ENZYMES INVOLVED IN THE PHOSPHATIDYLINOSITOL CYCLE

EXAMINATION OF ENZYMATIC ACTIVITY AND SUBSTRATE SPECIFICITY IN ENZYMES INVOLVED IN THE PHOSPHATIDYLINOSITOL CYCLE

By

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ABSTRACT

Phosphatidylinositol (PI) is a phospholipid that constitutes only a minor component of eukaryotic membranes. However, they are critical in many fundamental cellular processes, such as signal transduction pathways, vesicular trafficking and actin cytoskeletal dynamics. PI is highly enriched in specific acyl chains at both the *sn-1* and *sn-2* positions, the major species being 1-stearoyl-2-arachidonoyl. Enzymes required for PI synthesis are believed to play a major role in this enrichment through the selective catalysis of specific substrates. We have studied several aspects of two enzymes involved in PI synthesis, Diacylglycerol kinase ε (DGKε) and CDP-Diacylglycerol synthases (CDS). We have studied the role of the ATP-binding motif of DGKε and showed that this enzyme is not only required for enzymatic activity, but substrate specificity and sub-cellular localization. We have also looked at the region adjacent to the catalytic site, containing a cholesterol recognition motif, and determined that this also affects the enzymes activity and substrate specificity. Finally, we have characterized the enzymatic properties of two CDS isoforms *in vitro* and demonstrated that these isoforms exhibit different substrate specificities. Taken together, our results serve to further our understanding of both DGKε and CDS1/2 and their roles in PI synthesis and enrichment with specific acyl chains.

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PREFACE

This following work is presented as a sandwich thesis. Chapter 1 provides an introduction to the field of phosphatidylinositol metabolism using a review that we published in 2013. Chapter 2 is a manuscript that we submitted that looks at the enzymatic activity and substrate specificity of DGKε, an enzyme in the PI cycle. Chapter 3 is a published manuscript of a minor project on DGKε that further looks at enzymatic activity and substrate specificity. Chapter 4 is composed primarily of a manuscript in preparation of the substrate specificities of CDS, another enzyme in the PI cycle. All published papers are presented as the submitted word document. All chapters have been reproduced with consent of all co-authors.

CHAPTER 1: Introduction

The Enrichment of Phosphatidyinositols with Specific Acyl Chains[1][2]

Kenneth D'Souza and Richard M. Epand

This paper provides an introduction to phosphatidylinositol, its physiological and cellular relevance and the enzymes that contribute to its acyl chain enrichment. My contribution to this paper was to write the manuscript, prepare all the figures and edit.

[1] Dsouza, K. and Epand, R. M. (2013). Enrichment of phosphatidylinositols with specific acyl chains. Biochem Biophys Acta. pii: S0005-2736(13)00356-8. doi: 10.1016/j.bbamem.2013.10.003. [Epub ahead of print]

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Abstract: There are six major species of phospholipids in eukaryotes, each of which play unique structural and functional roles. One species, phosphatidylinositol (PI) only contributes about 2-10% of the total phospholipid pool. However, they are critical factors in the regulation of several fundamental processes such as in membrane dynamics and signal transduction pathways. Although numerous acyl species exist, PI species are enriched with one specific acyl chain composition at both the *sn-1* and *sn-2* positions. Recent work has identified several enzymes that act on lipids to lead to the formation or interconversion of PI species that exhibit acyl chain specificity. These enzymes contribute to this lipid's enrichment with specific acyl chains. The nature of the acyl chains on signaling lipids have been shown to contribute to their specificity. Here we review some of the critical functions of PI and the multiple pathways in which PI can be produced and metabolized. We also discuss a common motif that may confer arachidonoyl specificity to several of the enzymes involved.

Keywords: Phosphatidylinositol, phosphatidylinositol cycle, arachidonoyl, lipid signals, acyl chains

Abbreviations used: 2-AG, 2-arachidonoylglycerol; CDS, CDP-diacylglycerol synthase; CDP-DAG, CDP-diacylglycerol; DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic **r**eticulum; IP_3, inositol triphosphate; LPA, lysophosphatidic acid; LPIAT1, lysophosphatidylinositol acyltransferase 1; PA, phosphatidic acid; PI, phosphatidylinositol; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; PI4P5K, phosphatidylinositol-4-phosphate 5-Kinase; PIP_n, phosphorylated forms of PI; PIS, PI synthase; PKC, protein kinase C; PLC, phospholipase C; PM, plasma membrane; PUFA, polyunsaturated fatty acids; SAG, 1-stearoyl-2-arachidonoyl glycerol

1. Introduction

The major focus regarding the lipid composition of biological membranes and the roles of lipids in signal transduction has been on the nature of the lipid headgroup. However, it is well established that the acyl chain composition of lipids also has an important biological role. This is at first surprising since acyl chains are only hydrocarbons and do not contain a variety of polar groups that can result in specific interactions. An example of a specific role of an acyl chain is the finding that one specific acyl chain, the arachidonoyl (20:4) chain (Table 1 shows some acyl chain structures) attached to phosphatidylcholine oscillates during the cell cycle and delays cell cycle progression as a consequence of inhibiting the binding of Akt [1]. There are several mechanisms by which acyl chains can modulate function. One mechanism is by changing the physical properties of the membrane, with no specific requirements for a particular chemical structure. One example of this is tafazzin, an acyl transferase that enriches cardiolipin with linoleoyl (18:2) chains, yet exhibits no substrate specificity for particular acyl chains [2]. In contrast to this, there are very specific structural requirements of certain enzymes for particular lipids, such as the specificity of certain lipoxygenases for arachidonic acid [3].

Another indication that acyl chains play a functional role is the observation that they are very unevenly distributed among lipids of different classes, *i.e.* among lipids containing different headgroups. Even within the same organism, the acyl chain composition of specific lipids is different in different organs. Furthermore, changes in the acyl chain composition of a lipid can affect its function and even lead to disease states. The factors determining the specific incorporation of particular acyl chains in certain lipids and the consequences of the loss of this specificity are only recently attracting more attention. One lipid class that is highly enriched in specific acyl chains is phosphatidylinositol (PI).

1.1. Properties of Phosphatidylinositols

Phosphatidylinositol (PI) is composed of a glycerol backbone, with an inositol ring and a phosphate at the *sn-3* position and two acyl chains esterified at the *sn-1* and *sn-2* positions [4]. The inositol ring can be phosphorylated at multiple positions, which can yield seven unique species known as phosphoinositides (PIP_n) (Figure 1) [5]. PI species are spatially and temporally maintained in distinct sub-cellular compartments through the concerted actions of PI-kinases and phosphatases. For example, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) species are primarily enriched in the plasma membrane (PM), whereas phosphatidylinositol-4-phosphate (PI4P) is high in the Golgi [6,7].

PIP_n species control several different cellular processes such as the regulation of ion channels, actin-cytoskeleton dynamics, vesicular transport, endocytosis, exocytosis and signal transduction pathways [8-10]. The interactions between PIP_n and their downstream targets are numerous and complex, so only an overview of their interactions will be discussed. Simply, downstream targets of PIP_n are recruited and/or activated at specific sub-cellular compartments through phosphoinositide binding motifs. These interactions are primarily mediated through a combination of electrostatic and hydrophobic interactions [11]. The role of the headgroup structure in these interactions is currently better understood, however evidence is accumulating to indicate that the acyl chain composition also has an important role. Currently, ten phosphoinositide binding motifs have been characterized, each showing specificity for different PI species [12]. For example, although the adaptor proteins AP1 and AP2 bind similar cargo proteins, AP1 binds PI4P and localizes in the Golgi. AP2 on the other hand, binds $PI(4,5)P_2$ and is enriched in the PM [13,14].

1.2. Phosphatidylinositol biosynthesis

The *de-novo* biosynthesis of PI occurs exclusively in the endoplasmic reticulum (ER) and begins with the precursors, glycerol-3-phosphate or dihydroxyacetonephosphate (Figure 2) [15]. These molecules undergo two sets of acylations through the actions of acyltransferases; the first acylation forms lysophosphatidic acid (LPA), whereas the second acylation step produces phosphatidic acid (PA) [16]. PA can also be formed through the actions of diacylglycerol kinase (DGK) on diacylglycerol (DAG) and by the action of phospholipase D on certain phospholipids, such as phosphotidylcholine. PA is then converted to CDP-diacylglycerol (CDP-DAG), through CDP-DAG synthases (CDS), from PA and CTP. In the final step, PI synthase (PIS) catalyzes the coupling of CDP-DAG to *myo*-inositol to form PI [15]. Once formed in the ER, the PI is distributed to other locations in the cell, including the plasma membrane (PM) resulting in the recycling of lipid between the PM and ER forming the PI cycle as described below.

1.2.1. Acyl-chain enrichment PI is highly enriched at both the *sn-1* and *sn-2* positions with specific acyl chains; the major species being 1-stearoyl, 2-arachidonoyl PI (18:0 *sn-1*/20:4 *sn-2* PI) [17-19]. Depending on the particular tissue, up to 70% of all PI species have this acyl chain composition [18]. Interestingly, studies have shown that the acyl chain composition of PI synthesized through the *de novo* pathway, in contrast to the lipids in the PI cycle, contains mainly saturated and monounsaturated acyl chains [20-23]. So, how do PI species become highly enriched with a different acyl chain composition? There are two potential enzymatic processes that can result in this selective acyl chain incorporation. One involves the cyclical pathway known as the PI cycle [24]. The substrate specificity of the enzymes involved in this

cycle can lead to acyl chain enrichment. Since the processes is cyclical and all the lipid intermediates of the cycle are regenerated, any partial enrichment occurring in one cycle will be multiplied by the number of times the cycle repeats. The second pathway, known as the Land's cycle, involves acyl chain remodeling of PI species through acylation and deacylation reactions [25].

1.2.2. The PI cycle Upon stimulation by growth factors, PI-specific isoforms of phospholipase C (PI-PLC) cleave PI(4,5)P_2 in the PM into inositol triphosphate (IP_3) and DAG (Figure 2) [24]. IP_3 is a water soluble signaling molecule which can activate Ca^{2+} channels and release Ca^{2+} from the ER. DAG itself is a potent lipid secondary messenger which can activate several different DAG-binding proteins such as PKC, PKD, Munc13 and RasGRP [26]. The DAG that is a component of the PI cycle is phosphorylated to PA by DGK in either the ER or the PM [27]. This PA can then be fed into the PI cycle [24]. PA is also produced by the action of phospholipase D, but this PA is not enriched in stearoyl-arachidonoyl acyl chains and therefore must be segregated from the PI cycle. The PI produced in the ER is then transported to the PM, undergoing two phosphorylations to generate PI(4,5)P2[27]. The cyclical nature of the PI cycle suggests that acyl chain enrichment of PI species could occur if one or more enzymes show acyl chain specificity.

1.2.3 The Land's cycle. All phospholipids undergo acyl chain remodeling through the actions of acyltransferases and phospholipases. This process is collectively known as the Land's cycle (Figure 3) [25,28-30]. Remodeling allows certain lipids to maintain specific acyl chain compositions, which is important for signaling functions. Several classes of enzymes that are needed for remodeling have been characterized. Lysophosphatidylinositol acyltransferases are enzymes that transfer an acyl group from acyl-CoA to the *sn-2* position of a lysophospholipid

[31]. Other enzymes required for remodeling include the phospholipase A1 and A2 families, which cleave acyl chains off phospholipids [32,33]. The Land's cycle could result in acyl chain enrichment through the selective incorporation and/or removal at acyl chains of PI.

2. Evidence for the importance of enrichment of DAG with specific acyl chains

The DAG species produced from PC has distinct acyl chain compositions compared with the DAG produced from PI(4,5)P_2 [34,35]. DAG produced by cleavage of PC is either saturated or monounsaturated at its *sn-2* position. The principle form of DAG produced from PI(4,5)P_2, as a step in the PI cycle, is 1-stearoyl-2-arachidonoyl glycerol (SAG) formed by the action of PI-PLC on PI(4,5)P_2. The nature of the acyl chains on DAG determine its potency in stimulating protein kinase C (PKC) [36]. Sustained PKC activation has been shown to be oncogenic and contributes to malignant phenotypes seen in cancers [37,38]. Only polyunsaturated DAG species (derived from PI(4,5)P_2) were shown to be able to bind and activate PKC (α, ε, δ) isoforms both *in vitro* and *in* vivo [34,35,39]. In particular, SAG is more effective in activating PKCδ compared with other DAG species. The relative activating potency of different DAG species is different for different PKC isoforms [35], showing that the acyl chain composition of DAG affects its signaling properties. Other evidence of acyl chain specificity of DAG comes from recent studies of the activation of caged DAGs with different acyl chains [40]. It was demonstrated that the photoactivation to liberate SAG, and not other DAGs, resulted in a massive increase of intracellular Ca^{2+} levels. This can be understood in terms of the PI cycle. SAG is an intermediate in the PI cycle and its liberation will activate the cycle. This is because in biochemical cycles all of the intermediates in the cycle act as catalysts for the cycle because they are regenerated by the cycle itself. While all of the lipid intermediates are regenerated by the cycle, the overall process utilizes nucleotide triphosphates and inositol to generate IP_3, a ligand

for opening calcium channels in the ER and raising the intracellular levels of Ca^{2+}. Thus, these experiments tie in specific acyl chains with signaling resulting from the PI cycle.

3. Phospholipids as precursors of bioactive lipids

Phospholipids themselves can act as signaling molecules, as well as being a precursor for other bioactive lipids through the action of phospholipases. For example, arachidonoic acid, liberated by hydrolysis of phospholipids, is a critical precursor for a large family of inflammatory compounds known as eicosanoids [41,42]. Free fatty acids and lysophospholipids have roles as lipid signaling agents or as precursors to such lipids. However, fatty acids and lysolipids also have detergent-like properties and are toxic to cells at high concentration. Hence they are maintained at low concentrations in the cell. They are produced as signaling agents from phospholipid precursors and the quantity of fatty acids and lysolipids that can be generated is highly dependent on the presence of certain acyl chains in the phospholipid. Hence the amount of phospholipids in a cell with certain acyl chains, in particular arachidonoyl and docohexanoyl chains, will determine the ability to form downstream lipid signaling molecules.

3.1. Arachidonic acid as a precursor of bioactive lipids.

Polyunsaturated acyl chains also serve as precursors to several classes of lipid modulations. These lipid modulators play critical roles in processes such as inflammation, blood pressure, immune function and neuroprotection [43,44]. Arachidonic acid, derived from the *sn-2* acyl chain of PI and phosphatidylethanolamine (PE), are precursors to a family of oxygenated derivatives known as eicosanoids. Eicosanoids encompass a large family of lipid molecules, most of which are pro-inflammation [41,42]. Chronic inflammation is implicated in several diseases, such as fever, arthritis, atherosclerosis and cancer [41,44,45].

