PHOSPHORYLATION OF LIPIN 1 BETA PHOSPHATIDATE PHOSPHATASE BY CASEIN KINASE II

By

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A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Nutritional Sciences

Written under the direction of

Dr. George M. Carman

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New Brunswick, New Jersey

October 2018

ABSTRACT OF THE THESIS

Phosphorylation of lipin 1 beta phosphatidate phosphatase by casein kinase II

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Lipin is a phosphatidate phosphatase enzyme that catalyzes the penultimate step in triacylglycerol synthesis, the conversion of phosphatidic acid to diacylglycerol. By controlling this step, lipin regulates both triacylglycerol and phospholipid synthesis, and it also acts as a transcriptional regulator. It is known that phosphorylation controls the subcellular localization of lipin, and its activity as both a phosphatidate phosphatase and a transcriptional regulator. Multiple phosphorylation sites within lipin have been identified, but the identity of the protein kinases involved is largely unknown. In this work, we sought to identify and characterize kinases that play a role in lipin's regulation.

Using bioinformatics, we found that lipin 1β has a high probability of being phosphorylated by casein kinase II. Using purified lipin 1β, we showed that casein kinase II catalyzed the incorporation of the γ-phosphate of [γ-^{32}P]ATP into the protein. We then focused on the serine-rich domain of lipin 1β, which in lipin 1α has been shown to be important in subcellular localization through interaction with 14-3-3 protein. We generated a 22-residue peptide that included 7 putative serine/threonine phosphorylation sites, and found that this peptide was phosphorylated by casein kinase II. Phosphorylation analysis

of the peptide with alanine substitutions for the putative sites indicated that Ser-285 and Ser-287 were the targets of the kinase. In full-length lipin 1β, the S285A mutation caused a greater degree of phosphorylation, whereas the extent of phosphorylation of the S285A/S287A mutant was about the same as that of the control wild type protein. In phosphopeptide mapping analysis, two phosphopeptides present in the wild type protein were absent in both mutant proteins, but one phosphopeptide present in the mutants was absent in the wild type protein. These observations indicate that the phosphorylation by casein kinase II is complex. This work increases our understanding of the posttranslational modification of lipin 1β by phosphorylation.

ACKNOWLEDEMENTS

I would like to thank my advisor, Dr. George M. Carman, for his guidance and support. I am grateful to Dr. Thurl Harris and Mitchell Granade at the University of Virginia for providing the protein preparations used in this research. I would also like to thank my laboratory colleagues Dr. Gil-Soo Han, Dr. Wen-Min Su, Dr. Prabuddha Dey, Dr. Yeonhee Park, Dr. Joanna Kwiatek, and Dr. Mona Mirheydari, for their assistance with this work. Special thanks to Dr. Azam Hassaninasab for her assistance with phosphopeptide mapping. Lastly, I am grateful to my committee members, Dr. Judith Storch and Dr. Malcolm Watford. This work was supported by National Institute of Health grant GM050769.

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LIST OF ABBREVIATIONS

ACC1	Acetyl-CoA carboxylase
AFLD	Alcoholic fatty liver disease
AGPAT	Acylglycerol 3-phosphate acyltransferase
aP2	Adipocyte protein 2
ATP	Adenosine triphosphate
BMI	Body mass index
CCT	CTP-phosphocholine cytidylyltransferase
CDG-DAG	Cytidine diphosphate diacylglycerol
CDK	Cyclin-dependent kinase
C/EBPα	CCAAT/enhancer-binding protein α
CK	Choline kinase
CKI	Casein kinase I
CKII	Casein kinase II
CL	Cardiolipin
CPT	Choline phosphotransferase
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DGK	Diacylglycerol kinase
ECT	CTP-phosphoethanolamine cytidylyltransferase
EK	Ethanolamine kinase
EPT	Ethanolamine phosphotransferase

FA	Fatty acid
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthase
fld	Fatty liver dystrophy
GPAT	Glycerol 3-phosphate acyltransferase
Gro-3-P	Glycerol 3-phosphate
HAD	Haloacid dehydrogenase
HIV	Human immunodeficiency virus
KO	Knockout
Lyso-PA	Lysophosphatidic acid
MAG	Monoacylglycerol
MGAT	Monoacylglycerol acyltransferase
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
NAFLD	Non-alcoholic fatty liver disease
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PEPCK	Phosphoenolpyruvate carboxykinase
PG	Phosphatidylglycerol

PGC-1α	PPARγ coactivator-1α
PI	Phosphatidylinositol
PKA	Protein kinase A
PKC	Protein kinase C
PPARα	Peroxisome proliferator-activated receptor α
PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
PSS	Phosphatidylserine synthase
RNA	Ribonucleic acid
RNAi	RNA interference
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerol
UAS_{INO}	Inositol-sensitive upstream activating sequence
WT	Wild type

CHAPTER 1

INTRODUCTION

Triacylglycerol (TAG) is a storage lipid that serves as an energy reservoir; its structure consists of a glycerol backbone with fatty acids esterified at all three positions (1,2). TAG is the major neutral lipid stored in lipid droplets, along with small amounts of cholesteryl esters and retinyl esters (3,4). Under normal conditions, lipid droplets are stored mainly within adipose tissue, where they can be hydrolyzed to release fatty acids for energy. However, in the obese condition, there is an excessive amount of TAG synthesis and a resulting need for its storage; excessive amounts of lipid droplets are then stored in the muscle, liver, pancreas, and other tissues, leading to disease.

Obesity is a global health crisis. The World Health Organization (WHO) reports that in 2016, a total of 1.9 billion adults worldwide were overweight (defined as a body mass index [BMI] > 25 kg/m^2), while 650 million were obese (BMI > 30 kg/m^2) (5). Furthermore, a total of 41 million preschool-aged children worldwide were overweight (5). Within the United States, in 2016, all states had a rate of adult obesity greater than 20%, with five states having a rate of adult obesity greater than 35% (**Fig. 1**) (6). Obesity was once thought of as a problem mainly in developed countries, but due to the increasing availability of "Western-style" processed and convenience foods, this epidemic has spread to developing nations as well, where traditional diets are becoming increasingly rare (5).

Obesity has a number of significant physiological consequences. It increases the risk for multiple serious and potentially fatal diseases, including cardiovascular disease,

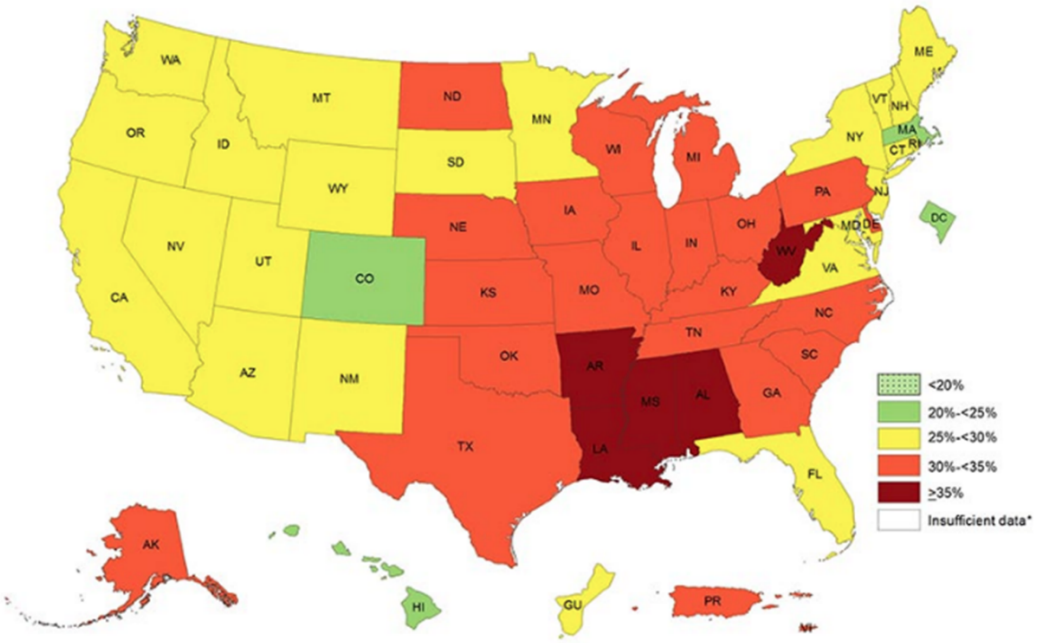

Figure 1. **Prevalence of self-reported obesity among U.S. adults in 2016.** Data taken from Ref. (6).

diabetes, non-alcoholic fatty liver disease, and some types of cancer. To give just one example of the prevalence of obesity-related disease, within the United States alone, a total of 30.3 million individuals are estimated by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) to have diabetes, with a resulting economic burden of $327 billion (7). The prevalence of obesity and obesity-related co-morbidities has made research into the mechanisms involved crucial.

The glycerol 3-phosphate pathway, initially described by Eugene Kennedy, is the major pathway to TAG synthesis in mammals (8). This pathway, illustrated in **Fig. 2**, consists of four steps: the conversion of glycerol 3-phosphate (Gro-3-P) to lysophosphatidic acid (LysoPA) via acylation by glycerol 3-phosphate acyltransferase (GPAT); the conversion of LysoPA to PA via acylation by acylglycerol 3-phosphate acyltransferase (AGPAT); the conversion of PA to DAG (diacylglycerol) via dephosphorylation by PAP (phosphatidic acid phosphatase); and the conversion of DAG to TAG via acylation by diacylglycerol acyltransferase (DGAT). Mammals also have a secondary pathway to TAG synthesis, which takes place in the small intestine, where dietary monoacylglycerol (MAG) is esterified in enterocytes by monoacylglycerol acyltransferase (MGAT) and DGAT (**Fig. 2**) (9).

PAP, the enzyme that catalyzes the penultimate step in the glycerol 3-phosphate pathway, has emerged as a major regulator of TAG levels, and, potentially, of adiposity and obesity. This work focuses on PAP, also called lipin (encoded in mammals by the gene *LPIN*), and its critical role in lipid metabolism and maintenance of lipid homeostasis.

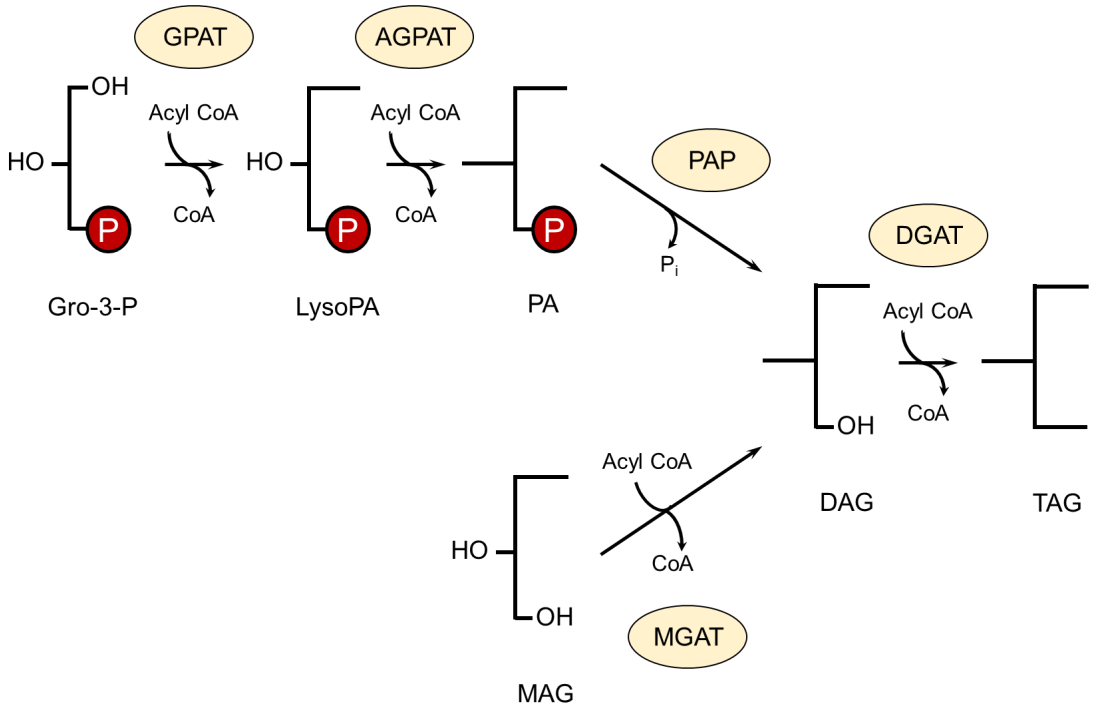

Figure 2. Synthesis of TAG. In the *de novo* pathway, Gro-3-P is converted to LysoPA via acylation by GPAT. LysoPA is converted to PA via acylation by AGPAT. PA is converted to DAG via dephosphorylation by PAP. DAG is converted to TAG via acylation by DGAT. In the MAG pathway, MAG is converted to DAG via acylation by MGAT; this DAG is then acylated to TAG via DGAT.

1.1. Role of PAP in Lipid Metabolism

1.1.1. The dual function of the PAP enzyme

As discussed above, PAP is the enzyme responsible for catalyzing the dephosphorylation of PA to DAG (10,11). In addition to forming TAG as part of the *de novo* synthesis pathway, DAG can also be used to form the zwitterionic phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE); PC and PE can then be converted to phosphatidylserine (PS), as discussed in further detail below (10,12). The PAP substrate PA can also be converted to CDP-DAG (cytidine diphosphate diacylglycerol), which is then used to form the anionic phospholipids phosphatidylglycerol (PG), phosphatidylinositol (PI), or cardiolipin (CL) (13). PAP's role in phospholipid synthesis is discussed in detail below.

In addition to its role as a PAP enzyme, lipin also plays a role in the control of lipid metabolism through transcriptional regulation. For example, lipin is able to regulate fatty acid oxidation through interaction with peroxisome proliferator-activated receptor α (PPARα) and PPARγ coactivator-1α (PGC-1α) (14,15). This direct interaction activates PPARα-PGC-1α genes during fasting, promoting hepatic fatty acid oxidation (15). Lipin can also act as a gene repressor; an example is the inhibition of nuclear factor of activated T cells c4 (NFATc4) in adipocytes (16,17). In most cases, the transcriptional regulatory role of lipin is separate from its role as a PAP enzyme, and this regulatory role can be carried out in the absence of PAP activity; however, the ability of lipin to repress sterol regulatory element binding protein 1 (SREBP1) requires both functions to be intact (18,19). In addition to directly interacting with target genes, lipin PAP activity also plays a role in

transcriptional regulation through the control of its intermediates, PA and DAG, which then act as signaling molecules in lipid metabolism (12).

This dual function of lipin (as a PAP enzyme and a transcriptional regulator) has led to its emergence as a key regulator of lipid metabolism.

1.1.2. PAP in phospholipid synthesis

PA and DAG serve as the precursors for the phospholipids, a class of lipids that are the major structural components of cell membranes (**Fig. 3**) (10). As part of the membrane bilayer, phospholipids help to determine the fluidity of a membrane, as well as to regulate the proteins that are able to bind to a given membrane (20). In addition, phospholipids also have regulatory functions due to their ability to be converted into such secondary messengers as arachidonic acid and the phosphatidylinositol phosphates (20-22).

In eukaryotes, the major phospholipid in cell membranes is PC, followed by PE, while PE makes up the majority of cell membranes in prokaryotes (20). These phospholipids are both derived from DAG via parallel pathways (**Fig. 4**). Choline is phosphorylated by choline kinase (CK), and then converted to CDP-choline by CTP-phosphocholine cytidylyltransferase (CCT). CDP-choline is then combined with DAG to form phosphatidylcholine. Similarly, ethanolamine is phosphorylated by ethanolamine kinase (EK), converted to CDP-ethanolamine by CTP-phosphoethanolamine cytidylyltransferase (ECT), and then combined with DAG to form PE. PE can also be converted to PC via a series of methylations catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT).

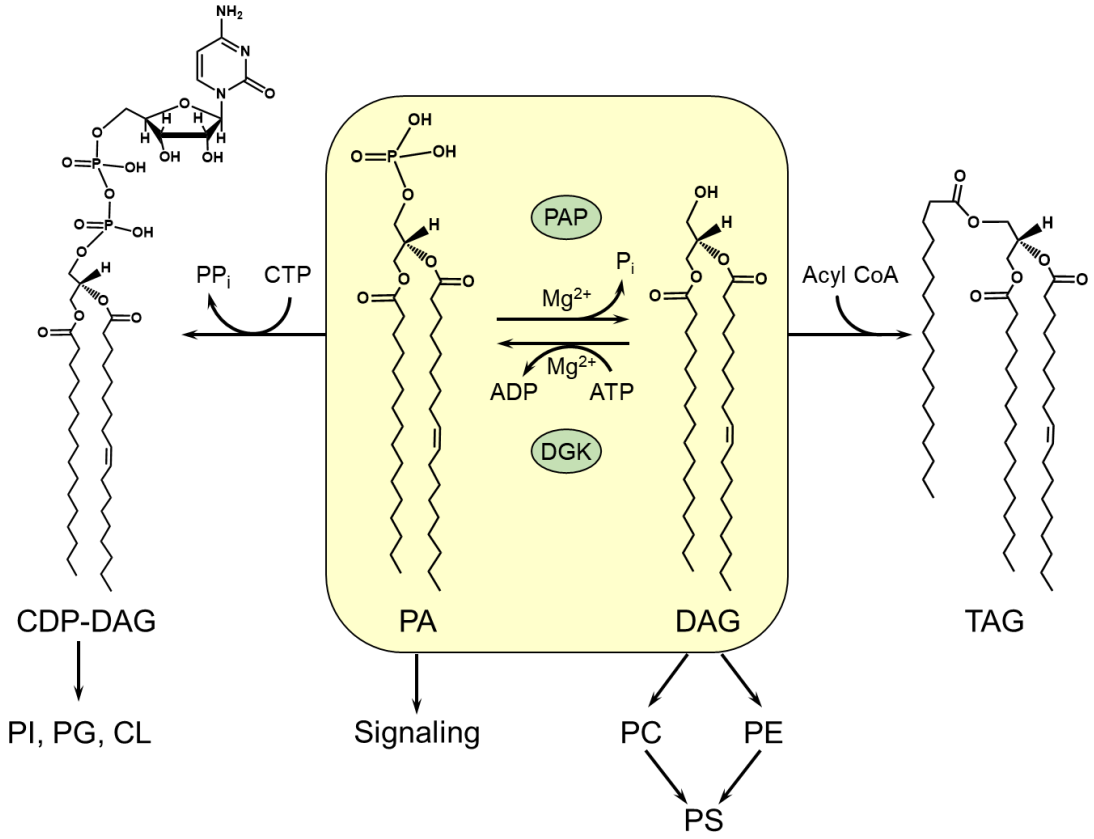

Figure 3. Overview of the role of PAP in lipid metabolism. PAP controls the conversion of PA to DAG, a reaction that is a branchpoint in lipid metabolism. PA can be converted to CDP-DAG, which can then be used to make the phospholipids PI, PG, and CL. DAG can be converted to TAG or used to make the phospholipids PC, PE, and PS. PA also functions as a signaling molecule. Diacylglycerol kinase (DGK) catalyzes the conversion of DAG to PAP.

