less than 10% of the lysates overexpressing DGKs and did not change in the presence of inhibitors. DGK specific activity was normalized to GFP specific activity. Each bar is mean of triplicate ± SEM of a representative experiment. One-way ANOVA was used to test statistical significance between DGK activity with inhibitor and 100% activity - DGK activity without inhibitor, followed by Tukey's post-hoc analysis. *$p<0.05$. A log dose-dependent curve of DGKα activity using with (H) R59022 and (I) Ritanserin. A log dose-dependent curve of DGKι activity with (J) R59022 and (K) Ritanserin. The assays contained cell homogenate, 1 mM ATP and 10 mol% DAG. Veh represents enzyme activity with no drug. The data points on the graphs were fitted to linear interpolation.

Table 3.2 The affinity of various DGK Isoform for ATP and DAG. GraphPad Prism software was used to measure Michaelis-Menten kinetic constants and SEM.

	K_m^{app}	
	ATP (mM)	**DAG (mM)**
DGKβ	0.06 ± 0.04	0.09 ± 0.06
DGKδ	0.13 ± 0.04	0.05 ± 0.006
DGKι	0.09 ± 0.03	0.03 ± 0.003
DGKθ	0.08 ± 0.03	0.08 ± 0.04

Table 3.3 The effect of R59022 and R59949 on a panel of serotonin receptors.

	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_{3}	5-HT_{5A}	5-HT_{6}	5-HT_{7}
				Percent Inhibition of Binding						
R59949	61.1	20.3	76.7	94.2	96.9	72.2	0.7	71.6	71.8	82.8
R59022	82.4	83.5	90.4	98.2	100.5	81.7	10.9	92.0	96.9	98.5
					K_i(nM)					
R59949	2002.0		64.5	9.2	80.0	46.0		3248.5	3070.0	681.0
R59022	410	655.5	12.3	2.2	2.7	0.8		18.0	55.3	14.2
				Percent Inhibition of Agonist Response						
R59949			135.9	100.7	99.4	28.1				32.9
R59022			108.0	99.9	100.2	99.0				99.5

Table 3.3 The effect of R59022 and R59949 on a panel of serotonin receptors.

Data in first two rows are shown as mean % inhibition of binding (n=4) with 10 μM test compounds using radioligand competition assays. Significant inhibition was considered to be >50% and K_i values were determined at these receptors from non-linear regression of radioligand competition binding isotherms. The K_i values are calculated from the best-fit IC_{50} values using the Cheng-Prusoff equation (122). For experimental details, see (121). The data in the last two rows are results from antagonist functional assays. They represent percent inhibition, using 10 μM compounds, of the response to an EC90 concentration (empirically determined) of a reference agonist. Response elicited by reference antagonist was set to 100% and response elicited by vehicle was set to 0%. For experimental details see (123).

Figure 3.6 Ritanserin and R59022 activate PKC in HeLa and U87 cells but not in U251 cells.

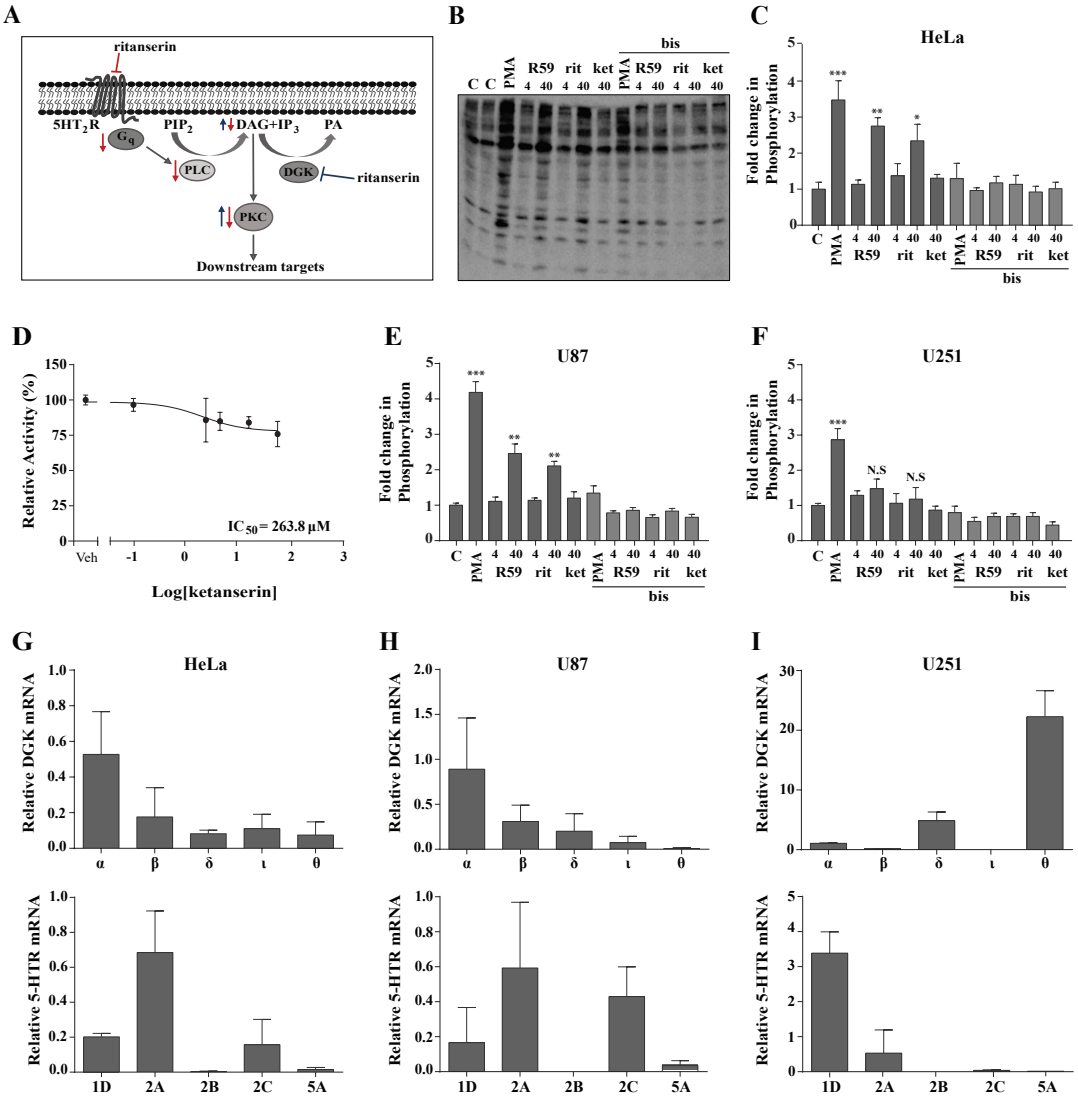

Figure 3.6 Ritanserin and R59022 activate PKC in HeLa and U87 cells but not in U251 cells. (A) Schematic outlining the rationale and signaling pathway relevant to the experiment. (B) A representative western blot of HeLa cell extracts treated with PKC activator (PMA), PKC inhibitor (bis), R59022, ritanserin, and ketanserin probed with the phosphor-(Ser) PKC substrate antibody. (C) Quantitation of four independent experiments in HeLa cells. (D) A ketanserin dose-dependent curve of purified DGKα activity. (E) A quantitation of three independent experiments in U87 cells. (F) A quantitation of three independent experiments in U251 cells. The mRNA expression of indicated DGKs (top) and indicated 5-HTRs (bottom) in (G) HeLa, (H) U87 and (I) U251 cells. Each bar is mean quantitation of three or four independent experiments ± SEM. One-way ANOVA was used to analyze statistical significance between control and each treatment, followed by Dunnett's post-hoc analysis: ***$p<0.0001$, **$p<0.0005$, *$p<0.005$, N.S= no statistical significance.

Figure 3.7 Ketanserin reverses the activation of PKC by TCB-2.

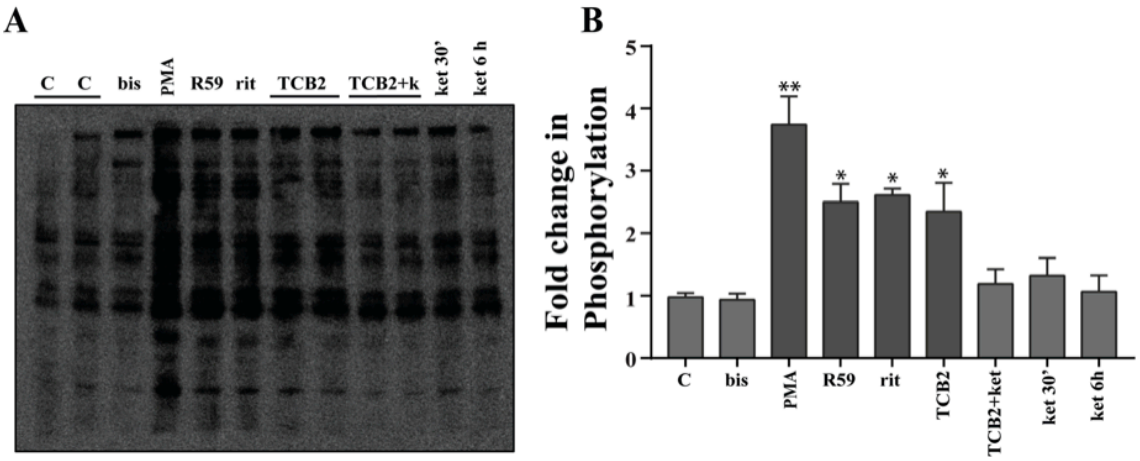

Figure 3.7 Ketanserin reverses the activation of PKC by TCB-2. (A) A western blot of HeLa cell extracts treated with PKC activator PMA, PKC inhibitor bis, 40 μM R59022, ritanserin, ketanserin and the 5-HTR_{2A}R agonist TCB-2 with and without 40 μM ketanserin. (B) A quantitation of four independent experiments. Each bar represents mean quantitation of four experiments ± SEM. One-way ANOVA was used to determine statistical significance between control and each treatment, followed by Dunnett's post-hoc analysis. **$p = 0.0001$, *$p < 0.005$.

CHAPTER 4: THE ACTIVATION OF PKC INHIBITS THE PHOSPHORYLATION OF AKT ON SERINE 473

ABSTRACT

Diacylglycerol kinase alpha (DGKα), which converts diacylglycerol (DAG) to phosphatidic acid (PA), was identified as an important regulator of cancer signaling pathways. It down-regulates key oncogenes, including phosphorylation of Akt on serine 473 (S473), a site downstream of the mammalian target of rapamycin complex II (mTORC2). We demonstrated that inhibition of DGKα with small molecules, R59022 and ritanserin, activates protein kinase C (PKC), probably by causing elevations in DAG. We wondered whether suppression of Akt phosphorylation on S473 was a result of DGKα inhibition and a subsequent increase of PKC activity acting on mTORC2. While PKC activity has been linked to Akt phosphorylation, DGK function has never been directly implicated in the PKC-mTOR-Akt axis. In the present study, I show that augmentation of PKC activity by DGK inhibitors greatly reduces the phosphorylation of Akt on S473 but has no effect on T308, suggesting that under these conditions, PKC impinges on Akt activity through mTORC2.

INTRODUCTION

Diacylglycerol kinase alpha (DGKα) controls the levels of two important signaling lipids, diacylglycerol (DAG) and phosphatidic acid (PA). Both lipids have been implicated in the activation of oncogenes and as such, DGKα is emerging as a regulator of pathways involved in cancer (21). Recently, DGKα was shown to be a potential drug target in GBM (23). The genetic and pharmacological attenuation of this kinase resulted in the apoptosis of GBM cells and a decrease in the expression and activation of several oncogenes, including a partial inhibition of Akt through a reduction in the phosphorylation of serine 473 (S473) (23). Akt is a serine/threonine kinase with a many substrates through which it controls cell growth. As such, Akt underlies the pathophysiology of many diseases, including cancer (148). The activation of Akt depends primarily on the phosphorylation of two sites by phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2): T308 and S473, respectively (148, 149). While the phosphoinositide 3-kinase (PI3K)-Akt-mTORC1 axis has been well characterized both in normal and cancerous cells, the interplay between mTORC2, Akt and other interacting proteins remains relatively elusive. In an effort to reposition the antipsychotic, ritanserin, as a DGKα inhibitor we discovered that activation of PKC leads to a decrease in the phosphorylation of Akt on S473, as well as a decrease in the phosphorylation of S2481 on mTOR (an autophosphorylation site important for mTROC2 activity) but has no effect on phosphorylation of T308. The stimulation of PKC by phorbol esters and a subsequent effect on Akt activation has been previously reported, but the results are conflicting, and to date the exact mechanism has not been elucidated (150-153). In addition, some PKCs are mTORC2 substrates, but there is no evidence of

the reverse scenario (154). The findings here present the first experimental evidence suggesting that the decrease in S473 upon DGKα knockdown observed by Dominguez *et al*. may occur through the activation of PKC, which by an unknown mechanism inhibits mTORC2 and partially attenuates Akt. These data suggest a novel pathway between PKC, mTORC2 Akt and perhaps some Akt substrates.

RESULTS

Activation of PKC eliminates the phosphorylation of Akt on S473 in HeLa cells

Cells were treated with 40 μM R59022 and ritanserin for 30 min, which have previously been shown to activate PKC (Chapter 3). I observed a statistically significant decrease in the phosphorylation of Akt on S473 with the latter compound (**Fig. 4.1**). HeLa cells were also treated with a potent PKC activator, PMA, and this almost completely eliminated S473 phosphorylation (**Fig. 4.1**). Pre-treatment of cells with a PKC inhibitor Bis reversed these effects, further indicating that it is PKC activation which causes changes in S473 phosphorylation (**Fig. 4.1**). Interestingly, none of the treatments had any effect on the phosphorylation of T308 an activating phosphorylation site of Akt. Additionally, the treatments did not alter the phosphorylation of S2448 on mTOR, a site important for mTROC1 activity (**Fig. 4.1** and (149, 155)). Additionally, the treatments did not change the phosphorylation of the Akt substrate glycogen synthase kinase 3 (GSK3) on S21 or S9 (**Fig. 4.1**).