3.2. Polyunsaturated acids (PUFA) as precursors of anti-inflamatory lipids.

Many of the eicosanoid lipids are pro-inflammatory. Polyunsaturated fatty acids are also precursors for anti-inflammatory lipids. Arachidonic acid is an n-3 PUFA. Another common n-3 PUFA is eicosapentaenoic acid (20:5) is a precursor for the E-resolvins, and docosahexaenoic acid (22:6) is a precursor for the D-resolvins and for protectin D1 [46]. All of these products are anti-inflammatory and reverse the inflammation caused by eisosanoids [47].

3.3. 2-arachidonoylglycerol (2-AG) as a signaling lipid.

2-arachidonoylglycerol (2-AG) is derived from the cleavage of SAG by DAG lipases [48]. 2-AG is a known ligand for the CB_1 and CB_2 cannabinoid receptors, which are implicated in the regulation of food intake, neurotransmitter release and pain [49-51]. Aberrant 2-AG signaling has also been clinically linked to the contribution of Alzheimer's disease in patients [52].

4. Enzymes contributing to the enrichment of lipids with arachidonoyl groups

While several different PUFA have specific roles in lipid signaling, in this review we wish to focus on the role of arachidonoyl enrichment which is important for the lipid intermediates of the PI cycle, as well as for the generation of eicosanoids. Several enzymes show arachidonoyl specificity and could play significant roles in PI's acyl chain enrichment. In the following sections, we will examine several of these enzymes, with specific emphasis on the physiological significance of DGKε, phosphatidylinositol-4-phosphate 5-Kinase (PI4P5K) and lysophosphatidylinositol acyltransferase 1 (LPIAT1).

4.1. Diacylglycerol Kinase Epsilon (DGKε). DGKε catalyzes the phosphorylation of DAG to PA using ATP as a phosphate donor [53]. Out of the ten mammalian isoforms of DGK, DGKε is the only isoform that shows specificity for the acyl chains of the substrate [54]. *In vitro* and *in vivo* experiments have shown that DGKε prefers DAG substrates with an *sn-1* stearoyl and an *sn-2* arachidonoyl group, the species being SAG (18:0 *sn-1*/20:4 *sn-2* DAG) [54-56].

Since the acyl chain composition of SAG is also predominant in PI species, it is believed that DGKε is committed to PI re-synthesis. We should point out that although DGKε can contribute significantly to acyl chain enrichment in PI, it is not the only factor. There are tissues in which DGKε is not highly expressed, yet they still can synthesize PI. However, in tissues such as the brain in which DGKε is highly expressed, acyl chain enrichment, with *sn-1* stearoyl and *sn-2* arachidonoyl, is particularly large. Studies in DGKε$^{-/-}$ MEFs have indicated that there is a roughly 30% decrease in the content of PA and PI the in plasma membrane of these cells as compared to wild type MEFs [24]. Similar lipidomic experiments in DGKε$^{-/-}$ MEFs have also shown a reduction in the levels of both stearoyl and arachidonoyl content in PI lipids [17,54]. The decrease in arachidonoyl content also extends to other lipid classes, including PC [17]. This change in arachidonoyl-PC would be expected to increase cell cycling and promote Akt activation [1]. Over-expression of DGKε in porcine aortic endothelial cells, that would phosphorylate SAG to make the corresponding PA, results in significant decreases in polyunsaturated DAGs [34]. In DGKε$^{-/-}$ mouse models, electrical stimulation of these mice resulted in decreases in the levels of arachidonyl-PI(4,5)P_2 and free arachidonic acid [57]. Thus it appears that DGKε not only contributes to the re-synthesis of PI, but also it also increased its arachidonoyl content.

The functional importance of DGKε in PI-synthesis and acyl chain enrichment has been demonstrated through several studies, most notably in neural function. DGKε appears to regulate seizure susceptibility and long term potentiation through PI signalling [43,57,58]. Using electroconvulsive shocks, DGKε$^{-/-}$ mice were shown to have reduced levels of free arachidonic acid, 20:4-DAG and 20:4-PI(4,5)P_2. These lipids were also shown to take longer to return to resting levels. Physiologically, DGKε$^{-/-}$ mice were shown to be resistant to electroconvulsive shock and kindling, conditions tied to deficiencies in long-term neural potentiation. DGKε$^{-/-}$ mice also showed a lack of morphological changes in hippocampal glial cells, including hypertrophied cell bodies and elongated processes [57]. The involvement of the PI-cycle and DGKε in kindling and long-term potentiation make it an attractive target for epilepsy.

DGKε also appears to be an attractive target for the attenuation of Huntington's disease. Blocking DAG-activated transient receptor potential has been shown to block Huntington's neurotoxicity [59]. Mutant Huntington (htt) protein also appears to bind subsets of PIPs more strongly that the wild type protein. Inhibition of DGKε using siRNAs reduced caspase activity and striatal toxicity of a Hdh$^{111Q/111Q}$ cell line. Similarly, DGKε protein levels were also increased in the striatum of HD transgenic mice, which suggests a potential role for this protein in the observed toxicity of mutant Htt.

Recent work has also identified roles for DGKε in renal function; mutations in the *DGKE* gene were identified in membranoproliferative-like glomerular microangiopathy (MPGN) and atypical hemolytic-uremic syndome (HUS) [60,61]. Both MPGN and HUS present with several renal phenotypes, such as thrombosis and frequently leading to renal failure. DAG signaling is believed to be key for the development of these diseases through the production of pro/anti-thrombic factors and the regulation of slit diaphragm function in endothelial cells and podocytes,

respectively. These mutant DGKε variants were primarily localized to its catalytic domain and were shown to result in increasing DAG levels.

DGKε restores cardiac dysfunction and improves survival under chronic pressure overload by controlling cellular DAG levels and TRPC-6 expression. It is suggested that DGKε may be a novel therapeutic target to prevent cardiac hypertrophy and progression to heart failure [62].

4.2. CDP-diacylglycerol Synthase 2 (CDS2). CDS2 is involved in the next step of PI re-synthesis after DGKε, catalyzing the conversion of PA to CDP-diacylglycerol [63]. There are two CDS isoforms, both of which are found in the ER [64,65]. CDS2 appears to show substrate specificity for PA species, preferentially acting on 1-stearoyl-2-arachidonoyl PA *in* vitro [66]. It is not currently known whether CDS1 also shows the same specificity. There is evidence that antidepressant drugs affect CDS activity [67].

4.3. Phosphatidylinositol-4-phosphate 5-Kinase (PI4P5K). PI4P5K phosphorylates PI4P to $PI(4,5)P_2$ using ATP as a phosphate donor. These enzymes are mainly responsible for the production of $PI(4,5)P_2$ and are the rate limiting step in the PI cycle [68]. $PI(4,5)P_2$'s regulate several critical processes such as actin polymerization and re-organization, vesicular trafficking, neurotransmitter release and signal transduction pathways [69,70]. Deregulation of $PI(4,5)P_2$ metabolism has been demonstrated in a wide range of neuronal disorders, including Down Syndrome, psychiatric disorders, Alzheimer's and Huntington's disease [71]. Additionally, $PI(4,5)P_2$ serves as a precursor to PIP_3, a potent signaling lipid implicated in cell survival, growth and migration. PIP_3 is up-regulated in several cancers and PTEN, a PIP_3 phosphatase is frequently mutated in cancers [72].

There are three PI4P5K isoforms (α, β, γ), all of which have been shown to prefer SAPI4P (18:0-*sn-1*/20:4-*sn-2* PI4P) and SOPI4P (18:0-*sn-1*/18:1-*sn-2* PI4P) species when compared to DPPI4P (16:0-*sn-1*/16:0-*sn-2* PI4P) [55,73]. Kinetic analysis also shows that these isoforms have the highest activity for SAPI4P, with the difference in selectivity being largest for the γ isoform [73]. PI4P5Ks also show acyl chain specificity for its lipid activator, the major activator being unsaturated PA species, such as DAPA (20:4-*sn-1*/20:4-*sn-2* PA) [73]. There thus appears to be more arachidonoyl specificity for the PA activation of PI4P5K than there is for the PI4P substrate of this enzyme [24,39,54].

Through its production of $PI(4,5)P_2$, PI4P5K play critical roles in many physiological processes; like DGKε and LPIAT1, these enzymes play especially critical roles in neural function and development. For example, PI4P5Kα is involved in ganglioside-stimulated astrocytes, helping to contribute and modulate the cell's inflammatory response [74]. Additionally, the α isoform was also shown to mediate TLR-dependant inflammation in microglial cells [75]. Prolonged inflammation by astrocytes is also recognized to play a role in the progression of neurodegenerative diseases [76]. It is currently not known whether regulation of these inflammatory processes is due to $PI(4,5)P_2$'s acyl chain composition or through interaction with $PI(4,5)P_2$ binding interaction.

PI4P5Kγ was shown to be important for both cardiovascular and neuronal development, being essential for cell junction formation in myocardiocytes and neural tube closure in the cranial region of mice [77]. Mutations in PI4P5Kγ were found to be present in Lethal Congenital Contractural Syndrome, a disease characterized by muscle contractures, wasting and atrophy [78]. A cluster of PI-metabolizing genes have also been mapped to chromosomal regions linked

to psychiatric diseases [78]. It has been suggested that these enzymes may play a potential role in the progression of these diseases; however, no evidence for this correlation exists.

4.4. Lysophosphatidylinositol acyltransferase 1 (LPIAT1). LPIAT1, also known as membrane bound O-acyltransferase containing domain 7 (also referred to as MBOAT7), catalyzes the transfer of an acyl-CoA to lysoPI [79]. As an acyltransferase, LPIAT1 is involved in the Land's cycle of acyl chain remodeling. Recently, it was shown that LPIAT1 has a high preference for arachidonoyl-CoA [79]. It is not know whether LPIAT1 also shows preference for the lyso-PI species as this species was not varied. However, LPIAT1's arachidonoyl specificity contributes to both the production and enrichment of arachidonoyl-PI.

Recently, two papers have characterized LPIAT1$^{-/-}$ KO mice and have demonstrated that this enzyme is critical for neural function. Both studies showed that knocking out LPIAT1 resulted in a significant decrease in arachidonoyl-containing PI, and PI(4,5)P_2 [80,81]. LPIAT1 appears to be the major arachidonoyl-CoA acyltransferase, as loss of this enzyme resulted in an almost complete loss of activity in the brain, liver, kidney and testis of mice. LPIAT1 also appears to be critical for neural development of mice; LPIAT$^{-/-}$ mice were viable up to 30 days after birth, but exhibited a smaller, atrophied cerebral cortex and hippocampus. The laminal structure of the neocortex was also disordered due to delayed neural migration, which indicated a role for LPIAT1 in cortical lamination [81].

4.5. DDHD domain containing 1 (DDHD1). DDHD1 belongs to a family of intracellular phospholipase A1's, which act to remodel acyl chains [82,83]. DDHD1 cleaves the *sn-1* acyl chain of both PI and PA, forming two important bioactive lipids, LPI and LPA [84]. LPI is an activator of a proposed cannabinoid receptor, GPR55, which can activate ERK signaling

pathways and intracellular Ca^{2+} levels [85]. LPA too, can stimulate cell proliferation through binding several GPCRs and activating Rho GTPase [86]. DDHD1 was shown to have higher activity for cleaving PA species over PI. Although DDHD1's substrate specificity has been poorly characterized, initial experiments have shown that it forms arachidonoyl-LPI at a much higher rate than stearoyl-LPI [84]. It is currently unknown whether DDHD1 shows substrate specificity for its substrate's *sn-1* chain or what impact this has on PI enrichment.

5. A common motif for arachidonoyl specificity?

Could there be a common amino acid motif that contributes to arachidonoyl specificity of several diverse enzymes? In the case of the family of enzymes known as lipoxygenases, several crystal structures have been solved that have identified a potential arachidonoyl preferring motif [87]. Lipoxygenases catalyze the dioxygenation of arachidonic acid; these enzymes are required for the synthesis of both inflammatory and anti-inflammatory leukotrienes and lipoxins, respectively [88]. In crystal structures of an 8R-lipoxygenase from *Plexaura homomalla* and human 5-lipoxygenase, the authors noted a U-shaped channel that allowed arachidonic acid access to the catalytic site [3,88]. In lipoxygenases, the segment with the required residues forms an arched helix, with the side chains of each of the required residues projecting into the channel [3,88]. This motif is highly conserved in certain lipoxygenases through evolution [88] (see Fig. 4).

We have referred to the conserved pattern of amino acid residues in the lining of this channel as the LOX-like motif. A similar consensus sequence $I/L\text{-}X_{(3\text{-}4)}\text{-}R\text{-}X_{(2)}\text{-}L\text{-}X_{(4)}\text{-}G$, where X can be any amino acid residue, is found not only in lipoxygenases, but also in the amino acid sequence of these enzymes discussed above, including DGKε, CDS2 and LPIAT1. A recent

study also concluded that the LOX-like motif is critical for DGKε's arachidonoyl specificity [55]. Similar studies on CDS2 and LPIAT1 also support a role for the LOX-like motif in the function of these enzymes (K.D., unpublished results).

6. Enrichment of PI and the brain

There are multiple factors contributing to the specific acyl chain content of PI. This includes the specificity of DGKε for SAG. This does not mean that DGKε is the only isoform of DGK that participates in the PI cycle. Other DGK isoforms will also phosphorylate SAG, they are just not specific for SAG. DGKε is preferentially expressed in certain organs of mammals, such as the brain. Brain PI is also more highly enriched with stearoyl-arachidonoyl forms than that of other organs. It is likely that this is a result of the high expression of DGKε in the brain. However, we do not believe DGKε is essential for the functioning of the PI cycle, since some cells survive with minimal expression of DGKε and DGKε-knock out mice have a rather mild phenotype. In addition to DGKε, we have shown that CDS2 also has arachidonoyl-specificity that can contribute to enrichment of this acyl chain in PI. However, it is not known if CDS has any stearoyl specificity for the sn-1 position, nor has it yet been determined what the role of CDS1 is with regard to acyl chain selectivity. In the case of PI4P5K there is little acyl chain specificity for the substrate. This is not surprising since both the substrate, PI4P, as well as the product, $PI(4,5)P_2$, are both enriched with stearoyl-arachidonoyl acyl chains to comparable extents. However, activation of PI4P5K by PA is acyl chain specific. This would increase the incorporation of stearoyl-arachidonoyl acyl chains into PI because of activation of the PI cycle. In addition, LPIAT1 will further enrich PI with these acyl chains as a result of acyl chain transfer from arachidonoyl-CoA to LPI. In addition to enzyme specificities, there could also be

compartmentalization within the cell, causing segregation of certain lipids. This has not yet been explored.