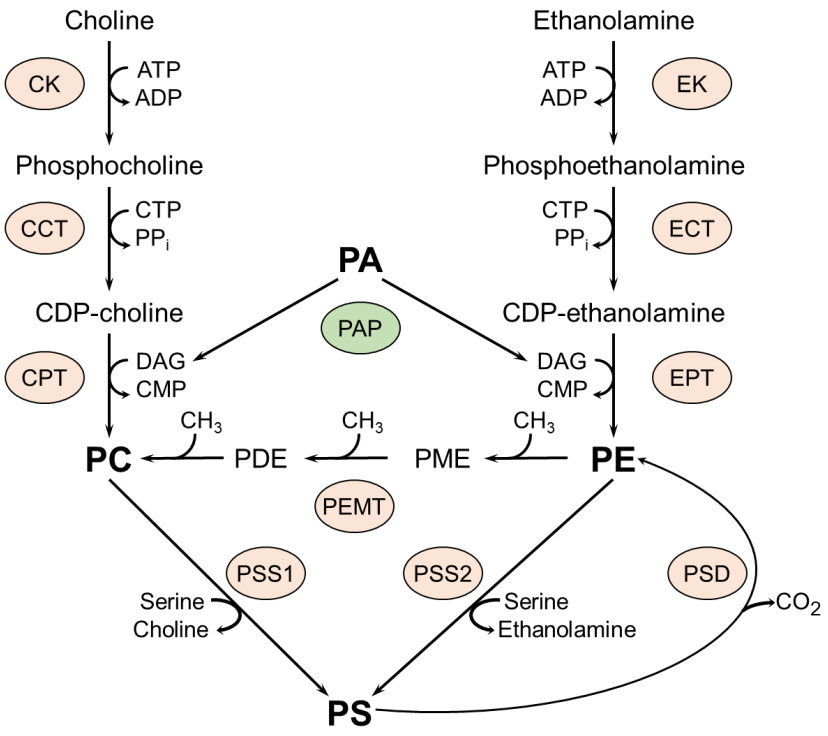

Figure 4. Synthesis of PC and PE by the Kennedy pathway, and synthesis of PS. PC and PE are formed by parallel pathways wherein choline and ethanolamine, respectively, are combined with DAG. PE can be converted to PC via a series of methylations catalyzed by PEMT. PC and PE can be converted to PS via base-exchange reactions catalyzed by PSS-1 and PSS-2, respectively. PS can be decarboxylated to PE by PSD.

In mammals, phosphatidylserine is synthesized from either PC or PE via calcium-dependent base-exchange reactions (23). These reactions are catalyzed by PS synthase 1 or 2; PSS-1 converts PC to PS, while PSS-2 converts PE to PS (24-26). Tissue distribution of the two PS synthases varies, with PSS-1 mRNA being most highly expressed in brain, kidney, and liver, and PSS-2 mRNA being most abundant in Sertoli cells (27,28). Additionally, PS can be converted to PE via a decarboxylation reaction catalyzed by PS decarboxylase (PSD) (29).

PA can also be converted to CDP-DAG by CDP-DAG synthase, a reaction that takes place primarily in the ER (30). CDP-DAG is used to make PI (by PI synthase in ER membranes) and CL (by CL synthase in mitochondrial membranes) (30). CDP-DAG is also the precursor of PG via a two-step reaction that consists of the synthesis of phosphatidylglycerol phosphate (PGP) by PGP synthase, followed by the dephosphorylation of PGP to PG by PGP phosphatase (30). These three phospholipids that arise from CDP-DAG are less abundant than PC, PE, and PS, but still play critical roles in membrane function.

As discussed above, PAP controls the flux between PA (the precursor to CDP-DAG, PG, PI, and CL) and DAG (the precursor to PE, PC, and PS), and in doing so, is a major regulator of phospholipid synthesis. Phospholipid membrane composition, which varies between cell types, subcellular organelles, and even membrane bilayers, is a crucial determinant of cell function (30). By controlling available levels of precursors, PAP plays a critical role in phospholipid synthesis and homeostasis.

1.2. Basic Structure and Function of Lipin

1.2.1. Structure and localization

Unlike the other proteins involved in TAG synthesis, lipin is not an integral membrane protein (12,31). Lipin 1 is found in the endoplasmic reticulum (ER), nucleus, and cytosol; its localization is determined primarily by its phosphorylation state, with phosphorylated lipin being found primarily in the cytosol and dephosphorylated lipin being found primarily in the membrane fraction (14,32,33). Insulin- and mTOR (mechanistic target of rapamycin)-dependent phosphorylation decreases nuclear import of the enzyme as a result of interactions with 14-3-3 proteins (16,34). It appears that the nuclear localization signal (NLS) for the enzyme is located within its polybasic domain (16,35).

The subcellular localization of lipin is related to its role as both a PAP enzyme and a transcriptional regulator. The PAP reaction takes place at the ER membrane, and *in vitro*, the presence of fatty acids causes lipin to translocate to the membrane (12,36,37). Conversely, the transcriptional regulation role of lipin requires it to be able to translocate to the nucleus.

1.2.2. Types of lipin and their biochemical regulation

In mammals, lipin is present in three paralogues: lipins 1, 2, and 3, expressed by the genes *LPIN1, LPIN2,* and *LPIN3*. All three members of the lipin family are able to dephosphorylate PA, but lipin 1 has significantly higher PAP activity than the other two lipins (31,38). The three family members act cooperatively, but silencing lipin 1 has been shown to decrease PAP activity by 95% (39). All three lipin PAP enzymes are specific for PA as a substrate (38).

Structurally, the lipin proteins contain two highly conserved domains, the NLIP domain (located in the NH_2-terminal region) and the CLIP domain (located in the COOH-terminal region) (40) (**Fig. 5**). The PAP active site motif, D*X*D*X*T, is contained within the CLIP domain; this motif marks the lipin enzymes as belonging to the haloacid dehalogenase enzyme superfamily (11) (41). Both the CLIP and NLIP domains are necessary for PAP activity (33).

Lipin 1, the best characterized of the mammalian lipins, is present in three isoforms as a result of alternative splicing: α, β, and γ. Lipin 1α has the highest specific activity of the three isoforms; however, lipin 1γ has the greatest affinity for PA (14). All three isoforms are dependent on Mg^{2+} or Mn^{2+} (with Mg^{2+} being preferred), and all are inhibited by N-ethylmaleimide, Ca^{2+}, and Zn^{2+} (14). All three isoforms have molecular masses between 100 and 115 kDa (14). The isoforms differ in their predominant cellular localization, tissue distribution, and functions. Unlike lipin 1α and lipin 1β, which are found in most tissues that express lipin 1 (although their relative expression levels vary), lipin 1γ is found mainly in brain tissue (12) (42).

In studies performed in mature 3T3-L1 adipocytes, lipin 1α is primarily found in the nucleus, where it induces genes involved in adipocyte differentiation (such as *PPARγ, C/EBPα,* and *aP2*); lipin 1β is primarily found in the cytoplasm, where it induces genes required for lipid biosynthesis (such as *ACC1*, *FAS*, *SCD1*, *PEPCK*, and *DGAT*) (16) (43). Lipin 1β contains a 33-amino acid stretch, located from residues 242 – 275, that is lacking in lipin 1α, a result of alternative splicing of exon 7 (43). In mammals, this alternative splicing is regulated by the RNA splicing factor SFRS10, a homolog of drosophila

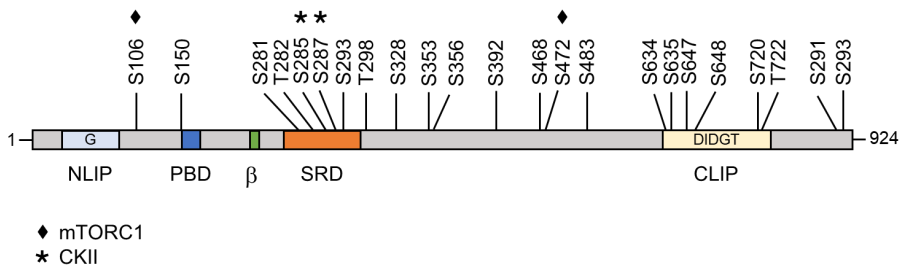

Figure 5. Structure of murine lipin 1. G refers to a conserved glycine in the NLIP region. PBD refers to the polybasic domain containing the nuclear localization sequence. β refers to the stretch of amino acids (residues 242-275) that is present in lipin 1β but lacking in lipin 1α. SRD refers to the serine-rich domain, which contains Ser-285 and Ser-287. DIDGT refers to the HAD-like domain in the CLIP region. Ser-106 and Ser-472 have been shown to be phosphorylated by mTORC1. In this work, Ser-285 and Ser-287 have been shown to be phosphorylated by CKII.

transformer-2 (Tra-2) (44). It has been shown that obese humans have lower levels of *SFRS10* in liver and skeletal muscle; this correlates with an increase in the proportion of lipin 1β isoform in cultured HepG2 cells with an siRNA-mediated knockdown of SFRS10, and a resulting increase in lipogenic gene expression (44).

1.2.3. Degradation of lipin 1

Lipin 1 is degraded by the ubiquitin-proteasome system (45) (46). Specifically, lipin 1 is a substrate for β-transducin repeat-containing protein (β-TRCP), a variable F-box protein that is a subunit of the SCF (Skp1/Cullin1/F-box protein) E3 ubiquitin ligase complex (45). It has been shown that β-TRCP interacts with lipin 1 via lipin 1's degron motif, and requires serine residues within this degron motif to be phosphorylated by mTORC1 (mTOR complex 1) (45). Glycogen synthase kinase-3 (GSK-3), a ubiquitously expressed serine/threonine kinase similar to CKI, may also be able to phosphorylate this motif and play a role in lipin 1 stability (46). The β-TRCP degron motif, DSG*XX*S, is located between amino acids 448 and 453 in human lipin 1, and is highly conserved among mammals (46). β-TRCP, therefore, may play a role in upregulating hepatic lipogenesis by promoting degradation of lipin 1, which leads to a dysregulation of SREBP-dependent transcription, particularly when mTORC1 activity is high (45). However, a *β-TRCP*$^{-/-}$ knockout mouse model shows no difference from wild-type mice in its fasting glucose or insulin concentration or body weight under normal conditions, indicating that this pathway may be more important in conditions of metabolic stress (45).

1.3. Mutations in Lipin Proteins

1.3.1. Lpin1 mutation in mice (fld phenotype)

In mice, a naturally occurring mutation of *Lpin1* resulting in a complete loss of lipin 1 protein leads to a fatty liver dystrophy (*fld*) phenotype (40). These mice display a loss of body fat, insulin resistance, hepatic steatosis, and neurological symptoms such as peripheral neuropathy and decreased nerve conduction velocity (40) (47). These neurological symptoms are the result of the buildup of PA activating the MEK-ERK pathway, leading to degradation of myelin and proliferation of Schwann cells (48) (49). Conversely, overexpression of the *Lpin1* gene in mice causes the mice to become obese, but, interestingly, also improves their insulin sensitivity (43) (48).

The lipodystrophy phenotype shown by *fld* mice is severe, with a 90% loss of total white adipose tissue when compared with wild type (WT) mice (12,50). The adipocytes of *fld* mice are unable to differentiate as a result of a decreased expression of transcription factors such as PPARγ and the buildup of PA in preadipocytes (12) (51). It has been shown that adipocyte differentiation can be restored in these *fld* preadipocytes by forced expression of lipin 1, but not by expression of a lipin 1 protein that lacks PAP activity (12) (52). Studies done in mice that express a variant of lipin 1 with intact coactivator function but no PAP activity further confirm that it is the PAP activity of lipin 1 that is necessary for adipocyte differentiation (12) (53). In addition to this decrease in adipocyte differentiation, *fld* mice also show an increase in PA and a decrease in TAG (12) (52).

In rats, a *Lpin1*^{1Hubr} model animal has been generated that produces a lipin 1 protein that retains transcriptional regulation activity but has no PAP activity (54). These rats show a mild lipodystrophy, peripheral neuropathy, and muscle wasting that is less severe than

the phenotype shown by *fld* mice (54). Interestingly, these rats show an improvement in their phenotypes as they age, which does not occur in *fld* mice (54). It is possible that this attenuation of symptoms may be the result of compensation by other biochemical pathways as the animal ages (54).

1.3.2. LPIN1 mutations in humans

Unlike in mice, the loss of lipin 1 in humans does not lead to changes in adipose tissue development; instead, it causes recurrent episodes of rhabdomyolysis in childhood. These episodes, often brought on by metabolic stress such as febrile illnesses, fasting, anesthesia, or exercise, are severe and are fatal in approximately one-third of cases; the cause of death is cardiac arrest (55) (56). Most patients with this condition have null allele mutations, meaning that they produce no lipin 1. However, there have been rare missense mutations (such as R725H) described in which a lipin 1 protein is made that is able to act as a co-activator, but has no PAP activity; this mutation leads to the same disease state as the null allele mutation, indicating, as in mice, that it is the lack of PAP activity that causes the symptoms (12) (57).

Lipin 1 is the main lipin isoform expressed in skeletal muscle and the myocardium (12) (58) (59). Muscle biopsies of patients affected by lipin 1 deficiency show an increase in the amount of neutral lipid droplets within their muscle cells, particularly during episodes of inflammation. These biopsies also revealed a significant increase in free fatty acids within the myoblasts, but no change in total TAG or phospholipid content (60). Similarly, *fld* mice subjected to a fasting and refeeding protocol demonstrated an accumulation of lipid droplets in muscle tissue; however, their muscle tissue contained very little TAG while levels of cholesteryl esters were nearly double that of WT mice (56). The ability of

human muscle cells to maintain normal levels of TAG despite loss of lipin 1 may be due to an upregulation of lipin 2, which also has PAP activity, in the muscle cells of these patients (60). It is thought that under normal conditions, this upregulation of lipin 2 is sufficient to compensate for the lack of lipin 1, but as energy needs increase under conditions of metabolic stress and inflammation, this compensation is no longer adequate. In addition to the increase in neutral lipid droplets, muscles of patients with lipin 1 deficiency also show an upregulation in expression of *ACACB,* the gene encoding acetyl-CoA carboxylase beta (60). ACACB is a key regulator in the balance between fatty acid oxidation and synthesis, and its upregulation leads to an accumulation of free fatty acids in muscle cells (60). It appears that the upregulation of inflammatory cytokines during periods of metabolic stress is the trigger for the symptoms experienced by these patients (60).

As discussed above, *fld* mice show primary defects in adipose and nerve tissue rather than in muscle tissue. However, it has been shown that subjecting these mice to metabolic stress can lead to symptoms similar to those found in humans with lipin 1 deficiency. Fasting and refeeding causes *fld* mice to show increased levels of creatine kinase, as well as buildup in the myocytes of cholesteryl esters and PA (12) (56). Additionally, mouse studies have shown that lipin 1 is needed for autophagy, a process by which abnormal organelles are removed from cells (56). These studies identified lipin 1 as an activator of the protein kinase D-Vps34 phosphatidylinositol 3-kinase signaling cascade, which is needed for autolysosome maturation and effective autophagy (56). Mice with lipin 1-related myopathy demonstrate misshapen and enlarged mitochondria, which are unable to be properly cleared due to the dysfunction in the autophagy pathway (56). A possible explanation for why human patients lacking lipin 1 have normal muscle function in

between episodes of rhabdomyolysis is that their mitochondria are able to keep up with everyday demand, but that metabolic stress creates a demand for autophagy that they are unable to meet (56).

Although cardiac arrest is the main cause of death during crisis episodes, when patients with *LPIN1* mutations are in between episodes they typically show no obvious cardiac pathology (59). A recent study of eight pediatric patients with *LPIN1* mutations found that seven of these patients, when examined outside of a rhabdomyolysis episode, had normal cardiac function at rest; the eighth patient had signs of minor ventricular systolic dysfunction on echocardiogram (59). However, four of these patients also underwent cardiac ^{1}H-magnetic resonance spectroscopy and were found to have significant myocardial steatosis, as determined by comparing their triglyceride/water ratio to controls; in unaffected individuals, the triglyceride/water ratio was approximately 0.3, while in affected patients, it was approximately 0.95 (59) The reason for this accumulation of TAG in the heart is unclear. Previous work has also shown that *fld* mice have decreased cardiac function, although interestingly, ex vivo studies of perfused *fld* hearts have found normal cardiac function as well as normal glucose and oleate oxidation, indicating that the in vivo cardiac dysfunction is related to systemic effects of lipin 1 deficiency (61).

The lack of lipodystrophy or obvious histological adipocyte changes in humans without lipin 1 has been a matter of some speculation. Recently, a gene expression analysis of adipose tissue from patients with inactivating *LPIN1* mutations found that their adipocytes showed normal lipid composition and normal differentiation, but were slightly smaller than controls (62). These adipocytes showed no upregulation of the expression of *LPIN2* or *LPIN3,* but did show increased expression of *SREBP1, PPARG,* and *PGC1A,* indicating

that increased levels of these adipogenic regulators may be compensating for the loss of *LPIN1* and allowing for normal adipocyte differentiation (62).

Interestingly, a role for lipin 1 in lipodystrophy has recently been elucidated in HIV patients undergoing antiretroviral therapy (ART). HIV patients being treated with ART commonly show changes in adipose tissue distribution, with a characteristic reduction of subcutaneous fat in the face and limbs; some also show increased fat tissue deposition in the neck and abdomen (63). It has been shown that patients that display this lipodystrophy have lower levels of lipin mRNA expression when compared with patients who maintain normal fat distribution despite ART treatment (63). Affected lipodystrophic patients show similar lipin mRNA expression profiles in adipose tissue to *fld* mice. In addition, lipodystrophic patients with lower lipin expression also had higher levels of inflammatory cytokines in their adipose tissue, reinforcing a role for lipin in inflammation which will be further discussed below (63).

1.3.3. Lpin2 mutations in mice

A mouse model of lipin 2 deficiency (*Lpin2*KO mice) has elucidated further information about the physiological roles of lipin 2. Lipin 2 is the most abundant lipin protein in mouse liver (37); however, in *Lpin2*KO mice, hepatic PAP activity is increased despite the full loss of lipin 2 (64). This is the result of compensation by lipin 1, which occurs at the protein level rather than the mRNA level, and leads to hepatic lipid homeostasis in *Lpin2*KO mice fed a chow diet (64). However, when *Lpin2*KO mice were fed a high-fat diet, they showed greater hepatic stores of TAG when compared to WT mice (64). It appears from these results that in normal feeding conditions, lipin 1 is able to

compensate for the loss of lipin 2 in the liver, but under dietary stress, the compensation is no longer adequate (64).

*Lpin2*KO mice are phenotypically normal until about six months of age, at which time they develop ataxia, tremors, and difficulty maintaining balance (64). It was found that although young *Lpin2*KO mice had normal levels of PA in their cerebellum, older symptomatic *Lpin2*KO mice had a 50% increase in cerebellar PA, although their cerebellar DAG levels remained normal (64). When the cerebellum of normal WT mice were examined, it was found that when these mice were young, their cerebellum contained both lipin 1 and lipin 2, but as they aged (10-12 months old), lipin 1 levels were nearly undetectable while lipin 2 remained (64). This indicates that with age, lipin 2 becomes the predominant lipin enzyme in the brain; in *Lpin2*KO mice, lipin 1 expression levels did not increase to compensate, leading to a buildup of PA and resulting neurological symptoms (64).

Although mutations causing the loss of either lipin 1 or lipin 2 result in pathology, which can be quite severe particularly in the case of lipin 1 loss in humans, there is sufficient compensation in both cases to allow survival. However, the loss of both lipin 1 and lipin 2 in mice leads to embryonic lethality, with embryos dying by approximately 12 days post conception, indicating that lipin 3 is not able to compensate for the loss of both lipin 1 and lipin 2 (64).

1.3.4. LPIN2 mutations in humans (Majeed syndrome)

The loss of lipin 2 in humans causes Majeed syndrome, a rare auto-inflammatory disease with such symptoms as bone lesions, fevers, skin lesions, and anemia (12) (65). As with lipin 1 deficiency, it has been shown that these symptoms are the result of the lack of

PAP function, as the missense mutation S734L, which leads to the loss of lipin 2 PAP activity but the retention of transcriptional coactivator function, is one of the mutations that leads to this syndrome (12) (37). Additional mutations have also been identified, such as a frame shift mutation that causes a stop codon to be inserted at residue 180; this leads to production of a non-functional truncated protein (37) (66).

The auto-inflammatory nature of Majeed syndrome has led to an understanding of the role of lipin in inflammation. Studies have shown that knocking down lipin 2 in macrophages increases the production of the pro-inflammatory cytokine interleukin 1β (IL-1β) by activating the NLRP3 inflammasome (67). Lipin 2 mediates this activation at both the first priming signal step, by regulating MAPK (mitogen-activated protein kinase) signaling, and at the second signal step by influencing sensitization of the purinergic receptor $P2X_7$ (67). This correlates with an improvement in symptoms of Majeed syndrome patients when treated with IL-1β blockers (12) (68).