The activation of PKC causes an inhibition of Akt phosphorylation prior to inhibition of mTORC2 phosphorylation

To further investigate the decrease in S473 phosphorylation, I treated HeLa cells with PMA over a course of 80 min alongside a pan-mTOR inhibitor, Torin 1. PMA eliminated S473 phosphorylation by 5 min, which began to reappear by 80 min of treatment (**Fig. 4.2**). Upon activation, mTOR is phosphorylated on S2481, a site that regulates the kinase activity of mTROC2 (155). Treatment with PMA did not significantly decrease phosphorylation on S2481 until 10 min of treatment, which was back to control levels by

40 min (**Fig. 4.2**). I had difficulty detecting the mTORC1 target S6K, but its phosphorylation on T389 appeared to increase with prolonged PMA treatment, suggesting that its activity was also elevated (**Fig. 4.2**). To verify that these observations are in fact correlated with an increase in PKC activity, I showed an elevation in the phosphorylation of PKC downstream targets with PMA treatment but not with the treatment of Torin 1. These preliminary data suggest that the effects on Akt and mTORC2 phosphorylation are by a mechanism involving PKC (**Fig. 4.1**). The increase in S6K phosphorylation with PMA treatment does not make sense and needs to be verified.

DISCUSSION

The identification of DGKα as a therapeutic target in GBM sparked an interest in ritanserin, which is a compound structurally similar to known DGKα inhibitors (23). We demonstrated that ritanserin is an attenuator of DGKα kinase activity and showed that treatment of HeLa and U87 cells with either ritanserin and R59022 leads to an elevation in DAG levels. This was demonstrated with an increase in PKC activity (**Fig. 3.6B, C and E and Fig. 3.7**). A major mechanism underlying the ability of DGKα inhibition to cause GBM cell death was its regulation of expression of a key oncogene, mTORC1 (23). However, additional mechanisms were indicated as perhaps also being at play. I wondered whether the activation of PKC might be involved, since in some cases, PKC has shown to be a tumor suppressor (156). As such, I investigated the effects of DGK inhibition on other proteins implicated in cancer signaling, namely Akt. The knockdown of DGKα decreases the phosphorylation of Akt on S473, but since this is not a target of mTORC1, the exact mechanism of this effect remains unclear (23). The phosphorylation of Akt on this site is important for its full activation, although without it Akt can still act on several, but not all of its downstream targets (157). I showed that treatment of HeLa cells with ritanserin significantly reduced phosphorylation of S473 but had no effect on T308 (**Fig. 4.1**). Since S473 is downstream of mTORC2 and T308 is a PI3K substrate, these results implicated the role of the former enzyme in the observed phenomena (149). As further evidence of this, upon activation of PKC, phosphorylation of mTOR on S2448, a site important for mTORC1 activation, also remained unchanged. Additionally, so did the phosphorylation of GSK3, a downstream target of Akt, which only requires partial Akt activity through the phosphorylation of T308 (**Fig. 4.1A** and (149, 155, 157)).

An important phosphorylation site for the activity of mTORC2 is S2481 and this was also diminished with PMA treatment. Interestingly, PMA did not result in a decrease in S2481 phosphorylation until after that of S473, suggesting that in the context of PKC activation, mTORC2 activation may be downstream of S473 phosphorylation (**Fig. 4.2**). This is contrary to the canonical understanding of the relationship between these two enzymes, where S473 on Akt is an mTORC2 substrate (157). However, recent data suggest that Akt may actually be involved in a positive-feedback loop to stimulate mTORC2 activity and cause its own activation (158). It is possible that PKC inhibits Akt by phosphorylating a yet unidentified site and disables Akt from activating mTORC2, which is reflected in the elimination of S473 phosphorylation. The data connecting PKC activity to direct or indirect regulation of Akt through phosphorylation is contested (150, 153). Further, treatment with Torin 1, which directly inhibits mTOR, also resulted in the decrease of Akt phosphorylation prior to that of mTORC2 phosphorylation (**Fig. 4.2**). The present data is preliminary and the kinetics of Akt and mTORC inhibition by PKC need to be more rigorously characterized before any mechanism can be concretely elucidated.

AUTHOR CONTRIBUTIONS

Dr. Salome Boroda conceived, designed and performed the experiments in Figs. 1 and 2.

Figure 4.1 Ritanserin causes a decrease in the phosphorylation of Akt on S473.

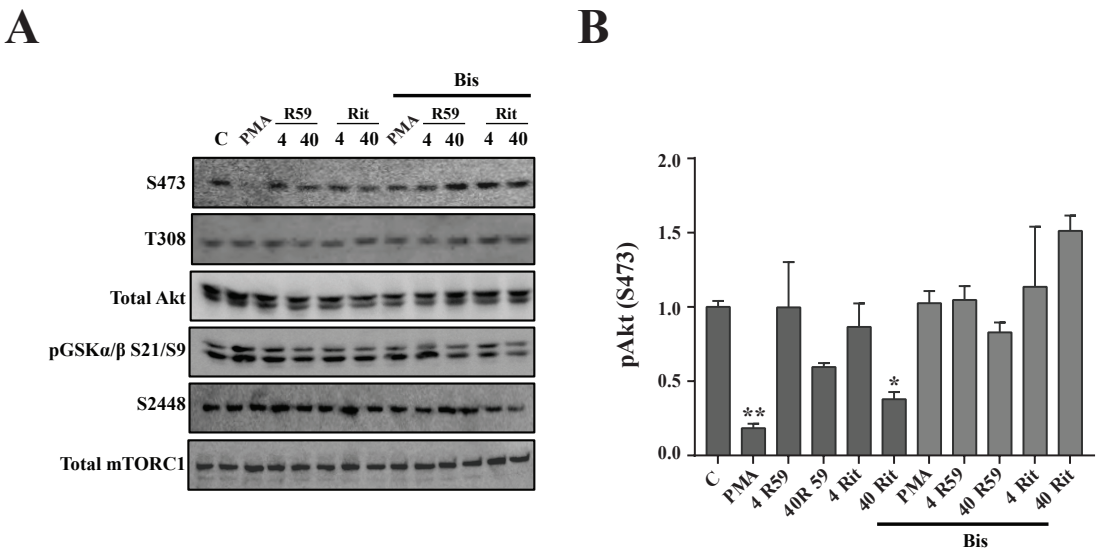

Figure 4.1 Ritanserin causes a decrease in the phosphorylation of Akt on S473. HeLa cells were treated as indicated, harvested and proteins were separated on SDS-PAGE gel and transferred to PVDF membrane. Western blot analysis and anti-rabbit antibodies against the indicated proteins were used to detect expression. (A) Image showing the presence of Akt phosphorylation on S473 and T308, mTORC1 phosphorylation on S2448 and GSK3 phosphorylation on S21 and S9. The unchanged expression of total Akt and mTOR proteins is also shown. (B) Quantitation of three independent experiments demonstrating the strong decrease in Akt S473 phosphorylation with PMA treatment and a statistically significant decrease with ritanserin treatment. The treatment of cells with R59022 resulted in a decrease in S473 phosphorylation that was trending towards significant but more experiments are needed to verify this result. The elimination of S473 phosphorylation was reversible by pre-treatment of cells with a PKC inhibitor, Bis. **$p = 0.0005$, *$p < 0.05$.

Figure 4.2 PMA treatment eliminates S473 phosphorylation and decreases phosphorylation of S2481 on mTORC2.

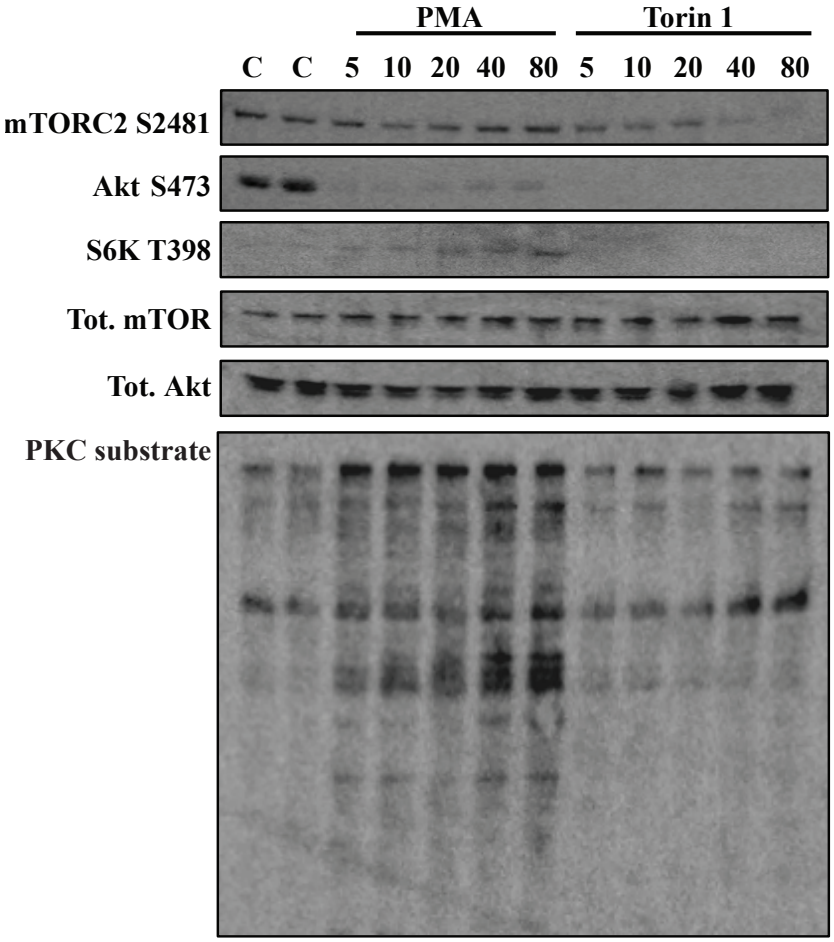

Figure 4.2 PMA treatment eliminates S473 phosphorylation and decreases phosphorylation of S2481 on mTORC2. HeLa cells were treated as indicated and phosphorylation sites on proteins were detected as in **Fig. 4.1**. PMA treatment eliminates phosphorylation of Akt on S473 within 5 min and S2481 phosphorylation on mTORC2 by 10 min. The phosphorylation of T389 on S6K was increased over the course of PMA treatment, as was the phosphorylation of PKC downstream targets. Torin 1 is a known inhibitor of mTOR and its treatment of HeLa cells completely abolished S473 phosphorylation and partially eliminated S2481.

CHAPTER 5: THE PHOSPHATIDIC ACID BINDING, POLYBASIC DOMAIN IS RESPONSIBLE FOR THE DIFFERENCES IN THE PHOSPHOREGULATION OF LIPINS 1 AND 3

ABSTRACT

Lipins 1, 2 and 3 are Mg^{2+} dependent phosphatidic acid phosphatases (PAP) and catalyze the penultimate step of triacylglycerol (TAG) synthesis. We have previously investigated the biochemistry of lipins 1 and 2 and shown that di-anionic phosphatidic acid (PA) augments their activity and lipid binding, yet only in lipin 1 is activity negatively regulated by phosphorylation. In the present study, I show that phosphorylation does not affect the catalytic activity of lipin 3 or its ability to associate with PA *in vitro*. The lipin proteins each contain a conserved polybasic domain (PBD) composed of nine lysine and arginine residues located between the conserved amino- (NLIP) and carboxy-terminal (CLIP) domains. In lipin 1, the PBD is the site of PA binding and sensing of the PA electrostatic charge. The specific arrangement and number of the lysines and arginines of the PBD varies among lipins. we show that the different PBDs of lipins 1 and 3 are responsible for the presence of phosphoregulation on the former, but not the latter enzyme. To do so, we generated lipin 1 that contained the PBD of lipin 3 and vice versa. The lipin 1 enzyme with the lipin 3 PBD loses its ability to be regulated by phosphorylation but remains downstream of phosphorylation by mTOR. Conversely, the presence of the lipin 1 PBD in lipin 3 subjects the enzyme to negative intramolecular control by phosphorylation. These results indicate a mechanism for the observed differences in lipin phosphoregulation *in vitro.*

INTRODUCTION

In many organisms, the lipin family of Mg^{2+} dependent phosphatidic acid (PA) phosphatases consists of three members (lipins 1-3), which form diacylglycerol (DAG) from PA in neutral and phospholipid synthesis (5, 87, 90, 91, 159, 160). Lipins are cytosolic enzymes that must associate with membranes in order to access substrate (5, 55, 95, 161). Their spatial regulation has most extensively been studied in the context of insulin signaling (66, 93, 96, 97). More specifically, inhibition of mTOR and reduction in the phosphorylation of lipin 1, allow its translocation to the ER membrane and nucleus (66, 85, 93, 96, 97). We have shown that while lipin 1 is regulated by phosphorylation *in vitro*, the activity and localization of lipin 2 are not (94).

Lipins are PA effector proteins and are regulated by the lipid's intrinsic chemical properties (76, 79). The PA head-group is a negatively charged phosphomonoester and contains two dissociable protons with pKa values of 3 and around 7.5, pKa_1 and pKa_2, respectively (4, 80). At the physiological pH, PA always carries a charge of at least -1. However, at least two phenomena can cause PA to become di-anionic: proximity to a hydrogen bond donor, which lowers the pKa_2 below the physiological pH, or increase in the membrane pH above that of pKa_2 (80).

Basic amino acids and phosphatidylethanolamine (PE), an abundant membrane lipid, are sources of hydrogen bond donors (1, 4). While there is no canonical lipid-binding site on PA effector proteins, many of them contain a cluster of lysines and/or arginines critical for binding to PA (76). Kooijman *et al*. postulated that these residues are responsible for the initial attraction of PA effectors to the negatively charged membrane. The proteins sample the surface until they encounter a mono-anionic PA.

They hydrogen bond with the PA head-group and cause a dissociation of the final proton. This switches the charge from mono to di-anionic and locks the protein onto its lipid-ligand through strengthened electrostatic interactions. This model was termed the hydrogen bond-switch mechanism, and has been demonstrated experimentally by an induction in lipin activity in the presence of di-anionic PA (77, 85, 94).

Each lipin contains a cluster of nine lysines and arginines known as the polybasic domain (PBD). This region on lipin 1 was identified as the primary PA binding site and a membrane/nuclear localization sequence (NLS) (60, 96, 99). In addition, it is likely to also be the site of negative regulation of lipin 1 by phosphorylation (85). The PBD is conserved among PAP enzymes but the specific arrangement and number of lysines and arginines varies between the mammalians lipins 1 – 3.