While several defects have been associated with enzymes contributing to acyl chain enrichment in PI, these enzymes are frequently associated with defects in brain function. Thus, DGKε has a role in epilepsy [57,58] and in Huntington's disease [59]. DGKε has also recently been shown to play a role in light-dependent mechanisms in mammalian photoreceptor cells [89]. CDS is activated by anti-depressants [67], PI4P5K is associated with schizophrenia [90] and loss of LPIAT1 results in severe developmental brain defects [80]. It is thus suggestive that modulation of arachidonoyl-enrichment of PI can be developed as a method for treating psychiatric disorders.

PI in the brain can also be a source of free arachidonic acid. It is found that arachidonic acid enhances brain healing following injury [91] and can reverse age-related brain decline [92].

7. Future Directions

Acyl chain enrichment is a critical determinant in many of PI's cellular processes, including activation of downstream proteins and generation of precursor molecules. However, several questions remain about acyl chain enrichment. Several of these enzymes, such as DGKε, also shows stearoyl preference at the *sn-1* position [55]. However, there have been no motifs identified that can confer this specificity. Changes in the acyl chain at the *sn-1* position are more tolerable than at the *sn-2* position [93]. What role does the *sn-1* acyl chain play in substrate recognition? Could it just serve to determine the depth of burial of these substrates in membranes or help position the substrate for catalysis?

Similarly, does the LOX-like motif form a similar channel in all enzymes that show arachidonoyl specificity? There are key differences between the substrates of lipoxygenases and enzymes involved in PI synthesis. For one, arachidonoyl containing substrates are only preferred substrates of enzymes involved in PI synthesis, whereas lipoxygenases generally only show activity for arachidonic acid. The arachidonoyl group in these substrates is also esterified to a glycerol backbone, unlike arachidonic acid. How then does the LOX-like motif guide these bulkier substrates to the catalytic site? The LOX-like motif could allow transfer of the sn-2 arachidonoyl chain from the bilayer to the catalytic site, thus extracting this group from the membrane; however other explanations are possible.

Finally, are there similar motifs that also confer acyl chain specificity for other enzymes involved in phospholipid synthesis? For example, PE and PS species show high enrichment in both arachidonoyl and docosahexanoyl acyl chains at its *sn-2* position [94]. How does this enrichment occur? Are there enzymes in PE and PS synthesis that show dual acyl chain preference or multiple enzymes that show either arachidonoyl or docosahexanoyl selectivity? Why don't other phospholipids, like PC, show similar acyl chain enrichment?

The answers to these questions can provide insight into the incredibly complex process of phospholipid biosynthesis and acyl chain enrichment. Continued research into PI's stearoyl and arachidonoyl enrichment can also prove invaluable, as these lipids are involved in critical biochemical processes and their alteration results in disease states.

Graphical Abstract

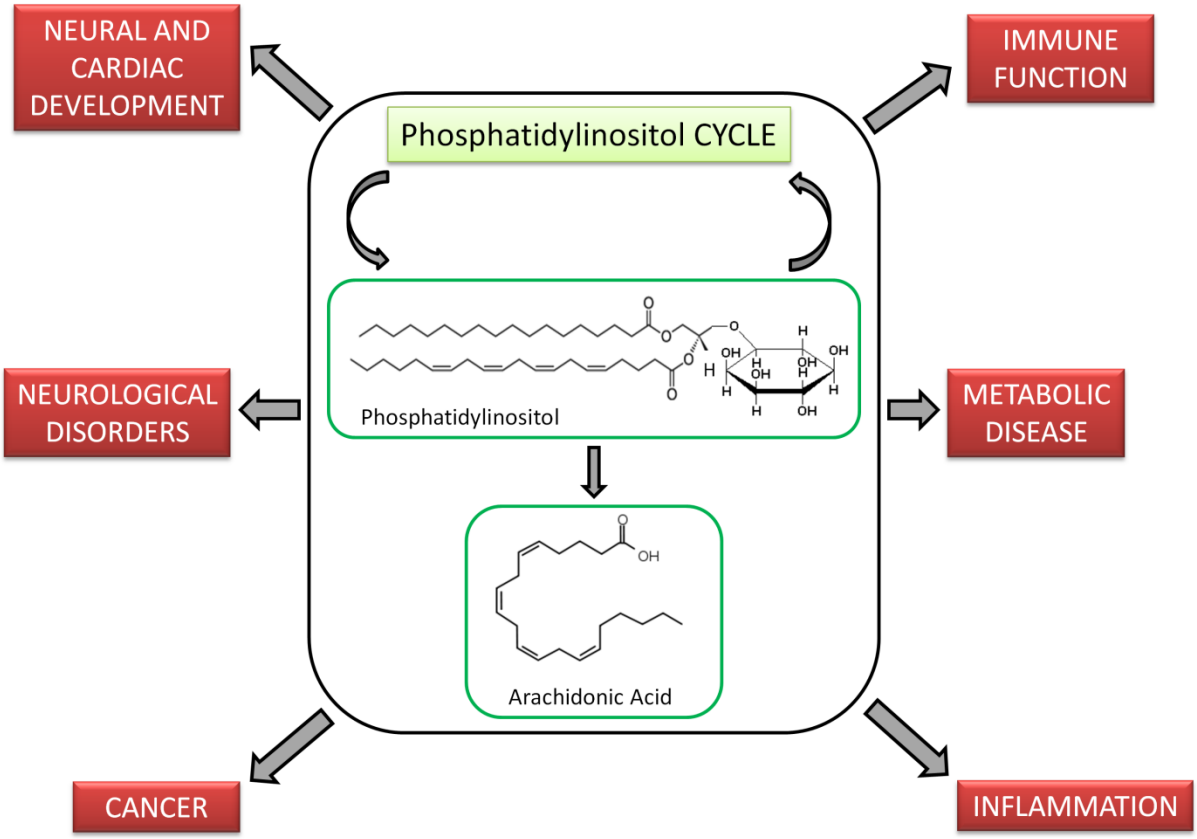


Figure 1: The struture and production of phosphatidylinositol. Phosphatidylinositol is composed of a glycerol backbone (blue), two acyl chains at the *sn-1* and *sn-2* position (red) and an inositol headgroup (green). The hydroxyl groups can be phosphorylated at position 3, 4 and 5, which can yield up to seven unique phosphoinositide species. The production of these species is tightly regulated through the actions of PI kinases and phosphatases. PIP_n species can also be interconverted, as indicated by the arrows.

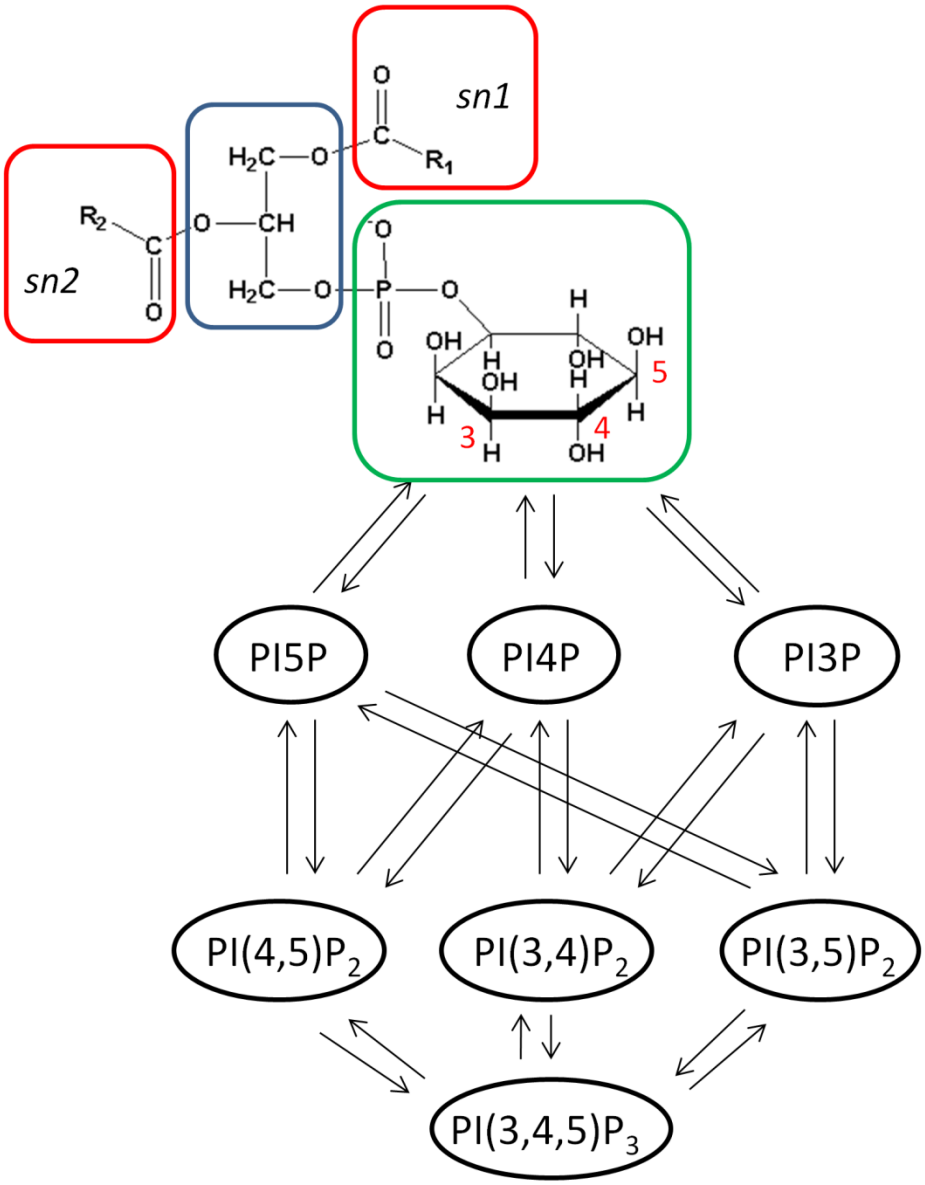


Figure 2. **The multiple pathways of PI synthesis.** The *de novo* synthesis of PI begins with G3P, which is converted to PA. CDS1/2 convert PA to CDP-DAG and PI is formed through PIS enzymes. The *de novo* pathway takes place in the ER. The PI cycle also contributes to PI synthesis, feeding PA into the *de novo* pathway. PA is produced through the phosphorylation of DAG by DGKε, which is formed by $PI(4,5)P_2$ cleavage. $PI(4,5)P_2$ is re-synthesized in the PM. The PI cycle requires both the ER and PM. Enzymes that have arachidonoyl specificity are highlighted in red and marked with an *.

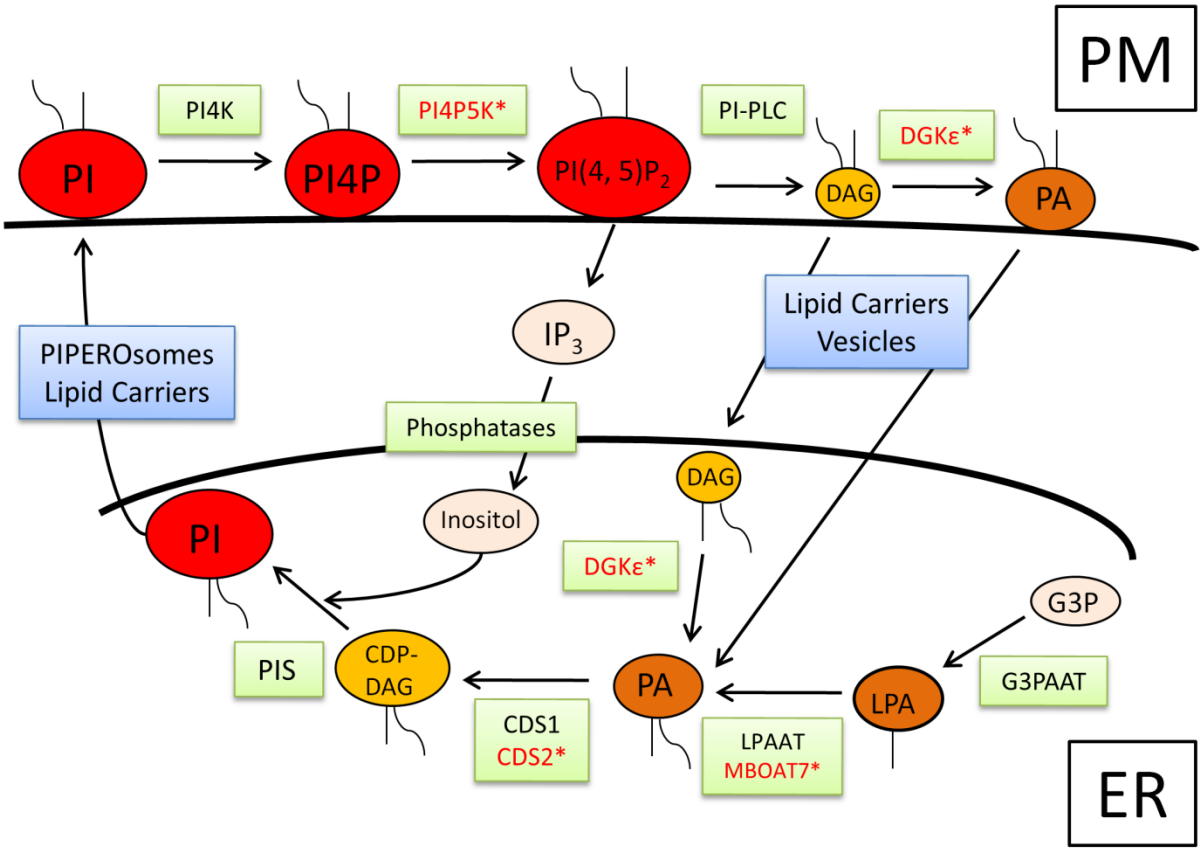


Figure 3. **The Land's cycle of acyl chain remodelling for PA and PI species.** The acyl chains of PI can be remodelled through phospholipases and acyltransferases. DDHD1 is a phospholipase A1 that has dual specificity for PA and PI. Other phospholipases, both A1 and A2, cleave acyl chains of phospholipids with varying specificity for the headgroup. Conversely, acyltransferases can transfer an acyl chain to lysophospholipids from other phospholipids or from acyl-CoA, creating new phospholipids. Both phospholipases and acyltransferases are critical as they not only maintain lipid asymmetery, but also regulate the levels of phospholipids, lysophospholipids and fatty acids. Enzymes that may contribute to arachidonoyl enrichment in PI are highlighted in red and marked with an *.