Mice that lack lipin 2 also show a mild anemia and skin lesions, but have not shown bone lesions; it is possible that because these lesions are episodic, they simply have not been observed in mice (12) (64). Unlike in mice, humans with Majeed syndrome have not shown neurological defects (65) (66). However, Majeed syndrome has only been described relatively recently, and the patients studied have been young; it is not known if these patients will demonstrate similar neurological symptoms as they age (64) (66).

The differences between the physiological effects of lipin 1 and lipin 2 mutations in humans serve to illustrate their unique functions. It has been postulated that these differences are based on the different tissue localizations of the two enzymes (12). As discussed above, lipin 1 is the main lipin gene expressed in skeletal muscle, which

coincides with the dysfunction in muscle tissue seen in lipin 1 mutations. However, this correlation is not as clear with lipin 2, which is expressed at its highest levels in intestine and liver; these tissues are not affected by Majeed syndrome (12) (58).

1.3.5. Lpin3 mutations in mice

Lipin 3, the least studied of the three lipin enzymes, may be critical to adipose tissue function. Like lipin 1, lipin 3 is expressed in adipose tissue, and its expression is induced during adipogenesis (69). Mice with a lipin 3 knockout (*Lpin3*KO) do not show an obvious phenotypic difference when compared to wild type mice, and display comparable body and tissue weights on both chow and high-fat diets; however, as the mice age, they show slightly reduced body weights at around 14-16 months old (69). When stromal vascular cells taken from *Lpin3*KO mice were analyzed, it was found that they had fewer and smaller lipid droplets than WT cells, and *Lpin3*KO adipocytes also had lower expression of adipogenic genes such as *PPARγ* and *FABP4* (69). A double knockout mouse model (*Lpin1/3*KO) lacking both lipin 1 and lipin 3 showed a more severe lipodystrophy phenotype than *Lpin1*KO mice, and PAP activity and TAG accumulation in adipose tissue of these mice were completely abolished (69). These results suggest a cooperative role of lipin 1 and lipin 3 in normal adipogenesis (69). Currently, it is not known whether lipin 3 mutations cause disease in humans (12).

1.4. Physiological Roles

1.4.1. Diabetes and Obesity

Because of lipin's role in TAG synthesis, it has been implicated in the pathogenesis leading to both obesity and diabetes. However, there is relatively little research at this time into the mechanisms behind this association, and results of the recent studies performed

have been somewhat mixed. In one recent study, *LPIN1* mRNA levels were shown to be lower in subcutaneous and visceral adipose tissue in obese human subjects and those with type 2 diabetes, while *LPIN2* mRNA levels were not altered and *LPIN3* mRNA levels were increased (39). *LPIN1* mRNA levels were found to be negatively correlated in these human subjects with BMI, insulin resistance, and plasma TAG levels (39).

Another study examining extremely obese (mean BMI = 60.8 kg/m^2) subjects found that *LPIN1*β mRNA expression in liver and adipose tissue was inversely correlated with plasma insulin levels, insulin resistance, and BMI (70). When these subjects underwent gastric bypass surgery and resulting significant weight loss (mean weight loss = 34.5% of body weight), *LPIN1*β mRNA expression markedly increased by approximately 73% in liver and 2.6-fold in adipose tissue (70). This increase in *LPIN1*β expression was accompanied by a mean reduction of 66.8% in plasma insulin concentration and a mean reduction of 70.7% in HOMA-IR values (a measure of insulin resistance) (70). However, *LPIN1*α mRNA levels did not correlate with BMI or insulin levels, and were not affected by surgery-induced weight loss. These results correlate with a study that found that treatment of insulin-resistant individuals with thiazolidinediones increases *LPIN1*β mRNA levels while improving insulin sensitivity (71). In addition, as discussed previously, *fld* mice, which produce no lipin 1, are insulin resistant, and overexpressing lipin 1 leads to improved insulin sensitivity. These results point to a possible protective role of lipin in insulin homeostasis.

Other studies, however, have shown an opposite role for lipin in obesity and diabetes. *LPIN1* has been shown to be a target gene of pregnane X receptor (PXR), a nuclear receptor that has recently been elucidated as having a role in the regulation of lipid metabolism and

the pathogenesis of type 2 diabetes. Knocking out PXR in mice leads to the downregulation of lipin 1 expression, and also attenuates obesity, insulin resistance, and hepatic steatosis in $PXR^{-/-}$ mice fed a high-fat diet (72). Conversely, over-activation of PXR led to an upregulation of lipin 1 coincident with a worsening of these symptoms. This effect on lipin 1 may be one of the mechanisms by which PXR regulates metabolism (72).

As discussed above, lipin 1 is known to be an activator of the PGC-1α/PPARα pathway (15). Mouse models of type 1 and type 2 diabetes (streptozotocin injection and *db/db,* respectively) were both found to have higher levels of hepatic lipin 1 mRNA than WT mice (15). This upregulation of PGC-1α and lipin 1 in the liver during diabetes is thought to be induced by the increased delivery of fatty acids to the liver in this condition, as it is a similar response to that induced by fasting, which also leads to increased need for hepatic fatty acid oxidation (15). In other words, the increase in hepatic lipin 1 during diabetes may be a pathophysiologic adaptation stemming from the normal physiologic role of lipin 1 in the liver (15).

1.4.2. Cancer

Enzymes involved in the regulation of lipid metabolism have emerged as possible targets for cancer treatment, due to the fact that cancer cells have been shown to have altered lipid metabolism. One of the factors leading to this lipid metabolism dysregulation is the need of cancerous tumors for increased lipid biosynthesis to provide rapidly proliferating cells with building materials for membranes, as well as energy storage and signaling molecules (73) (74). This is evidenced by some types of prostate cancer cells, which do not demonstrate the Warburg effect (a high rate of glycolysis) typically seen in cancer cells, leading them to rely on increased lipid metabolism (73). Lipin 1 has been

found to be upregulated in several prostate cancer cell lines. In a PC-3 prostate cancer cell line, inhibiting lipin 1 led to a decreased cell proliferation rate; similar results were also found in a Hs578T line of breast adenocarcinoma cells (73). Lipin 1 silencing by RNAi also had a negative effect on cell migration in the PC-3 cell line (73). When lipin 1 and lipin 2 were both silenced, the inhibition of proliferation was increased over lipin 1 silencing alone (73). When combined with rapamycin treatment, lipin 1 silencing almost completely prevented PC-3 and Hs578T cells from proliferating; combining propranolol (an inhibitor of the PAP activity of lipin) and rapamycin (an inhibitor of mTOR) had the same effect (73).

LPIN1 expression, as well as the expression of several other phospholipid synthesis genes, is also upregulated in basal-like triple-negative breast cancer (TNBC) cells (75). This upregulation has been shown to correlate with a poor prognosis for TNBC patients. Knocking down *LPIN1* in these TNBC cells leads to cell death, apparently by inducing apoptosis (75). It is thought that the increase in apoptosis is caused by activation of the IRE1a-JNK pathway. These *LPIN1* knockdown TNBC cells also showed a decrease in phospholipid synthesis in the absence of lipin 1, particularly synthesis of sphingomyelin, cardiolipin, and cholesteryl ester (75). Similarly, lipin 1 protein has been found to be upregulated in lung adenocarcinoma (LUAD) cells, and, as in TNBC patients, its upregulation correlates with poor prognosis (76). When LUAD cells were treated with shRNA to knock down lipin 1, the cells demonstrated an increase in apoptosis, dysregulation of autophagy, and increased ER stress (76). Silencing lipin 1 with shRNA and the inhibitor propranolol increased the sensitivity of LUAD cells to treatment with cisplatin, a common chemotherapy drug (76).

Lipin 1 is known to be induced by hypoxia, a condition of low oxygen that is found in cancer cells as well as tissue ischemia, in an HIF (hypoxia-inducible factors)-mediated manner (74). As cancer cells are proliferating in low oxygen conditions (caused by outgrowing their blood supply), they display an increase in lipid droplet accumulation resulting from increased de novo fatty acid synthesis as the cells shift towards an increased level of glycolysis; cancer cells also show an increased uptake of fatty acids and a reduction in β-oxidation of fatty acids (74). In order to avoid lipotoxicity as a result of these metabolic shifts, these cells must store these fatty acids as TAG, a process requiring lipin (74). It has been shown that casein kinase 1 δ (CKIδ) inhibits the activation of HIF-1 under conditions of low oxygen, and this in turn leads to a decrease in lipin 1 expression (74). Inhibiting CKIδ, in turn, was found to increase proliferation of cancerous cells in hypoxic conditions. These results indicate that in addition to its role in providing cancerous cells with membrane components, lipin 1 is also required for the adaptation of cancerous cells to low oxygen conditions (74).

1.4.3. Liver disease

Within the liver, the lipin enzymes are crucial to the regulation of energy homeostasis. Their PAP activity is responsible for the esterification of fatty acids into TAG, which can be stored within the liver or secreted as very low density lipoproteins (VLDL) to be used as an energy source in the peripheral tissues during fasting conditions (77). In addition, as discussed above, lipin is a coactivator of PPARα and PGC-1α, both of which increase the expression of genes that promote fatty acid oxidation (77). Both PGC-1α and hepatocyte nuclear factor 4α (HNF4α) have been shown to regulate the expression of *LPIN1* in HepG2 cells (77). HNF4α is the main transcriptional regulator of hepatic gene expression,

controlling expression of enzymes involved in fatty acid oxidation and VLDL metabolism as well as mediating the hepatic fasting response (77). In addition, overexpression of lipin 1 leads to a modulation of the effects of HNF4α, promoting its fatty acid oxidation effects while decreasing its effects on apolipoprotein synthesis genes, leading to an increase in lipid catabolism (77).

The lipin enzymes are also thought to play a role in hepatic steatosis, a condition in which lipids accumulate in the parenchymal cells of the liver, due to their effects on TAG synthesis (18). Hepatic steatosis is an advanced form of both non-alcoholic fatty liver disease (NAFLD), which is common in obese individuals and is becoming a major cause of liver disease in the US, and alcoholic fatty liver disease (AFLD), further discussed below (18). The majority of PAP activity in the liver is encoded by lipin 2, but hepatic lipin 1 expression is upregulated by such stimuli as fasting, ethanol consumption, and glucocorticoid administration; this is likely due to the need to increase hepatic triglyceride synthesis in these conditions (18). Mice who express a liver-specific truncated lipin 1 protein that has no PAP activity but is still able to co-activate PPARα had no change in their ability to synthesize triglycerides or in their hepatic TAG levels, even when hepatic steatosis was induced with a high-fat diet (18). These mice demonstrated an increase in hepatic expression of lipin 2, the lipin enzyme that is most highly expressed in the liver in normal conditions, and so were able to carry out the PAP reaction in the liver, albeit at <50% of normal rates (18). From these results, the authors concluded that lipin 1's role as a transcriptional regulator, which was intact in these mice, may be as crucial as its PAP activity in hepatic TAG synthesis (18).

Lipin 1 also plays a role in AFLD. Patients with alcoholic liver disease have hepatic inflammation and liver injury, which can progress to cirrhosis and liver failure and can be fatal (78). Lipin 1 mRNA expression has been shown to be upregulated in AFLD, accompanied by an increase in intrahepatic lipid accumulation (79) (80). However, results of studies of the effect of lipin 1 on AFLD have been mixed. A 2013 study by Hu et.al. found that chronic ethanol exposure in mice with a liver-specific *Lpin1*KO lipin 1 knockout led to an increase in hepatic TAG accumulation and a worsening of liver injury when compared with wild type mice, indicating that lipin 1 may play a protective role in AFLD (79). These knockout mice showed a decrease in PGC-1α and a corresponding decrease in PGC-1α target genes, indicating that it may be lipin 1's role as a transcriptional regulator that is protective (79). This same study showed that liver-specific *Lpin1*KO mice showed an increase in pro-inflammatory cytokines and hepatic oxidative stress regardless of whether the mice were exposed to ethanol, pointing to a potential anti-inflammatory role of lipin 1 in the liver (79). Conversely, however, a 2016 study by Wang et.al. demonstrated that mice with a myeloid cell-specific knockout of lipin 1 showed less hepatic inflammation and liver injury than wild type mice after being subjected to a chronic + binge ethanol feeding protocol; however, steatosis was not affected (78). These results point to possible cell type-specific roles of lipin 1 in the inflammatory process.

Lastly, lipin 1 may also mediate the development of liver fibrosis as a result of chronic liver inflammation or injury. Liver injury leads to the activation of hepatic stellate cells (HSCs); in a healthy liver, these cells are storage depots for vitamin A and lipid droplets, but upon activation, they produce the extracellular matrix proteins and α-smooth muscle actin proteins that cause fibrosis (81). Quiescent HSCs have been shown to express high

levels of lipin 1, while lipin 1 expression is downregulated in activated HSCs; this is regulated by transforming growth factor β (TGF-β), which promotes downregulation of lipin 1 via the ubiquitin-proteasome system (81). This study also found that treatment of HSCs with ectopic lipin 1 decreased TGF-β-mediated fibrogenesis, and this effect was increased when HSCs were treated with both ectopic lipin 1 and resveratrol, a natural antioxidant that has shown promise in treating liver injury (81). The authors suggested that lipin 1 may be a potential therapeutic target in liver fibrosis (81).

1.4.4. Inflammation and atherosclerosis

Lipin 1 is also involved in the macrophage-mediated inflammatory responses implicated in atherosclerosis (82) (83). Atherosclerosis results from the creation of foam cells by the internalization of modified low-density lipoproteins (modLDL) by macrophages, leading to inflammation (83). It has been shown that lipin 1 contributes to the glycerolipid synthesis needed for modLDL to be converted to foam cells (82) (83). When mice with a myeloid cell-specific loss of lipin 1 were fed a high fat diet, they showed reduced rates of atherosclerosis when compared with controls, as well as lower levels of circulating TNF-α (tumor necrosis factor α), an inflammatory cytokine (83). It appears that lipin 1 modulates foam cell formation by regulating a DAG-mediated signaling cascade that leads to macrophages responding to and engulfing modLDL (83).

1.5. Posttranslational Modifications

1.5.1. Phosphorylation

As discussed above, the localization and activation of lipin is determined largely by its phosphorylation state. Phosphorylated lipin is found primarily in the cytosol, while dephosphorylated lipin is found primarily in the membrane fraction (48). Because the PAP

reaction occurs at the ER membrane, phosphorylation deactivates lipin by preventing its association with the ER membrane (33) (48). Mammalian lipin has multiple phosphorylation sites, and its phosphorylation has been shown to be dependent on the insulin and mTOR signaling pathways (33) (48) (84). At least 25 phosphorylation sites in lipin 1 have currently been identified, and a number of potential kinases have been proposed (48) (33) (85). A study performed with a murine lipin 1 enzyme that had 21 serine/threonine phosphorylation sites mutated to alanine showed that this 21xA mutant had similar activity as lipin 1 that had been dephosphorylated by λ-phosphatase, indicating that phosphorylation is a major regulator of PAP activity (85). Lipin is dephosphorylated in mammals by the Dullard-TMEM188 complex (also known as CTDNEP1-NEP1R1) (86).

In addition to controlling subcellular localization of lipin, phosphorylation also affects lipin's ability to recognize and bind to PA. Lipin 1 has been shown to have greater affinity for the di-anionic form of PA (85). PA, a phosphomonoester, has a second pK_a within the physiologic range; hydrogen bonding within the phospholipid bilayer results in the deprotonation of the phosphate of PA and creates a charge of -2 at the PA head group. This hydrogen bonding within the phospholipid bilayer is affected by bilayer composition, specifically the ratio of PC to PE (85) (87). Lipin's greater affinity for di-anionic PA, therefore, means that lipin 1 binding and activity can be affected by both membrane composition and intracellular pH. Phosphorylation of lipin 1 has been proposed to decrease lipin activity both by preventing it from recognizing this di-anionic PA and by inhibiting the PA-binding region found in the polybasic domain of lipin 1. Interestingly, it has been shown that obesity increases the ratio of PC to PE at the ER membrane (from 1.3 to 2.0);

this may alter the charge of PA and lead to aberrations in lipin 1 localization and function (85) (88).

As discussed above, mammalian lipin 1 has multiple phosphorylation sites, two of which (Ser-106 and Ser-472) have been shown to be affected by mTOR. However, because inhibition of mTOR leads to a significant increase in lipin 1 activity, it is unclear whether these other putative phosphorylation sites play a large role in localization of lipin 1 (85). In addition, a lipin 1 protein that has had all of its known phosphorylation sites mutated showed a similar k_{cat} to a lipin 1 protein that had only its mTOR phosphorylation sites mutated, indicating that mTOR may be the most important kinase affecting lipin 1's PAP activity (85).

Lipin 1 contains a serine-rich domain, located from residues 218-260 in murine lipin 1α and from residues 251-293 in murine lipin 1β, that contains a number of putative serine/threonine phosphorylation sites (34). This region has been shown in lipin 1α to be phosphorylated in response to insulin, although the kinase involved is not currently known (34). Phosphorylation of this serine-rich domain leads to interaction of lipin 1α with 14-3-3 proteins, a class of ubiquitously expressed proteins that bind to phosphoserine and phosphothreonine proteins and have roles in cell signaling, control of the cell cycle, apoptosis, and cellular stress response (34). This interaction with 14-3-3 promotes cytosolic retention of lipin 1α, preventing its association with membranes (34).

1.5.2. Acetylation

In addition to phosphorylation, recent research has shown that lipin 1 is also regulated by acetylation. This acetylation is done by the catalytic subunit of the NuA4 acetyltransferase complex, HIV-1 Tat-interacting protein 60 kDa (Tip60) (89). An

experiment with mice who express an S86A mutation in Tip60 (*Tip60*$^{SA/SA}$) that results in a less active form of the protein show significant decreases in adiposity, particularly when fed a high-fat diet (89). Interestingly, *Tip60*$^{SA/SA}$ mice fed a high-fat diet appear to also be resistant to developing hepatic steatosis, and, when compared to wild-type mice on the same high-fat diet, had improved glucose and insulin sensitivity, lower respiratory exchange ratio, and increased energy expenditure (89). Additionally, lactating *Tip60*$^{SA/SA}$ females are unable to produce milk with enough TAG content for their pups to survive, indicating a need for Tip60 in TAG synthesis (89). It appears that Tip60-mediated acetylation is necessary for lipin 1 to be able to translocate to the ER membrane; this acetylation may cause a disorder-to-order shape change in lipin 1 that allows membrane interaction (89).

1.6. Lipin in Yeast

1.6.1. Yeast Pah1

In yeast, the ortholog of lipin, Pah1, has been extensively studied. It was the discovery by Han et.al. in 2006 that the yeast gene *PAH1* encoded a PAP enzyme that led to the identification of the lipins as the enzymes responsible for carrying out the PAP reaction in mammals (11). Unlike in mammals, yeast has only one lipin homolog protein. Structurally, yeast Pah1 shows some homology with mammalian lipin in that both enzymes have conserved NLIP (NH_2-terminal) and CLIP (COOH-terminal) domains (31) (40); also, both the mammalian and yeast enzymes contain a catalytic D*X*D*X*T region within an HAD-like domain (11) (31). Yeast Pah1 has been shown to be inhibited by sphingoid bases, ATP, and CTP (48) (90), while its activity is increased via lowering of its K_m by PI, CL, and CDP-DAG (48) (91).

The role of Pah1 varies according to phases of the yeast cell cycle. During the exponential phase of growth, it shows fairly low activity, with PA going mainly to form phospholipids via CDP-DAG (92) (93) (94). However, during the stationary phase of growth, its activity is higher, with PA going mainly to form TAG via DAG (92) (95). By regulating the cellular pool of PA, Pah1 also controls other phospholipid synthesis genes (48) (96) (97) (98) (99) (100).