The present study is the first to investigate the *in vitro* activity of purified lipin 3 against PA-containing liposomes in the context of phosphorylation and the electrostatic charge of PA. Additionally, we present evidence that the specific PBDs of lipins 1 and 3 are responsible for the observed differences in their *in vitro* phosphoregulation.

RESULTS

Purification and enzymatic activity of recombinant lipin 3

Adenovirus was used to express *Mus musculus* FLAG-lipin 3 in HeLa cells. After two days of expression lipin 3 was affinity purified, eluted with FLAG peptide and dialyzed (**Fig. 5.1A**). The activity and biochemistry of purified lipin 3 was investigated using 0.5 mM PA solubilized in Triton X-100 micelles (162, 163). Lipin 3 activity was linear with time (**Fig. 5.1B**). Work by Carman *et al*. and our laboratory demonstrated that lipin 1 and 2 adhere to surface dilution kinetics (94, 162, 163). The principles of this model can be demonstrated experimentally by testing the detergent concentration-dependent changes in enzyme activity while maintaining substrate at a constant bulk concentration (162). We show that lipin 3 activity decreases with increasing Triton X-100 concentration, suggesting that it also adheres to surface dilution kinetics (**Fig. 5.1C**). The optimal pH for lipin 3 function was determined, with the peak activity at pH 8.0 (**Fig. 5.1D**). Maximal lipin 3 PAP activity was reached at 0.5 mM Mg^{2+} (**Fig. 5.1E**). Addition of 0.01 mM Mn^{2+}, another divalent cation, increased lipin 3 activity by only 35%, relative to the maximum activity of lipin 3 at 0.5 mM Mg^{2+} (**Fig. 5.1F**). The PAP enzymes are characterized by their sensitivity to an alkylating agent, N-ethylmaleimide (NEM) (55, 56). The addition of 2 mM NEM resulted in a near complete inhibition of lipin 3 PAP activity, suggesting that it contains NEM sensitive cysteine residues (**Fig. 5.1G**, IC_{50} = 0.4 mM) as is the case for lipins 1 and 2 (66, 94, 163). Addition of reducing agents stimulates the activity of lipin 1 and 2 (94, 163). Indeed, 20 mM β-mercaptoethanol (β-ME) maximally stimulates lipin 3 activity (**Fig. 5.1H**).

Phosphorylation of lipin 3 and sensing of PA charge

Like the other mammalian lipin proteins, lipin 3 migrates well above its predicted size as a somewhat diffuse band in the 120-130 kDa range (**Fig. 5.1A**). It is likely that part of the aberrant migration pattern in lipin 1 and 2 proteins is due to phosphorylation, which plays important roles in lipin 1 activity (66, 85, 93). To investigate the phosphorylation of lipin 3, the purified protein was subjected to liquid chromatography – tandem mass spectrometry (LC-MS/MS). We obtained 92.3% sequence coverage of lipin 3 and found twenty-three novel phosphorylation sites throughout the length of the protein (**Fig. 5.2A and Table 5.1**). Two sites are predicted to be phosphorylated in all three lipins: S106 and S218 (S243 on lipin 2 and S285 on lipin 1) (**Table 5.1** and (66, 85)). The S218 residue is in the same region as the lipin 1 serine rich domain (SRD). This domain contains several 14-3-3 consensus-binding motifs and is important for mediating lipin 1 localization (96). In lipin 3, the region corresponds to amino acids S185 – S228, of which S198, T203, S215, S218 and S220 are phosphorylated (**Fig. 5.2A and Table 5.1**).

To determine whether phosphorylation affects lipin 3 enzymatic activity, recombinant lipin 3 was purified using affinity chromatography. Prior to protein elution, lipin 3 was incubated in phosphatase buffer with and without protein lambda phosphatase, eluted and dialyzed. This yielded phosphorylated (-λ) and dephosphorylated (+λ) lipin 3 (**Fig. 5.2B**). I verified that all the phosphates were removed by overexpressing and isolating lipin 3 from ^{32}P-radiolabeled HeLa cells expressing FLAG-lipin 3. A 30 min treatment with lambda protein phosphatase *in vitro* removed almost all phosphates (+λ, **Fig. 5.2C**). Next, purified protein and either phosphatidylcholine

(PC):PA liposomes or Triton X-100/PA mixed micelles were used to show that removal of phosphates from lipin 3 does not affect its basal PAP activity or affinity for substrate (**Fig. 5.2D and E and Table 5.2**). The K_m^{app} values of phosphorylated (-λ) and dephosphorylated (+λ) lipin 3 for PA in liposomes were around 24.6 ± 4 μM and 22.5 ± 5 μM, respectively (**Table 5.2**).

Under these conditions, the majority of PA is mono-anionic (4, 77). Phosphatidylethanolamine causes the pKa_2 of PA to decrease to 6.9 and switches the charge of PA to -2 at the physiological pH (4). The incorporation of 30 mol% PE in the PC:PA liposomes resulted in a two-fold augmentation of phosphorylated and dephosphorylated lipin 3 PAP activity, and an increase in k_{cat} (**Fig. 5.2D and Table 5.2**). These data suggest that lipin 3 has greater activity towards di-anionic than mono-anionic PA independent of phosphorylation. Proximity to a hydrogen bond donor is not the only mechanism whereby PA can regulate association with to its own effector proteins. The charge of the PA head-group can also be altered by changes in the pH (77, 85). For example, lowering the pH below the pKa_2 of PA will result in the protonation of the head-group due to an increase in the concentration of hydrogen ions. Conversely, an increase in pH would result in stabilization of di-anionic PA. Indeed, the activity of lipin 3 against Triton X-100/PA mixed micelles was more than two-fold greater at pH 8.0 than at pH 7.5.

As a more direct measure of association with PA, the ability of phosphorylated and dephosphorylated lipin 3 to bind PC:PA and PC:PE:PA liposomes were investigated using liposome flotation on a sucrose gradient. It was found that in the absence of PE, the binding of lipin 3 to PC:PA liposomes was unaffected by dephosphorylation. In the

presence of 30 mol% PE, the binding of lipin 3 was enhanced by 50%, regardless of its phosphorylation state (**Fig. 5.2F and G**). These data further support phosphorylation-independent augmentation of lipin 3 PAP activity under these conditions.

Phosphorylation of lipin 3 in 3T3-L1 cells

Lipin 1 is phosphorylated and negatively regulated upon acute insulin treatment, in a rapamycin and Torin 1 sensitive manner (66, 85, 93, 96). I sought to determine if lipin 3 is similarly affected by phosphorylation. Lipin 1 and 3 were expressed in differentiated 3T3-L1 cells by adenoviral transduction. Two days post-infection, the cells were serum starved in low phosphate buffer for two hours, incubated with ^{32}P orthophosphate, treated with vehicle or a pan mTOR inhibitor, Torin 1 for 45 min plus 10 mU/ml insulin for the last 15 min or insulin alone, and phosphate incorporation was detected (**Fig. 5.3A and B**). Concurrent with previous studies we show that insulin treatment stimulated the phosphorylation of lipin 1 in a Torin 1 sensitive manner (66, 97). However, insulin and Torin 1 treatment had no effect on the levels of phosphate incorporation into lipin 3.

The PAP activity of PBD mutants with liposomes

All three lipin family members contain homologous PBDs immediately C-terminal to the NLIP domain at residues 153-161 in lipin 1 and 136-144 in lipin 3 (**Fig. 5.4A** and (60, 163)). In lipin 1, deletion of this site excluded the enzyme from the nucleus (99). The mutation of the PBD to alanines confirmed that it is an NLS and further that it is critical for PA binding but not for PAP activity (96, 99). Our laboratory has shown that PBD is important for sensing of the PA electrostatic charge, and phosphorylation sites within lipin 1 may hinder PA association with the PBD (85). Our data thus far contrasts that of

lipin 1 and indicates that under the present conditions, lipin 3 phosphorylation does not prevent its recognition and binding to di-anionic PA (**Fig. 5.2**).

We wondered about the molecular basis for such differences in the phosphoregulation of lipin enzymes. One possibility is that the distinct PBDs of lipins 1 and 3 are at least partially responsible. To test this hypothesis, constructs exchanging the PBD of WT lipin 1 with that of WT lipin 3 (lipin 1 (3PBD)) and the PBD of WT lipin 3 with that of WT lipin 1 (lipin 3 (1PBD)) were expressed. The PBD mutant constructs (**Fig. 5.4A**) were purified from HeLa cells, to yield phosphorylated (-λ) and dephosphorylated (+λ) lipin proteins (**Fig. 5.4B**). The PAP activities of the PBD exchange mutants were tested in the context of PC:PA and PC:PE:PA liposomes. Insertion of the lipin 3 PBD in lipin 1 eliminated the ability of phosphorylation to hinder the recognition of the electrostatic state of PA, and PE significantly augmented specific activity of both phosphorylated and dephosphorylated enzyme (**Fig. 5.4C and D**). As seen in Table 3, the K_m^{app} did not significantly decrease with 30% PE, but the catalytic efficiency and turnover number of the lipin 1 (3PBD) mutant increased independent of phosphorylation.

In contrast, only dephosphorylated lipin 3 (1PBD) activity was significantly augmented in the presence of 30 mol% PE but the activity of phosphorylated lipin 3 (1PBD) was not (**Fig. 5.4E and F**). The k_{cat} of dephosphorylated lipin 3 (1PBD) increased by about 2.5-fold, compared to 1.3-fold for the phosphorylated enzyme (**Table 5.3**). These data suggest that the phosphoregulation of lipin 1 primarily occurs because of its specific PBD. Further, that the differences in the composition of residues of the lipin 3 PBD, compared to those of lipin 1, are sufficient to hinder this intramolecular control.

The PAP activity of PBD mutants with micelles

In the context of Triton X-100/PA mixed micelles, the activity of WT lipin 1 is significantly higher than its specific activity in liposomes (85). In contrast, lipin 3 activity is closely matched between these two modes of substrate presentation. It is possible that lipin 3 enzymatic activity is sensitive to inhibition by detergent. To test whether the differences in lipin 1 and 3 PAP activity in micelles is due to the PBD, I measured the activity of the lipin PBD mutants under the described conditions. The lipin 1 (3PBD) displayed comparable specific activity to that of WT lipin 1 (**Fig. 5.5A** and (85)). Lipin 3 (1PBD) activity was also similar to WT lipin 3 (**Fig. 5.2E and Fig. 5.5B**). Similar results were obtained using TWEEN-20 (data not shown) to solubilize PA, suggesting that this is not a function of a specific detergent but may be a consequence of micellar presentation of substrate. Therefore, the functional portion of the lipin proteins that defines the high PAP activity is not within the PBD.

The binding of lipin 1 and 3 PBD mutants to PC:PA liposomes

The lipin PBDs are the main site of substrate interaction (85, 99). It is likely that changes in activity caused by the PBD mutants, are due to altered association of the mutant enzymes with PA. Using liposome flotation assays, we investigated the ability of phosphorylated and dephosphorylated Venus-lipin 1 (3PBD) and Venus-lipin 3 (1PBD) purified enzymes to associate with PA containing liposomes. Venus is a yellow fluorescent protein and is easily detectable by spectrometry. The binding of Lipin 1 (3PBD) increased by about 25 – 30% in the presence of 30 mol% PE regardless of phosphorylation state (**Fig. 5.6A and B**). The binding of phosphorylated lipin 3 (1PBD) increased slightly from 0 to 30 mol% PE while the dephosphorylated enzyme binding

increased by approximately 40% from PC:PA to PC:PE:PA liposomes (**Fig. 5.6C and D**). It should be pointed out that the PBD exchange mutants were catalytically active but displayed significantly reduced K_m^{app} for PA, compared to WT lipins (**Fig. 5.4C-F, Table 5.3** and (85)). suggesting the PBD exchange did not affect affinity to bulk substrate. However, K_m^{app} takes into account both bulk and surface concentration.

Phosphorylation of S106 on lipin 1 (3PBD)

There are two possibilities by which exchanging the PBDs alters the ability of phosphorylation to regulate enzymatic activity. Either the phosphorylation sites themselves are altered by the generation of the PBD mutants or the existing phosphorylation sites can regulate the PBD of lipin 1 but not lipin 3. Serine 106 is a conserved site among the lipins and in lipin 1 is phosphorylated in response to insulin, downstream of mTOR (66, 97). S106 on lipin 2 is not responsive to insulin stimulation or Torin 1 treatment (94). I attempted to investigate the phosphorylation of this site on lipin 3 and the PBD mutants, but could not detect phospho- S106 on lipin 3 or lipin 3 (1PBD) using western blot analysis. Lipin amino acid alignments revealed that the region surrounding S106 which was used to generate phospho-specific antibodies are conserved within lipins 1 and 2 but not in lipin 3. Therefore, we investigated the regulation of S106 phosphorylation in lipin 1 (3PBD).

Wild type lipin 1 and lipin 1 (3PBD) proteins were overexpressed in HeLa cells and treated with vehicle or 250 nM Torin 1. Changes in the phosphorylation of S106 were detected using the phospho-S106 antibody. Under basal conditions, the residue S106 on lipin 1 (3PBD) was phosphorylated (**Fig. 5.7A**). Treatment with Torin 1 decreased phosphorylation of S106 on WT lipin 1, as previously reported (**Fig. 5.7A and**

B and (97)). Exchanging the lipin 1 PBD with the PBD of lipin 3 had no effect in the ability of Torin 1 to inhibit phosphorylation of this site (**Fig. 5.7A and B**). In addition, gel mobility shifts between phosphorylated and dephosphorylated PBD mutants, were similar to what has been seen with the WT enzymes (**Fig. 5.7C**). Together, these data suggest that generation of the lipin 1 (3PBD) mutant does not alter the phosphorylation of S016 and does not eliminate it as a downstream substrate of mTOR. A more detailed biochemical analysis is needed to know for certain whether other S/T sites are responsible for the results outlined above.

DISCUSSION

The lipins are enzymes with complex and multifunctional roles in cellular lipid metabolism. While lipins serve the same catalytic function to dephosphorylate PA, their roles on the physiological level are distinct. Deleterious mutations in *Lpin1* gene result in hepatic and adipose irregularities in mice; while in humans they cause muscle rhabdomyolysis (60, 64, 70). Mice lacking lipin 2 appear to have age-dependent neuronal dysfunction while human *LPIN2* mutations have been linked to the rare immunological disorder, Majeed Syndrome (164). Lipin 3 deficient rodents do not show an obvious phenotype (75). These findings are perhaps a reflection of the intricate and distinct means by which these enzymes are regulated on the molecular level. One of the best understood is the *in vitro* control of lipin 1 localization and activity by phosphorylation and the electrostatic charge of PA. In these contexts, lipin 3 is relatively understudied.