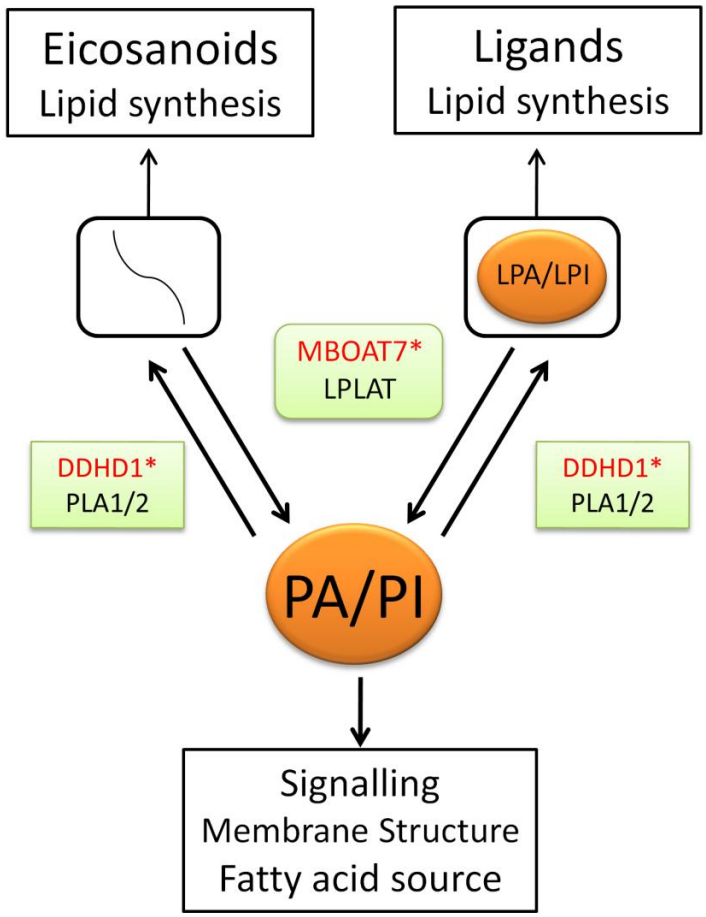
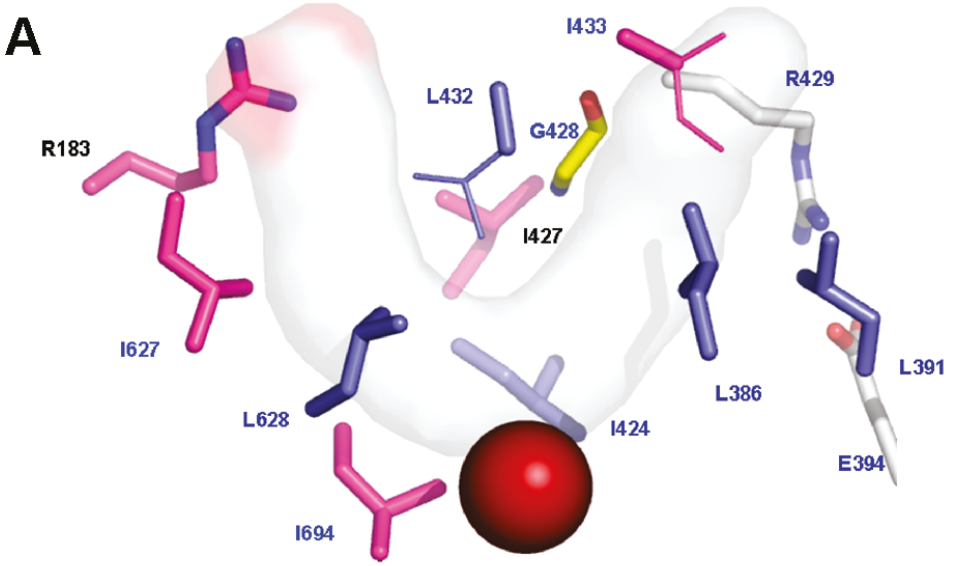


Figure 4. **Channel in lipoxygenase** to which arachidonic acid binds. Below the figure are the sequences of lipoxygenase from different species, with the residues that are invariant in evolution shown in red. Taken from [88] with permission.



B

8*R*-LOX	LLQPHIYGVLAIDTIGRKELIGSGGIVDQSLSLGGGGHVTFMEK	456
Q5DWF4_9CNID	LLQPHIYGVLAIDTIGRKELIGSGGIVDKSLSLGGGGHVEFMEK	430
Q2N410_9CNID	LLYPHTKGIMAINTLGRNDLIPTGGAADKVLSIGGGGQVTLMQK	400
Q4RJE8_TETNG	LLIPHTRYTLQINLLARIRLISETGVFSKFTASGGEGMLSILKK	320
LOX15_HUMAN	LIIPHLRYTLEINVRARTGLVSDMGIFDQIMSTGGGGHVQLLKQ	326
LOX15_PONPY	LIIPHLRYTLEINVRARTGLVSDMGIFDQIMSTGGGGHVQLLKQ	326
LOX12_BOVIN	LLIPHLRYTMEINIRARTGLVSDSGVFDQVVSTGGGGHVELLQR	326
LOX12_PIG	LLIPHFRYTMEINVRARNGLVSDLGIFDQVVSTGGGGHVELLRR	326
LOX15_RABIT	LIVPHLRYTLEINVRARNGLVSDFGIFDQIMSTGGGGHVQLLQQ	326
LX12L_RAT	LLVPHLLYTMEINVRARSDLISERGFFDKAMSTGGGGHLDLLKQ	326
LX12L_MOUSE	LLVPHLLYTMEINVRARSDLISERGFFDKVMSTGGGGHLDLLKQ	325
LOX12_HUMAN	FLIPHIRYTMEINTRARTQLISDGGIFDKAVSTGGGGHVQLLRR	329
Q95103_BOVIN	LLMPHIRYTMEINTRARTQLISDGGIFDKAVSTGGGGHVHLLRR	295
LOX12_MOUSE	LLVPHIRYTMEINTRSRTQLISDGGIFDQVVSTGGGGHVQLLTR	329
Q7T2A9_DANRE	LLTPHLRYTLEINCRGRTQLLSPEGIFKRVVSTGGEGLLILAQR	392
LOX5_RAT	LLVAHVRFTIAINTKAREQLNCEYGLFDKANATGGGGHVQMVQR	392
LOX5_MOUSE	LLVAHVRFTIAINTKAREQLICEYGLFDKANATGGGGHVQMVQR	399
LOX5_MESAU	LLVAHVRFTIAINTKAREQLICEYGLFDKANATGGGGHVQMVQR	398
LOX5_HUMAN	LLVAHVRFTIAINTKAREQLICECGLFDKANATGGGGHVQMVQR	400
A0JM77_XENTR	LLIAHVRFTIAINTKAREQLICECGLFDKANATGGGGHVELLLR	284
Q503R7_DANRE	LLIPHVRFTIAINTSARQTLINDTGVFNKANSTGGIGVLQVVHR	325
LOX1_ AT	LLEPHFRDTMNINALARQILINGGGIFEITVFPSKYA-MEMSSF	589
L1_SOYBEAN	LLTPHYRNMMNINALARQSLINANGIIETTFLPSKYS-VEMSSA	571
LOXA_TOMATO	LLYPHFRDTMNINALARQILINAGGVLESTVFPSKFA-MEMSAV	591

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CHAPTER 2:

The ATP Binding Site of the Arachidonoyl Specific Diacylglycerol Kinase[3]

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This paper shows that the motif, defined by the consensus sequence *GGDG*, is responsible for ATP binding in DGKε. The *GGDG* motif, also known as the phosphate binding motif, or P-motif is critical for DGKε's enzymatic activity. Mutation of these residues also lead to a loss in acyl chain specificity. My contribution to this paper was performing all experiments (Figures 1-5), writing the manuscript for publication, drawing the graphical abstract, preparing the tables and helping in the editing process.

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The ATP Binding Site of the Arachidonoyl Specific Diacylglycerol Kinase[†]

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[1]Abbreviations: ATP, adenosine tri-phosphate; BHT, butylated hydroxytoluene; DG, 1,2-diacyl-sn-glycerol; DGK, diacylglycerol kinase; DGKε, diacylglycerol kinase epsilon; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; KO, knock-out; MEF, mouse embryo fibroblasts; P-motif, phosphate binding motif; PA, phosphatidic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PM, plasma membrane; PS, phosphatidylserine; WT, wild type. Refer to Table 2 for full list of lipid abbreviations.

Abstract

Little is known about the ATP binding or catalytic site of diacylglycerol kinases (DGKs). Two alternative ATP-binding motifs have been proposed for DGKε, *GGDG* and *GxGxxG*. We demonstrate through mutational studies and sequence variation among species that *GGDG* is the ATP-binding motif of DGKε. The segment *GGDG* is critical for the enzymatic activity of DGKε. This nucleotide binding motif is conserved across all mammalian species and DGK isoforms as well as several other enzyme families. Mutation of residues within the *GGDG* motif of DGKε greatly reduces enzymatic activity resulting in a greatly reduced V_{max} for ATP. Interestingly, these changes in the ATP binding site also reduce the enzyme's acyl chain specificity for its substrate diacylglycerol. This result indicates that there is an interaction between the ATP and lipid substrate binding sites of the active site of DGKε. The loss of enzymatic activity of these weakly active DGKε mutants affects their subcellular localization.

Introduction

Diacylglycerol kinase (DGK) catalyzes the phosphorylation of diacylglycerol (DG) to phosphatidic acid (PA) using ATP as a phosphate donor[1, 2]. Both DG and PA are important secondary messengers implicated in a variety of signalling pathways, as well as playing structural roles and being precursors to phospholipid biosynthesis. DGs, for example, have been shown to recruit and/or regulate the activity of several different classes of proteins including chimaerins, protein kinase C (PKC) and RasGRP[3,4]. Similarly, PA has been shown to play a role in the modulation of several proteins including phosphatidylinositol-4-phosphate-5-kinase, protein phosphatase 1, mammalian target of rapamycin and in the recruitment of enzymes such as Raf-1-kinase[5-8]. DGs are precursors to phospholipids including PC, PE and PS while PAs are

precursors to PI, PG and cardiolipin[4]. DGKs play critical roles in regulating the levels of these two lipids. To date, ten mammalian isoforms have been identified and characterized[9-11].

DGKε is unique among the DGK isoforms for several reasons. First, it is the only isoform believed to be committed to PI synthesis and its enrichment with arachidonoyl acyl chains. Although it is the smallest DGK isoform and the simplest in structure, DGKε is the only isoform that shows substrate specificity, preferring DG's that have a *sn-1* stearoyl and an *sn-2* arachidonoyl chains[12-14]. Coincidently, these are the acyl chains most enriched in lipid species of the intermediates in the PI cycle[15-16]. DGKε is also the only isoform predicted to be membrane bound and is restricted to the ER and PM, membranes in which PI synthesis occurs[17-18]. Finally DGKε is potently inhibited by anionic phospolipids, such as PI, which provides a potential feedback mechanism in which DGKε is most active when PI levels are low[12]. Several studies in DGKε$^{-/-}$ mouse embryo fibroblasts and DGKε knockout mice have demonstrated that this isoform is important for the arachidonoyl enrichment of PI[12, 19-21]. Physiologically, disruptions of DGKε has been shown to have several effects, such as deficiencies in long term potentiation in neural functions and the development of the renal diseases, membranoproliferative-like glomerular microangiopathy and atypical hemolytic–uremic syndrome[22-23].

As such, DGKε would result in PI enrichment with specific (i.e. arachidonoyl) acyl chains by supplying corresponding PA's precursors. However, as mentioned, PA has several additional roles in lipid metabolism, signal transduction and structure. One potentially interesting effect of an increase in PA production could be an increase in transport. PA is a highly fusogenic lipid, which due to its negative intrinsic curvature, is found at fusion contact sites in transport complexes[24]. So PA could also help drive the transport of lipids and proteins between membranes, including the ER and PM, required for completion of the PI-cycle.

Surprisingly, very little is known about DGKε's ATP-binding or catalytic site. No crystal structures of mammalian DGK's are available and only limited sequence alignments and mutagenesis experiments exist[25]. However, a crystal structure of a soluble DGK from *Staphylococcus aureus* was solved that provided insight into a potential ATP-binding site[26]. In the crystal structure of DGKB, the authors identified a motif, defined by the residues *GGDG* that was critical in binding an ATP molecule.

Sequence alignments of all mammalian DGK's and their isoforms have shown that the *GGDG* is universally conserved (Table 1); it is also universally conserved in DGKε within species from humans to invertebrates (data not shown). This motif is also found in several other enzyme families, such as 6-phosphofructokinases and polyphosphate/ATP NAD kinases (PPNKs)[27-28]. Therefore, the *GGDG* motif, hereby referred to as the phosphate binding motif (*P-motif*) could serve as a potential target to study the ATP binding and catalytic site in DGKε, and more broadly in other DGK's. Curiously, however, there is the presence of another common nucleotide binding motif, a glycine rich motif defined by the residues *GxGxxG* that overlaps with the *GGDG* motif. The *GxGxxG* motif is common in several nucleotide binding proteins such as PKCs and Ras[29-30]. This sequence is also highly conserved, but is not found in DGKκ (Table 1).

Materials and Methods

Materials- All lipids were purchased from Avanti Polar Lipids and were stored in a solution containing 2:1 (v/v) $CHCl_3/CH_3OH$ and 0.1% (w/v) butylated hydroxytoluene (BHT). All traces of the solvent phase were evaporated using N_2 gas and a vacuum desiccator and the lipid films were stored under argon gas for stability. All antibiodies were purchased from Santa Cruz Biotechnology unless otherwise noted. Most other chemicals and reagents were purchased from

Sigma unless otherwise noted. All lipids used in this study and summarized in Table 2, together with the abbreviations used.

DGKε constructs-DNA coding for human DGKε (kindly provided by M.K. Topham, University of Utah) was cloned into the p3xflag-cmv-7.1 vector as previously described[17]. Mutations were created using the Quikchange Site Directed Mutagenesis kit (Aligent Technologies) as per the manufacturer's instructions. All mutations were confirmed through sequencing.

Cell Culture and Tranfections- COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) and were supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO) and 1:100 (v/v) Pen/Strep (GIBCO). Cells were maintained at 37°C with 5% CO_2. For transfections, cells were grown to 90% confluency and tranfected using lipofectamine 2000 reagent (Invitrogen) as per the manfacturer's instructions. After 48 hours, cells were harvested by scraping into a solution of PBS with a 1:100 (v/v) protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma-Aldrich). Cells were spun at 1000× g for 5 min and the pellets were flash-frozen at -80°C.

Enzyme preparation for DGK activity assay- Cells pellets containing transfected constructs were re-suspended in an ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1:1000 (v/v) protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich), 1 mM β-glycerophosphate, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate) and lysed on ice for 10 min. Re-suspended cells were sonicated in ice cold water for 10 min and centrifuged at 100000× g for 30 min at 4°C. Supernatants were used for the mixed micelle-based enzymatic activity assay.