As in mammals, yeast Pah1 also has a role in transcriptional regulation. The promoter element UAS_{INO} controls transcription of the genes for enzymes involved in the Kennedy pathway and the CDP-DAG pathway (101) (102), and this promoter element is regulated by levels of PA and therefore by Pah1 (16) (103). Decreases in the levels of PA in the cell lead to the translocation of the PA-binding protein Opi1 into the nucleus; this translocation then leads to a repression in UAS_{INO}-dependent genes. In addition to being controlled by PA levels, the UAS_{INO} pathway is also controlled by the phosphorylation state of Pah1, with the dephosphorylated active form of Pah1 causing a decrease in PA leading to the repression of UAS_{INO} genes (16) (104). This is because a decrease in PA allows Opi1 to enter the nucleus to bind to Ino2 and thus repress expression of the genes (103) (104).

1.6.2. Phosphorylation of Pah1

In *Saccharomyces cerevisiae*, Pah1 is phosphorylated by multiple kinases, including Cdc28-cyclin B, Pho85-Pho80, PKA (protein kinase A), CKII (casein kinase II), and PKC (protein kinase C) (48) (105) (106) (107), and at least 30 phosphorylation sites have been identified (92) (108) (109) (110). Phosphorylation has an inhibitory effect on the enzyme, while dephosphorylation by the Nem1-Spo7 phosphatase complex activates it (92) (104). As with the mammalian enzyme, this inhibition and activation is based on subcellular

localization, with the phosphorylated form of the enzyme being unable to associate with the ER membrane where the PAP reaction takes place. It has been shown that yeast cells lacking the Nem1-Spo7 phosphatase complex show a phenotype similar to that of Pah1 knockout cells described below (48) (104), indicating that the inability to dephosphorylate the enzyme has a similar effect *in vivo* as the complete knockout of the *PAH1* gene. Phosphorylation also controls stability of Pah1, with the dephosphorylated form of the enzyme showing higher susceptibility to degradation by the 20S proteasome (92). It is thought that phosphorylation causes the enzyme to assume a more structured shape, since the phosphorylation target sites are located in unfolded regions of the protein and these regions are known to be more susceptible to degradation (92). However, it has also been found that phosphorylation by PKC has the opposite effect of phosphorylation with PKA, CKII, Pho85-Pho80, and Cdc28-cyclin B; phosphorylation with PKC increases susceptibility of the enzyme to degradation by the 20S proteasome (92).

1.6.3. pah1Δ mutations in yeast

In yeast, Pah1 mutants (*pah1*Δ) show altered lipid metabolism. Levels of PA, PI, and PE increase, while levels of PC, DAG, and TAG decrease (11) (31) (95). As a result, these cells display multiple phenotypic abnormalities. Increased phospholipid synthesis and PA levels lead to expansion of the nuclear and ER membranes (31) (104) (111), which is rescued by the loss of the *DGK1* gene, which catalyzes the conversion of DAG to PA (94) (112) (113) (114). Lipid droplet formation is decreased as a result of the repression of TAG synthesis (86) (94) (113). Additionally, *pah1*Δ cells have shorter life spans than WT cells (115), and show an increased sensitivity to oxidative stress and FA toxicity (94) (115). *Pah1*Δ cells are less able than WT cells to grow at high temperatures or on non-fermentable

carbon sources (11) (104) (116). Lastly, *pah1*Δ cells demonstrate deficiencies in autophagy, cell wall integrity, and vacuole fusion (117) (118) (119) (120) (121).

1.7. Purpose of Current Work

As discussed above, lipin 1 plays a critical role in lipid metabolism. By catalyzing the conversion of PA to DAG, it controls a major metabolic branchpoint. Because it influences TAG synthesis, it is an emerging target of obesity research. It has been implicated in such diverse disease states as diabetes, cancer, and atherosclerosis. The phenotypic severity of mutations in lipin proteins points to its central role not only in adipose tissue, but in whole-body metabolic homeostasis.

Although it is known that the subcellular localization of lipin 1 is largely controlled by phosphorylation, it is not known what kinases, other than mTOR, phosphorylate lipin 1. We hypothesized that lipin 1β would be phosphorylated by additional protein kinases, and that this phosphorylation would affect subcellular localization and, subsequently, PAP activity of lipin 1β. Previous work has shown that lipin 1α contains a serine-rich domain that is phosphorylated by an unknown kinase, and that this phosphorylation leads to interaction with 14-3-3 proteins and subsequent cytoplasmic retention of lipin 1α. We chose to focus on lipin 1β because it is the predominant lipin 1 isoform in adipocytes, and because we hypothesize that the serine-rich domain of lipin 1β would also be a target for phosphorylation as it is in lipin 1α. Three of the residues within the serine-rich domain of lipin 1α that have been shown to be required for interaction with 14-3-3 proteins (Ser-252, Ser-254, and Ser-260) are found within casein kinase II (CKII) motifs. Accordingly, we hypothesized that lipin 1β (where the corresponding residues Ser-285, Ser-287, and Ser-293 are also found within CKII motifs) would be phosphorylated by CKII.

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. Materials

All chemicals were reagent grade. CKII was purchased from New England BioLabs. Peptides were prepared by EZ BioLabs. Radiochemicals were purchased from PerkinElmer Life Sciences and National Diagnostics. Nitrocellulose paper and P81 phosphocellulose paper were purchased from Sigma-Aldrich and Whatman, respectively. Markers used in electrophoresis were purchased from Bio-Rad and Sigma-Aldrich. Bio-Rad was the source of the electrophoresis kits and Coomassie Brilliant Blue stain. PKA was purchased from Promega. CKI was purchased from ThermoFisher Scientific. PKC was purified from *S. cerevisiae* as described previously (122). Cellulose thin-layer chromatography plates and L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin used in phosphopeptide mapping were purchased from Merck and Sigma-Aldrich, respectively.

2.2. Purification of Lipin 1β Proteins

Lipin 1β proteins (wild type, S285A mutant, and S285A/S287A mutant) were purified by our collaborators, Mitchell Granade and Thurl Harris at the University of Virginia, using FLAG immunoprecipitation as described elsewhere (123). HeLa cells (human cervical cancer cells) were cultured in DMEM for 48-72 hours and infected with FLAG-tagged lipin constructs via an adenovirus vector (123). Cells were harvested, homogenized, and centrifuged, and the resulting lysates were incubated with FLAG beads for 4 hours (123). Beads were then separated, incubated in phosphatase buffer with or without λ protein phosphatase, loaded onto an affinity column, and washed 10 times in

buffer (123). To elute the lipin 1β proteins, five additions of FLAG peptide were performed, and the proteins were dialyzed without detergent (123). To determine protein concentrations, purified lipins were run on an SDS-polyacrylamide gel with bovine serum albumin standards and then stained with Coomassie Blue dye (123).

The S285A and S285A/S287A mutations were made using PCR site-directed mutagenesis in the pcDNA3 vector with a FLAG-tagged lipin 1β insert. The mutagenesis was confirmed by sequencing with a reverse primer starting at position 1400 in the lipin 1β sequence. Next, the mutated lipin 1β was placed into an adenoviral vector, using recombination between the pAdTRACK-CMV shuttle vector and pAdEasy; this produced the adenovirus used for expression and purification.

2.3. Phosphorylation Reactions

The protocol for phosphorylation reactions has been described previously (107) (122). Phosphorylation reactions were performed by analyzing the amount of radiolabeled phosphate transferred from [γ-^{32}P]ATP to the substrate by the relevant enzyme. For CKII, the reaction mixture contained a buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol) and indicated amounts of lipin 1β or peptide, CKII, and [γ-^{32}P]ATP in a total volume of 20 μL. For PKA, the reaction mixture contained a buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol), 100 μM peptide, indicated amounts of PKA, and 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) in a total volume of 20 μL. For PKC, the reaction mixture contained a buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5 mM PS, and 150 μM DG), 100 μM peptide, 2.5 ng PKA, and 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) in a total volume of 20 mL. For CKI, the reaction mixture contained a buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, and 10

mM β-mercaptoethanol), 100 μM peptide, 6.5-22.6 ng CKI, and 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) in a total volume of 20 μL.

For each reaction, tubes were equilibrated in a water bath at 30°C for at least five minutes. At set intervals, enzyme was added to each tube to start the reaction, and the reaction was run in a water bath at 30°C for 15 minutes (unless otherwise indicated). The reaction was terminated by transferring 15 μL from each reaction to paper (nitrocellulose paper was used for full-length lipin 1β and P81 phosphocellulose paper was used for peptides). This paper was allowed to dry for at least 15 minutes, and then was washed in a 75 mM phosphoric acid buffer to remove excess radiation. The buffer was changed at 2 minutes, 4 minutes, 30 minutes, and 60 minutes. After 60 minutes, the paper was added to scintillation vials with 4 mL of scintillation fluid added to each vial. The vials were then counted in a scintillation counter (Beckman), which determined radioactivity in counts per minute (cpm).

Alternatively, to stop the reaction, 5 μL of protein sample buffer (8% SDS, 4% β-mercaptoethanol, 240 mM Tris-HCl [pH 6.8], 0.004% bromophenyl blue, 40% glycerol) was added to each reaction tube. Samples were boiled for 5 minutes and centrifuged for 1 minute. Samples were then loaded onto the gel and run in a running buffer (0.025 M Tris, 1% SDS, 0.192 M glycine) at 35 milliamperes per gel for approximately 1 hour. Gels were imaged using a PhosphorImager (GE Storm 685) after exposing films to the gels (GE Healthcare Storage Phosphorscreen).

SDS-PAGE (polyacrylamide gel electrophoresis) gels were prepared using ddH20, Tris-HCl (1.5 M, pH 8.8 for separating gel; 1.0 M, pH 6.8 for stacking gel), 10% SDS, 40% acrylamide (37.5:1), 10% ammonium persulfate, and TEMED. Gels were prepared to

1.5 mM thick and either 8% (full-length lipin 1β) or 19% (peptides) polyacrylamide. Gels were run using a Bio-Rad Mini-PROTEAN Tetra Cell 2-gel system.

2.4. Analysis of Phosphopeptides

The methods used for phosphopeptide analysis has been described elsewhere (107) (122). Briefly, phosphorylated lipin 1β samples were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and proteolytically cleaved using L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin. These trypsin digests were then separated on cellulose plates, first by electrophoresis (Pharmacia Biotech Multiphor II) and then by thin-layer chromatography. Radioactive phosphopeptides were then visualized by phosphorimaging analysis.

2.5. Statistical Analysis

Statistics were performed using SigmaPlot software. All data is presented as mean ± standard deviation (S.D.). Kinetic data were analyzed according to the Michaelis-Menten equation using the enzyme kinetics module of SigmaPlot.

CHAPTER 3

RESULTS

3.1. Lipin 1β is a substrate for CKII

Our work focuses on the phosphorylation and subsequent regulation of lipin 1β. It is known that subcellular localization of lipin and its resulting ability to carry out the PAP reaction is determined by its phosphorylation state (48) (33) (85). Mammalian lipin 1 has been shown to have multiple phosphorylation sites (33), but the identity of the kinases that phosphorylate these sites is largely unknown. A bioinformatics analysis of lipin 1β indicates that the protein is phosphorylated by a plethora of protein kinases (124). Of these putative kinases, CKII was found to have a relatively high probability of phosphorylating lipin 1β (124). Accordingly, we chose to examine phosphorylation of lipin 1β with this protein kinase. For these experiments, we obtained a preparation of lipin 1β from our collaborators Mitchell Granade and Thurl Harris. SDS-PAGE analysis showed that the preparation of lipin 1β used in this work is highly purified (**Fig. 6**). The phosphorylation of lipin 1β by CKII was examined by following the incorporation of radioactive γ-phosphate from [γ-^{32}P]ATP into the protein, and the product of the phosphorylation reaction was analyzed by SDS-PAGE. Phosphorimaging analysis of the polyacrylamide gel showed that CKII phosphorylated lipin 1β, and that the amount of phosphorylation was dependent on the amount of lipin 1β in the reaction (**Fig. 7**). The same experiment was performed with a lipin 1β preparation that was treated with λ phosphatase, which was used to remove phosphates from sites that might be endogenously phosphorylated. There was no difference between the phosphorylation of the λ phosphatase-treated and -untreated

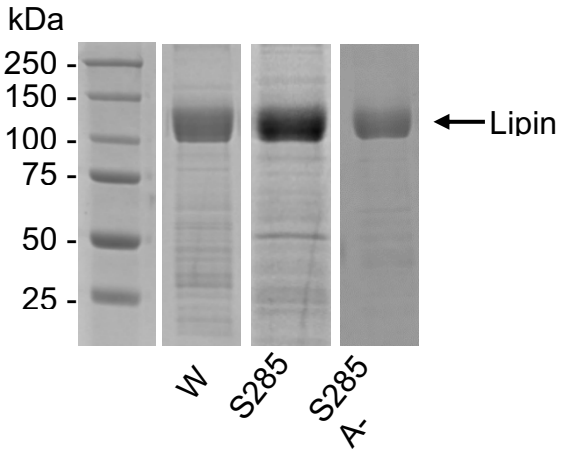

Figure 6. SDS-PAGE of purified lipin 1β. Samples of purified lipin 1β, S285A lipin 1β, and S285A-S287A lipin 1β were subjected to SDS-PAGE and stained with Coomassie blue. The position of the purified lipin 1β is indicated. Lipin 1β proteins and gel images supplied by Thurl Harris and Mitchell Granade.

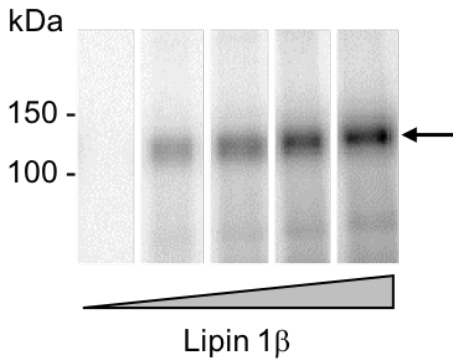

Figure 7. SDS-PAGE of phosphorylation of lipin 1β by CKII. Lipin 1β (0, 50, 100, 200, and 300 ng) was phosphorylated by 0.02 μg of CKII with 50 μM [γ-^{32}P]ATP (8000 cpm/pmol) for 15 min at 30°C. Following the kinase reaction, lipin 1β was separated from ATP and CKII by SDS-PAGE and subjected to phosphorimaging analysis. The position of phosphorylated lipin 1β is indicated by the arrow. The data are representative of three experiments.

preparations (data not shown). Thus, any endogenous phosphorylations did not interfere with the *in vitro* phosphorylation of lipin 1β by CKII.

With confidence that lipin 1β is phosphorylated by CKII, we next characterized this phosphorylation by analyzing the ^{32}P-labeled product on nitrocellulose paper. Following each reaction, the ^{32}P-labeled peptide was trapped on nitrocellulose filter paper followed by washing with phosphoric acid to remove the unreacted [γ-^{32}P]ATP. The filter paper was then subjected to scintillation counting and the radioactivity on the paper was used to calculate the moles of phosphate incorporated into the peptide. The phosphorylation of lipin 1β by CKII was characterized in detail to confirm that lipin 1β is a *bona fide* substrate of CKII. Indeed, the CKII activity was dependent on the amount of CKII (**Fig. 8**), the time of the reaction (**Fig. 9**), the concentrations of ATP (**Fig. 10**) and the amount of lipin 1β (**Fig. 11**). Analysis of the data for ATP according to the Michaelis-Menten equation yielded a V_{max} and K_m values, respectively, of 0.38 ± 0.03 nmol/min/mg and 1.8 ± 0.7 μM. The amount of lipin 1β was limited and data at concentrations needed to saturate the reaction were not obtained (**Fig. 11**).

3.2. Bioinformatics analysis of the lipin 1β serine-rich domain for putative protein kinases

The serine-rich region of lipin 1β contains a number of putative serine/threonine phosphorylation sites, and has been shown to be important in subcellular localization (34) (31). Previous work performed on lipin 1α, in which the serine-rich domain is located from residues 218-260, has shown that this region is phosphorylated in response to insulin, and this phosphorylation leads to interaction with 14-3-3 proteins and subsequent cytoplasmic retention of lipin 1α (34). In lipin 1β, this region is located from residues 251-293, and

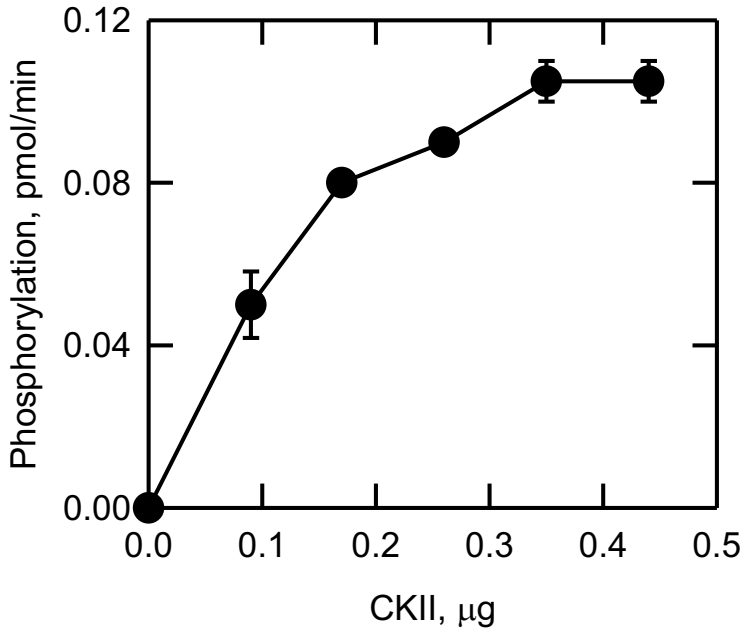

Figure 8. Effect of the amount of CKII on the phosphorylation of lipin 1β. The phosphorylation of lipin 1β by CKII was measured with the indicated amounts of the protein kinase, 2.5 ng of lipin 1β, and 50 μM [γ-^{32}P]ATP for 15 min at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the protein. The reaction mixtures were spotted onto nitrocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

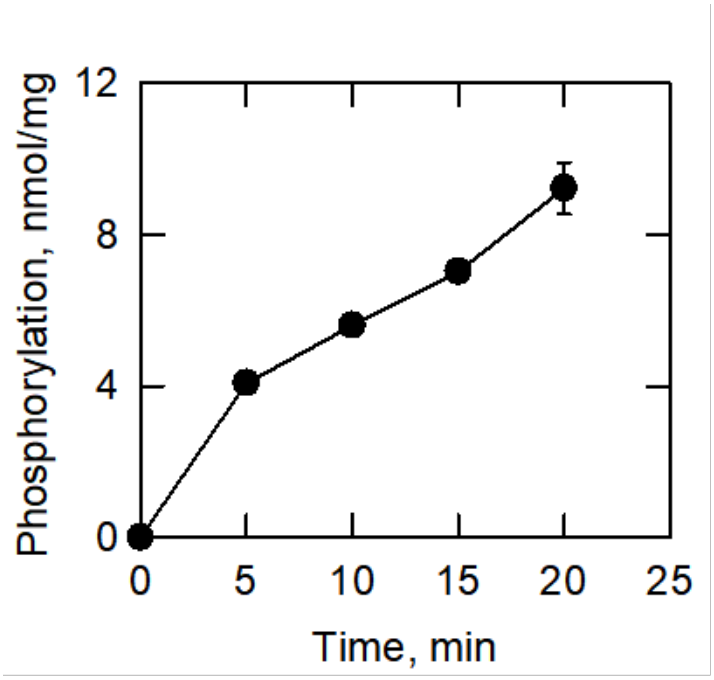

Figure 9. Effect of time on the phosphorylation of lipin 1β by CKII. The phosphorylation of 2.5 ng of lipin 1β by 0.2 μg CKII and 50 μM [γ-^{32}P]ATP was measured for the indicated time intervals at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the protein. The reaction mixtures were spotted onto nitrocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