In present work, we investigated lipin 3 activity, identified phosphorylated residues, showed that phosphorylation does not negatively regulate its ability to recognize the electrostatic charge of PA, and demonstrated that lipin 3 phosphorylation is not acutely controlled by insulin or mTOR inhibition *in vitro*. These findings are similar to what was observed for lipin 2, but contrast what is known of lipin 1 phosphoregulation (85, 94). The molecular basis for such differences among enzymes, which otherwise catalyze the same reaction, is unclear.

One possibility is that the protein-wide phosphorylation of lipins 1 and 3 are intrinsically unique. Both proteins are highly phosphorylated, as shown previously for lipin 1 and demonstrated herein by MS analysis and the incorporation of ^{32}P (**Fig. 5.2, Fig. 5.3, Table 5.1** and (66)). However, subtle differences phosphorylation may alter

enzyme activity and regulation. In lipin 1, the SRD is a region enriched in serines and involved in the nucleo-cytoplasmic trafficking of this enzyme (66, 96). Lipin 3 contains a homologous domain that contains nine serines and one threonine. Of these, we found four serines and one threonine to be phosphorylated (**Fig. 5.2A and Table 5.1**). One serine in this region, S226, is phosphorylated in lipin 1 but not in lipin 3. While this may play a role in the regulatory differences between lipins 1 and 3, it is likely not the only factor.

PA effector proteins, including lipins, do not contain a recognized lipid association motif but their recruitment and ability to interact with membranes is important for enzymatic activity (76, 79). Kooijman *et al*. showed that small peptides composed of lysines and arginines can alter the electrostatic charge of PA, by lowering pKa_2 below the physiological pH and causing a dissociation of the proton (4). Basic residues in native proteins demonstrate these principles. For example, the FRBP12 rapamycin-binding domain (FRB) of mTOR contains a single arginine, crucial for the interaction of FRB with PA (83). Other examples include Raf-1, which binds PA at a non-conserved sequence of 35 amino acids, which includes a cluster of four residues with positively charged side-groups three of which are critical for binding (84).

In yeast, the best-described example of protein-PA association is with Opi1, a gene repressor (165-167). The activity of Opi1 is regulated by its sequestration to the ER membrane, mediated by binding to PA (168). Young *et al*. showed that a basic domain in Opi1 is responsible for direct interaction to PA, which was diminished when cells were acidified (86). Further, they identified two lysines and one arginine as being critical for the pH-dependent electrostatic interactions with this lipid. A methyl-PA lacking the pKa_2, displayed weak and pH-independent binding to Opi1 (86, 88). These studies eloquently

demonstrate the dynamic nature of PA ionization state and the importance of the interactions between basic residues and PA, for proper protein function.

Due to the significance of basic residues in protein-PA association, we hypothesized that the unique PBD of lipin 1 plays an important role in the ability of phosphorylation to regulate its activity. In the present work, we show that replacing the lipin 1 PBD with that lipin 3 eliminates the ability of phosphorylation to negatively regulate lipin 1 activity (**Fig. 5.4C and D**). Conversely, the specific activity of lipin 3 (1PBD) increased significantly only after treatment with protein phosphatase, and only in the presence of PE, indicating that phosphorylation hinders its recognition of di-anionic PA (**Fig. 5.4E and F**). In accordance with this data, the k_{cat} of dephosphorylated lipin 3 (1PBD) increased to a much greater extent from 0 to 30% PE, than its phosphorylated counterpart (**Table 5.3**).

What remains unclear is how minor changes in the amino acid arrangement of the lipin PBD can drastically alter the ability of phosphorylation to control lipin activity. Kooijman *at el*. demonstrated that the increase in the PA charge induced by lysine residues is greater than the increase induced by arginines (77). It is possible that lysines more readily hydrogen bond with the phosphorylated amino acids within the protein, which weakens their interaction with PA. Interestingly, lipin 1 contains lysines on three sites within its PBD where lipin 3 contains arginines (**Fig. 5.4A**). Perhaps this underlies the mechanism whereby lipin 1 but not lipin 3 is negatively regulated by its own phosphorylation. In addition, it may be that not the number of lysines but a very precise arrangement of basic residues, both lysines and arginines, is necessary for phosphorylation to hinder recognition of PA charge and substrate association.

Unfortunately, there is no structural data available for lipins and the region between the carboxy-terminal (CLIP) and amino-terminal (NLIP) domains does not conform to any known protein fold (91). As such, the precise mechanisms whereby the lipin 1 PBD allows for phosphoregulation is speculative. The elimination of phosphoregulation of lipin 1 (3PBD) could alternatively be explained by inherent changes in the overall phosphorylation of the mutant enzyme. However, the observed changes in gel mobility upon lambda phosphatase treatment of the purified PBD mutants, suggest that this is not the case (**Fig. 5.7C**). Additionally, S106 of lipin 1 (3PBD) is phosphorylated under basal conditions and remains downstream of mTOR *in vitro*, as demonstrated by reduction of pS106 with Torin 1 treatment (**Fig. 7B**). These data suggest that the lipin 1 (3PBD) retains the phosphorylation pattern of the WT lipin 1, yet these residues can no longer negatively regulate enzymatic activity.

It is curious whether the lipin PBD was always subject to this intermolecular regulatory mechanism rather than gaining this function later, during the emergence of more complex organisms. The PBD does not exist in yeast and the recruitment of the yeast Pah1 is dependent on an amino-terminal amphipathic helix rather than a PBD-like domain (169). The PBD domain may have first appeared around the time of *Ciona intestinalis*, before the appearance of vertebrate species and the three distinct lipins. Whether the single lipin PBD in these early chordates was regulated by phosphorylation is unknown. However, while the lipin 1 domain notably changed from its first appearance to mammals, it is highly conserved, hinting at its important role in lipin function. Conversely, the lipin 3 PBD sequence is more variable in its evolution, at least from reptiles to humans. These observations suggest that perhaps lipin 3 PBD evolved to

function under different cellular conditions and its recognition of substrate charge was fine-tuned to be unresponsive to control by the enzyme's phosphorylation sites.

Another feature of the lipin PBDs is their unusual length. The regions implicated in PA association in most PA effector proteins are composed of no more than 2-5 basic residues, and often just one lysine or arginine is critical for binding (76). Lipins, however, have a nine-residue PBD and the differences between lipin 1 and 3 PBDs are in the first five residues. Interestingly, mutation of the first four amino acids in this domain (KKRR) perturbed the binding of lipin 1 to PA. Mutations of the remaining five amino acids did not negatively affect binding to any further extent but abolished nuclear localization (99). Lipins are known to be multifunctional: for example, they are PA phosphatases but also transcriptional co-regulators (5, 90, 91, 159, 170). In the context of the literature, our data suggest that while the PBDs are critical for substrate recognition and association, they are likely involved in other lipin function. These, and maybe yet unknown roles for lipins, are perhaps reasons for the evolution of a long PBD. The nuances surrounding the differences in the molecular regulation of lipins may be indicative of the specialization of their activities, or perhaps speak to their tissue specific functions and necessity for either more lax or alternative regulatory mechanisms (65, 75, 160).

Our findings elucidate a novel intramolecular mechanism of lipin 1 regulation, implicating the PBD as the determinant factor in the ability of lipin activity to be controlled by phosphorylation.

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AUTHOR CONTRIBUTIONS

Dr. Salome Boroda and Dr. Thurl Harris conceived, coordinated and designed the study. Dr. Harris also significantly contributed to the editing of the manuscript. Dr. Boroda designed, performed and analyzed the experiments shown in Fig. 1C, D and H, Fig. 2B-E, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig.7, Table 2, Table 3 and wrote the manuscript. Sankeerth Tekkellapati designed, performed and analyzed the experiments shown in Fig. 2F and G. Dr. Robert Lawrence and Dr. Judit Villen designed and performed the experiments shown in Fig. 2A and Table 1. Jennifer Pearson performed and analyzed the experiments shown in Fig. 1A, B and E-F. Dr. James Eaton designed, performed and analyzed the experiments shown in Fig. 1A. Dr. Garrett Mullins assisted in the experiments performed in Fig. 2B and made significant contributions to the editing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Figure. 5.1 Purification and biochemical characterization of FLAG-lipin 3.

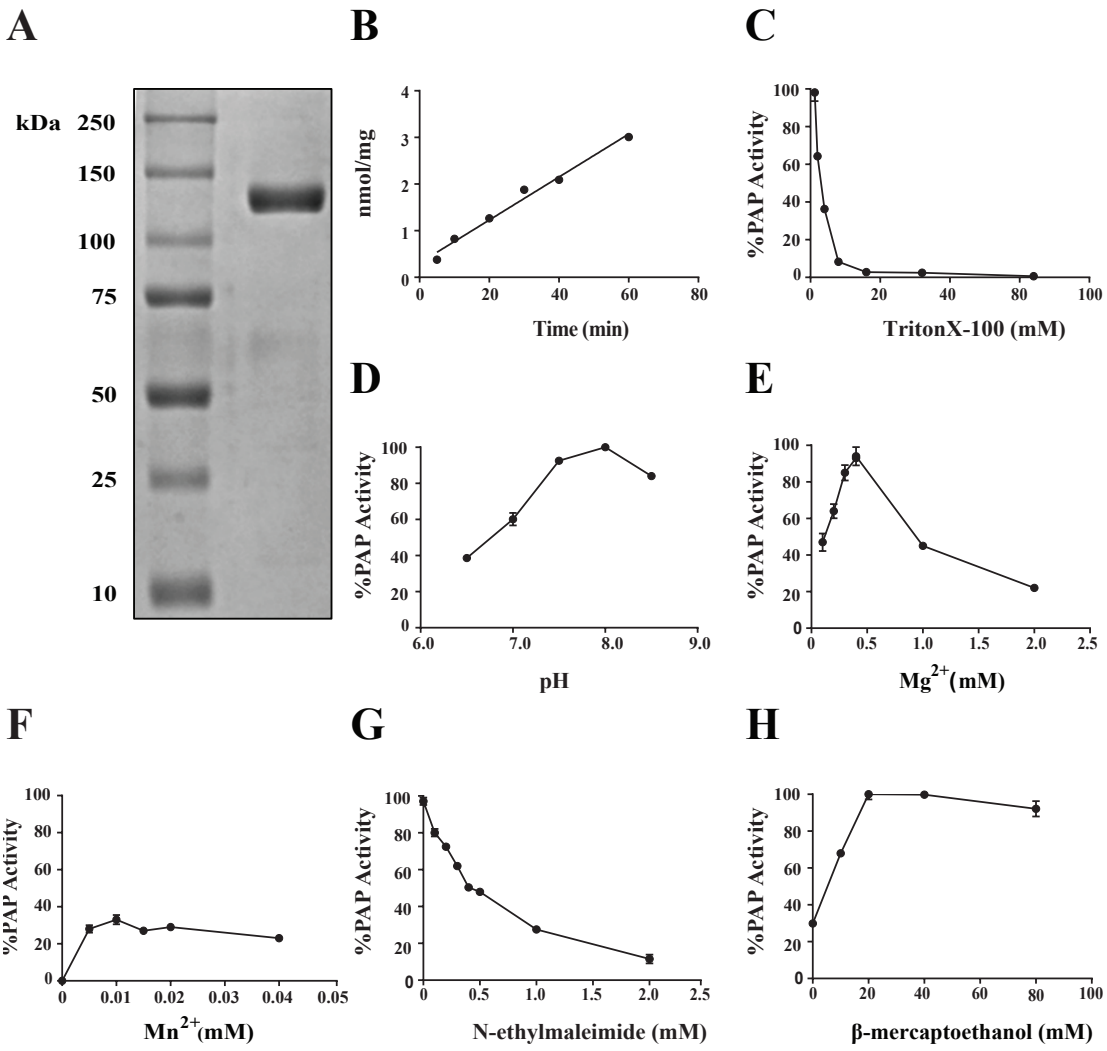

Figure. 5.1 Purification and biochemical characterization of FLAG-lipin 3. (A) HeLa cells were infected with adenovirus expressing FLAG-lipin 3. After two days, cells were homogenized in buffer A and the lipin 3 protein was affinity purified, eluted with FLAG peptide, and dialyzed. An aliquot of the purified FLAG-tagged lipin 3 was resolved by SDS-PAGE and the gel was stained with Coomassie blue. (B) PAP enzymatic activity of 50 ng of purified lipin 3 was measured as a function of time in Triton X-100/PA mixed micelles. (C) PAP activity of lipin 3 against an increasing concentration of Triton X-100 while maintaining PA at 0.5 mM. (D) PAP activity of lipin 3 as a function of pH. (E) PAP activity of lipin 3 as a function of $MgCl_2$. (F) PAP activity of lipin 3 against as a function of $MnCl_2$. (G) PAP activity of lipin 3 with increasing concentrations of NEM. (H) PAP activity of lipin 3 with increasing concentrations of β-ME. Experiments shown in panels (D-H) were performed using Triton X-100/PA mixed micelles with a surface concentration and bulk concentration of PA at 10 mol% and 0.5 mM, respectively. Each data point is a mean of triplicate ± SEM.

Figure. 5.2 Phosphorylation, enzymatic activity and PA-binding of lipin 3.