Detergent-phospholipid-mixed micelle-based enzymatic activity assay- Enzymatic activity was determined as previously described[31]. Lipid films were prepared by solvent evaporation from a chloroform-methanol solution of the diacylglycerol substrate, along with 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Lipid films were suspended in 50 µl of 4× assay buffer (200 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.2, 400 mM NaCl, 20 mM MgCl, 4 mM ethylene glycol tetraacetic acid (EGTA), 60 mM Triton X-100) by vortexing for 2 minutes. Supernatants from DGKε transfected cells were added to the mixed micelles, along with 10mM dithiothreitol to a final volume of 180 µl. The reaction was initiated by adding 20 µl of 1 mM [γ-^{32}P]-ATP and incubated at room temperature for 10 min. The reaction was terminated by adding 2 ml of stop solution (1:1 (v/v) $CHCl_3/CH_3OH$, 0.25 mg/ml dihexadecyl phosphate). The organic phase was washed three times with wash solution (7:1 v/v ddH_20/CH_3OH, 1% v/v $HClO_4$, 0.1% H_3PO_4). 400 µl of the organic phase was used to determine the level of incorporation of ^{32}P into DG to form PA using Cerenkov counting. The data from these assays are presented as the mean ± S. D. To account for endogenous levels of DGKε and background activity, lysates with mock transfected COS7 cells (empty vector) were used as a negative control, after normalizing for the actin content. The mock transfected lysates were included with each activity assay and had significantly lower activities than DGKε transfected constructs. Since DGKε is restricted to the interface of the detergent-phospholipid mixed micelle, concentrations of individual lipid components in mixed micelles are used instead of the bulk concentrations of lipid components. All lipid concentrations are expressed as mol % of the components of the mixed micelle, rather than their bulk concentrations.

Kinetic Analysis of DGKε activity-To determine V_{max} and K_m parameters, activity assays were performed using various concentrations of SAG lipid substrates. V_{max} and K_m parameters were

determined using nonlinear regression analysis by plotting initial enzyme velocity (v_o) against substrate concentration ([S]). Data was analyzed using Origin8.

Quantification of phosphatidic acid- The concentration of all PA stocks used in this study was determined based on its phosphate content. Briefly, 30 µl of 10% (w/v) $Mg(NO_3)_2$ in 95% (v/v) ethanol was added to PA samples or K_2PO_4 standards (up to 80 nmol) in acid-washed Pyrex tubes. The solution was flamed until the organic phosphate was completely ashed. 350 µl of 0.5 M HCl was added, the mixture was refluxed for 15 min and 750 µl of a 1:6 (v/v) mixture of 10% (w/v) L-ascorbic acid and 0.42% (w/v) ammonium molybdate tetrahydrate in 0.5 M H_2SO_4 was added. The mixture was incubated at 60 °C for 10 min, allowed to cool to room temperature and the absorbance at 820 nm was measured.

Sub-cellular Fractionation- Sub-cellular fractionation was adapted from a previously described procedure[18]. The fractionation was performed using the OptiPrep gradient (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, COS7 cells were transfected with either WT or D280A DGKε. Eight dishes (10 cm^2) of each construct were washed twice with ice-cold PBS and scraped into PBS containing a protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich). The cells were collected by centrifugation at 1000×g for 5 min at 4°C and re-suspended in 1ml of ice-cold homogenization buffer [0.25 M sucrose, 10 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM KCl, 20 mM NaCl, and 1× protease inhibitor cocktail]. The cells were broken by 30 passages through a 25-gauge needle syringe. Unbroken cells and nuclei were removed from the cell homogenate by centrifugation at 1000g for 10 min at 4°C. The crude microsomal sample was diluted with the 50% Optiprep Density Gradient Medium (Iodixanol, from Sigma) to a final concentration of 25% Optiprep. The vesicle suspension was layered underneath an OptiPrep gradient consisting of 3, 6.5, 10, 13.5, 17, and 20.5% (w/v)

iodixanol solutions. Gradients were centrifuged using a SW41Ti rotor in a Beckman Optima L-100 XP ultracentrifuge at 50000g for 18 h at 4°C. Eighteen fractions were collected and concentrated using Vivaspin-500 columns (5 kDa cutoff, GE Healthcare). The presence of organelle specific proteins was detected by SDS-PAGE and Western blotting.

Immunoblot Analysis- Immunoblotting was used to quantify the amount of protein from transfected COS7 cells. Samples were prepared by mixing equal volume of lysate and Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and were boiled at 60°C for 5 min. The samples were run on a 7.5% Tris-glycine SDS-PAGE gel for 30 min at 200V. The proteins were then transferred onto a polyvinylidene fluoride membrane (Bio-Rad). The antibodies used to detect the flag epitope on DGKε constructs were a mouse anti-FLAG M2 antibody (Sigma). The antibodies used to detect actin were the mouse anti-actin antibody (Life Technologies). For sub-cellular localization experiments, the PM fraction was determined using the anti-Na/K^+ ATPase polyclonal antibody and the ER fraction was determined using the anti-calnexin antibody (BD Biosciences). The secondary antibodies used were the horseradish peroxidise (HRP)-conjugated goat anti-mouse antibody and the HRP-conjugated goat anti-rabbit. The immunoblot was detected using ECL Western Blotting Detection Reagents (GE Healthcare) on XAR Biofilm (Kodak). To determine the amount of flag-DGKε in each of the lanes, a known amount of 3× flag-tagged bacterial alkaline phosphatase (BAP) was run along with the samples.

Statistical Analysis- Where necessary, statistical analysis was performed using the one way analysis of variance (ANOVA) tests followed by a Tukey's test. Analysis was performed using Origin Pro 8. Experiments were repeated at least three times independently in triplicates. Data is presented as mean ± SEM.

Results

The *P-motif* is Required for DGKε's Enzymatic Activity

We initially created alanine point mutations in each of the residues of the *P-motif* of DGKε (G^{278}GDG) and tested the mutant's enzymatic activity using SAG as a DG substrate. Enzymatic assays with these mutants showed varying effects (Figure 1A). G279A and G281A mutants retained approximately 20% and 50% activity of WT, respectively. D280A showed a large loss in activity, having 10% of activity as compared to WT DGKε. The G278A mutant did not show a significant loss in activity; however a more drastic mutant, G278D, showed almost a complete loss of activity, with ~5-10% activity of WT DGKε.

In addition to assaying mutants in only the *P-motif,* we also create mutants in an adjacent glycine residue (G284A/D). G284 would be the final residue in the alternative ATP-binding motif, corresponding to *GxGxxG* (Table 1). The crystal structure of DGKB also identified a putative catalytic glutamic acid base, which we mutated in DGKε (E523A) (Figure 1B). The G284A mutant resulted in no loss of activity as compared to WT. The more drastic G284D mutation had about 25% activity as WT. Mutation of the catalytic base abolished DGKε's activity, having only about 5% of activity as WT DGKε (Figure 1B). As an additional experiment, we also wanted to see whether mutations in the P-motif can act in a dominant negative manner. So, we incubated WT DGKε and E523A (which showed the largest loss in activity), in an approximately 1:1 ratio and tested the activity. The WT:E523A DGKε mixture had roughly half the activity of WT DGKε, indicating that this mutation does not act in a dominant negative manner (data not shown). Since G278D and D280A showed a large, but measurable loss in activity, we decided to focus on these mutants going forward.

Next the kinetics of WT DGKε and mutants were measured, using SAG as a substrate (Figure 2). All three constructs showed typical saturation kinetics and the data was fit using the Michaelis-Menten equation to calculate V_{max} and K_m parameters. There are no significant changes in the K_m of the constructs, at least within the inherent large error of K_m values of the weakly active mutants. However, there are significant decreases in the V_{max} and k_{cat} values of the G278D and D280A mutants, with an ~ 27 and ~8 fold decrease in k_{cat} compared to WT DGKε. These values show that the G278D and D280A mutants only have minimal activity and turnover rate (Figure 2).

DGKε *P-motif* mutants show losses in activity against DG substrates

DGKε is the only isoform that shows acyl chain specificity for the substrate. We tested the effect that mutations in the ATP-binding *P-motif* has on acyl chain specificity. First, WT DGKε, G278D and D280A were tested against a variety of different DG substrates with differing *sn-1* and *sn-2* acyl chain compositions and degrees of unsaturation (Table 2). WT DGKε exhibits acyl chain specificity, showing maximal activity for its preferred substrate SAG. The enzyme also is able to distinguish between SAG and DGs with similar acyl chain compositions, such as DAG (Figure 3). In contrast, the *P-motif* mutants, show a large loss in activity against all DGs. The results indicate a large loss in substrate specificity in the mutants, with SAG, DAG and DLG showing similar activity for the two mutants (Figure 3).

PA inhibits DGKε *P-motif* mutants

As a complementary experiment, we also tested the inhibition of DGKε with different PA's. Previous studies have indicated that inhibition is also acyl chain specific, with the greatest inhibition seen for *sn-1* stearoyl-*sn-2*-arachidonoyl PAs (SAPA). WT DGKε shows a subtle, but

significant acyl chain dependant inhibition with different PAs (Figure 4). Of the three PAs test, SAPA showed the greatest inhibition of DGKε. *P-motif* mutants exhibit a similar loss of activity in the presence of any of the three PA's. As the PAs still inhibit these mutants, it indicates that some inhibition is due to non-acyl chain dependant factors, such as its headgroup (Figure 4).

DGKε uses ATP almost exclusively as a phosphate donor

Some lipid kinases have been shown to utilize more than one nucleotide as a phosphate donor[32]. We tested to see whether WT DGKε can also use GTP as a phosphate donor and whether *P-motif* mutants affect the enzyme's preference for one phosphate donor over another. DGKε shows almost no activity when GTP is used as a phosphate donor. Additionally, *P-motif* mutants do not affect the enzymes preference for ATP over GTP (data not shown).

DGKε mutants show subtle shifts in the sub-cellular localization profile

We next sough to determine whether the loss in the enzymatic production of PA affects the enzyme's sub-cellular localization. We were able to separate the ER and PM (Figure 5); however, some overlap of the two membranes is expected as these membranes are highly dynamic and are constantly involved in lipid and protein transfer. There are also PM specific fractions (fractions 1-4) and ER specific fractions (fractions 13-16). WT DGKε, as expected, shows a diffuse localization profile that extends throughout both the ER and the PM fractions. The enzyme is found in both PM and ER specific fractions. D280A, while it shows a similar localization profile, is also subtly shifted towards the ER fraction (Figure 5). This mutant is also not found in PM specific fractions, which could indicate that movement of the enzyme is restricted to the ER or PM-ER mixed fractions.

Discussion

The phosphate binding motif (*P-motif*) is conserved in several kinase families, including DGK's. Using DGKε as an example, mutations in the conserved residues of the *P-motif* generally resulted in a large loss of activity. Two mutations in particular, G278D and D280A, resulted in activities that were roughly 5-10% as the wild type protein. In the simplest terms, these mutations likely abolish DGKε's enzymatic activity by disrupting the ability of these enzymes to properly position or 'house' a negative phosphate group. The G278D mutant does this by introducing another negative charge to the catalytic site and the D280A does this by likely preventing a Mg^{2+} ion from stabilizing the β and γ phosphates of an ATP molecule. We were also able to identify the likely catalytic base of DGKε through comparisons with DGKB. Although there are several differences between the bacterial and mammalian DGK's, many of the core features of DGK, including their ATP-binding motif and catalytic base are conserved. Therefore, the structure of DGKB can be used to provide valuable insight into some of the common mechanisms of catalysis with DGKs. We have identified E523 as a catalytic base. Interestingly, a shorter active fragment of DGKε found in the testes and having a mass of only 58.9 kDa, and therefore if truncated at the carboxyl terminus, would contain E523, has been found to be enzymatically active[31].

Interestingly, there are two common nucleotide-binding motifs found in DGK's; the *GGDG* motif, which was the focus of this study, and the *GxGxxG* motif, a glycine rich motif common in several nucleotide binding proteins such as PKCs and Ras. Both motifs share some overlap; the $G^{278}GDG$ and $G^{279}xGxxG$ share glycines at positions 279 and 281. Mutations in the unique residues of each motif, G278 and D280 in the *GGDG* motif and G284 in the *GxGxxG* motif yielded loss in enzymatic activity. The G284A mutation in the *GxGxxG* motif showed no loss of

activity in contrast to G to A mutations in the *GGDG* motif that in all cases studied, showed a marked loss of activity (Figure 1). In addition, the G278D mutation in the *GGDG* motif had a more drastic effect on enzymatic activity, resulting in a ~95% loss in activity, while the corresponding G284D mutant of the *GxGxxG* motif had a ~75% loss in activity. It is possible that both glycine residues play some role in nucleotide binding and/or flexibility in the nucleotide binding site; however, more concrete conclusions cannot be made without a crystal structure of a mammalian DGK.

DGKε was chosen due to its unique ability in distinguishing between DG substrates with differing acyl chains. We were able to show that *P-motif* mutants affect not only DGKε's preference for its preferred substrate, SAG, but other DG's as well. The G278D mutants show a similar low activity towards all DG substrates, even those with significantly different acyl chains. The D280A mutant, conversely, is able to differ among substrates that have an arachidonoyl acyl chain at the *sn-2* position (SAG/DAG) over a DG that does not (SLG). However, we still see that the loss in activity seen for this mutation is not limited only to SAG. Additionally, a loss in acyl chain dependant inhibition by PA's are also seen, regardless of the acyl chain composition, for both G278D and D280A. Crystal structures of other arachidonoyl preferring enzymes, such as lipoxygenases have identified a U-shaped channel, known as the lipoxygenase (LOX)- motif that allows access of the substrate to the catalytic site[34]. This motif is also found in DGKε, and is proposed to most effectively allow access of arachidonoyl containing DG substrates or optimally position them for catalysis[35]. Mutations in the ATP-binding site of the catalytic center could affect access and the positioning of the substrate SAG, so that it is no longer the most preferred substrate. Our results also indicate a close interaction between the ATP

and lipid binding site. Both enzyme catalysis and substrate specificity are closely linked as both substrates must be close to each other for PA formation.

Finally, *P-motif* mutants appear to affect the sub-cellular localization of the enzyme. A loss of enzyme activity resulted in a subtle shift in the localization profile towards the ER (WT vs. D280A mutant). A loss in enzymatic activity with the D280A mutant (Figure 1), would suggest a decrease in the amount of PA available to be utilized. While some of the PA would be funnelled for PI and other phospholipid synthesis, some PA may be used as a fusogenic lipid for transport. Therefore, transport of DGKε from the ER to the PM and the overall localization profile of DGKε within these membranes may be subtly affected.