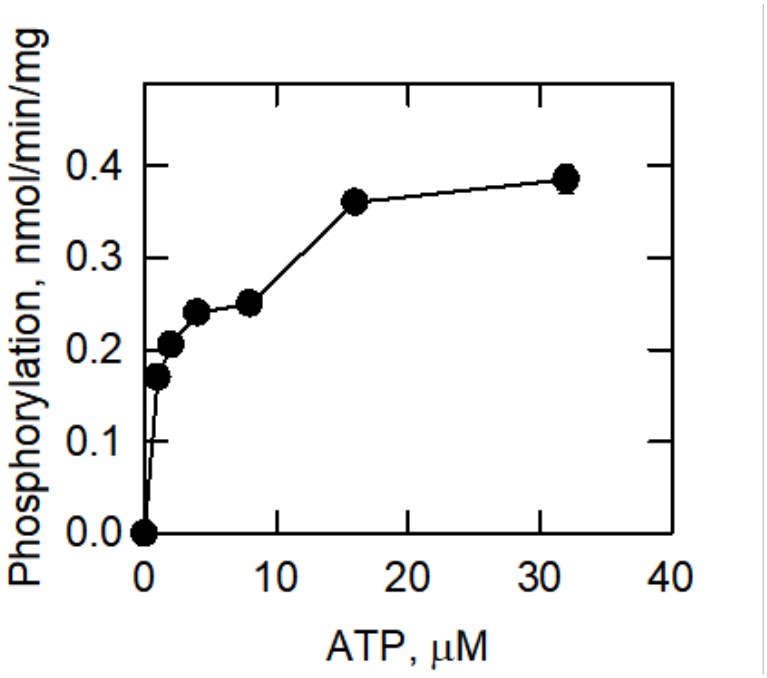

Figure 10. Effect of the concentration of ATP on the phosphorylation of lipin 1β. The phosphorylation of 2.5 ng of lipin 1β by 0.2 μg of CKII and [γ-^{32}P]ATP was measured with the indicated concentrations of ATP. Reactions were run for 15 minutes at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the protein. The reaction mixtures were spotted onto nitrocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

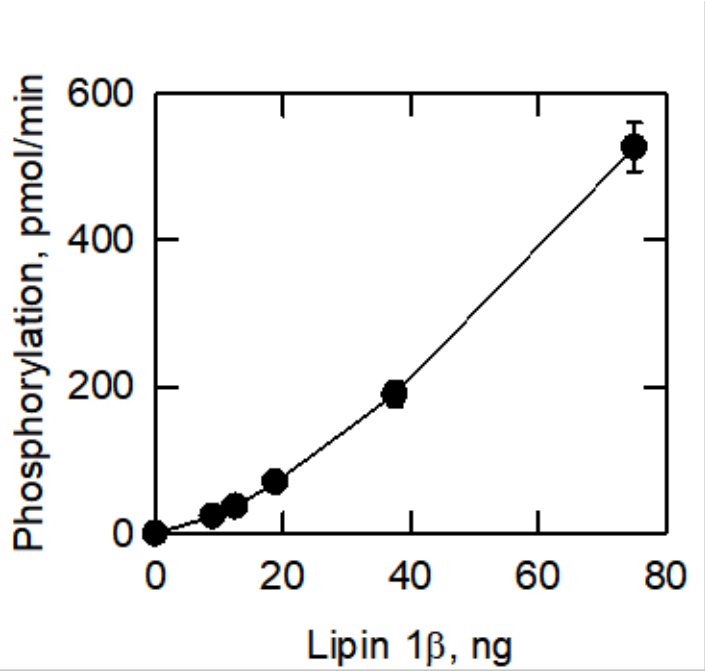

Figure 11. Effect of the amounts of lipin 1β on the phosphorylation by CKII. The phosphorylation of lipin 1β by 0.2 μg of CKII and 50 μM [γ-^{32}P]ATP was measured with the indicated amounts of lipin 1β. Reactions were run for 15 minutes at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the protein. The reaction mixtures were spotted onto nitrocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

```
Lipin 1β    1 MNYVGQLAGQ VFVTVKELYK GLNPATLSGC IDIIVIRQPN GSLQCSPFHV RFGKMGVLRS
           61 REKVVDIEIN GESVDLHMKL GDNGEAFFVQ ETDNDQEIIP MYLATSPILS EGAARMESQL
          121 KRNSVDRIRC LDPTTAAQGL PPSDTPSTGS LGKKRRKRRR KAQLDNLKRD DNVNTSEDED
          181 MFPIEMSSDE DTAPMDGSRT LPNDVPPFQD DIPKENFPSI STYPQSASYP SSDREWSPSP
          241 SSLVDCQRTP PHLAEGVLSS SCPLQSCHFH ASESPSGSRP STPKSDSELV SKSADRLTPK
          301 NNLEMLWLWG ELPQAAKSSS PHKMKESSPL GSRKTPDKMN FQAIHSESSD TFSDQSPTMA
          361 RGLLIHQSKA QTEMQFVNEE DLESLGAAAP PSPVAEELKA PYPNTAQSSS KTDSPSRKKD
          421 KRSRHLGADG VYLDDLTDMD PEVAALYFPK NGDPGGLPKQ ASDNGARSAN QSPQSVGGSG
          481 IDSGVESTSD SLRDLPSIAI SLCGGLSDHR EITKDAFLEQ AVSYQQFADN PAIIDDPNLV
          541 VKVGNKYYNW TTAAPLLLAM QAFQKPLPKA TVESIMRDKM PKKGGRWWFS WRGRNATIKE
          601 ESKPEQCLTG KGHNTGEQPA QLGLATRIKH ESSSSDEEHA AAKPSGSSHL SLLSNVSYKK
          661 TLRLTSEQLK SLKLKNGPND VVFSVTTQYQ GTCRCEGTIY LWNWDDKVII SDIDGTITRS
          721 DTLGHILPTL GKDWTHQGIA KLYHKVSQNG YKFLYCSARA IGMADMTRGY LHWVNERGTV
          781 LPQGPLLLSP SSLFSALHRE VIEKKPEKFK VQCLTDIKNL FFPNTEPFYA AFGNRPADVY
          841 SYKQVGVSLN RIFTVNPKGE LVQEHAKTNI SSYVRLCEVV DHVFPLLKRS HSCDFPCSDT
          901 FSNFTFWREP LPPFENQDMH SASA

Lipin 1α 241 S--------- ---------- ---------- ----PSGSRP STPKSDSELV SKSADRLTPK 267
Lipin 1β 241 SSLVDCQRTP PHLAEGVLSS SCPLQSCHFH ASESPSGSRP STPKSDSELV SKSADRLTPK 300
```

Figure 12. Sequence of murine lipin 1β with the synthesized portion of the serine-rich domain underlined, and alignment of this region with murine lipin 1α. The region used to synthesize the 22-residue peptide of lipin 1β is designated in *bold* letters with putative phosphorylation sites in *red* (*upper*). Lipin 1α is aligned with lipin 1β to show the conserved residues in the serine-rich region (*lower*). The serine residues shown in *red* for lipin 1α are phosphorylated by an unknown protein kinase, whereas the residues shown in *red* for lipin 1β are phosphorylated by CKII (this work).

includes seven putative serine/threonine phosphorylation sites: Ser-281, Thr-282, Ser-285, Ser-287, Ser-291, Ser-293, and Thr-298 (**Fig. 12**). To examine the serine-rich domain of lipin 1β, we specifically selected the stretch of amino acids from residues 271-310 (**Fig. 12**). Using the Phosphomotif Finder from the Human Protein Reference Database (*http://www.hprd.org/PhosphoMotif_finder*) (125) and the NetPhos program from the Center for Biological Sequence Analysis (*http://www.cbs.dtu.dk/services/NetPhos/)* (124), a number of putative kinases, common between both programs, were found (**Table 1**). A 22-residue peptide with the sequence RPSTPKSDSELVSKSADRLTPK (residues 279-300; hereafter referred to as WT peptide) was synthesized by EZ Biolabs and used for examination of phosphorylation.

3.3. A 22-residue peptide is a substrate for CKII and PKA, but not for CKI or PKC

Of the protein kinases that are predicted to phosphorylate the lipin 1β WT peptide, PKA, PKC, CKI, and CKII are widely expressed mammalian serine/threonine protein kinases. We questioned whether these kinases phosphorylate the WT peptide by following the incorporation of the γ-phosphate of [γ-^{32}P]ATP into the WT peptide. Following the reaction, the ^{32}P-labeled peptide was trapped on P81 phosphocellulose filter paper followed by extensive washing with phosphoric acid to remove the unreacted [γ-^{32}P]ATP. The filter paper was then subjected to scintillation counting and the radioactivity on the paper was used to calculate the moles of phosphate incorporated into the peptide. The WT peptide was shown to be phosphorylated by PKA and CKII, but not by CKI or PKC (**Fig. 13**). Because we had already shown that lipin 1β is a substrate for CKII, we chose to characterize the CKII phosphorylation of the peptide in detail. Characterization of the PKA

phosphorylation of the peptide, along with the evaluation of other protein kinases that were found to have a high probability of phosphorylating the serine-rich region of lipin 1β, were not addressed in this thesis.

3.4. Characterization of phosphorylation of WT peptide by CKII

The phosphorylation of the WT peptide by CKII was characterized in detail. Under the conditions of our experiments, CKII activity was dependent on the amount of the protein kinase (**Fig. 14**) and the time of the reaction (**Fig. 15**). Based on these experiments, subsequent phosphorylation reactions were routinely measured with 0.2 μg CKII and for 15 min. The CKII activity was also examined with respect to the concentrations of ATP (**Fig. 16**) and the WT peptide (**Fig. 17**). In both cases, CKII activity followed typical saturation kinetics. The kinetic data were analyzed according to the Michaelis-Menten equation using the Enzyme Kinetics module of Sigma Plot software. This analysis yielded apparent V_{max} and K_m values, respectively, for ATP of 10.7 ± 0.8 nmol/min/mg and 6.2 ± 1.5 μM, and for the WT peptide of 45 ± 5 nmol/min/mg and 146 ± 40 μM. CKII catalyzes a Bi Bi reaction (e.g., two substrates and two products), and thus, determination of true kinetic constants would require an analysis of one substrate dependence at a series of concentrations of the other substrate (126). Since this type of analysis was not conducted here, the kinetic constants obtained must be considered apparent values under the conditions employed. Nonetheless, these data provided confidence that the WT peptide is a *bona fide* substrate for CKII.

Phosphorylation site	Putative protein kinase
Ser-281	PKC, GSK-3, CaM-II, PKA, CKI, DNAPK, CKII, CDK-5
Thr-282	CDK-5, GSK-3, CaM-II, CKI, DNAPK, PKC, CKII, PKA
Ser-285	CKII, CKI, PKA, PKC
Ser-287	GSK-3, CKII, PKC, PKA
Ser-291	CKII, GSK-3, CKI
Ser-293	CKII
Thr-298	GSK-3, CKI, CDK-5, PKC, PKA

Table 1. Putative protein kinases determined for the known phosphorylation sites within the serine-rich domain of lipin 1β. The portion of the serine-rich domain (residues 271-310) was analyzed by the Phosphomotif Finder and NetPhos programs, and the putative kinases predicted for each phosphorylation site are listed. Kinases are listed in order of probability as determined by the NetPhos program. PKA, protein kinase A; PKC, protein kinase C; CaM-II, Ca^{2+}/calmodulin- dependent protein kinase II; DNAPK, DNA-dependent protein kinase; GSK-3, glycogen synthase kinase 3; CDK-5, cyclin-dependent kinase 5.

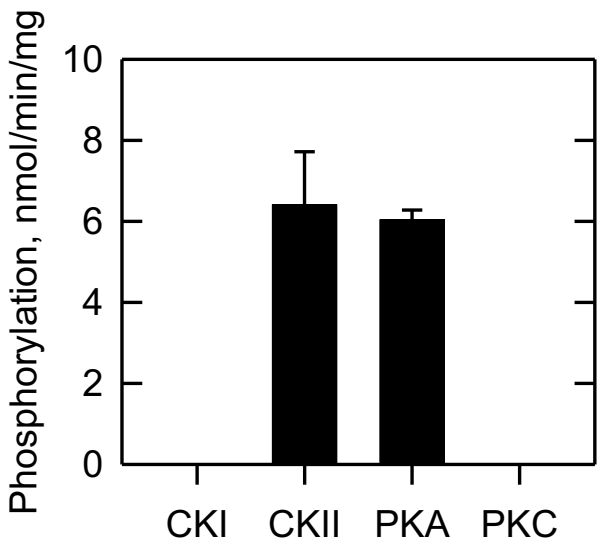

Figure 13. Analysis of protein kinases for their ability to phosphorylate the serine-rich lipin 1β WT peptide. The 100 μM WT peptide was incubated with PKA, PKC, CKI, or CKII and [γ-^{32}P]ATP for 15 min at 30°C. Following the incubation, the phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

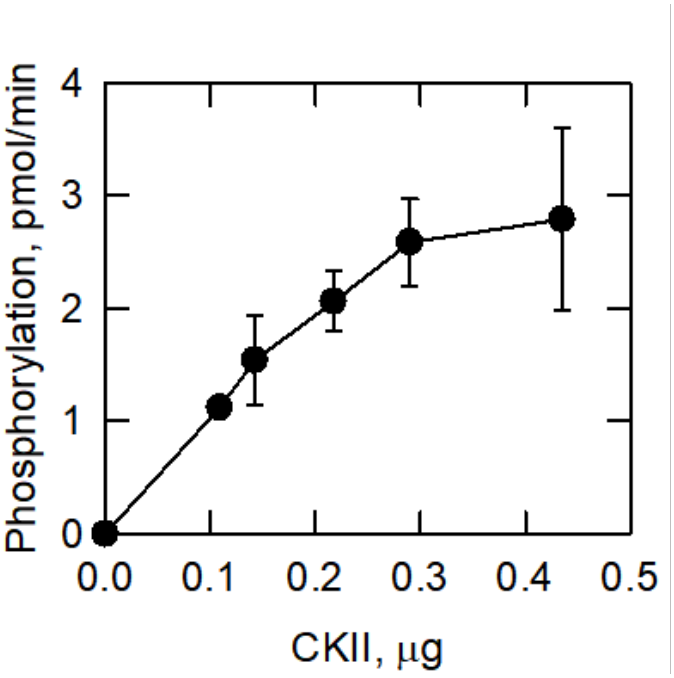

Figure 14. Effect of the amount of CKII on the phosphorylation of WT peptide. The phosphorylation of 100 μM WT peptide by CKII was measured with the indicated amounts of the protein kinase and 100 μM [γ-^{32}P]ATP for 15 min at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

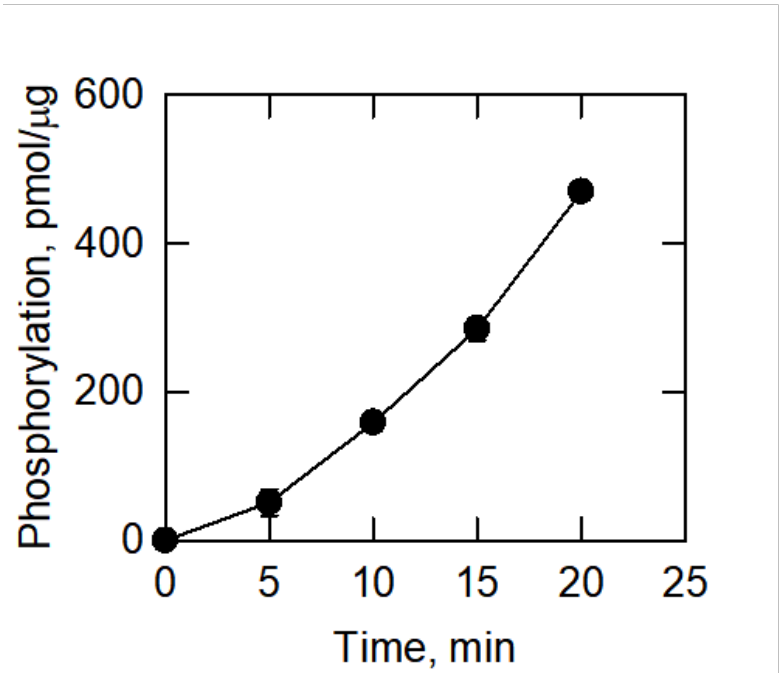

Figure 15. Effect of time on the phosphorylation of WT peptide by CKII. The phosphorylation of the 100 μM WT peptide by 0.2 μg CKII and 100 μM [γ-^{32}P]ATP was measured for the indicated time intervals at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

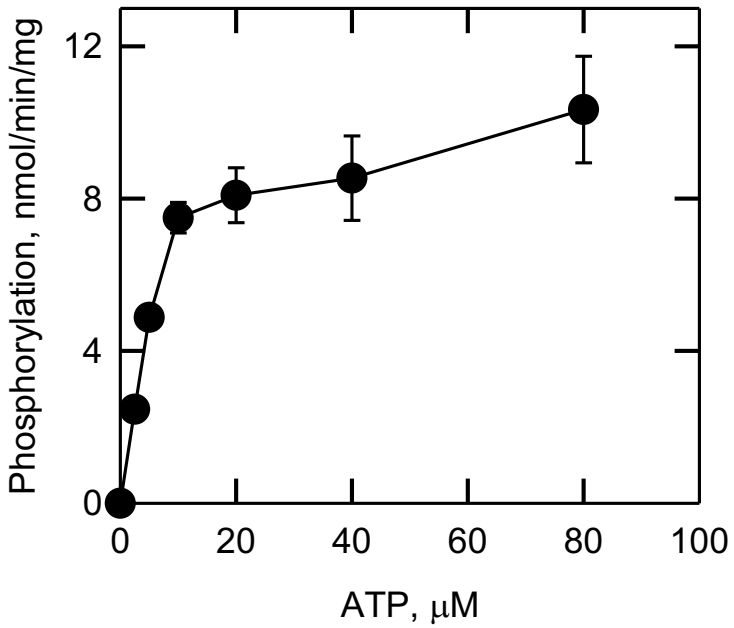

Figure 16. Effect of the concentration of ATP on the phosphorylation of WT peptide. The phosphorylation of the 100 μM WT peptide by 0.2 μg CKII and [γ-^{32}P]ATP was measured with the indicated concentrations of ATP. Reactions were run for 15 minutes at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

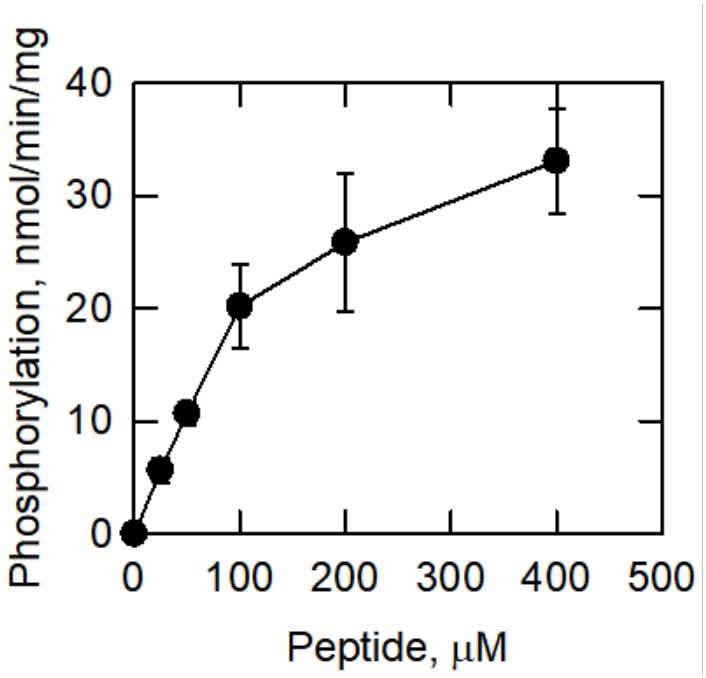

Figure 17. Effect of the concentration of peptide on the phosphorylation of WT peptide by CKII. The phosphorylation of the WT peptide by 0.2 μg CKII and 100 μM [γ-^{32}P]ATP was measured with the indicated concentrations of the WT peptide. Reactions were run for 15 minutes at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

3.5. Mutagenesis of the 22-residue peptide identifies Ser-285 as the main site of phosphorylation by CKII

After determining that the WT peptide was indeed a substrate for CKII, we next wanted to determine which of the seven putative serine/threonine phosphorylation sites was being phosphorylated. In order to determine this, fourteen variants of a 22-residue peptide that contained the seven sites (residues 279-300) were synthesized (EZ Biolabs). These variants included two groups of mutations: Group 1, in which six of the seven serine/threonine phosphorylation sites were changed to alanine and the remaining site was left available for phosphorylation; and Group 2, in which one phosphorylation site was changed to alanine and the remaining six sites were left intact (**Fig. 18**). A mutant peptide (referred to as 7A) was also synthesized in which all seven sites were changed to alanine; this peptide served as a negative control for phosphorylation.