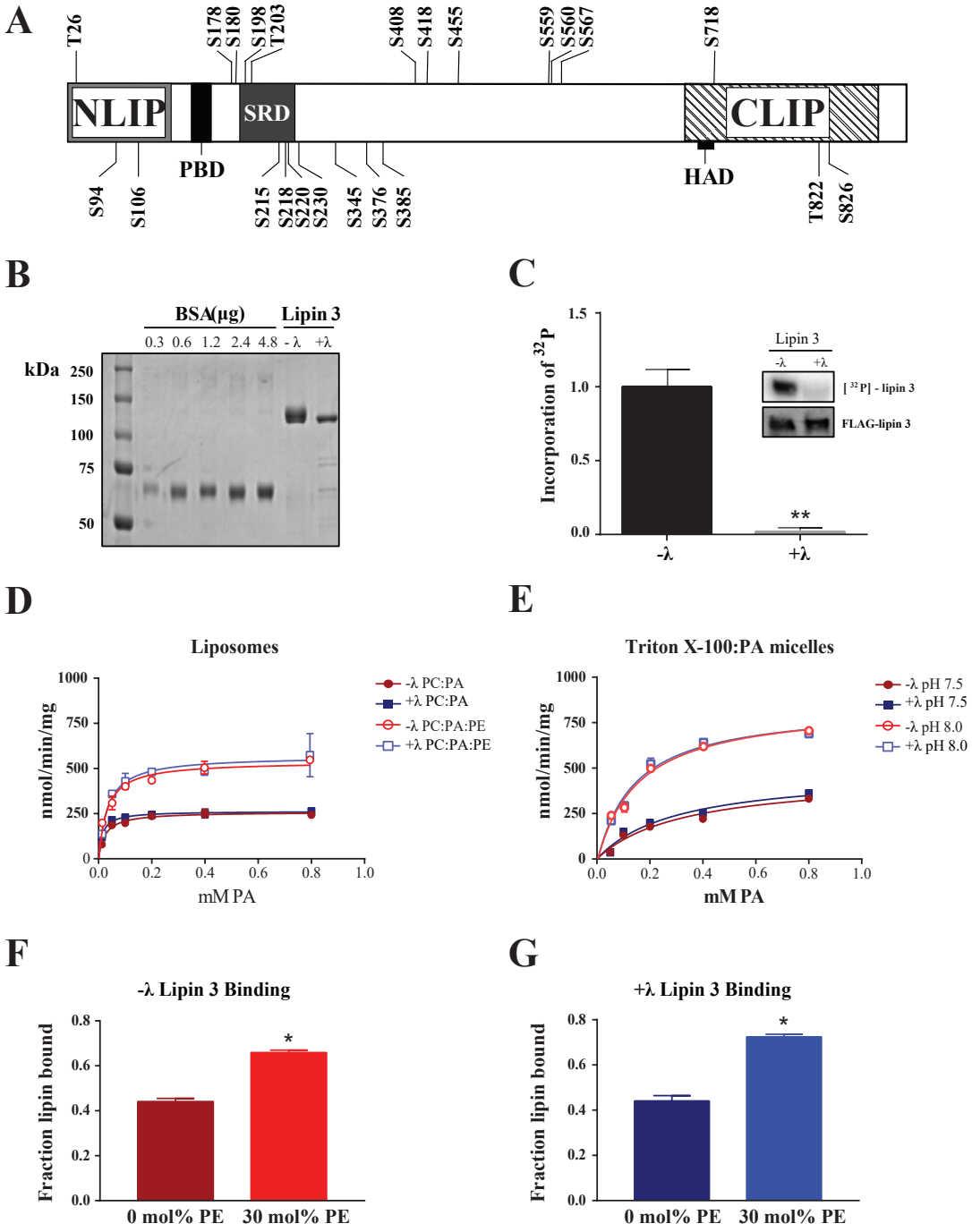

Figure. 5.2 Phosphorylation, enzymatic activity and PA-binding of lipin 3.

(A) Affinity purified lipin 3 was digested into peptide fragments and subjected to LC-MS/MS to identify phosphorylated residues. Shown is a cartoon schematic of the identified phosphorylation sites. (B) Lipin 3 was affinity purified from HeLa cells with and without prior incubation with 2000 units of lambda protein phosphatase. Phosphorylated (-λ) and dephosphorylated (+λ) lipin 3 proteins were separated on an SDS-PAGE gel along with BSA standards and stained with Coomassie Blue dye (C) to verify that +λ lipin 3 residues were deficient of phosphorylation, HeLa cells over-expressing FLAG-lipin 3 were radiolabeled. Cells were harvested, homogenized and incubated with anti-FLAG beads for 4 h. After immunoprecipitation, the radiolabeled lipin 3 was incubated with 200 units of lambda phosphatase. The protein was separated on an SDS-PAGE and incorporation of ^{32}P was visualized using autoradiography (inset top, [^{32}P]-lipin 3) and then immunoblotted for FLAG (inset bottom, FLAG-lipin 3). The bar graph displays the amount of ^{32}P in phosphorylated and dephosphorylated lipin 3 normalized to the amount of total lipin 3 in the immunoprecipitation. The bars are a mean of triplicate experiments ± SEM. (D) PAP activity of purified lipin 3 using PC:PA (90 mol% PC:10 mol% PA) and PC:PE:PA (30 mol% PE) liposomes. – λ and +λ lipin 3 activities were assayed at pH 7.5. (E) - λ and + λ lipin 3 PAP activity was measured using Triton X-100/PA mixed micelles. (F) Phosphorylated and (G) dephosphorylated lipin 3 were subjected to liposome floatation assays using PC:PA (20 mol% PA) and PC:PE:PA (30 mol% PE:20 mol% PA) liposomes. Each data point is a mean of triplicate ± SEM. Student's t-test was used to analyze statistical significant of phosphate removal and binding between 0 and 30 mol% PE. * $p < 0.0005$, **$p < 0.0001$.

Table 5.1 Lipin 3 phospho-peptide analysis by LC-MS/MS.

Modified Sequence Lipin 3	Residue	A-score	(ph)-S/T found in other isoforms
GLNPAT(ph)LSGGIDVLVVR	T26	26.6	
LGDSGEAFFVQELDS(ph)DEEDVPPR	S94	84.4	
LCTS(ph)PIPWGGLSGFPSDSQIGTASEPEGLVITGR	S106	14.0	lipin 1, lipin 2
PTPES(ph)PSAQEAEEPSSQPK	S178	20.4	
PTPESPS(ph)AQEAEEPSSQPK	S180	20.4	
DIHPYS(ph)DGECTPQANLSSGDLM(ox)SPK	S198	23.3	lipin 2
DIHPYSDGECT(ph)PQANLSSGDLMSPK	T203	19.3	
DIHPYSDGECTPQANLSSGDLMS(ph)PK	S215	19.4	
S(ph)DSELELR	S218	29.3	lipin 1, lipin 2
SDS(ph)ELELR	S220	26.3	lipin 1,
LRSLEPS(ph)PLRAE	S230	38.0	
SWS(ph)WTTPESHTPSGHPQVSR	S345	26.3	lipin 2
RNQHLGPS(ph)DIYLDDLPSLDSENVALYFPK	S376	28.2	
NQHLGPSDIYLDDLPS(ph)LDSENVALYFPK	S385	19.7	
RWS(ph)EPSNQK	S408	32.7	
LLES(ph)PNPEHIAECTLDSVDK	S418	55.0	lipin 1
FT(ph)QHMVSYEDLTK	T455	22.1	
TEVLS(ph)SDDDVPDSPVILEVPPLPSSTPGYVPTYKK	S559	18.4	
TEVLS(ph)S(ph)DDDVPDSPVILEVPPLPSSTPGYVPTYKK	S560	23.0	
TEVLSSDDDVPDS(ph)PVILEVPPLPSSTPGYVPTYKK	S567	63.6	lipin 1
GPILLS(ph)PSSLFSALHR	S718	14.2	lipin 2
GPST(ph)DLASPEYSNLSYWR	T822	14.5	
LLFPPVVRGPSTDLAS(ph)PE	S826	73.7	

Table 5.1 Lipin 3 phospho-peptide analysis by LC-MS/MS. The first column indicates the phospho-peptide identified by mass spectrometry, which contain an S/T residue predicted as phosphorylated, annotated as (ph) The annotation of (ox) represents methionine oxidation. The second column indicates the identity and the position of the phosphorylated residue. Ascore is a measure of sequence position confidence. The final column indicates whether the predicted phosphorylated residues on lipin 3 are conserved and phosphorylated on lipins 1 and 2. Only amino acids with PEP score of < 0.05 and Ascore of > 13 are listed.

Table 5.2 The steady state kinetic data for phosphorylated (-λ) and dephosphorylated (+λ) lipin 3.

		K_m^{app}(μM)	K_{cat} (s^{-1})	K_{cat} / K_m^{app} ($\mu M^{-1} s^{-1}$)
-λ	**0**	24.6 ± 4	0.50 ± 0.02	0.020
-λ	**30**	37.3 ± 3	0.78 ± 0.12	0.023
+λ	**0**	22.5 ± 3	0.51 ± 0.01	0.023
+λ	**30**	31.9 ± 9	0.91 ± 0.05	0.028

Table 5.2 The steady state kinetic data for phosphorylated (-λ) and dephosphorylated (+λ) lipin 3. The data was generated from assays using PC:PA or PC:PE:PA liposomes containing 90 mol% PA, 10 mol% PA and the indicated mol% PE. The specific activity was measured as V_{max}/mg protein.

Figure 5.3 Phosphorylation of lipin 3 in 3T3-L1 cells.

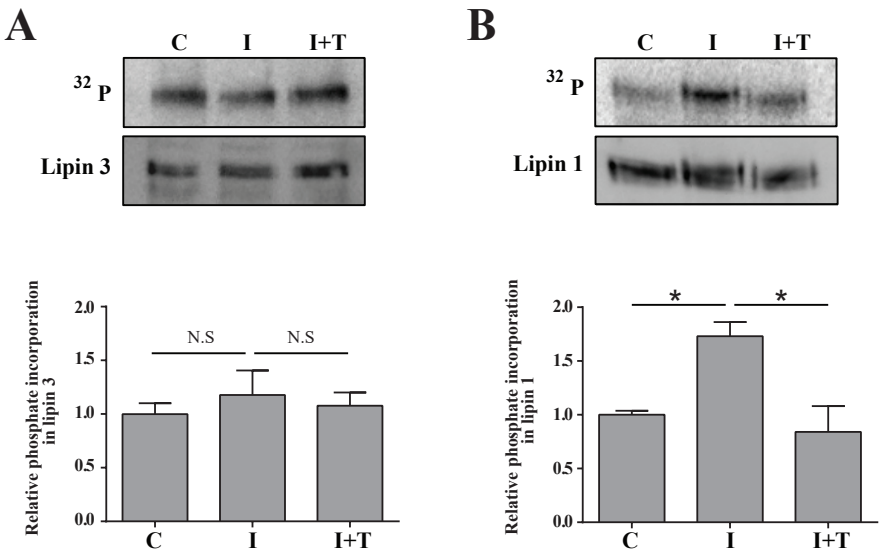

Figure 5.3 Phosphorylation of lipin 3 in 3T3-L1 cells. (A) 3T3-L1 cells infected with adenovirus expressing FLAG-lipin 3 were serum starved in low phosphate buffer for 2 h, incubated with orthophosphate/ml and treated as indicated. The cells were harvested, incubated with anti-FLAG beads for 4 h, displaced from beads and separated on SDS-PAGE gel and transferred to PVDF. Top: A phospho-image of ^{32}P incorporation into lipin 3 (upper panel, ^{32}P) and an immunoblot image of total lipin 3 protein (bottom panel, lipin 3). Bottom: Quantitation of three independent experiments. (B) Experiment was performed as in (A) but with FLAG-lipin 1. Each bar is a mean of quantitation from three separate experiments ± SEM. One-way ANOVA was used to analyze statistical significance followed by Tukey's post hoc analysis. *$p < 0.05$, N.S = no statistical significance.

Figure 5.4 Enzymatic activities of lipin PBD exchange mutants in liposomes.

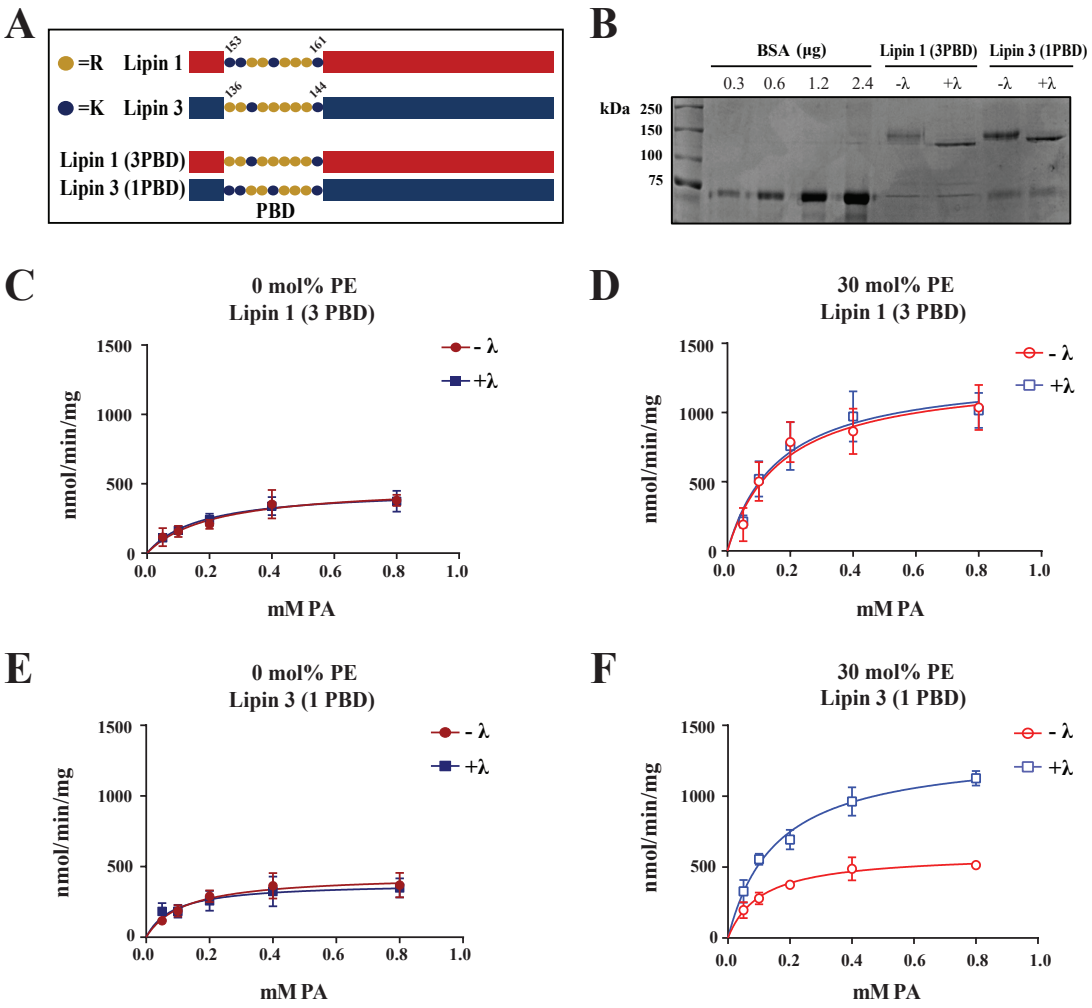

Figure 5.4 Enzymatic activities of lipin PBD exchange mutants in liposomes.