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Tables

Table 1: **Sequence alignments of the putative ATP binding sites in mammalian DGKs**.

Unique residues of the *GGDG* motif are highlighted in red, whereas unique residues of the *GxGxxG* are highlighted in blue. Shared residues between the two motifs are highlighted in green Sequence alignments of all DGK show that the *GGDG* motif is universally conserved in all DGK isoforms and their splice variants, whereas the *GxGxxG* motif is universally conserved in all DGK isoforms except DGKκ.

DGKε	|	VLVCGGDGTVGWVL
DGKα	|	ILVCGGDGTVGWIL
DGKβ	|	VLACGGDGTVGWVL
DGKγ	|	VLACGGDGTVGWIL
DGKγ2	|	VLACGGDGTVGWIL
DGKδ1	|	ILVCGGDGSVGWVL
DGKδ2	|	ILVCGGDGSVGWVL
DGKθ	|	VLVCGGDGTVGWVL
DGKι	|	ILACGGDGTVGWIL
DGKζ1	|	ILACGGDGTVGWIL
DGKζ2	|	ILACGGDGTVGWIL
DGKζ3	|	ILACGGDGTVGWIL
DGKη1	|	ILVCGGDGSVGWVL
DGKη2	|	ILVCGGDGSVGWVL
DGKη3	|	ILVCGGDGSVGWVL
DGKκ	|	ILVCGGDGSVSWVL

Table 2: Lipids used in the study.

Abbreviation	Full Name	*sn*-1/*sn*-2 Notation
	Diacylglycerol (DG)	
SAG	1-Stearoyl-2-arachidonoyl-*sn*-glycerol	18:0/20:4 DG
SLG	1-Stearoyl-2-linoleoyl-*sn*-glycerol	18:0/18:2 DG
DLG	1, 2-Dilinoleoyl-*sn*-glycerol	18:2/18:2 DG
DAG	1, 2-Diarachidonoyl-*sn*-glycerol	20:4/20:4 DG
	Phosphatidic Acid (PA)	
SAPA	1-Stearoyl-2-arachidonoyl-*sn*-phosphatidic acid	18:0/20:4 PA
SLPA	1-Stearoyl-2-linoleoyl-*sn*-phosphatidic acid	18:0/18:2 PA
DLPA	1, 2-Dilinoleoyl-*sn*-phosphatidic acid	18:2/18:2 PA
	Phosphatidylcholine (PC)	
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	18:1/18:1 PC

Table 3: **Summary of Vmax and Km parameters for wild type DGKε, G278D and D280A mutants**. The results indicate that both the G278D and D280A mutants show a significant decrease in K_{cat} values when compared to WT DGK. That data is shown as mean ± SEM and is represented graphically in Figure 2. n = 9, N= 3.

DGKε	**V_{max} (nmol PA/min/ng)**	**K_m (mol % SAG)**	**K_{cat} (s^{-1})**
WT	0.0100 ± 0.0003	2.0 ± 0.2	10.7 ± 0.4
G278D	0.0004 ± 0.0002	1.0 ± 2.0	0.4 ± 0.2
D280A	0.0012 ± .00003	1.6 ± 1.1	1.3 ± 0.3

Figure Legends

Graphical Abstract

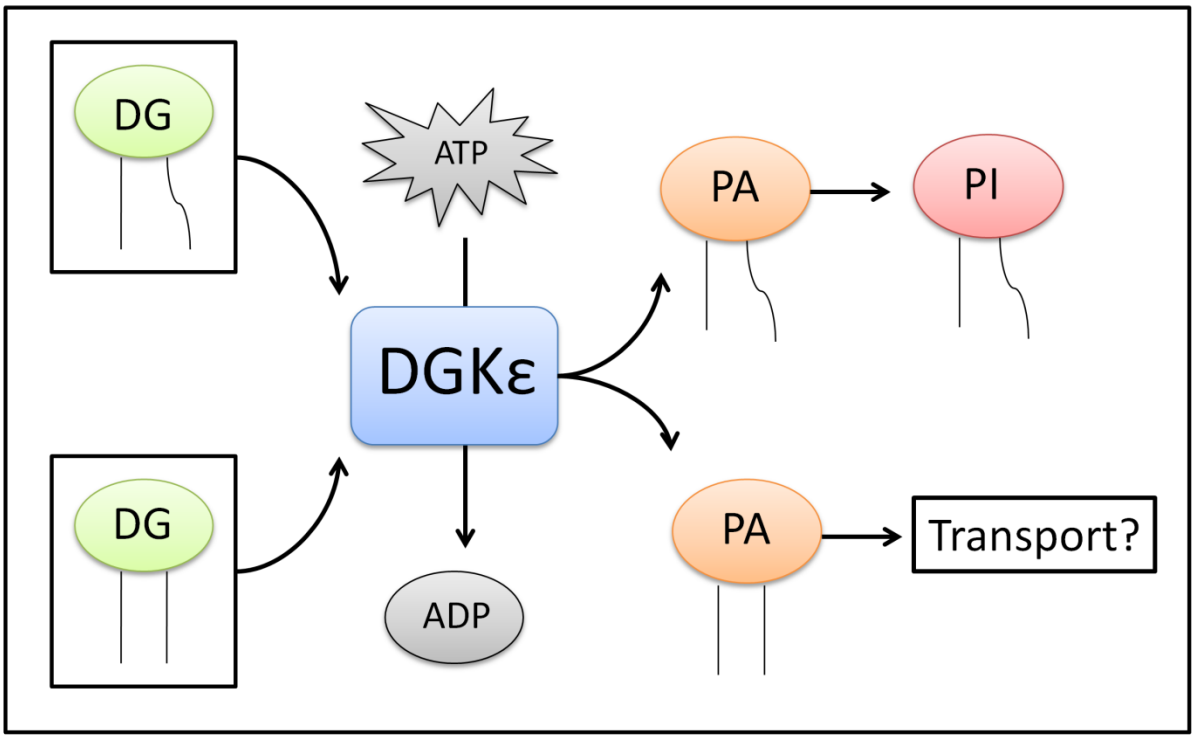


Figure 1: **Comparison of enzymatic activity for DGKε *P-motif* mutants.** Mixed-micelle based enzymatic activity of wild type DGKε and P-motif mutants using SAG as a DG substrates. Enzymatic activity is adjusted using mock transfected Cos-7 cells (EV control) to account for endogenous DGKε activity. *A*, P-motif mutants. *B*, Adjacent glycine residue and proposed catalytic base. The bars with an * were determined to be statistically different from wild type protein ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.

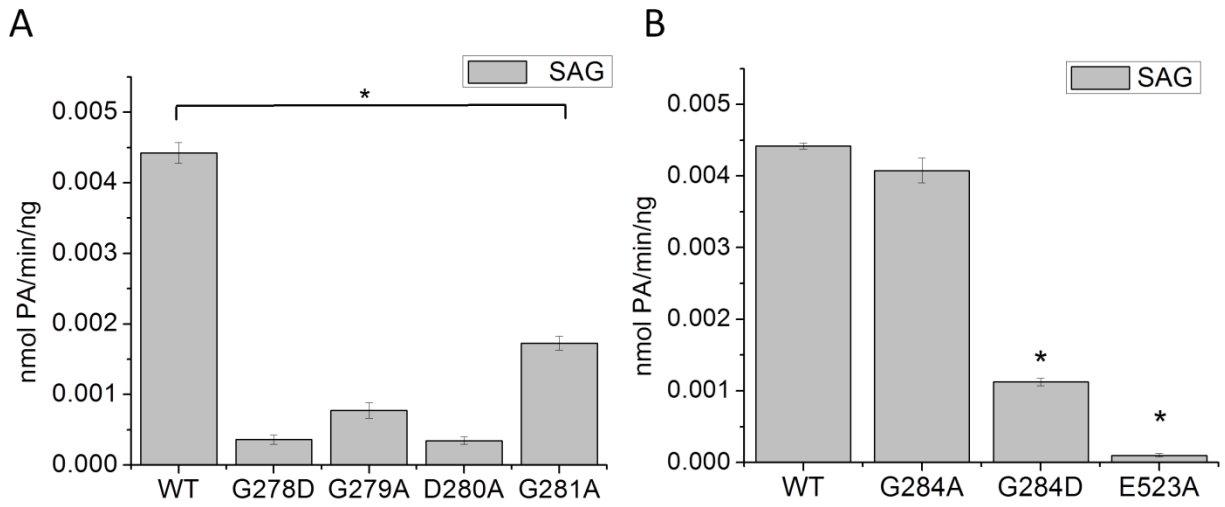


Figure 2: **Kinetic analysis of wild type, G278D and D280A mutants.** A mixed-micelle assay was used to test enzymatic activity over a series of substrate concentrations. The total lipid concentrations of DOPC and substrate were adjusted to remain constant at 4.75mM. Substrate concentrations are represented as mol % SAG. Enzymatic activity is adjusted using mock transfected Cos-7 cells (EV control). For K_m, V_{max} and K_{cat} values, refer to Table 3. Data is represented as means ± SEM, *n* = 9, *N*= 3.

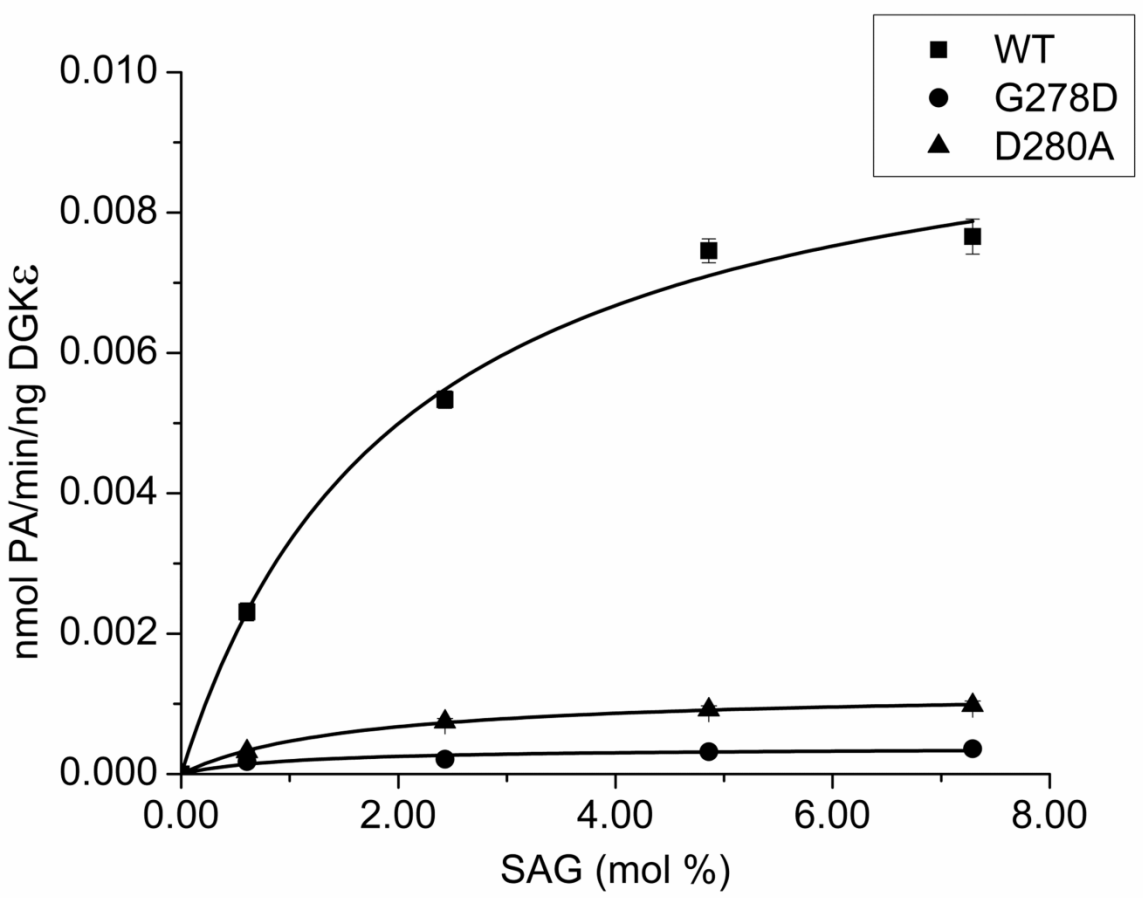


Figure 3: **Substrate specificity assays of mutations involving the *P-motif*.** Enzymatic activity was tested using four different substrates: SAG, DAG, DLG and SLG. The total lipid concentrations of DOPC and substrate were constant at 4.75mM. The bars with an * were determined to be statistically different from SAG ($P < 0.05$). Data is represented as means ± SEM. n.s. non-significant, $n = 9$, $N = 3$..

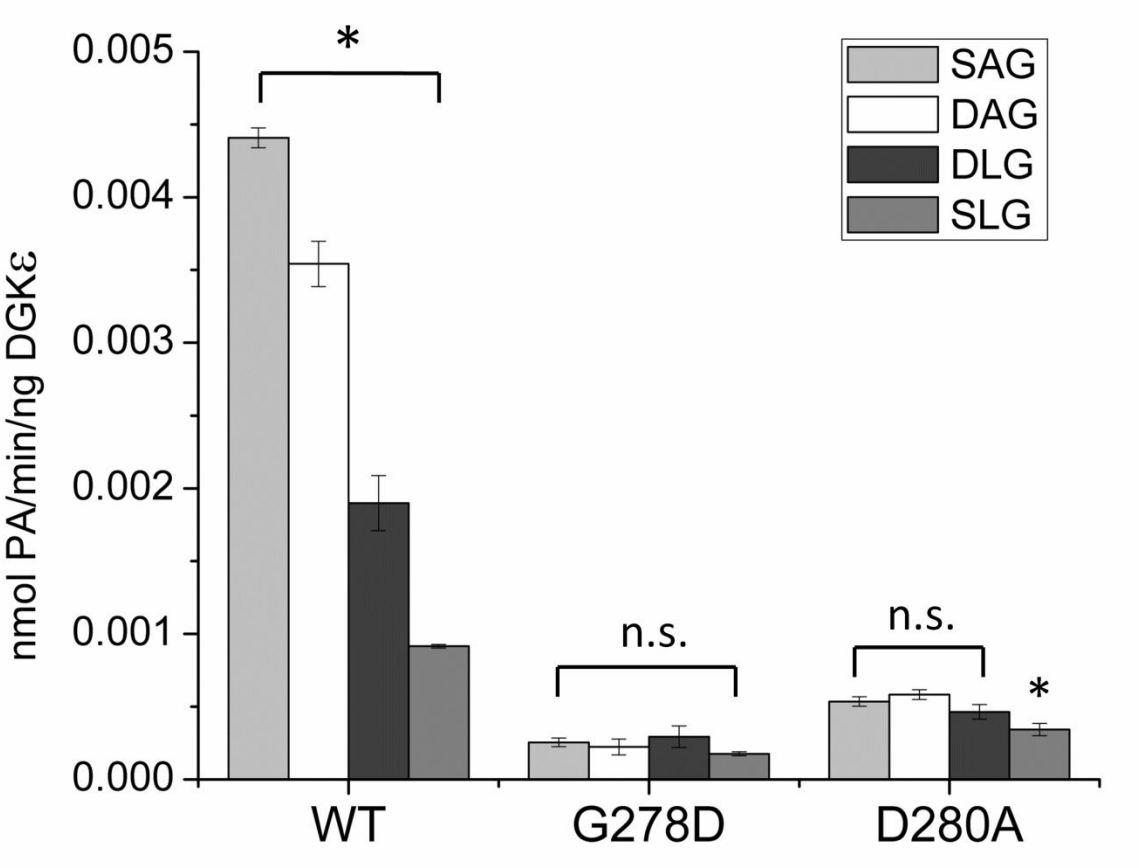


Figure 4: **Inhibition of DGKε and *P-motif* mutants by PA.** The inhibition of WT and P-motif mutants were tested using three different PAs and SAG as a substrate. An equal mole percent of PA (2%) as substrate SAG was used. The total lipid concentrations of DOPC, SAG and PA were constant at 4.75mM. The bars with an * were determined to be statistically different from SAG + SAPA ($P < 0.05$). Data is represented as means ± SEM. n.s. non-significant, $n = 9$, $N = 3$.