Phosphorylation reactions were conducted with each of the 14 mutated versions of the 22-residue peptide. When the Group 1 peptides (one site intact) were phosphorylated by CKII, only two mutants, Ser-285 and Ser-287, showed any significant phosphorylation when compared with the WT peptide **(Fig. 19)**. The CKII activity using the peptides with intact Ser-285 and Ser-287, respectively, was approximately 75% and 25% of the activity observed with the WT peptide. When the Group 2 peptides (one site mutated) were phosphorylated by CKII, all of them showed some degree of phosphorylation when compared to WT except for S285A, which showed no phosphorylation **(Fig. 20)**. These results indicate that Ser-285 is the main site of phosphorylation by CKII within the 22-residue peptide, with Ser-287 also showing some degree of phosphorylation.

Group 1 (1 site intact):

WT	RPSTPKSDSELVSKSADRLTPK
Ser-281	RPSAPKADAELVAKAADRLAPK
Thr-282	RPATPKADAELVAKAADRLAPK
Ser-285	RPAAPKSDAELVAKAADRLAPK
Ser-287	RPAAPKADSELVAKAADRLAPK
Ser-291	RPAAPKADAELVSKAADRLAPK
Ser-293	RPAAPKADAELVAKSADRLAPK
Thr-298	RPAAPKADAELVAKAADRLTPK

Group 2 (1 site mutated):

WT	RPSTPKSDSELVSKSADRLTPK
S281A	RPATPKSDSELVSKSADRLTPK
T282A	RPSAPKSDSELVSKSADRLTPK
S285A	RPSTPKADSELVSKSADRLTPK
S287A	RPSTPKSDAELVSKSADRLTPK
S291A	RPSTPKSDSELVAKSADRLTPK
S293A	RPSTPKSDSELVSKAADRLTPK
T298A	RPSTPKSDSELVSKSADRLAPK
7A	RPAAPKADAELVAKAADRLAPK

Figure 18. Sequence of mutant peptides used for phosphorylation experiments. Two groups of peptides were synthesized for mutagenesis experiments: Group 1, in which six of the seven putative serine/threonine phosphorylation sites were mutated to alanine, while the one remaining site was left intact; and Group 2, in which one of the seven phosphorylation sites was mutated to alanine, while the remaining six sites were left intact. A WT peptide (no mutations) and a 7A peptide (all seven sites mutated to alanine) were also synthesized.

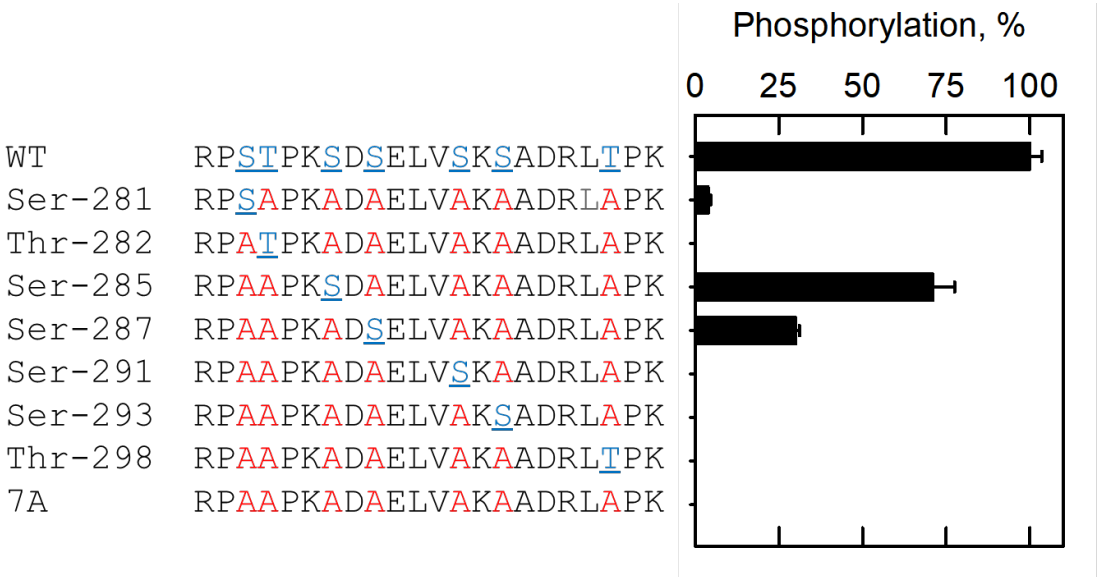

Figure 19. Phosphorylation of Group 1 peptides by CKII. The phosphorylation of the indicated 100 μM peptides by 0.2 μg CKII and 100 μM [γ-^{32}P]ATP was measured by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. Reactions were run for 15 minutes at 30°C. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The levels of the peptide phosphorylation were normalized to the maximum phosphorylation of the WT peptide. The data shown are means ± S.D. (*error bars*) from triplicate assays.

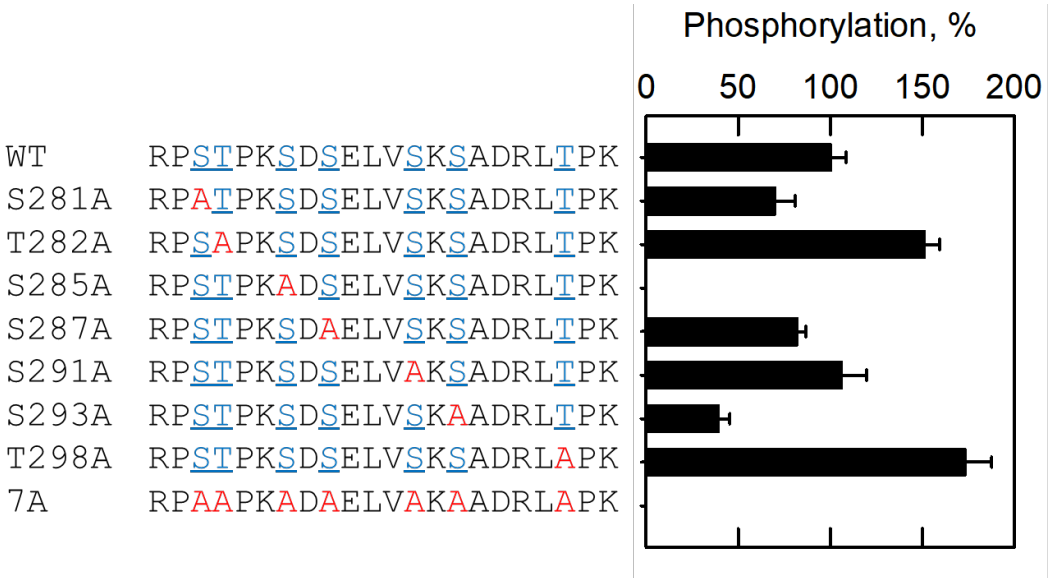

Figure 20. Phosphorylation of Group 2 peptides by CKII. The phosphorylation of the indicated 100 μM peptides by 0.2 μg CKII and 100 μM [γ-^{32}P]ATP was measured by following the incorporation of the radioactive γ-phosphate from ATP into the peptide. Reactions were run for 15 minutes at 30°C. The reaction mixtures were spotted onto P81 phosphocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The levels of the peptide phosphorylation were normalized to the maximum phosphorylation of the WT peptide. The data shown are means ± S.D. (*error bars*) from triplicate assays.

3.6. The effects of the S285A and S285A/S287A mutations on the phosphorylation of lipin 1β by CKII

We tested two mutated versions of full-length lipin 1β, one in which Ser-285 was mutated to alanine (S285A) and one in which both Ser-285 and Ser-287 were mutated to alanine (S285A/S287A), and compared their phosphorylation to WT lipin 1β. Increasing amounts of S285A, S285A/S287A, and WT lipin 1β were phosphorylated by CKII, spotted onto nitrocellulose paper, and subjected to scintillation counting. Based on the results obtained from the peptide mutagenesis experiments, described above, we expected that S285A lipin 1β would show a decrease in phosphorylation when compared with WT lipin 1β. Unexpectedly, the S285A lipin 1β showed a greater degree of phosphorylation than its WT counterpart at each protein amount tested (**Fig. 21**). Conversely, the S285A/S287A mutant showed a similar degree of phosphorylation as the WT.

Phosphopeptide mapping analysis was performed on the WT and phosphorylation-deficient forms of lipin 1β (**Fig. 22**). The map of the WT protein showed multiple phosphopeptides, indicating that there are more than two sites of phosphorylation by CKII within lipin 1β. Two phosphopeptides (labeled as *7* and *10* in **Fig. 22**) present in the wild type protein were absent in both mutant proteins, but one phosphopeptide (labeled as *3* in **Fig. 22**) present in the mutants was absent in the wild type protein. Based on the map of the S285A mutant, we concluded that the phosphopeptides labeled *7* and *10* likely contain Ser-285. Since the same two phosphopeptides were missing from the map of the S285A/S287A double mutant and Ser-287 is close to Ser-285, it is likely that these peptides also contained Ser-287.

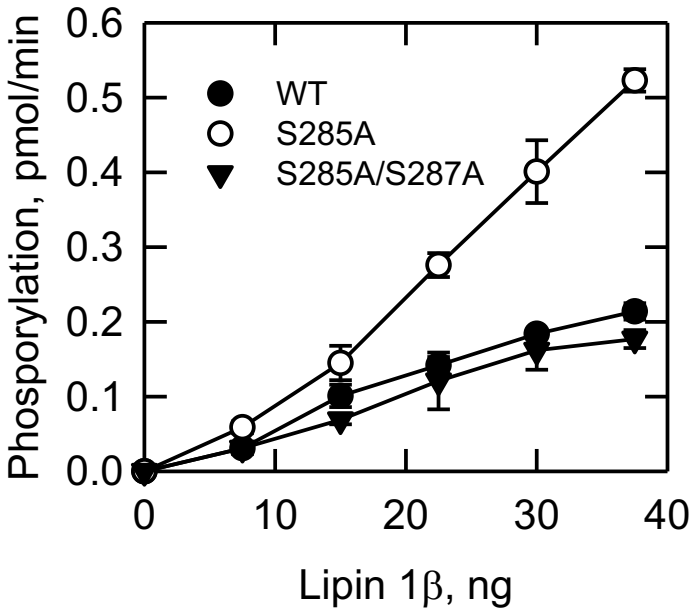

Figure 21. Effect of the amount of WT, S285A, and S285A/S287A lipin 1β on phosphorylation by CKII. The phosphorylation of WT, S285A, and S285A/S287A lipin 1β by 0.2 μg CKII and 50 μM [γ-^{32}P]ATP was measured with the indicated amounts of protein. Reactions were run for 15 minutes at 30°C. The phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate from ATP into the protein. The reaction mixtures were spotted onto nitrocellulose paper, which was washed with 75 mM phosphoric acid and subjected to scintillation counting. The data shown are means ± S.D. (*error bars*) from triplicate assays.

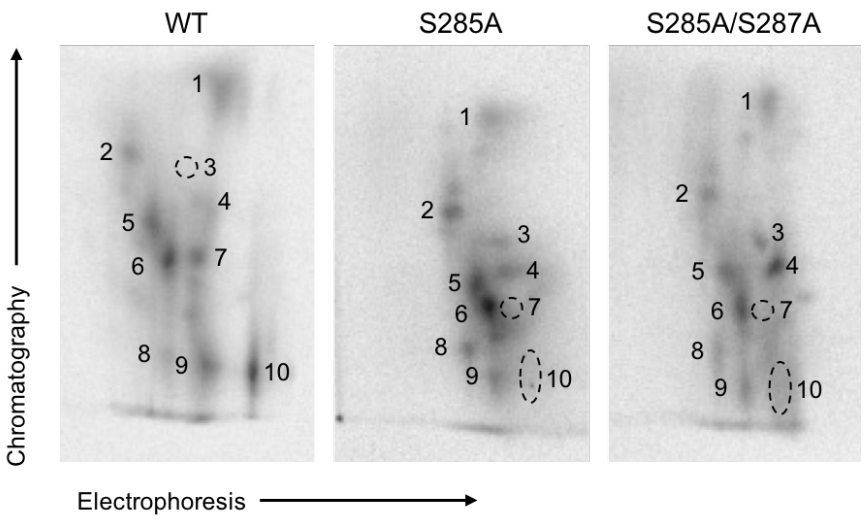

Figure 22. Phosphopeptide mapping analysis of WT, S285A, and S285A/S287A lipin 1β. 100 ng of WT, S285A, and S285A/S287A lipin 1β were phosphorylated with 0.2 μg CKII and 50 μM [γ-^{32}P]ATP (10,000 cpm/pmol). The phosphorylated samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The ^{32}P-labeled lipin 1β from the polyvinylidene difluoride membrane was subjected to phosphopeptide mapping. The positions of the phosphopeptides that were absent in one map, but present in another map, are indicated by a *dotted line circle*.

CHAPTER 4

DISCUSSION AND FUTURE WORK

Phosphorylation is a critical regulator of the subcellular localization of lipin, and, in turn, of its activity as a PAP enzyme. Phosphorylation increases the negative charge on lipin, thus decreasing its ability to interact with negatively charged membrane surfaces; therefore, phosphorylated lipin is retained in the cytoplasm (127). Because the PAP reaction takes place at the ER membrane, phosphorylation prevents lipin from carrying out the PAP reaction, effectively inactivating it (14) (32). In addition, the role of lipin as a transcriptional regulator requires it to be able to translocate to the nucleus, again requiring its dephosphorylation.

The importance of the phosphorylation state of lipin 1 is underscored in yeast, where Pah1, the yeast homolog of lipin, is dephosphorylated and therefore activated by the Nem1-Spo7 phosphatase complex. Cells lacking Pah1 (*pah1*Δ cells) show distinctive phenotypes, including aberrant nuclear/ER membrane expansion and dysregulation of phospholipid synthesis (104) (48) (112). These phenotypes are also exhibited in cells lacking the Nem1-Spo7 phosphatase complex, indicating that the inability to dephosphorylate Pah1 has similar consequences to the full loss of Pah1 (104) (48) (112). In addition, a mutant yeast cell in which seven serine/threonine phosphorylation sites in Pah1 that are known to be targeted by Pho85p-Ph080p are mutated to alanine has been shown to have PAP activity that is 1.8 times greater than a WT cell, again reiterating the importance of the phosphorylation state for the activity of the enzyme (128). In yeast, multiple kinases, including Pho85p-Pho80p (128), Cdc28-cyclin B (129), PKA (130),

CKII, and PKC (131), are known to phosphorylate Pah1; however, less is known about the phosphorylation of mammalian lipin 1.

In this work, we have demonstrated that lipin 1β is a substrate for the mammalian serine/threonine protein kinase CKII. This phosphorylation was time- and CKII-dependent and dependent on the concentrations of ATP and lipin 1β. This is a novel finding; to our knowledge, the only kinase that has conclusively been shown to phosphorylate lipin is mTORC1, which phosphorylates Ser-106 and Ser-472 (19) (33). Although it is known that mammalian lipin 1 contains at least 25 likely sites of phosphorylation, it was not previously known what kinases, other than mTORC1, are involved in its phosphorylation. The work presented here, that lipin 1β is phosphorylated by CKII, increases our understanding of the regulation of this important PAP enzyme. CKII is a ubiquitously expressed mammalian serine/threonine protein kinase, and has three phosphorylation motifs: (S/T)*XX*(E/D), (S/T)*X*(E/D), and (S/T)(E/D) (132).

As discussed above, it is known that mammalian lipin is a target of the mTOR pathway, and that Ser-106 is phosphorylated by mTORC1. mTOR is a downstream target of insulin, and insulin stimulation has been shown to increase the phosphorylation of lipin (33). However, it is unlikely that Ser-106 is the only insulin-mediated phosphorylation site within lipin 1. Experiments comparing a WT lipin 1 protein and an S106A mutant lipin showed that upon insulin stimulation, the increase in phosphorylation was only slightly lower in the S106A mutant than in the WT, indicating that there must be other sites that are phosphorylated in response to insulin (33). In the future, it would be interesting to examine whether phosphorylation of lipin 1β by CKII is increased in response to insulin, as CKII has also been shown to be a target of insulin in adipocytes (34,133,134).

In this work, we do not examine the effects of CKII phosphorylation on the subcellular localization or PAP activity of lipin 1β. Future work will seek to determine whether phosphorylation by CKII affects these attributes, and whether the mutation of Ser-285 and Ser-287 to alanine would abolish these effects. Entering the sequence for murine lipin 1β into the NetPhos program indicated that there are a total of 156 predicted serine/threonine CKII phosphorylation sites, so it is likely that there are sites other than Ser-285 and Ser-287 being phosphorylated by CKII in the full-length protein (124). The phosphopeptide mapping analysis of the WT protein supports the assertion that there are additional sites. The identity of these sites and their importance will require additional studies.

After determining that full-length lipin 1β was a substrate for CKII, we next examined a 22-residue peptide that makes up part of lipin 1β's serine-rich domain. The serine-rich domain has been shown to be important in subcellular localization, as sites located within this domain interact with 14-3-3 protein and promote cytosolic retention of lipin 1 (34). We determined that the 22-residue WT peptide is also a substrate for CKII, and as with full-length lipin 1β, the phosphorylation was shown to be dependent on the concentrations of ATP and WT peptide, as well as time- and CKII-dependent. The activity of CKII using the WT peptide followed typical saturation kinetics, and we were able to determine Michaelis-Menten constants for the reaction. The K_m value for ATP (6.2 ± 1.5 μM) was in the range of that determined for full-length lipin 1β (1.8 ± 0.7 μM); these values are also in the range of the K_m value for ATP when the yeast homolog of lipin, Pah1, is phosphorylated by CKII (107). Unfortunately, we did not have sufficient full-length protein to saturate the CKII reaction for determination of kinetic constants.

Lastly, we demonstrated that Ser-285 and Ser-287 within the 22-residue peptide are major sites for CKII phosphorylation. In order to determine this, we used a novel mutagenesis approach, in which two groups of peptides were synthesized: one group in which one of seven serine/threonine phosphorylation sites was left intact, and a second group in which one of these seven sites was mutated to alanine. This approach allowed us to effectively determine which site within the peptide was being phosphorylated by CKII. When testing the first group of peptides, Ser-285 was phosphorylated at approximately 75% of WT, while Ser-287 was phosphorylated at approximately 25% of WT; none of the other peptides within this group showed any significant phosphorylation when compared with the WT. When testing the second group, S285A was not phosphorylated; the other six peptides within the group all showed some degree of phosphorylation when compared with the WT peptide. Unexpectedly, S287A was phosphorylated to nearly the same degree as the WT peptide; we had expected, based on the Group 1 results, that it would show decreased phosphorylation. Further work would be needed to determine the significance of this result. Ser-285 and Ser-287, respectively, in lipin 1β correspond with Ser-252 and Ser-254 in lipin 1α (**Fig. 12**), which have been shown to be two of the phosphorylation sites that are critical to the insulin-stimulated interaction of lipin 1α with 14-3-3 proteins (34). Therefore, it is possible that phosphorylations of Ser-285 and Ser-287 by CKII are involved in subcellular localization of lipin 1β by regulating interaction of the enzyme with 14-3-3 proteins. However, Ser-293, which corresponds to Ser-260 in lipin 1α and is one of the sites within lipin 1α shown to be crucial for 14-3-3 protein interaction, was not phosphorylated by CKII, although S293A did show a decrease of about 60% compared with WT peptide. This site does fall within a CKII phosphorylation motif, so future work

will also examine whether an S293A mutation in full-length lipin 1β has any effect on its phosphorylation.