(A) Schematic of lipin PBD exchange mutants. (B) PBD mutants were expressed, affinity purified, incubated in phosphatase buffer with (+λ) or without (-λ) lambda phosphatase and eluted as described before. Proteins were separated on an SDS-PAGE gel along with BSA standards and stained with Coomassie Blue dye. The PAP activities of purified phosphorylated (– λ) and dephosphorylated (+ λ) lipin 1 (3PBD) were measured with (C) PC:PA and (D) PC:PE:PA liposomes. The PAP activities of - λ and + λ purified lipin 3 (1PBD) with (E) PC:PA and (F) PC:PE:PA liposome. Activities were assayed at pH 7.5. Each data point is a mean of triplicate ± SEM.

Table 5.3 The steady state kinetic data for phosphorylated (-λ) and dephosphorylated (+λ) lipin PBD mutants.

		K_m^{app}(μM)	K_{cat}(s^{-1})	K_{cat} / K_m^{app}($\mu M^{-1} s^{-1}$)
Lipin 1 (3PBD)				
-λ	**0**	211 ± 170	0.76 ± 0.23	0.003
-λ	**30**	191 ± 88	1.66 ± 0.17	0.008
+λ	**0**	170 ± 99	0.64 ± 0.06	0.004
+λ	**30**	146 ± 54	1.64 ± 0.34	0.011
Lipin 3 (1PBD)				
-λ	**0**	120 ± 69	0.67 ± 0.09	0.005
-λ	**30**	117 ± 31	0.92 ± 0.05	0.007
+λ	**0**	107 ± 40	0.78 ± 0.19	0.007
+λ	**30**	155 ± 38	2.02 ± 0.26	0.014

Table 5.3 The steady state kinetic data for phosphorylated (-λ) and dephosphorylated (+λ) lipin PBD mutants. The data was generated from assays using PC:PA or PC:PE:PA liposomes containing 90 mol% PC, 10 mol% PA and the indicated mol% PE. The specific activity was measured as V_{max}/mg protein.

Figure. 5.5 Enzymatic activity of lipin PBD exchange mutants in Triton X-100/PA micelles.

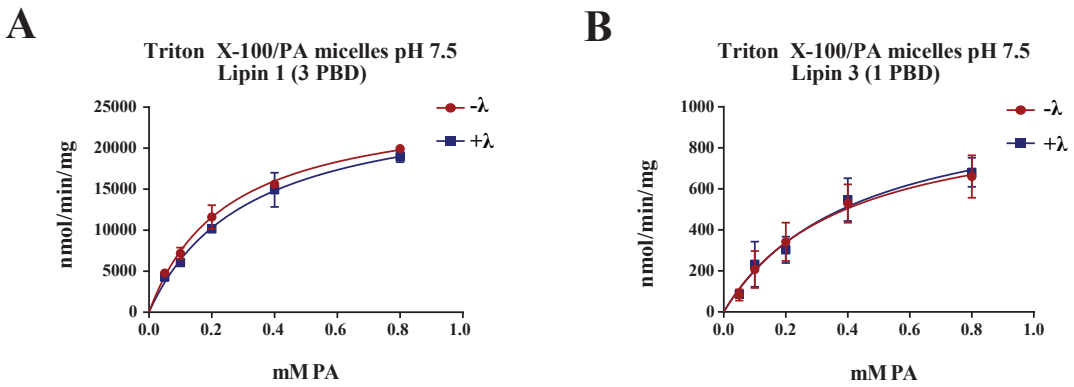

Figure. 5.5 Enzymatic activity of lipin PBD exchange mutants in Triton X-100/PA micelles. Phosphorylated (-λ) and (+λ) dephosphorylated (A) Lipin 1 (3PBD) and (B) Lipin 3 (1PBD) activities were assayed using Triton X-100/PA mixed micelles at pH 7.5. The surface concentration of PA was 10 mol%. Each data point is a mean of triplicate ± SEM.

Figure. 5.6 Binding of Venus-lipin PBD exchange mutants with PA containing liposomes.

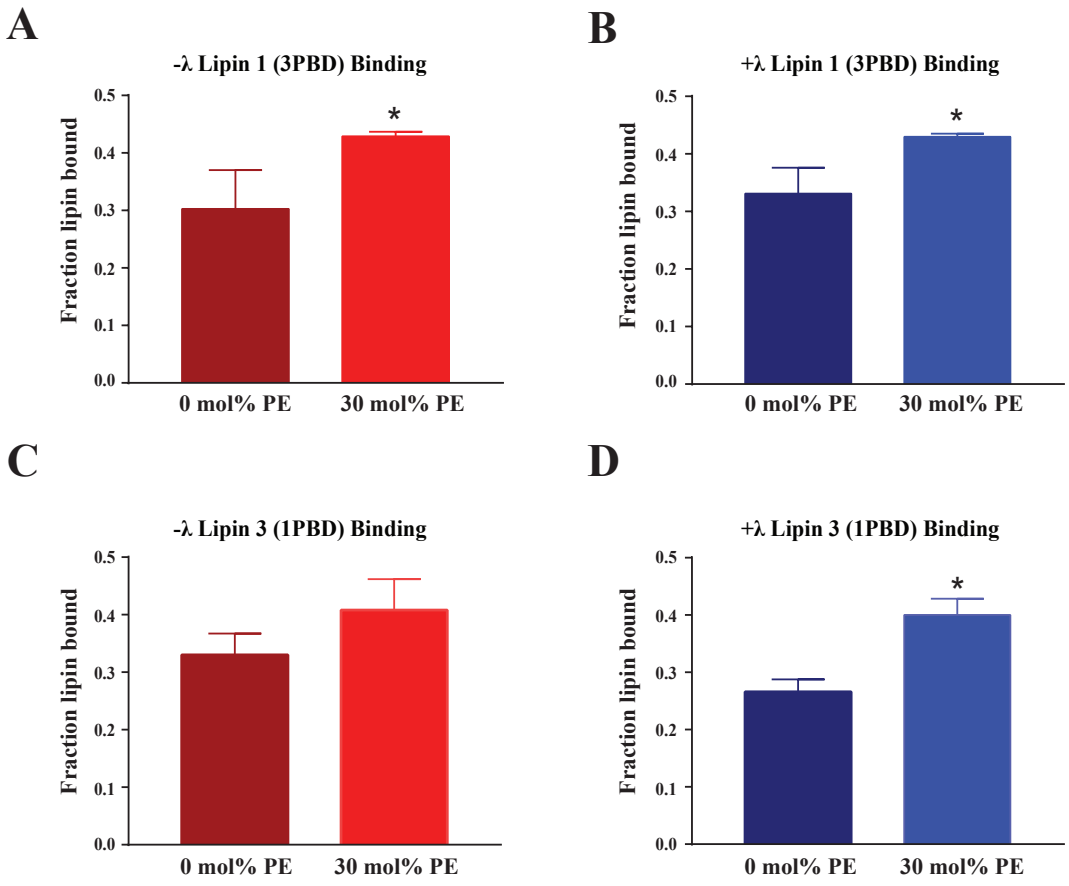

Figure. 5.6 Binding of Venus-lipin PBD exchange mutants with PA containing liposomes. (A) Phosphorylated (- λ) Venus-tagged lipin 1 (3PBD) (B) Dephosphorylated (+ λ) Venus-tagged lipin 1 (3PBD) (C) - λ Venus-tagged lipin 3 (1PBD) and (D) + λ Venus-tagged lipin 3 (1PBD) were subjected to liposome floatation assays using PC:PA and PC:PE:PA (30 mol% PE) liposomes, containing 20 mol% PA and 0.1 mol% PC-Pyrene. The final concentration of PA in all assays was 2 mM. Student's t-test was used to analyze statistical analysis. *$p < 0.05$. Each bar is a mean of triplicate ± SEM.

Figure. 5.7 Phosphorylation of lipin 1 (3PBD) at S106.

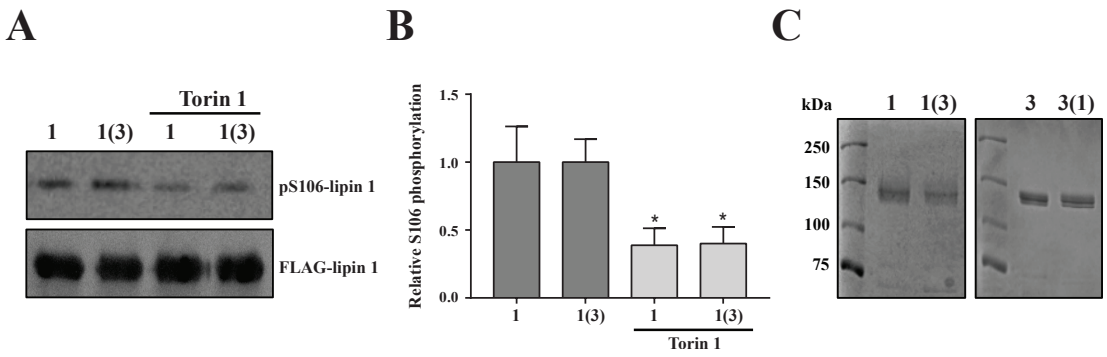

Figure. 5.7 Phosphorylation of lipin 1 (3PBD) at S106. HeLa cells infected with adenovirus expressing either FLAG- lipin 1 or FLAG-lipin 1 (3PBD) were treated with vehicle or Torin 1, harvested and incubated with anti-FLAG beads for 4 h. The enzymes were displaced, separated on SDS-PAGE gel and probed for phospho S106 or total lipin protein. (A) Image of phosphorylated S106 and total enzyme. (B) Quantitation of three independent experiments. (C) (left panel) Coomassie image of purified phosphorylated lipin 1 and lipin 1 (3PBD), (right panel) Coomassie image of purified phosphorylated lipin 3 and lipin 3 (1PBD). One-way ANOVA was used to analyze statistical significance. *$p < 0.05$. Each bar is a mean of quantitation from three separate experiments ± SEM.

CHAPTER 6: PHOSPHATIDYLSERINE PREVENTS THE PHOSPHATIDYLETHANOLAMINE INDUCED AUGMENTATION OF LIPIN 1 ENZYMATIC ACTIVITY

ABSTRACT

Lipin 1 dephosphorylates PA to yield DAG in the pathway of *de novo* TAG synthesis. The enzyme is regulated by phosphorylation, the electrostatic charge and the concentration of PA on the ER. The charge of PA is affected by changes in membrane pH and proximity to hydrogen bond donors such as PE, which can augment lipin activity by lowering the pKa_2 and making PA di-anionic, thus increasing the protein-lipid attractions. Lipin has only been reported to traffic between the cytosol and the ER membrane and is excluded from the plasma membrane (PM). Due to the important role that molecules, such as PE play in the modulation of PAP activity, we postulated that varying lipid compositions of the PM and ER membrane influence the specificity of lipin localization. The ER is enriched in PE, while the PM contains the greatest concentration of the mono-anionic PS of any cellular membrane. In the present work, I show that PS significantly reduces the PE-induced augmentation of lipin activity *in vitro*, suggesting that the high concentration of PS may play a role in directing lipin localization away from the PM.

INTRODUCTION

All enzymes of triacylglycerol (TAG) synthesis are anchored to the membrane of the endoplasmic reticulum (ER), save lipin. The latter is a soluble protein and must be recruited from the cytosol to dephosphorylate phosphatidic acid (PA) and form diacylglycerol (DAG) in the penultimate step of TAG synthesis (5). PA exists in the membranes of various organelles including the plasma membrane, but lipin localizes from the cytosol only to the ER/nucleus (1, 97, 99). Although the underlying mechanism for lipin 1's exclusive trafficking between the cytosol and the ER is unknown, one explanation could be the heterogeneity of the lipid composition of the different cellular membrane surfaces. For example, phosphatidylethanolamine (PE) is highly enriched in the ER membrane (although it is abundant in almost all membranes), and it has been shown to augment lipin PAP activity (1, 85). This lipid contains a positive charge in its head-group, which can hydrogen bond with PA and cause it to become di-anionic, increasing the attraction of lipin to its substrate, as outlined in the previous chapters.

Phosphatidylserine (PS) is enriched in the inner-leaflet of the mammalian as well as the yeast plasma membranes (PM). Here, it comprises over 30% of the total phospholipids and plays a critical role in apoptosis during which it flips to the outer-leaflet and signals for the phagocytosis of the dying cell (1, 171). PS is mono-anionic and not a phosphomonoester and as such, under physiological conditions it carries a maximum charge of -1 (79). Lipin does not bind PS, however, the electrostatic nature of this lipid and its enrichment in the PM may be one underlying mechanism whereby the cell excludes lipin from this region of the cell (99).

Specifically, we hypothesized that the negative charged head-group of PS might interfere with the hydrogen bonding between PE and PA. Indeed, I show that the presence of PS in PE-containing liposomes reverses the augmentation of lipin PAP activity *in vitro*. Although preliminary, these data suggest that high concentrations of PS in the PM may be involved in the indirect regulation of lipin localization.

RESULTS

The presence of PS in PC:PE:PA liposomes reverses the augmentation of lipin activity

I generated the following sets of liposomes: PC:PA (90 mol% PC:10 mol% PA), PC:PE:PA (60 mol% PC:30 mol% PE:10 mol% PA), PC:PS:PA (50 mol% PC:40 mol% PS:10 mol% PA), and PC:PE:PS:PA with 30 mol% PE, 10 mol% PA and varying PS concentrations (10-40 mol%), at the expense of PC (**Fig. 6.1**). The activity of both phosphorylated and dephosphorylated lipin 1 was tested against these liposomes. Concurrent with previous reports, the enzymatic activity of lipin 1 increased in the presence of 30 mol% PE, with the activity of dephosphorylated lipin being about 2-fold higher than that of the phosphorylated enzyme (**g. 6.1A-B**). Incorporation of 40 mol% PS alone did not significantly alter lipin 1 activity (**Fig. 6C**). The presence of PS together with PE reversed the PE-induced increase in lipin 1 activity in a PS concentration dependent manner (**Fig. 6.1D-F**). More specifically, 10 mol% PS abolished the activity of dephosphorylated lipin 1 (**Fig. 6.1D**), with the effect on phosphorylated lipin 1 being more modest (**Fig. 6.1D-F**). The attenuation of dephosphorylated lipin 1, however, was significant from 0 to 10 mol% PS and was even more notable by 30 mol% PS (**Fig. 6.1D and E**). In the presence of 40 mol% PS, the activity of dephosphorylated enzyme was less than half of what it was with 30 mol% PE alone, although still higher than with PC:PA liposomes (**Fig. 6.1 A, B and F**).