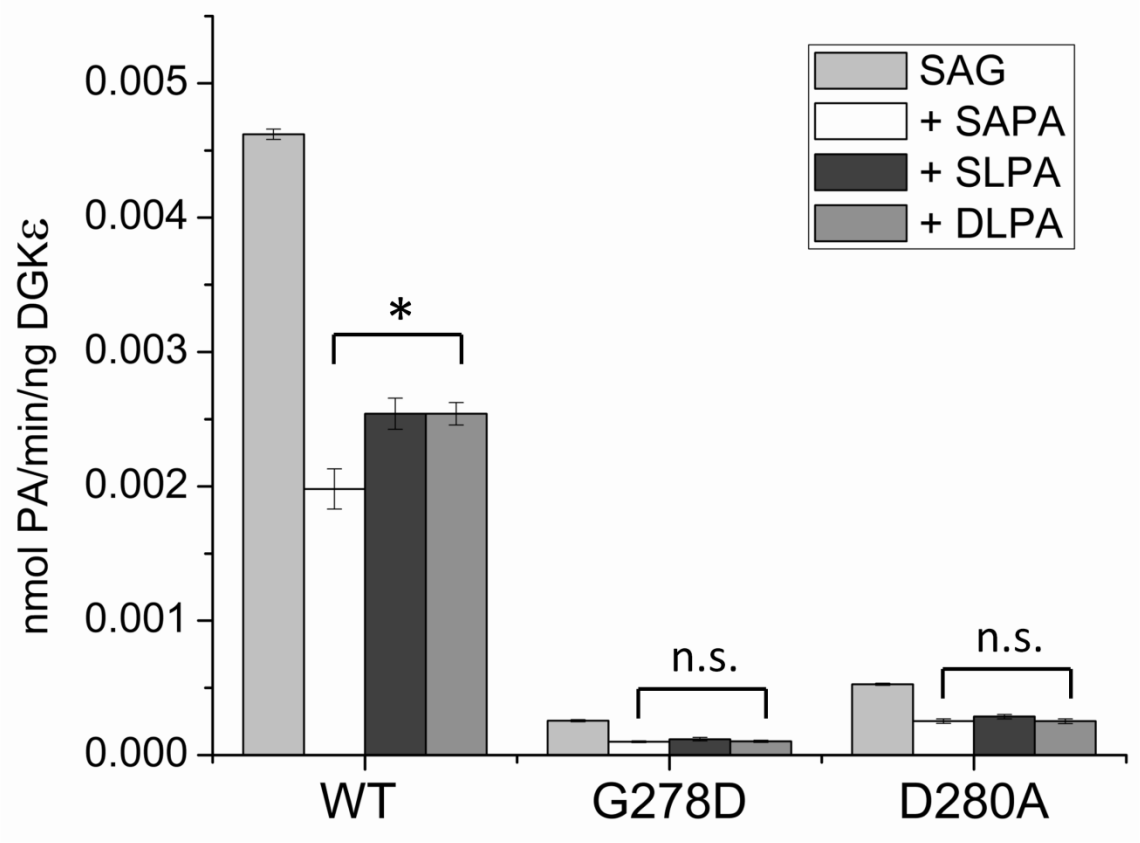
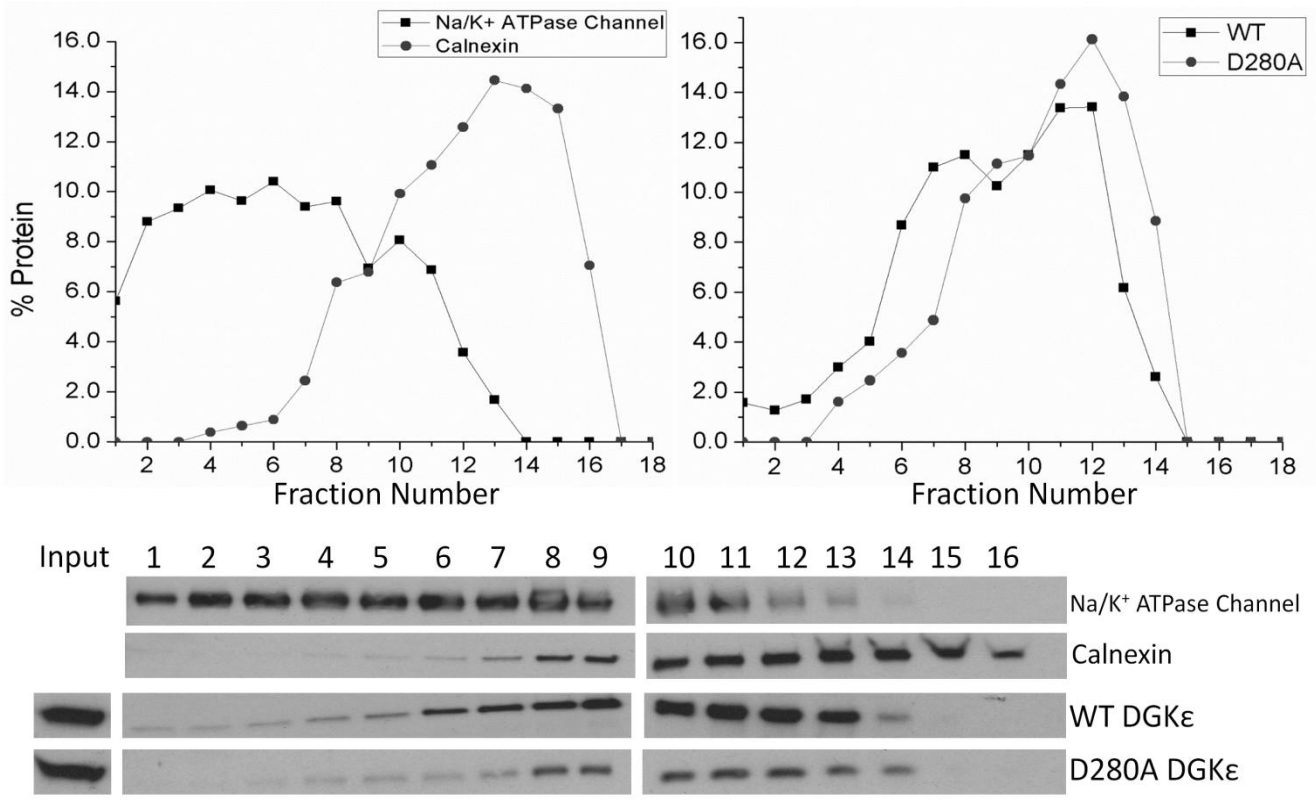


Figure 5: Sub-cellular profile of WT DGKε and D280A mutant. Density gradient separation of membranes. COS-7 cells were transfected with either WT DGKε or D280A DGKε and the lysed cell membranes were separated on a 3%–25% continuous iodixanol gradient by overnight ultracentrifugation. Eighteen total fractions were collected with fraction 1 being the top of the gradient proper. The distributions of selected markers are shown. Fractions 17 and 18 are not included in the Western blots as none of the control proteins and DGK constructs appeared in these fractions. The input lane shows that a similar amount of DGK appears in both samples. Graphs are representative of multiple replicates.



CHAPTER 3:

Catalytic Activity and Acyl Chain Selectivity of Diacylglycerol Kinase ε is Modulated by Residues In and Near the Lipoxygenase-like Motif[4][5]

Kenneth D'Souza and Richard M. Epand

This is a minor project. The paper shows that the region adjacent to the LOX-like motif also affect DGKε's enzymatic activity and substrate specificity. There is a cholesterol recognition motif in this region, known as the CRAC motif; we mutated the invariant residues of the CRAC motif. My contribution to this paper was in performing all the experiments (Figures 1-5), drawing the graphical abstract, preparing the tables, the writing of the manuscript and was involved in the editing process.

[4] Dsouza, K. and Epand, R. M. (2012) Catalytic Activity and Acyl Chain Selectivity of Diacylglycerol Kinase ε is Modulated by Residues In and Near the Lipoxygenase-like Motif. J Mol Biol. 416, 619-28

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REGIONS IN THE ACCESSORY DOMAIN OF DIACYLGLYCEROL KINASE ε CONFER ACYL CHAIN SELECTIVITY

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Abstract

Diacylgylcerol kinase ε (DGKε) plays an important role in the re-synthesis of phosphatidylinositol by mediating the phosphorylation of diacylglycerol to phosphatidic acid. DGKε is unique among mammalian DGK isoforms in that it is the only one that shows acyl chain selectivity, preferring diacylglycerols with an *sn-2* arachindonoyl group. The region responsible for this arachidonoyl specificity is the lipoxygenase (LOX)-like motif that is in the accessory domain, adjacent to DGKε's catalytic site. Many mutations within the LOX-like motif result in a loss in enzyme activity. However, the few mutants that retain significant activity exhibit some decrease in selectivity for the arachidonoyl chain. In the present work we have explored mutations in a region adjacent to the LOX-like motif, which is also contained within the same hydrophobic segment of the protein. This adjacent region also contains a cholesterol recognition/interaction amino acid consensus (CRAC) motif. Being outside of the LOX-like motif, this region likely has less direct contact with the substrate and more activity is retained with mutations. This has allowed us to probe in more detail the relationship between this region of the protein and substrate specificity. We demonstrate that this CRAC domain also plays a role in acyl

chain selectivity. Despite the high degree of conservation of the amino acid sequence in this region of the protein, certain mutations result in proteins with higher activity than the wild type protein. These mutations also result in a selective gain of acyl chain preferences for diacylglycerols with different acyl chain profiles. In addition to the LOX-like motif, adjacent residues also contribute to selectivity for diacylglycerols with specific acyl chains compositions, such as those found in the PI cycle.

Keywords: LOX motif, CRAC motif, Substrate Specificity, PI Cycle

Introduction

Phosphatidylinositol (PI) and its phosphorylated forms play critical roles in lipid signalling, signal transduction pathways, membrane dynamics and membrane trafficking[1-2]. The process of the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to form diacylglycerol and the re-synthesis of PIP_2 from diacylglycerol is collectively known as the PI cycle. Apart from phosphoinositides, other lipids of the PI cycle also play important roles in many cellular processes. One especially important lipid intermediate is diacylglycerol, formed from the hydrolysis of PIP_2 by isoforms of phospholipase C[3-4]. Diacylglycerol has multiple functions, resulting in diverse effects within cells. In addition to being a secondary lipid messenger and intermediate in metabolic reactions, it also recruits and modulates multiple classes of proteins such as protein kinase C (PKC) and chimaerins[5-8]. As expected, diacylglycerols are tightly regulated within cells.

A characteristic of the lipid intermediates of the PI-cycle is that they are highly enriched with specific acyl chains, the major species being the 1-stearoyl-2-arachidonoyl form. This includes the major diacylglycerol intermediate of the PI cycle, 1-stearoyl-2-arachidonoylglycerol (SAG).

The acyl chain composition of lipids has an important role in signal transduction such as determining the specific isoform of PKC that is activated[9]. One of the primary ways in which diacylglycerol concentrations are regulated is through DGKs. DGK's phosphorylate diacylglycerol to PA using ATP as a donor[10-11]. As one of at least ten mammalian isoforms, DGKε is the only isoform that shows acyl chain specificity, preferentially phosphorylating diacylglycerols with an arachidonoyl group at the *sn-2* position[11-13]. This acyl chain specificity has also been shown to extend to the *sn-1* position, with DGKε preferring a stearoyl group[13]. Thus, one of the preferred substrates of DGKε is SAG, the species of diacylglycerol produced in the PI cycle through PIP_2 hydrolysis[14-17].

Recently it has been shown that a segment of human DGKε comprising residues L^{431} to G^{443} plays an important role in substrate recognition[18]. The sequence of this segment is LDGERVALPSLEG. This amino acid pattern corresponds to a consensus sequence *L-*X_{3-4}*-R-*X_2*-L-*X_4*-G*, in which X can be any amino acid residue, that is found in DGKε as well as in other enzymes of the PI cycle[18-19] and has suggested to be responsible for arachidonoyl specificity. This motif is very similar to one that was first identified by analysis of the crystal structure of the 8R-lipoxygenase from *Plexaura homomalla* that shown to contribute to arachidonoyl specificity by lining a U-shaped channel that allows the substrate access to the catalytic iron site[20]. In human 5-lipoxygenase, residues of this motif are predicted to allow access of O_2 to the substrate or to position the substrate[21]. The motif responsible for the recognition of polyunsaturated fatty acid in DGKε is very similar to that in LOX itself, except that the first leucine in the DGKε motif is replaced with an isoleucine in LOX itself[20]. In DGKε, the LOX motif is positioned close to catalytic domain in a region known as the accessory domain (Figure 1). As in the case of LOX, we believe that the LOX domain in DGKε may form part of a substrate binding pocket that

allows diacylglycerols with an arachidonoyl group near the catalytic site or may serve to properly position susbtrates.