Surprisingly, when the phosphorylation of S285A and S285A/S287A lipin 1β were compared with the phosphorylation of WT lipin 1β, it was found that the S285A mutant showed a greater degree of phosphorylation than the WT. Conversely, the double mutant, S285A/S287A, showed the same degree of phosphorylation as the WT protein. Phosphopeptide mapping analysis showed that two phosphopeptides present in WT lipin 1β were missing in the maps of the S285A and S285A/S287A mutant proteins. We believe that these phosphopeptides contain Ser-285 and Ser-287. The maps of the mutant proteins contained a phosphopeptide that is not present in the map of the WT protein. This raised the suggestion that the phosphorylations of Ser-285 and Ser-287 prevent the phosphorylation of a site that is contained in this phosphopeptide. When considering the phosphopeptide mapping analysis in conjunction with the phosphorylation experiments showing the relative extents of phosphorylation (**Fig. 21**) of the WT and mutant forms of lipin 1β, it appears that there is a site within the protein that is able to be phosphorylated only when Ser-285 is not present; this would account for the presence of the additional phosphopeptide in the map of S285A as well as the greater degree of phosphorylation in this mutant. This effect of Ser-285 was not seen in the experiments with the 22-residue peptide, leading us to speculate that the phosphorylation of Ser-285 may lead to a shape change in the full-length protein that does not occur in the 22-residue peptide. Similar experiments will need to be performed on an S287A single mutant as a control to better understand these results.

Taken together, these results indicate that two sites within the serine-rich domain of lipin 1β are phosphorylated by CKII, and these sites correspond with sites in lipin 1α that have been shown to be necessary for interaction of lipin 1α with 14-3-3 proteins. In order to fully understand the significance of these results, future work will focus on this interaction. It will be enlightening to determine if mutating Ser-285 and Ser-285 in lipin 1β with CKII has an effect *in vivo* on interaction of the enzyme with 14-3-3 proteins, and whether this leads to a change in the subcellular localization or PAP activity of lipin 1β. Additionally, experiments could be performed on lipin 1α with S252A and S254A mutations to see if these mutations affect the ability of lipin 1α to be phosphorylated or to interact with 14-3-3 proteins.

We hypothesized that lipin 1β is phosphorylated by a serine/threonine kinase, and this hypothesis was shown to be correct. Indeed, CKII phosphorylates lipin 1β. However, there are multiple other putative kinases proposed by bioinformatics, and further work would be needed to determine if one or more of them is also able to phosphorylate lipin 1β. We demonstrated that the WT 22-residue peptide also appears to be a substrate for PKA. It would be interesting to see if PKA phosphorylates the full-length protein, and to determine which sites within lipin 1β it phosphorylates.

The importance of lipin in lipid homeostasis is demonstrated by its dual function as both a PAP enzyme and a transcriptional regulator controlling lipid metabolism. It has been implicated in such diverse disease states as obesity, diabetes, cancer, and atherosclerosis, yet comparatively little is known at the biochemical level about its regulation. The findings in this work contribute to our knowledge about the posttranslational modification of this crucial enzyme by phosphorylation, and this information may lead to greater understanding

of how the regulation of lipin 1β contributes to lipid metabolism, and ultimately, the cellular processes involved in obesity.

REFERENCES

1. Ahmadian, M., Duncan, R.E., Jaworski, K., Sarkadi-Nagy, E., and Sul, H.S. (2007) Triacylglycerol metabolism in adipose tissue. *Futur. Lipidol.* **2**, 229-237

2. Wang, H., Airola, M.V., and Reue, K. (2017) How lipid droplets "TAG" along: Glycerolipid synthetic enzymes and lipid storage. *Molecular and Cell Biology of Lipids* **1862**, 1131-1145

3. Fujimoto, T. and Parton, R.G. (2011) Not just fat: the structure and function of the lipid droplet. *Cold Spring Harbor Perspectives in Biology* **3**

4. Bartz, R., Li, W.H., Venables, B., Zehmer, J.K., Roth, M.R., Welti, R., Anderson, R.G., Liu, P., and Chapman, K.D. (2007) Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *Journal of Lipid Research* **48**, 837-847

5. World Health Organization. (2017) Ten facts on obesity. http://www.who.int/features/factfiles/obesity/en/. Accessed 6/17/18.

6. Center for Disease Control and Prevention. (2017) Adult obesity prevalence maps. https://www.cdc.gov/obesity/data/prevalence-maps.html. Accessed 6/19/18.

7. National Institute of Diabetes and Digestive and Kidney Diseases. (2017) Diabetes statistics. https://www.niddk.nih.gov/health-information/health-statistics/diabetes-statistics. Accessed 6/18/18.

8. Smith, S. W., Weiss, S. B., and Kennedy, E. P. (1957) The enzymatic dephosphorylation of phosphatidic acids. *J. Biol. Chem* **228**, 915-922

9. Kayden, H. J., Senior, J.R., and Mattson, F.H. (1967) The monoglyceride pathway of fat absorption in man. *Journal of Clinical Investigation* **46**, 1965-1703

10. Kennedy, E. P. (1986) The biosynthesis of phospholipids. In *Lipids and membranes: Past, present and future* (Op den Kamp, J. A. F., Roelofsen, B., and Wirtz, K. W. A. eds.), Elsevier Science Publishers B.V., Amsterdam. pp 171-206

11. Han, G.-S., Wu, W.-I., and Carman, G. M. (2006) The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. *J. Biol. Chem* **281**, 9210-9218

12. Zhang, P. and Reue, K. (2017) Lipin proteins and glycerolipid metabolism: Roles at the ER membrane and beyond. *Biochim Biophys Acta*

13. Brindley, D. N. (1984) Intracellular translocation of phosphatidate phosphohydrolase and its possible role in the control of glycerolipid synthesis. *Prog. Lipid Res* **23**, 115-133

14. Han, G.-S. and Carman, G. M. (2010) Characterization of the human *LPIN1*-encoded phosphatidate phosphatase isoforms. *J. Biol. Chem* **285**, 14628-14638

15. Finck, B. N., Gropler, M. C., Chen, Z., Leone, T. C., Croce, M. A., Harris, T. E., Lawrence, J. C., Jr., and Kelly, D. P. (2006) Lipin 1 is an inducible amplifier of the hepatic PGC-1α/PPARα regulatory pathway. *Cell Metab* **4**, 199-210

16. Siniossoglou, S. (2013) Phospholipid metabolism and nuclear function: roles of the lipin family of phosphatidic acid phosphatases. *Biochim. Biophys. Acta* **1831**, 575-581

17. Kim, H. B., Kumar, A., Wang, L., Liu, G. H., Keller, S. R., Lawrence, J. C., Jr., Finck, B. N., and Harris, T. E. (2010) Lipin 1 represses NFATc4 transcriptional activity in adipocytes to inhibit secretion of inflammatory factors. *Mol. Cell Biol* **30**, 3126-3139

18. Schweitzer, G. G., Chen, Z., Gan, C., McCommis, K.S., Soufi, N., Chrast, R., Mitra, M.S., Yang, K., Gross, R.W., and Finch, B.N. (2015) Liver-specific loss of lipin-1-mediated phosphatidic acid phosphatase activity does not mitigate intrahepatic TG accumulation in mice. *Journal of Lipid Research* **56**, 848-858

19. Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., Guertin, D. A., Madden, K. L., Carpenter, A. E., Finck, B. N., and Sabatini, D. M. (2011) mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146**, 408-420

20. Vance, J. (2018) Historical perspective: phosphatidylserine and phosphatidylethanolamine from the 1800s to the present. *Journal of Lipid Research* **59**, 923-944

21. Berridge, M. J. and Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transductino. *Nature* **312**, 315-321

22. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614

23. Hubscher, H. G., Dils, R.R., and Pover, W.F. (1959) Studies on the biosynthesis of phosphatidylserine. *Biochim Biophys Acta* **36**, 518-528

24. Ansell, G. B. and Chojnakci, T. (1966) The incorporation of the phosphate esters of N-substituted aminoethanols into the phospholipids of brain and liver. *Biochem J* **98**, 303-310

25. Kuge, O. and Nishijima, M. (1997) Phosphatidylserine synthase I and II of mammalian cells. *Biochimica et Biophysica Acta: Lipids and Lipid Metabolism* **1348**, 151-156

26. Voelker, D. R. and Frazier, J. L. (1986) Isolation and characterization of a Chinese hamster ovary cell line requiring ethanolamine or phosphatidylserine for growth and exhibiting defective phosphatidylserine synthase activity. *J. Biol. Chem* **261**, 1002-1008

27. Stone, S. J. and Vance, J.E. (1999) Cloning and expression of murine liver phospatidylserine synthase (PSS)-2: differential regulation of phospholipid metabolism by PSS1 and PSS2. *Biochem J* **342**, 57-64

28. Sturbois-Balcerzak, B., Stone, S.J., Sreenivas, A., and Vance, J.E. (2001) Structure and expression of the murine phosphatidylserine synthase-1 gene. *Journal of Biological Chemistry* **276**, 8205-8212

29. Kanfer, J. N. and Kennedy, E. P. (1964) Metabolism and function of bacterial lipids. II. Biosynthesis of phospholipids in *Escherichia coli*. *J. Biol. Chem* **239**, 1720-1726

30. Vance, J. E. (2015) Phospholipid synthesis and transport in mammalian cells. *Traffic* **16**, 1-18

31. Harris, T. E. and Finck, B. N. (2011) Dual function lipin proteins and glycerolipid metabolism. *Trends Endocrinol. Metab*

32. Grimsey, N., Han, G.-S., O'Hara, L., Rochford, J. J., Carman, G. M., and Siniossoglou, S. (2008) Temporal and spatial regulation of the phosphatidate phosphatases lipin 1 and 2. *J. Biol. Chem*, 29166-29174

33. Harris, T. E., Huffman, T. A., Chi, A., Shabanowitz, J., Hunt, D. F., Kumar, A., and Lawrence, J. C., Jr. (2007) Insulin controls subcellular localization and multisite phosphorylation of the phosphatidic acid phosphatase, lipin 1. *J Biol. Chem* **282**, 277-286

34. Péterfy, M., Harris, T. E., Fujita, N., and Reue, K. (2009) Insulin-stimulated interaction with 14-3-3 promotes cytoplasmic localization of lipin-1 in adipocytes. *J. Biol. Chem* **285**, 3857-3864

35. Ren, H., Federico, L., Huang, H., Sunkara, M., Drennan, T., Frohman, M. A., Smyth, S. S., and Morris, A. J. (2010) A phosphatidic acid binding/nuclear localization motif determines lipin1 function in lipid metabolism and adipogenesis. *Mol. Biol. Cell* **21**, 3171-3181

36. Hopewell, R., Martin-Sanz, P., Martin, A., Saxton, J., and Brindley, D. N. (1985) Regulation of the translocation of phosphatidate phosphohydrolase between the cytosol and the endoplasmic reticulum of rat liver. *Biochem. J* **232**, 485-491

37. Donkor, J., Zhang, P., Wong, S., O'Loughlin, L., Dewald, J., Kok, B. P., Brindley, D. N., and Reue, K. (2009) A conserved serine residue is required for the phosphatidate phosphatase activity but not transcriptional coactivator functions of lipin-1 and lipin-2. *J. Biol. Chem* **284**, 29968-29978

38. Donkor, J., Sariahmetoglu, M., Dewald, J., Brindley, D. N., and Reue, K. (2007) Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J. Biol. Chem* **282**, 3450-3457

39. Temprano, A., Sembongi, H., Han, G. S., Sebastian, D., Capellades, J., Moreno, C., Guardiola, J., Wabitsch, M., Richart, C., Yanes, O., Zorzano, A., Carman, G. M., Siniossoglou, S., and Miranda, M. (2016) Redundant roles of the phosphatidate phosphatase family in triacylglycerol synthesis in human adipocytes. *Diabetologia* **59**, 1985-1994

40. Péterfy, M., Phan, J., Xu, P., and Reue, K. (2001) Lipodystrophy in the *fld* mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat. Genet* **27**, 121-124

41. Koonin, E. V. and Tatusov, R. L. (1994) Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. Application of an iterative approach to database search. *J Mol. Biol* **244**, 125-132

42. Wang, H., Zhang, J., Qiu, W., Han, G.-S., Carman, G. M., and Adeli, K. (2011) Lipin-1γ isoform is a novel lipid droplet-associated protein highly expressed in the brain. *FEBS Lett* **585**, 1279-1284

43. Péterfy, M., Phan, J., and Reue, K. (2005) Alternatively spliced lipin isoforms exhibit distinct expression pattern, subcellular localization, and role in adipogenesis. *J. Biol. Chem* **280**, 32883-32889

44. Pihlajamaki, J., Lerin, C., Itkonen, P., Boes, T., Floss, T., Schroeder, J., Dearie, F., Crunkhorn, S., Burak, F., Jimenez-Chillaron, J.C., Kuulasmaa, T., Miettinen, P., Park, P.J., Nasser, I., Zhao, Z., Zhang, Z., Xu, Y., Wurst, W., Ren, H., Morris, A.J., Stamm, S., Goldfine, A.B., Laakso, M., and Patti, M.E. (2011) Expression of the splicing factor gene SFRS10 is reduced in human obesity and contributes to enhanced lipogenesis. *Cell Metab* **14**, 208-218

45. Shimizu, K., Fukushima, H., Ogura, K., Lien, E.C., Nihira, N.T., Zhang, J., North, B.J., Guo, A., Nagashima, K., Nakagawa, T., Hoshikawa, S., Watahiki, A., Okabe, K., Yamada, A., Toker, A., Asara, J.M., Fukumoto, S., Nakayama, K.I., Nakayama, K., Inuzuka, H., and Wei, W. (2017) The SCFβ-TRCP E3 ubiquitin ligase complex

targets Lipin1 for ubiquitination and degradation to promote hepatic lipogenesis. *Science Signaling* **10**, 1-15

46. Ishimoto, K., Hayase, A., Kumagai, F., Kawai, M., Okuno, H., Hino, N., Okada, Y., Kawamura, T., Tanaka, T., Hamakubo, T., Sakai, J., Kodama, T., Tachibana, K., and Doi, T. (2017) Degradation of human Lipin-1 by BTRC E3 ubiquitin ligase. *Biochemical and Biophysical Research Communications* **488**, 159-164

47. Nadra, K., De Preux Charles, A.-S., Medard, J.-J., Hendriks, W. T., Han, G.-S., Gres, S., Carman, G. M., Saulnier-Blache, J.-S., Verheijen, M. H. G., and Chrast, R. (2008) Phosphatidic acid mediates demyelination in *Lpin1* mutant mice. *Genes Dev* **22**, 1647-1661

48. Carman, G. M. and Han, G.-S. (2009) Phosphatidic acid phosphatase, a key enzyme in the regulation of lipid synthesis. *J. Biol. Chem* **284**, 2593-2597

49. Langner, C. A., Birkenmeier, E. H., Roth, K. A., Bronson, R. T., and Gordon, J. I. (1991) Characterization of the peripheral neuropathy in neonatal and adult mice that are homozygous for the fatty liver dystrophy (*fld)* mutation. *J. Biol. Chem* **266**, 11955-11964

50. Reue, K., Xu, P., Wang, X.P., and Slavin, B.G. (2000) Adipose tissue deficiency, glucose intolerance, and increased atherosclerosis result from mutation in the mouse fatty liver dystrophy (*fld*) gene. *Journal of Lipid Research* **41**, 1067-1076

51. Phan, J., Peterfy, M., and Reue, K. (2004) Lipin expression preceding peroxisome proliferator-activated receptor-γ is critical for adipogenesis in vivo and in vitro. *J. Biol. Chem* **279**, 29558-29564

52. Zhang, P., Takeuchi, K., Csaki, L. S., and Reue, K. (2012) Lipin-1 phosphatidic phosphatase activity modulates phosphatidate levels to promote peroxisome proliferator-activated receptor γ (PPARγ) gene expression during adipogenesis. *J. Biol. Chem* **287**, 3485-3494

53. Mitra, M. S., Chen, Z., Ren, H., Harris, T. E., Chambers, K. T., Hall, A. M., Nadra, K., Klein, S., Chrast, R., Su, X., Morris, A. J., and Finck, B. N. (2012) Mice with an adipocyte-specific lipin 1 separation-of-function allele reveal unexpected roles for phosphatidic acid in metabolic regulation. *Proc. Natl. Acad. Sci. U. S. A* **110**, 642-647

54. Mul, J. D., Nadra, K., Jagalur, N. B., Nijman, I. J., Toonen, P. W., Medard, J. J., Gres, S., de Bruin, B. A., Han, G.-S., Brouwers, J. F., Carman, G. M., Saulnier-Blache, J. S., Meijer, D., Chrast, R., and Cuppen, E. (2011) A hypomorphic mutation in *Lpin1* induces progressively improving neuropathy and lipodystrophy in the rat. *J. Biol. Chem* **286**, 26781-26793

55. Michot, C., Hubert, L, Brivet, M., De Meirleir, L., Valayannopoulos, V., Muller-Felber, W., Venkateswaran, R., Ogier, H., Desguerre, I., Altuzarra, C., Thompson, E., Smitka, M., Huebner, A., Husson, M., Horvath, R., Chinnery, P., Vaz, F.M., Munnich, A., Elpeleg, O., Delahodde, A., de Keyzer, Y., and de Lonlay, P. (2010) *LPIN1* gene mutations: a major cause of severe rhabdomyolysis in early childhood. *Hum Mutat* **31**, E1564-1573

56. Zhang, P., Verity, M. A., and Reue, K. (2014) Lipin-1 regulates autophagy clearance and intersects with statin drug effects in skeletal muscle. *Cell Metab* **20**, 267-279

57. Schweitzer, G. G., Collier, S.L., Chen, Z., Eaton, J.M., Connolly, A.M, Bucelli, R.C., Pestronk, A., Harris, T.E., and Finck, B.N. (2015) Rhabdomyolysis-associated mutations in human *LPIN1* lead to loss of phosphatidic acid phosphohydrolase activity. *JIMD Report*, 113-122

58. Mele, M., Ferreira, P.G., Reverter, F., DeLuca, D.S., Monlong, J., Sammeth, M., Young, T.R., Goldmann, J.M., Pervouchine, D.D., Sullivan, T.J., Johnson, R., Segre, A.V., Djebali, S., Niarchou, A., Consortium, T.G., Wright, R.A., Lappalainen, T., Calvo, M., Getz, G., Dermitzakis, E.T., Ardlie, K.G., and Guigo, R. (2015) The human transcriptome across tissues and individuals. *Science* **348**, 660-665

59. Legendre, A., Khraiche, D., Ou, P., Mauvais, F.X., Madrange, M., Guemann, A.S., Jais, J.P., Bonnet, D., Hamel, Y., and de Lonlay, P. (2018) Cardiac function and exercise adaptation in 8 children with *LPIN1* mutations. *Molecular Genetics and Metabolism* **123**, 375-381

60. Michot, C., Mamoune, A., Vamecq, J., Viou, M. T., Hsieh, L.-S., Testet, E., Laine, J., Hubert, L., Dessein, A. F., Fontaine, M., Ottolenghi, C., Fouillen, L., Nadra, K., Blanc, E., Bastin, J., Candon, S., Pende, M., Munnich, A., Smahi, A., Djouadi, F., Carman, G. M., Romero, N., de Keyzer, Y., and de Lonlay, P. (2013) Combination of lipid metabolism alterations and their sensitivity to inflammatory cytokines in human lipin-1-deficient myoblasts. *Biochim. Biophys. Acta* **1832**, 2103-2114