DISCUSSION

A critical component of lipin function is its ability to translocate from the cytosol to the membrane. With the experiments outlined above, I present evidence for a unique and indirect regulation of lipin 1 activity that may play a role in the specificity of lipin 1 localization within the cell. More specifically, I demonstrate that PS can reverse the *in vitro* increase in PAP activity caused by the hydrogen bonding between the phosphomonoester of PA and the amine of PE.

The molecular mechanisms that underlie lipin 1 movement within the cell are multifaceted: PA charge, phosphorylation and metabolic signaling can all impinge on lipins catalytic activity in various ways. An additional layer of regulation, involving the complex interplay between the chemical properties of charged membrane lipids, may be also play a role. Here, I use an in vitro system to begin investigating the question of why lipin does not localize to the PM.

The ER and PM both contain PA, PE and PS. However, the former membrane is slightly enriched in PE, while the PM (especially that of yeast) has an abundance of PS (1). While PE augments lipin activity *in vitro,* there is no evidence suggesting that chemical properties inherent to PS negatively alter lipin activity directly, to prevent its localization to the PM, and our data show that PS alone does not significantly change PAP activity *in vitro* (**Fig. 6.1C** and (85)). However, it is plausible that on the PM, the negative charge of the PS head-group interferes with the hydrogen bonding between PE and PA making PA mono-anionic more often than di-anionic. Since lipin 1 shows increased binding to and activity against di-anionic PA, perhaps it prefers to localize to the ER where the PS to PE ratio is relatively low.

A well-studied PA effector protein is Raf-1, which translocates to the PM upon stimulation of PLD and the generation of PA (172). What causes one PA-binding protein to localize to the PM and another to the ER is unclear. However, it is important to note that while Raf-1 contains a PA binding domain at its C-terminal region, it also contains a PS-binding cysteine rich domain (like the C1 domains of PKC and DGK) and has shown to specifically associate with PS-containing liposomes (84, 173, 174). Further work is needed to elucidate the specific mechanisms of PA effector localization within the cell.

The data outlined above demonstrates a PS-dependent reversal of the PE induced increase in lipin 1 activity (**Fig. 6.1**). In addition, PS mostly eliminates the negative regulation of lipin 1 activity by phosphorylation (**Fig. 6.1B and D-F**). Although it does not appear that PS alone significantly affects lipin 1 activity, these data suggest that the lipid hinders the electrostatic interactions between PE and PA, and prevents the switch of PA from mono- to di-anionic. These findings may have a significant impact on the understanding of lipin regulation and the importance of membrane composition on protein localization. Future work could extrapolate these data in a more biologically relevant setting such as the localization of lipin 1 during various stages of cell apoptosis, when PS moves from the inner to the outer leaflet of the PM.

AUTHOR CONTRIBUTIONS

Dr. Thurl Harris and Dr. Salome Boroda conceived the experiment in Fig 1. Dr. Boroda designed and performed the experiment in Fig. 1.

Figure 6.1 Incorporation of PS into PC:PE:PA liposomes reverses lipin 1 activation

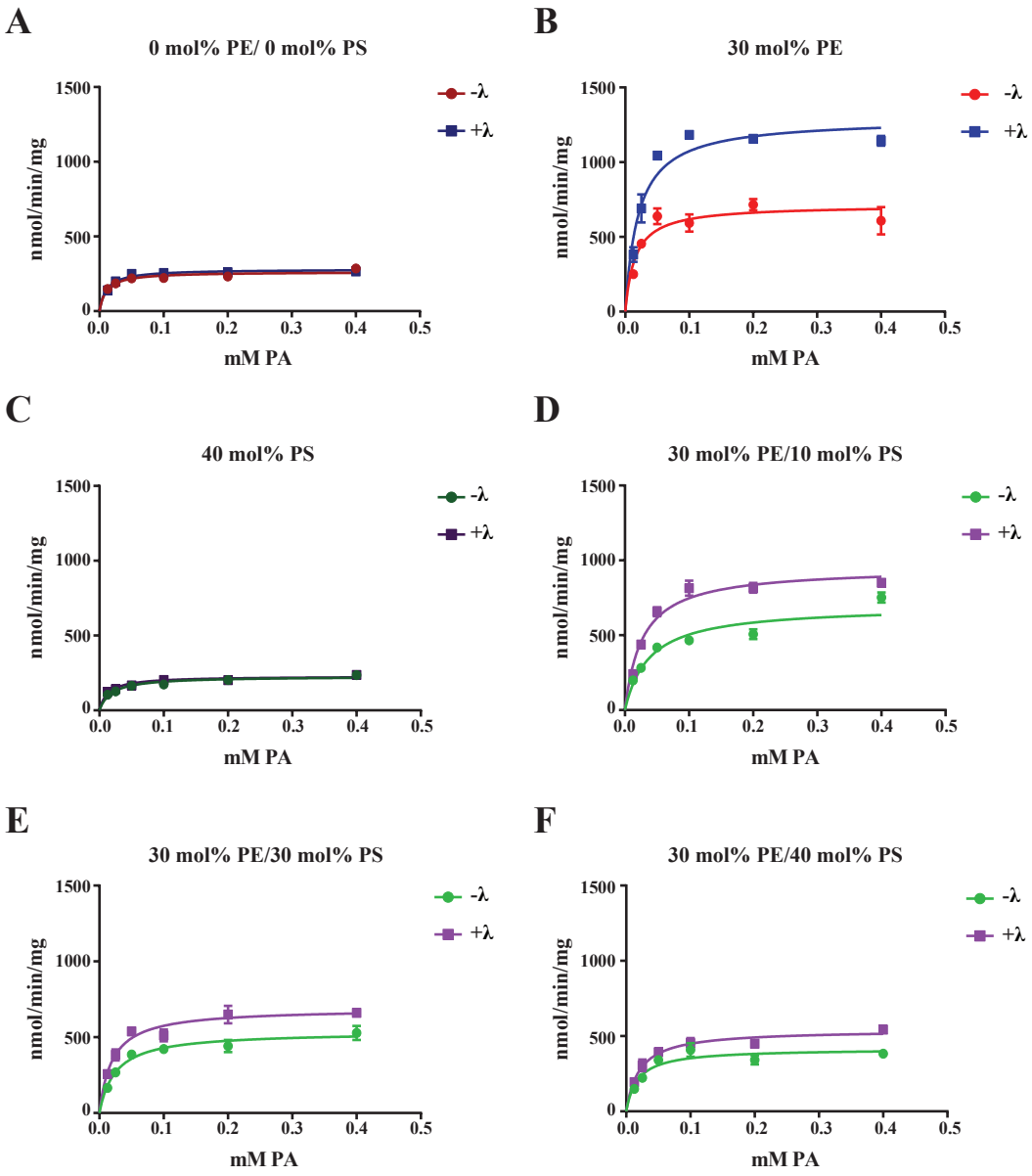

Figure 6.1 Incorporation of PS into PC:PE:PA liposomes reverses lipin 1 activation. Liposomes were generated as indicated and as described in materials and methods. The activity of purified lipin 1 was tested against (A) PC:PA liposomes (B) PC:PE:PA liposomes (C) PC:PS:PA liposomes (D) PC:PE:PS:PA liposomes with 10 mol% PS (E) PC:PE:PS:PA liposomes with 30 mol% PS and (F) PC:PE:PS:PA liposomes with 40 mol% PS. Each data point represents a mean of triplicate ± SEM.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Summary

The present dissertation outlines the study of two enzymes, DGK and lipin, which catalyze opposite reactions and can inversely control the levels of the second-messenger lipids: DAG and PA. We show that DGKα can be inhibited *in vitro* by ritanserin, a compound that was previously characterized as a 5-HTR antagonist and tested in clinical trials. Our data sets the stage for the *in vivo* repositioning of this compound as a treatment of GBM (work ongoing) and strongly implicates PKC activation as a potential method for verifying DGK inhibition in the whole cell (**Fig. 7.1**).

We also demonstrate that the difference observed among the regulation of lipins 1 and 3 by phosphorylation is due to their specific PBDs. The PBD of lipin 1 allows for phosphorylated residues within itself to compete with PA for association with the PBD. The PBD of lipin 3, on the other hand, associates with its substrate without hindrance from its phospho-sites (**Fig. 7.1**). The insertion of the lipin 3 PBD into the lipin 1 enzyme completely eliminates phosphoregulation. Conversely, the insertion of the lipin 1 PBD into lipin 3, subjects the protein to this intramolecular control. These findings implicate the PA-binding domain of lipins as the enzyme region responsible for conferring phosphoregulation on lipin 1 and suggest that the amino acid differences that exist in this region of lipin 3 are sufficient to render the phosphorylated residues unable to block recognition of PA charge.

7.2 Conclusions and Significance

The work presented here addresses important unanswered or insufficiently answered questions in DGK biology and fills significant gaps in our understanding of DGK inhibition. As it currently stands, patients diagnosed with GBM have very limited treatment options and rarely survive past 16 months post-diagnosis and treatment. Our work identifying ritanserin as a DGK inhibitor, has set the stage for its potential development as an alternative treatment option and for the development of other novel DGK inhibitors. If the work presented here in any way aids in the improvement of the quality of life of these patients, we can take pride in the fact that one of our primary goals as scientists in the field of biomedical research has been accomplished.

The present work elucidates previously unknown functions of R59022 and R59949 and shows that like ritanserin, they have a substantial polypharmacological profile, beginning with their affinity for 5-HT_2Rs (and other 5-HTRs) and DGKα. Interestingly, 5-HTRs activate PLC to generate DAG and subsequently PA. Like DGK inhibition, the attenuation of these receptors would also result in diminished PA levels. The *in vivo* use of R59022 and ritanserin in mouse cancer models has shown remarkable success in countering tumor growth and progression (*Olmez et al.* unpublished and (23)). If the oncogenic activity of DGKα is through PA, our data suggest that the polypharmacology is in fact favorable, as it inhibits PA production from two different sources. This may explain the ability of these compounds to markedly increase survival of tumor bearing mice.

Our work also provides an additional pharmacological tool for studying DGK biology *in vitro*, in cells and *in vivo*. Until now, the use of the classic DGK inhibitors *in*

vivo has been difficult due to their poor pharmacokinetics. Our discovery that they have a polypharmacological profile further complicates their application in mouse models and in cells. Since ritanserin has favorable pharmacokinetics ($t_{1/2}$ of ritanserin is 40 h in humans compared to $t_{1/2}$ of R59022 and R59949 in mice which is approximately 2 h), it may be a better alternative for probing DGK function.

Unfortunately, this polypharmacology also raises many concerns for the ability of ritanserin to become a cancer treatment option. We are concerned that this issue has not been sufficiently addressed during the use of R59022 and R59949 *in vivo*. This work underscores the importance of performing proper controls, and being cognizant of the fact that at the physiological level, the present DGK inhibitors may elicit their effects by interacting with multiple targets. To increase confidence that the desired target is being attenuated with R59022, R59949 and ritanserin, validation methods need to be developed. Our work has begun this journey by showing that treatment of cells with these compounds results in a very significant increase in PKC activity, suggesting that DGK is attenuated. These findings set the stage for more fine-tuned validation methods, which without doubt will be necessary for the progression of ritanserin to clinical trials as chemotherapy.

I also address the fundamental question of how lipins 1 and 3 maintain such striking differences in the ability of general protein phosphorylation to regulate their activity. To this end, we hypothesized that the difference emerges from the variation in the arrangement of basic amino acids within their PBDs. Quite remarkably, we were correct. This is an astonishing finding, as it implicates a region of just nine amino acids as the decisive factor in whether the entire protein becomes subject to phosphoregulation by

its own phospho-sites.

The question now remains as to what this means for lipins as an entire family of phosphatases. Until the evolution of the vertebra and jaw, the PBDs varied greatly between organisms in both length and amino acid composition (**Fig. 1.6**). With the three lipins, there came the evolution of the nine-residue domain, which is now readily recognized as the PBD (**Fig. 1.6**). However, while they catalyze the same reaction, lipins 1 – 3 display distinct biochemistry, ability to be regulated and tissue expression. Each lipin also contains a PBD that is notably different from the others in the specific arrangement and composition of basic residues. Our data supports the notion that multiple lipins evolved in order to serve different functions in the various tissues where they are predominantly expressed. It is possible that this is accomplished by their varying responsiveness to cellular regulatory mechanisms, perhaps subserved by the different PBDs of each lipin.

Furthermore, lipins display varying levels of activity *in vitro*, with purified lipin 1 having the highest activity in Triton X-100/PA micelles and lipin 2 having the lowest activity with liposomes and micelle assays (85, 94, 163). It is possible that the PBDs of lipins 2 and 3 diverged and became unresponsive to phosphoregulation in order to provide compensatory, albeit reduced, activity in the case of the loss of lipin 1. In other words, deleterious mutations or absence of lipin 1, may lead induction of lipin 2 or 3 and due to the apparently lower PAP activity of these enzymes, it is advantageous that they are “continuatively” active in the context of certain regulatory stimuli. It has also been hypothesized that the phenotypic consequences of the loss of lipin 1, that lead to increased lipin 2, might be due to this compensatory yet, non-regulatable activity (94). If

these hypotheses are at true, and the lipin 3 PBD evolved to evade regulation by certain cell signals, our findings provide the *in vitro* biochemical evidence needed to further pursue these studies in a more biologically relevant setting.

7.3 Limitations

Our work is limited from multiple perspectives. Although PKC activation may be a viable readout of DGK inhibition in cells, these results are cell-type dependent. However, we noted that in the cells where PKC was not activated by R59022 or ritanserin, the mRNA expression of DGKα, relative to other DGKs, was low. This data supports our previous results, which showed that the compounds are selective for DGKα and imply that prior to treatment of a new cell line with R59022, ritanserin or R59949, the expression of DGKα should be verified.