61. Kok, B. P. C., Kienesberger, P.C., Dyck, J.R.B., and Brindley, D.N. (2012) Relationship of glucose and oleate metabolism to cardiac function in lipin-1 deficient (*fld*) mice. *Journal of Lipid Research* **53**, 105-118

62. Pelosi, M., Testet, E., Le Lay, S., Dugail, I., Tang, X., Mabilleau, G., Hamel, Y., Madrange, M., Blanc, T., Odent, T., McMullen, T.P.W., Alfo, M., Brindley, D.N., and de Lonlay, P. (2017) Normal human adipose tissue functions and differentiation in patients with biallelic *LPIN1* inactivating mutations. *Journal of Lipid Research* **58**, 2348-2364

63. Lindegaard, B., Larsen, L. F., Hansen, A. B., Gerstoft, J., Pedersen, B. K., and Reue, K. (2007) Adipose tissue lipin expression levels distinguish HIV patients with and without lipodystrophy. *Int. J. Obes. (Lond)* **31**, 449-456

64. Dwyer, J. R., Donkor, J., Zhang, P., Csaki, I.S., Vergnes, I., Lee, J.M., Dewald, J., Brindley, D.N., Atti, E., Tetradis, S., Yoshinaga, Y., De Jong, P.J., Fong, L.G., Young, S.G., and Reue, K. (2012) Mouse lipin-1 and lipin-2 cooperate to maintain glycerolipid homeostasis in liver and aging cerebellum. *Proc Natl Acad Sci U S A* **109**, E2486-E2495

65. Majeed, H. A., Al-Tarawna, M., El-Shanti, H., Kamel, B., and Al-Khalaileh, F. (2001) The syndrome of chronic recurrent multifocal osteomyleitis and congenital dyserythropoietic anaemia. Report of a new family and a review. *European Journal of Pediatrics* **160**, 705-710

66. Ferguson, P. J., Chen, S., Tayeh, M.K., Ochoa, L., Leal, S.M., Pelet, A., Munnich, A., Lyonnet, S., Majeed, H.A., and El-Shanti, H. (2005) Homozygous mutations in *LPIN2* are responsible for the syndrome of chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia (Majeed syndrome). *Journal of Medical Genetics* **42**, 551-557

67. Lorden, G., Sanjuan-Garcia, I., de Pablo, N., Meana, C., Alvarez-Miguel, I., Perez-Garcia, M.T., Pelegrin, P., Balsinde, J., and Balboa, M.A. (2017) Lipin-2 regulates NLRP3 inflammasome by affecting P2X7 receptor activation. *Journal of Experimental Medicine* **214**, 511-528

68. Herlin, T., Fiirgaard, B., Bjerre, M., Kerndrup, G., Hasle, H., Bing, X., and Ferguson, P.J. (2013) Efficacy of anti-IL-1 treatment in Majeed syndrome. *Ann. Rheum. Dis.* **72**, 410-413

69. Csaki, L. S., Dwyer, J. R., Li, X., Nguyen, M. H., Dewald, J., Brindley, D. N., Lusis, A. J., Yoshinaga, Y., de, J. P., Fong, L., Young, S. G., and Reue, K. (2014) Lipin-1 and lipin-3 together determine adiposity in vivo. *Mol. Metab* **3**, 145-154

70. Croce, M. A., Eagon, J. C., LaRiviere, L. L., Korenblat, K. M., Klein, S., and Finck, B. N. (2007) Hepatic lipin 1β expression is diminished in insulin-resistant obese subjects and is reactivated by marked weight loss. *Diabetes* **56**, 2395-2399

71. Yao-Borengasser, A., Rasouli, N., Varma, V., Miles, L. M., Phanavanh, B., Starks, T. N., Phan, J., Spencer, H. J., III, McGehee, R. E., Jr., Reue, K., and Kern, P. A. (2006) Lipin expression is attenuated in adipose tissue of insulin-resistant human subjects and increases with peroxisome proliferator-activated receptor γ activation. *Diabetes* **55**, 2811-2818

72. He, J., Gao, J., Xu, M., Ren, S., Stefanovic-Racic, M., O-Doherty, R.M., and Xie, W. (2013) PXR ablation alleviates diet-induced and genetic obesity and insulin resistance in mice. *Diabetes* **62**, 1876-1887

73. Brohee, L., Demine, S., Willems, J., Arnould, T., Colige, A. C., and Deroanne, C. F. (2015) Lipin-1 regulates cancer cell phenotype and is a potential target to potentiate rapamycin treatment. *Oncotarget* **6**, 11264-11280

74. Kourti, M., Ikonomou, G., Giakoumakis, N. N., Rapsomaniki, M. A., Landegren, U., Siniossoglou, S., Lygerou, Z., Simos, G., and Mylonis, I. (2015) CK1δ restrains lipin-1 induction, lipid droplet formation and cell proliferation under hypoxia by reducing HIF-1α/ARNT complex formation. *Cell Signal* **27**, 1129-1140

75. He, J., Zhang, F., Tay, L. W., Boroda, S., Nian, W., Levental, K. R., Levental, I., Harris, T. E., Chang, J. T., and Du, G. (2017) Lipin-1 regulation of phospholipid synthesis maintains endoplasmic reticulum homeostasis and is critical for triple-negative breast cancer cell survival. *FASEB J*

76. Fan, X., Weng, Y., Bai, Y., Wang, Z., Wang, S., Zhu, J., and Zhang, F. (2018) Lipin-1 determines lung cancer cell survival and chemotherapy sensitivity by regulation of endoplasmic reticulum homeostasis and autophagy. *Cancer Medicine* **16**

77. Chen, Z., Gropler, M. C., Mitra, M. S., and Finck, B. N. (2012) Complex interplay between the lipin 1 and the hepatocyte nuclear factor 4 α (HNF4α) pathways to regulate liver lipid metabolism. *PLoS. One* **7**, e51320

78. Wang, J., Kim, C., Jogasuria, A., Han, Y., Hu, X., Wu, J., Shen, H., Chrast, R., Finck, B. N., and You, M. (2016) Myeloid cell-specific lipin-1 deficiency stimulates endocrine adiponectin-FGF15 axis and ameliorates ethanol-induced liver injury in mice. *Sci. Rep* **6**, 34117

79. Hu, M., Yin, H., Mitra, M.S., Liang, X., Ajmo, J.M., Nadra, K., Chrast, R., Finch, B.N., and You, M. (2013) Hepatic-specific lipin-1 deficiency exacerbates experimental alcohol-induced steatohepatitis in mice. *Hepatology* **58**, 1953-1963

80. Shen, Z., Liang, X., Rogers, C.Q., Rideout, D., and You, M. (2009) Involvement of adiponectin-SIRT1-AMPK signaling in the protective action of rosiglitazone against alcoholic fatty liver in mice. *Am J Physiol Gastrointest Liver Physiol* **298**, G364-G374

81. Jang, C. H., Kim, K. M., Yang, J. H., Cho, S. S., Kim, S. J., Shin, S. M., Cho, I. J., and Ki, S. H. (2016) The role of lipin-1 in the regulation of fibrogenesis and TGF-β signaling in hepatic stellate cells. *Toxicol. Sci* **153**, 28-38

82. Navratil, A. R., Vozenilek, A. E., Cardelli, J. A., Green, J. M., Thomas, M. J., Sorci-Thomas, M. G., Orr, A. W., and Woolard, M. D. (2015) Lipin-1 contributes to modified low-density lipoprotein-elicited macrophage pro-inflammatory responses. *Atherosclerosis* **242**, 424-432

83. Vozenilek, A. E., Navratil, A.R., Green, J.M., Coleman, D.T., Blackburn, C.M.R., Finney, A.C., Pearson, B.H., Chrast, R., Finck, B.N., Klein, R.L., Orr, A.W., and Woolard, M.D. (2018) Macrophage-associated lipin-1 enzymatic activity contributes to modified low-density lipoprotein-induced proinflammatory signaling and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **38**, 324-334

84. Huffman, T. A., Mothe-Satney, I., and Lawrence, J. C., Jr. (2002) Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin. *Proc. Natl. Acad. Sci U. S. A* **99**, 1047-1052

85. Eaton, J. M., Mullins, G.R., Brindley, D.N., and Harris, T.E. (2013) Phosphorylation of lipin 1 and charge on the phosphatidic acid head group control its phosphatidic acid phosphatase activity and membrane association. *Journal of Biological Chemistry* **288**, 9933-9945

86. Han, S., Bahmanyar, S., Zhang, P., Grishin, N., Oegema, K., Crooke, R., Graham, M., Reue, K., Dixon, J. E., and Goodman, J. M. (2012) Nuclear envelope phosphatase 1-regulatory subunit 1 (formerly TMEM188) is the metazoan Spo7p ortholog and functions in the lipin activation pathway. *J. Biol. Chem* **287**, 3123-3137

87. Kooijman, E. E., Carter, K. M., van Laar, E. G., Chupin, V., Burger, K. N., and de Kruijff, B. (2005) What makes the bioactive lipids phosphatidic acid and lysophosphatidic acid so special? *Biochemistry* **44**, 17007-17015

88. Fu, S., Yang, L., Li, P., Hofmann, O., Dicker, L., Hide, W., Lin, X., Watkins, S.M., Ivanov, A.R., and Hotamisligil, G.S. (2011) Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. *Nature* **473**

89. Li, T. Y., Song, L., Sun, Y., Li, J., Yi, C., Lam, S.M., Xu, D., Zhou, L., Li, X., Yang, Y., Zhang, C.S., Xie, C., Huang, X., Shui, G., Lin, S.Y., Reue, K., and Lin, S.C. (2018) Tip60-mediated lipin 1 acetylation and ER translocation determine triacylglycerol synthesis rate. *Nature Communications* **9**, 1-14

90. Wu, W.-I., Lin, Y.-P., Wang, E., Merrill, A. H., Jr., and Carman, G. M. (1993) Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by sphingoid bases. *J. Biol. Chem* **268**, 13830-13837

91. Wu, W.-I. and Carman, G. M. (1996) Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by phospholipids. *Biochemistry* **35**, 3790-3796

92. Hsieh, L.-S., Su, W.-M., Han, G.-S., and Carman, G. M. (2015) Phosphorylation regulates the ubiquitin-independent degradation of yeast Pah1 phosphatidate phosphatase by the 20S proteasome. *J. Biol. Chem* **290**, 11467-11478

93. Taylor, F. R. and Parks, L. W. (1979) Triacylglycerol metabolism in *Saccharomyces cerevisiae* relation to phospholipid synthesis. *Biochim. Biophys. Acta* **575**, 204-214

94. Fakas, S., Qiu, Y., Dixon, J. L., Han, G.-S., Ruggles, K. V., Garbarino, J., Sturley, S. L., and Carman, G. M. (2011) Phosphatidate phosphatase activity plays a key role in protection against fatty acid-induced toxicity in yeast. *J. Biol. Chem* **286**, 29074-29085

95. Pascual, F., Soto-Cardalda, A., and Carman, G. M. (2013) *PAH1*-encoded phosphatidate phosphatase plays a role in the growth phase- and inositol-mediated regulation of lipid synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem* **288**, 35781-35792

96. Exton, J. H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta* **1212**, 26-42

97. Exton, J. H. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem* **265**, 1-4

98. Testerink, C. and Munnik, T. (2005) Phosphatidic acid: a multifunctional stress signaling lipid in plants. *Trends Plant Sci* **10**, 368-375

99. Waggoner, D. W., Xu, J., Singh, I., Jasinska, R., Zhang, Q. X., and Brindley, D. N. (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *Biochim. Biophys. Acta* **1439**, 299-316

100. Sciorra, V. A. and Morris, A. J. (2002) Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta* **1582**, 45-51

101. Kodaki, T., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) Functional analysis of the regulatory region of the yeast phosphatidylserine synthase gene, *PSS*. *J. Bacteriol* **173**, 7992-7995

102. Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L., and Henry, S. A. (1991) Analyses of sequences in the *INO1* promoter that are involved in its regulation by phospholipid precursors. *Nucl. Acids Res* **19**, 1687-1693

103. Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., and Levine, T. P. (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. *Science* **304**, 1644-1647

104. Santos-Rosa, H., Leung, J., Grimsey, N., Peak-Chew, S., and Siniossoglou, S. (2005) The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth. *EMBO J* **24**, 1931-1941

105. Ubersax, J. A., Woodbury, E. L., Quang, P. N., Paraz, M., Blethrow, J. D., Shah, K., Shokat, K. M., and Morgan, D. O. (2003) Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**, 859-864

106. Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R. R., Schmidt, M. C., Rachidi, N., Lee, S. J., Mah, A. S., Meng, L., Stark, M. J., Stern, D. F., C., D. V., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. F., and Snyder, M. (2005) Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679-684

107. Hsieh, L.-S., Su, W.-M., Han, G.-S., and Carman, G. M. (2016) Phosphorylation of yeast Pah1 phosphatidate phosphatase by casein kinase II regulates its function in lipid metabolism. *J. Biol. Chem* **291**, 9974-9990

108. O'Hara, L., Han, G.-S., Peak-Chew, S., Grimsey, N., Carman, G. M., and Siniossoglou, S. (2006) Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg^{2+}-dependent phosphatidate phosphatase. *J. Biol. Chem* **281**, 34537-34548

109. Chi, A., Huttenhower, C., Geer, L. Y., Coon, J. J., Syka, J. E., Bai, D. L., Shabanowitz, J., Burke, D. J., Troyanskaya, O. G., and Hunt, D. F. (2007) Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A* **104**, 2193-2198

110. Li, X., Gerber, S. A., Rudner, A. D., Beausoleil, S. A., Haas, W., Villen, J., Elias, J. E., and Gygi, S. P. (2007) Large-scale phosphorylation analysis of alpha-factor-arrested *Saccharomyces cerevisiae*. *J. Proteome. Res* **6**, 1190-1197

111. Han, G.-S. and Carman, G. M. (2017) Yeast *PAH1*-encoded phosphatidate phosphatase controls the expression of *CHO1*-encoded phosphatidylserine synthase for membrane phospholipid synthesis. *J. Biol. Chem* **292**, 13230-13242

112. Pascual, F. and Carman, G. M. (2013) Phosphatidate phosphatase, a key regulator of lipid homeostasis. *Biochim. Biophys. Acta* **1831**, 514-522

113. Adeyo, O., Horn, P. J., Lee, S., Binns, D. D., Chandrahas, A., Chapman, K. D., and Goodman, J. M. (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. *J. Cell Biol* **192**, 1043-1055

114. Han, G.-S., O'Hara, L., Carman, G. M., and Siniossoglou, S. (2008) An unconventional diacylglycerol kinase that regulates phospholipid synthesis and nuclear membrane growth. *J. Biol. Chem* **283**, 20433-20442

115. Park, Y., Han, G. S., Mileykovskaya, E., Garrett, T. A., and Carman, G. M. (2015) Altered lipid synthesis by lack of yeast Pah1 phosphatidate phosphatase reduces chronological life span. *J. Biol. Chem* **290**, 25382-25394

116. Irie, K., Takase, M., Araki, H., and Oshima, Y. (1993) A gene, *SMP2*, involved in plasmid maintenance and respiration in *Saccharomyces cerevisiae* encodes a highly charged protein. *Mol. Gen. Genet* **236**, 283-288

117. Sasser, T., Qiu, Q. S., Karunakaran, S., Padolina, M., Reyes, A., Flood, B., Smith, S., Gonzales, C., and Fratti, R. A. (2011) The yeast lipin 1 orthologue Pah1p regulates vacuole homeostasis and membrane fusion. *J. Biol. Chem* **287**, 2221-2236

118. Sherr, G. L., LaMassa, N., Li, E., Phillips, G., and Shen, C. H. (2017) Pah1p negatively regulates the expression of V-ATPase genes as well as vacuolar acidification. *Biochem. Biophys. Res. Commun* **491**, 693-700

119. Rahman, M. A., Mostofa, M.G., and Ushimaru, T. (2018) The Nem1/Spo7-Pah1/lipin axis is required for autophagy induction after TORC1 inactivation. *FEBS J*

120. Lussier, M., White, A. M., Sheraton, J., di, P. T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F., Kapteyn, J. C., Roemer, T. W., Vo, D. H., Bondoc, D. C., Hall, J., Zhong, W. W., Sdicu, A. M., Davies, J., Klis, F. M., Robbins, P. W., and Bussey, H. (1997) Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* **147**, 435-450

121. Ruiz, C., Cid, V. J., Lussier, M., Molina, M., and Nombela, C. (1999) A large-scale sonication assay for cell wall mutant analysis in yeast. *Yeast* **15**, 1001-1008

122. Dey, P., Su, W.-M., Han, G.-S., and Carman, G. M. (2017) Phosphorylation of lipid metabolic enzymes by yeast Pkc1 protein kinase C requires phosphatidylserine and diacylglycerol. *J. Lipid Res* **58**, 742-751

123. Boroda, S., Takkellapati, S., Lawrence, R.T., Entwisle, S.W., Pearson, J.M., Granade, M.E., Mullins, G.R., Eaton, J.M., Villen, J., and Harris, T.E. (2017) The phosphatidic acid-binding, polybasic domain is responsible for the differences in the phosphoregulation of lipins 1 and 3. *J Biol Chem* **292**, 20481-20493

124. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**, 1633-1649

125. Prasad, T. S., Kandasamy, K., and Pandey, A. (2009) Human Protein Reference Database and Human Proteinpedia as discovery tools for systems biology. *Methods Mol Biol* **577**, 67-79

126. Cleland, W. W. (1970) Steady state kinetics. In *The enzymes* (Boyer, P. D. ed.), 2 Ed., Academic Press, New York. pp 1-65

127. Kok, B. P., Skene-Arnold, T. D., Ling, J., Benesch, M. G., Dewald, J., Harris, T. E., Holmes, C. F., and Brindley, D. N. (2014) Conserved residues in the N terminus of lipin-1 are required for binding to protein phosphatase-1c, nuclear translocation, and phosphatidate phosphatase activity. *J. Biol. Chem* **289**, 10876-10886

128. Choi, H.-S., Su, W.-M., Han, G.-S., Plote, D., Xu, Z., and Carman, G. M. (2012) Pho85p-Pho80p phosphorylation of yeast Pah1p phosphatidate phosphatase regulates its activity, location, abundance, and function in lipid metabolism. *J. Biol. Chem* **287**, 11290-11301

129. Choi, H.-S., Su, W.-M., Morgan, J. M., Han, G.-S., Xu, Z., Karanasios, E., Siniossoglou, S., and Carman, G. M. (2011) Phosphorylation of phosphatidate phosphatase regulates its membrane association and physiological functions in *Saccharomyces cerevisiae:* identification of Ser^{602}, Thr^{723}, and Ser^{744} as the sites phosphorylated by *CDC28* (*CDK1*)-encoded cyclin-dependent kinase. *J. Biol. Chem* **286**, 1486-1498

130. Su, W.-M., Han, G.-S., Casciano, J., and Carman, G. M. (2012) Protein kinase A-mediated phosphorylation of Pah1p phosphatidate phosphatase functions in conjunction with the Pho85p-Pho80p and Cdc28p-cyclin B kinases to regulate lipid synthesis in yeast. *J. Biol. Chem* **287**, 33364-33376

131. Su, W.-M., Han, G.-S., and Carman, G. M. (2014) Cross-talk phosphorylations by protein kinase C and Pho85p-Pho80p protein kinase regulate Pah1p phosphatidate phosphatase abundance in *Saccharomyces cerevisiae*. *J. Biol. Chem* **289**, 18818-18830

132. Litchfield, D. W. (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J* **369**, 1-15

133. Witters, L. A., Tipper, J.P., and Bacon, G.W. (1983) Stimulation of site-specific phosphorylation of acetyl coenzyme A carboxylase by insulin and epinephrine. *Journal of Biological Chemistry* **258**, 5643-5648

134. Diggle, T. A., Schmitz-Peiffer, C., Borthwick, A. C., Welsh, G. I., and Denton, R. M. (1991) Evidence that insulin activates casein kinase 2 in rat epididymal fat-cells and that this may result in the increased phosphorylation of an acid-soluble 22 kDa protein. *Biochem J* **279 (Pt 2)**, 545-551