In characterizing ritanserin, Leysen *et al*. demonstrated that the compound also targets histamine, dopamine and adrenergic receptors, albeit with reduced affinity. Our work has not taken these additional targets of ritanserin into account. Future work showing DGK inhibition with ritanserin, in particular in mouse models, would need to rule out these "off-target" effects.

Our work both with lipins and DGKs is also limited by its *in vitro* nature. We chose to use highly purified enzymes against liposomes containing the lipid substrates. We used this method for several reasons. First, it eliminates the contribution of other cellular proteins and components. Secondly, the use of liposomes removes any detergent effects on protein activity. Finally, liposomes may represent a slightly more biologically relevant setting since they are lipid bilayers. Using this method for testing enzymatic

activity, we noted some discrepancies from previously published data. For example, we demonstrated that ritanserin and R59022 are selective for DGKα compared to four other DGKs. Others have shown, however, that R59022 may attenuate DGKθ and DGKε. We additionally demonstrated that the activity of lipin 3 in liposomes is comparable to that of lipin 1. This is in conflict with previous reports where lipin 3 PAP activity is lower than that of lipins 1 and 2. To our knowledge, no other laboratories have used highly purified DGKα and lipins against substrate-containing liposomes to test activity, and although this method has its benefits, it may be the underlying reason for these discrepancies.

7.4 Future Directions

With the discovery that ritanserin can inhibit DGKα and DGK inhibitors can antagonize 5-HTRs *in vitro*, we have underscored the complex nature of the activity of these compounds. To date, there is little evidence that treatment of cultured cells or mouse models with ritanserin or R59022 and R59949 cause a reduction in PA. This work is a difficult undertaking since the inhibition of 5-HT_{2A-C} receptors might have the same result. However, alterations in the levels of DAG could differentiate between receptor antagonism and kinase inhibition. We have shown that indeed, treatment of cells with R59022 and ritanserin causes PKC activation, suggestive of DGK inhibition and DAG elevation. Future work should pursue a more direct form of validating DGK inhibition in the whole cell, by measuring changes in DAG upon inhibition of endogenous DGKα. This work involves the optimization of treatment conditions and extensive mass spectrometric analysis of cellular lipid DAG species.

Another difficulty with measuring DGK inhibition in cells is the presence of

multiple isoforms, which can serve compensatory roles in the case of the loss of DGKα activity. Additionally, the selectivity of these compounds against the DGK isoforms is still under investigation. While our data show that ritanserin and R59022 are selective for DGKα over four other DGKs, five more remain. Also, as noted above, these data are conflicting with others reports, which suggest that the compounds can inhibit several other mammalian DAG kinases (31). Future work will have to address this issue by identifying cell lines that express only a few DGKs and genetically eliminating the expression of all but DGKα. Such work will allow for the investigation of endogenous enzyme in mammalian cells, cancer cells or cells from specific tissues. An alternative method is to overexpress mammalian DGKα in organisms, such as yeast, which only contain one endogenous DAG kinase, which can be easily deleted. If these methods can be established, the next step may be to generate a DGKα that is resistant to R59022 and ritanserin inhibition. Although difficult and requiring structural information, this would be an excellent method for target validation.

The ability of DGK to impinge on PA and DAG levels makes it an indirect regulator of many oncogenes, since both lipids have been implicated in the control of cancer signaling pathways. Because of this, there may be multiple mechanisms at play in the ability of DGK inhibition to slow tumor progression in mouse models. Some of these mechanisms have been elucidated (23). Our discovery that DGK inhibition leads to PKC activation revealed that PKC might negatively regulate Akt through mTORC2. Our findings are preliminary, but warrant further investigation into the detailed signaling mechanism. I hypothesized that PKC inhibits mTORC2. This could be through complex dissociation, direct phosphorylation of mTORC2 by PKC at an unidentified site (or both),

or through an intermediate protein. Our laboratory has developed excellent assays for detecting mTORC dissociation using Venus mTOR and Cerulean-tagged Raptor/Rictor. The fluorescently tagged proteins can be spectrophotometrically detected (175). As such, mTROC2 kinase assays with and without PKC, may be a simple way to rule out or suggest PKC-caused dissociation of mTORC2. Additionally, an analysis of mTORC2 amino acid sequence may reveal the presence of PKC substrate consensus region, which once mutated, might no longer result in mTORC2 and Akt inhibition. If there is no effect on mTORC2 dissociation or phosphorylation using these methods *in vitro*, the presence of an intermediate regulatory protein should be considered.

Another hypothesis is that PKC in fact directly inhibits Akt, affecting the positive feedback loop between Akt and mTORC2, and resulting in mTORC2 inhibition (158). There is some evidence that PKC does in fact directly regulate Akt, but the reports are inconsistent, with some claiming direct phosphorylation of S473 and others claiming indirect dephosphorylation of this site (151, 153). An alternative answer could be that PKC phosphorylates an unidentified, inhibitory residue on Akt. If evidence does not suggest mTORC2 regulation, this may be an alternative avenue to pursue.

Phosphatidic acid is a unique lipid due to the chemistry of its phosphomonoester head-group. Our laboratory has revealed that the ability of PA to exist as a mono- or di-anionic substrate controls its association with lipin. Further, that this association is regulated in lipin 1, but not in either lipin 2 or lipin 3, by its phosphorylated residues (31, 94). We have now pinpointed that the varying lipin PBDs are the protein regions responsible for these differences. However, we have no insight into the specific residues, which are responsible.

The lipin 1 PBD contains lysines in three positions where the lipin 3 PBD contains arginines. Since the former amino acid is believed to form stronger hydrogen bonds with phosphomonoesters, it is possible that phosphorylation sites more readily interact with them than with arginines to hinder substrate association (77). Alternatively, it may not be individual residues that are responsible, but the very specific arrangement of lysines and arginines that renders one PBD subject to phosphoregulation and not the other. Biochemical characterization of lipins with various mutations within the PBD might be needed to answer this question. Other PA-binding proteins do not contain a PBD-like domain, but employ a few relatively dispersed amino acids for PA association (76). Even so, perhaps phosphorylation also plays a role in the ability of these basic residues to recognize PA charge. The identification of such phosphoregulation in PA effector proteins may suggest that this is an overarching mechanism of regulation for many if not all proteins that bind PA via electrostatic interactions.

Furthermore, while we have shown that phosphorylation in general interferes with the recognition of PA charge by lipin 1, we have not identified any specific phospho-sites. Lipins are all highly phosphorylated proteins, and they all contain a domain enriched in serines, which in lipin 1 it is called the serine rich domain (SRD) (66, 94). The residues in this region are important for lipin 1 interaction with the 14-3-3 proteins, and its subsequent sequestration in the cytosol (96). 14-3-3 is a phospho-serine/phospho-threonine binding protein that regulates many functions of its binding partners (176). Lipin 1 contains 14-3-3 binding sites within the SRD. Three residues within this site, which have shown to be phosphorylated by LC-MS analysis, abolished the 14-3-3 effects on lipin when they were mutated to alanines (96). These amino acids – serines 252, 254

and 260 (amino acid numbers as on lipin 1a) may be the culprits of negative lipin regulation. Perhaps mutation of just these sites to alanines will render the lipin 1 PBD able to recognize PA charge.

The investigation of the impact of the PBD switch on lipin biology is imperative. Mutation of the lipin 1 PBD to alanine residues impaired the ability of lipin 1 to rescue adipogenesis in *fld* mouse cells. It would be interesting to know whether the insertion of the lipin 3 PBD in the lipin 1 enzyme results in a similar alteration. The transcriptional co-activator activities of lipins has been investigated, but that of lipins 2 and 3 are relatively understudied. Further, while the role of the lipin 1 PBD in nuclear localization is defined, to my knowledge similar experiments with lipins 2 and 3 have not been performed. It would be interesting to probe the role of the PBDs of these two lipins in protein localization, transcriptional function, and cellular metabolism.

In the present dissertation, we also hypothesized that the phospholipid composition of various cellular membranes can regulate lipin localization. The *in vitro* evidence supports this hypothesis and prompts further investigation in a more biologically relevant setting. Specifically, our laboratory has demonstrated that PE augments lipin activity and I have shown that PS eliminates this augmentation. While convincing, these experiments were performed in an isolated system. Demonstrating these principles in the cell may be challenging but they need to be considered. For example, PS is enriched in the inner leaflet of the PM, induction of apoptosis will cause the lipid to flip to the outer leaflet and if done at the correct time, maybe localization of lipin 1 to this membrane can be detected. If so, it will further support our hypothesis.

Figure 7.1 The serotonin (5-HT) receptor (5-HTR) antagonist, ritanserin, inhibits DGKα activity and the lipin 3 PBD eliminates the phosphoregulation of lipin 1.

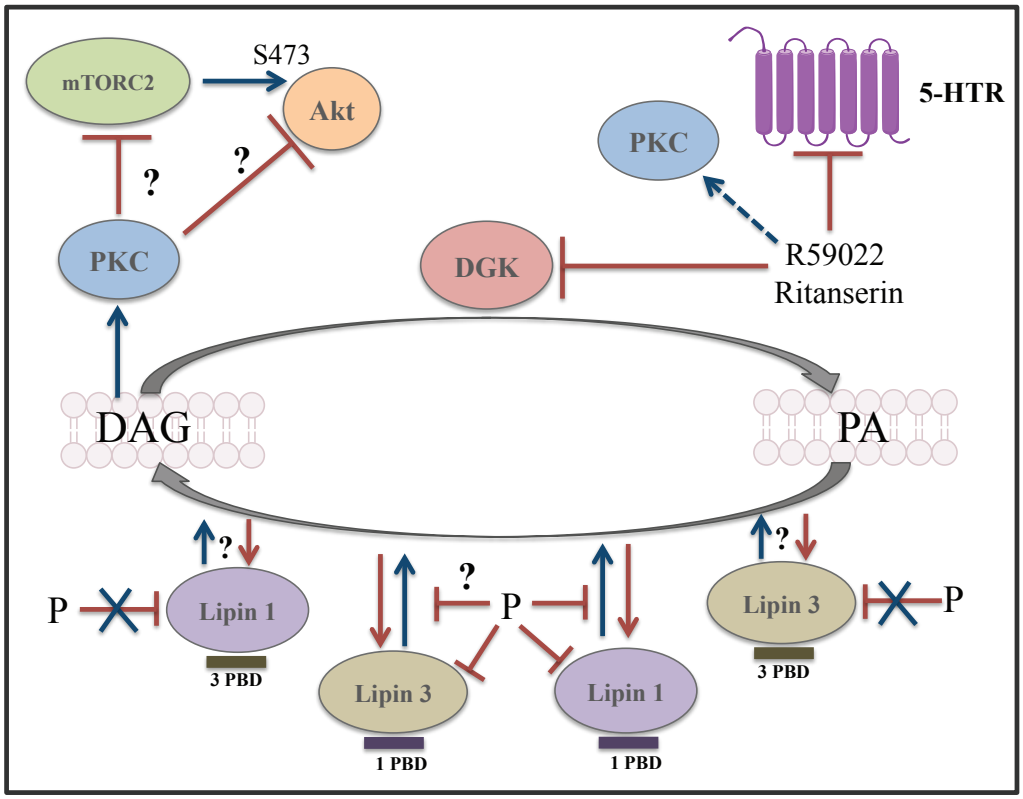

Figure 7.1 The serotonin (5-HT) receptor (5-HTR) antagonist, ritanserin inhibits DGKα activity and the lipin 3 PBD eliminates the phosphoregulation of lipin 1. This dissertation described the investigation of two lipid-modulating enzymes, DGK and lipin, from very different perspectives. First, I showed that like R59022, ritanserin inhibits DGKα and like ritanserin, R59022 also antagonizes 5-HTRs. The compounds indirectly activate PKC (dashed lines), by causing elevations in DAG levels. From here, I showed that the activation of PKC attenuates phosphorylation of mTORC2 and Akt, on sites that are important for activity. Exactly how this occurs remains to be investigated. Secondly, we demonstrated that while lipin 1 localization and substrate recognition are regulated by phosphorylation (indicated as the letter P), lipin 3 is not (indicated as the presence of lipin 3 enzyme closer to the gray arrow). Further, the inserting the lipin 3 PBD into lipin 1 eliminates the intramolecular phosphoregulation of lipin 1. Conversely, the presence of the lipin 1 PBD in the lipin 3 enzyme subjects it to phosphoregulation. We do not know whether lipin 3 localization is controlled by phosphorylation or whether the PBD mutations effect the movement of these enzymes between the cytosol and ER.

CHAPTER 8: PUBLICATIONS RESULTING FROM THIS WORK

Boroda S, Niccum M, Raje V, Purow BW, Harris TE. 2016. Dual activities of ritanserin and R59022 as diacylglycerol kinase inhibitors and serotonin receptor antagonists. *Biochemical Pharmacology.*

Boroda S, Takkellapati S, Lawrence RT, Pearson JM, Eaton JM, Mullins GR, Harris TE. The lipin polybasic domain (PBD) is responsible for *in vitro* lipin 1 regulation. *Under review.*

ADDITIONAL PUBLICATIONS

Olmez I, Love S, Xiao A, **Boroda S,** Randolph R, McKenna BD, Neal BP, Li M, Floyd D, Brenneman B, Abounader R, Gioeli D, Weber MJ, Lee J, Godlewski J, Nakano I, Bronisz A, Mayo M, Harris TE, Purow BW. 2017. Targeting the mesenchymal subtype in glioblastoma and other cancers via inhibition of diacylglycerol kinase alpha. *Neuro-oncology.*

He J, Feng Z, Lei W, Tay R, Nian W, **Boroda S**, Levental KR, Levental I, Harris TE, Chant JT, Guangwei D. 2017. Lipin-1 regulation of phospholipid synthesis maintains endoplasmic reticulum homeostasis and is critical for triple-negative breast cancer cell survival. *The FASEB Journal.*

Pagac M, Cooper DE, Qi Y, Lukmantara IE, Mak HY, Wu Z, Tian Y, Liu Z, Lei M, Du X, Ferguson C, Kotevski D, Sadowski P, Chen W, **Boroda S,** Harris TE, Liu G, Parton RG, Huang X, Coleman RA, Yang H. 2016. SEIPIN regulates lipid droplet expansion

and adipocyte development through modulating the activity of glycerol-3-phosphate acyltransferase. *Cell Reports.*

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