Evidence that this LOX-motif contributes to arachidonoyl specificity of the enzyme includes the fact that DGKε is the only DGK isoform with such a motif and also the only one that exhibits specificity for arachidonoyl groups. The motif was also shown to contribute to the arachidonoyl specificity of phosphatidylinositol-4-phosphate-5-kinase[18]. Most mutations of the LOX motif of DGKε resulted in proteins essentially devoid of enzyme activity[18]. This is similar to what was found with the 8-LOX[20] and suggests that this segment of DGKε is in close contact with the bound substrate. This contrasts sharply with the removal of 58 residues from the amino terminus of DGKε that had only very small effects on the kinetics of the enzyme[14]. Only two mutants of DGKε had sufficient expression level and activity to be studied in more detail. A complete kinetic analysis showed that both the L438I and L431I had decreased specificity for arachidonoyl groups as a result of the mutation, suggesting that this region of the protein was important not only for activity, but also for substrate specificity. This conclusion was supported by the observation that mutation of another isoform, DGKα, to introduce a LOX-motif that was not present in the wild type enzyme, also resulted in some increase in the selectivity of the modified enzyme for substrates with arachidonoyl groups.

The LOX domain in DGKε, comprising residues L^{431} to G^{443}, is at the amino terminal end of a hydrophobic segment that extends from residue V^{436} to C^{456}. This is the second most hydrophobic segment of the protein after the segment 20-40. It is not predicted to be a transmembrane helix by most predictive algorithms. At the carboxyl end of this hydrophobic segment there is a cholesterol recognition/interaction amino acid consensus (CRAC) motif. The CRAC motif has the consensus sequence $L/V\text{-}X_{1-5}\text{-}Y\text{-}X_{1-5}\text{-}R/K$, in which X can be any amino acid

residue[22- 23]. The CRAC motif, which is found in several different proteins such as the HIV fusion protein, gp41, flotillin, the translocator protein and caveolin helps sequester them to cholesterol rich areas in the membrane[22, 24-31]. DGKε has four CRAC motifs; one of these CRAC motifs is located in the accessory domain overlapping a hydrophobic segment that includes the LOX motif. This CRAC segment comprises residues L^{447} to R^{457}.The LOX domain and this CRAC segment make up an almost continual protein segment from residue L^{431} to R^{457} with only three residues separating the LOX and CRAC segments, i.e. I^{444}, I^{445} and V^{446}. In addition, these two domains overlap with and form part of a hydrophobic segment of the protein that should sequester both the LOX and this CRAC domain close to the membrane.

In this study, we examine the effect of mutating regions in the accessory domain, by not the LOX motif of DGKε. Furthermore, we test substrate specificity using several species of diacylglycerol. Until recently, only two species of diacylglycerol were known to be good substrates of this enzyme, i.e. SAG and SLG. Recently however, it was discovered that AAG (diarachidonoyl glycerol) is also a good substrate for this enzyme. In fact its activity is comparable to that of SAG (32). This has allowed us to compare the activity of mutants against AAG as well as SAG, SLG and other forms of diacylglycerol with lower activity.

RESULTS

Mutations of the CRAC motif in the segment L^{447} to R^{457} of DGKε- In order to test whether this CRAC motif close to, but separated from, the LOX motif, affected DGKε's enzymatic activity, mutations were made in the required, highly conserved amino acid residues of this CRAC motif (Table 1). The CRAC domain from L^{447} to R^{457} as well as surrounding residues is invariant among the forms that have been identified going from avian species to man.

Enzymatic activity was tested using the mixed micelle assay with DGKε's preferred substrate, SAG (for a list of different diacylglycerols used in this study refer to Table 2) compared with a less preferred substrate, SLG. As the data shows in Figure 2a, a range of effects are seen with different mutants, resulting in similar, higher or lower activities than wild type DGKε. Of interest, the R457Q mutation, which results in the loss of this CRAC consensus sequence, as well as the loss of a positively charged residue, resulted in a higher enzymatic activity than wild type DGKε. Similarly, the Y451F mutation, which resulted in a loss of a hydroxyl group and an essential residue of the CRAC domain, also resulted in a higher activity than the wild type protein. In contrast, mutations in the L447 residue, the other required residue of this CRAC motif resulted in a loss of enzymatic activity (Fig. 2A). As the motif studied is normally involved in cholesterol recognition and interaction, the effect of adding cholesterol to the mixed micelles on the DGK activity was measured. The addition of cholesterol has no effect on enzymatic activity of either the wild type protein or the R457Q mutant (Figure 2b).

Substrate specificity of mutants- Many of the mutations in the region adjacent to the LOX motif had significant effects on the substrate specificity of the enzyme. Thus, the mutants L447A and L447I had significantly greater selectivity for SAG over SLG, relative to the wild type protein (Table 3). In contrast, R457K had less specificity for the arachidonoyl-containing substrate. These results demonstrate that mutations near the LOX domain will affect the substrate binding site in different ways, depending on the specific mutation. The more highly active mutants, Y451F and R457Q, had little effect on substrate specificity.

Michaelis-Menten constants for the Y451F and R457Q mutations- Despite the fact that the region of this CRAC domain is conserved through evolution, we observed that two different single residue mutations could lead to expression of proteins with higher activity. We further

explored the basis of this phenomenon by determining the values of V_{max} and K_m against SAG. In order to test whether the Y451F and R457Q mutations resulted in a higher activity and/or higher affinity for SAG, a kinetic activity assay was performed over a range of different substrate concentrations. At higher concentrations of substrate (~9.75 mol % SAG), an inhibitory effect was seen, suggesting the possibility of product inhibition, i.e. that the PA produced was inhibiting the enzymatic activity; these points were excluded. As shown in Figures 3 a-c and summarized in Table 4, the Y451F and R457Q mutants both have a higher V_{max} than wild type DGKε, while their K_m's are within error of each other. This suggests that while the Y451F and R457Q mutants do not have higher affinities than SAG than the wild type DGKε, they do have a higher catalytic rate constant.

The relative activity of the Y451F and R457Q mutants against 6 different substrates- We tested the activity of the two mutants with higher than wild type activity against the preferred substrate, SAG and five less preferred substrates (Figure 4a and 4b). Surprisingly, the Y451F mutant resulted in a gain of preference for AAG, with activities comparable to SAG. The R457Q mutant resulted in higher preference for SDG, which wild type DGKε had essentially no activity for. Preferences for other diacylglycerols were all within error of each other (Table 5).

Activity of mutants against a group of different diacylglycerol sustrates- An additional mutant, A437E, was produced, since the A437 residue is found in higher primates, while an E437 residue is found in all other vertebrate species (Table 1), we thought the mutant could potentially highlight differences in diacylglycerol specificities between higher primates and other vertebrates. As seen in Figure 5a, mutations in the LOX motif resulted in a decrease in SAG activity when compared to wild type protein. When comparing percent activity to SAG, there were no difference in activities between wild type DGKε and the mutants (Figure 5b). Thus the

sequence change that occurred as a result of evolution to primates, also led to a more active form of DGKε, although without change in substrate specificity.

DISCUSSION

Mutations in the LOX domain only showed activity in a few cases. These examples were all conservative mutations, substituting one hydrophobic residue for another, generally with similar chemical structure[34]. In contrast, most of the constructs made in residues adjacent to the LOX domain were active. In particular, the R457Q mutation, that changes both charge and chemical structure of the side chain, gives a construct with higher activity. These results can be understood in terms of the LOX domain forming a lining of the channel in which the substrate binds, in analogy with LOX[20]. Hence, any mutation in this region will likely have large effects on substrate binding. The amino acid sequence of DGKε is highly conserved through evolution. This is particularly true of the segment of the protein in and around the LOX and CRAC domains studied in the present work (Table 1). Hence mutations will generally result in loss of enzyme activity. However, this loss of activity is greater when this domain is in the substrate binding site, as with the LOX domain, than when it is adjacent to this domain.

It was therefore of particular interest that we uncovered two mutants, Y451F and R457Q, whose activity was greater than that of the wild type enzyme (Fig. 2A). This is a consequence of a change in the catalytic rate constant, rather than a change in affinity (Table 3). Both mutations correspond to making this region of the protein more hydrophobic, in one case with the loss of a polar hydroxyl group and in the other case a loss of the positive charge in the side chain. It is possible that making this region of the protein more hydrophophic allows better access of the hydroxyl group of diacylglycerol to the catalytic site on the enzyme. None of the other mutants

have an amino acid change that would clearly correspond to an increase in hydrophobicity, although this suggestion must be taken with caution until more examples are tested.

The relative activity of the Y451F and R457Q mutants against SAG is similar to their increased activity against other diacylglycerol substrates, i.e. the ratio of activity with another diacylglycerol to its activity against SAG is constant (Table 5). There are, however, two significant exceptions to this. Y451F has relatively higher activity against AAG, while R457Q has relatively higher activity against SDG, a diacylglycerol that is essentially not a substrate for either the wild type enzyme or the Y451F mutant. Even more dramatic differences are observed when comparing the activity of a wider range of mutants against SLG versus SAG (Table 4). The specificity for SAG relative to SLG is several fold higher for L447A and lower for R457K. Thus, the specificity of the mutants for SAG can either increase or decrease, depending on the nature of the mutation (Table 4). In addition, the relative activity of different substrates will be affected differently by a particular mutation.

We previously stated that the wild type DGKε has a substrate binding site specific for arachidonoyl groups and that any mutation of this site will lead to a loss of specificity. From the results of the present study, we believe that a more accurate description is to state that the wild type protein has a particular substrate binding site. The shape and location in the membrane of this site will be affected by mutations both within and close to the site. This will alter the efficiency of the enzyme catalysis as well as the substrate specificity.

It is also unknown whether this CRAC motif also plays a role in cholesterol recognition in addition to being part of the lipid binding pocket. The addition of cholesterol to mixed micelles had no significant effect on enzymatic activity when comparing wild type DGKε to

mutants, but may still serve to sequester DGKε to cholesterol rich regions. Ideally, these experiments should be performed in belayed-based system instead of detergent-phospholipid mixed micelles. However, we have been unable to transfer DGKε to liposomes needed for these assays.

The series of mutations studied in the present work has allowed us to determine the consequences of mutating a region of DGKε that is close to, but not within the LOX motif of this enzyme. Making mutations outside of the LOX motif has resulted in higher enzymatic activity of the resulting mutations and allowing larger changes in the nature of the residue without loss of activity. This study is able to further define our understanding of DGKε substrate specificity by showing that regions of its accessory domain, specifically the CRAC motif, also play a role in acyl chain selectivity.

MATERIALS AND METHODS

Materials- All lipids were purchased from Avanti Polar Lipids and were stored in a solution containing 2:1 (v/v) $CHCl_3/CH_3OH$ and 0.1% (w/v) butylated hydroxytoluene (BHT). When using these lipids, the solvent phase was evaporated using N_2 gas, then placed in a vacuum desiccator for 2 hours and stored in argon gas for storage overnight. All antibiodies were purchased from Santa Cruz Biotechnology. Most other chemicals and reagents were purchased from Sigma unless otherwise noted.

DGKε constructs-Human DGK_ε was cloned into the p3xflag-cmv-7.1 vector as described[33]. Mutations were created using the Quikchange Site Directed Mutagenesis kit (Aligent Technologies) as per the manufacturer's instructions. All mutations were analyzed and confirmed through sequencing.

Cell Culture and Tranfections- COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) and were supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO) and 1:100 (v/v) Pen/Strep (GIBCO). Cells were maintained at 37°C with 5% CO_2. For transfections, cells were grown to 70-90% confluency and tranfected with expression vectors using lipofectamine 2000 reagent (Invitrogen) as per the manfacturer's instructions. After 48 hours, cells were harvested by scraping into a solution of PBS with a 1:100 v/v protease inhibitor cocktail for use with mammalian and tissue extracts. Cells were spun at 1000× g for 5 min and the pellets frozen at -80°C.

Enzyme preparation for DGK activity assay- Cells pellets containing transfected constructs were re-suspended in cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1:1000 v/v protease inhibitor cocktail for use with mammalian and tissue extracts, 1 mM β-glycerophosphate, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate) and left to lyse on ice for 10 min. Re-suspended cells were then sonicated in ice cold water for 10 min and centrifuged at 100000× g for 30 min at 4°C. The supernatants were used for the mixed micelle-based enzymatic activity assay.

Detergent-phospholipid-mixed micelle-based enzymatic activity assay- Enzymatic activity was determined using a protocol previously described [33-34]. Lipid films were prepared by solvent evaporation from a chloroform-methanol solution of the diacylglycerol substrate, along with the phospholipid 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). After drying under vacuum for at least two hours, lipid films were suspended in 50 µl of 4× assay buffer (200 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.2, 400 mM NaCl, 20 mM MgCl, 4 mM ethylene glycol tetraacetic acid (EGTA), 60 mM Triton X-100) by vortexing for 2 minutes to give a final total lipid concentration of 4.75 mM. . Supernatants from mock and DGKε transfected cells were

added to the mixed micelles, along with dithiothreitol (DDT) and $CaCl_2$ to give final concentrations of 10 mM DTT, 10 mM $CaCl_2$ and sufficient ddH_20 to a final volume of 180 µl. The reaction was initiated by adding 20 µl of 1 mM [γ-^{32}P] ATP and allowed to incubate at room temperature for 10 min. The reaction was terminated by adding 2 ml of stop solution (1:1 (v/v) $CHCl_3/CH_3OH$, 0.25 mg/ml dihexadecyl phosphate). The organic phase was washed three times with wash solution (7:1 v/v ddH_20/CH_3OH, 1% v/v $HClO_4$, 0.1% H_3PO_4). 400 µl of the organic phase was used to determine the level of incorporation of ^{32}P into DAG to form PA using Cerenkov counting. The formation of PA was linear over the 10 min reaction period, so enzymatic activity data was obtained from initial rate experiments. Each assay was performed in triplicates and repeated independently at least twice. The data from these assays will be presented as the mean ± S. D. To account for endogenous levels of DGKε and background activity, lysates with mock transfected COS7 cells (empty vector) were used as a negative control, after normalizing for the actin content. The mock transfected lysates were included with each activity assay and had significantly lower activities than DGKε transfected constructs. Since DGKε is restricted to the interface of the detergent-phospholipid mixed micelle, concentrations of individual lipid components in mixed micelles are used instead of the bulk concentrations of lipid components. All lipid concentrations are expressed as mol % rather than their bulk concentrations.

Kinetic Analysis of DGKε activity-To determine V_{max} and K_m parameters, activity assays were performed using various concentrations of SAG lipid substrates (Table 2). Substrate concentrations higher than 9.75 mol % showed inhibitory effects and were not included in V_{max} and K_m calculations. V_{max} and K_m parametes were determined using nonlinear regression

analysis by plotting initial enzyme velocity (v_o) against substrate concentration ([S]). Data was analyzed using Origin8.

Immunoblot Analysis- Immunoblotting was used to quantify the amount of protein from transfected COS7 cells. Samples were prepared by mixing equal volume of lysate and Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and were boiled at 60°C for 5 min. The samples were run on a 7.5% Tris-glycine SDS-PAGE gel for 30 min at 200V. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The antibodies used to detect the flag epitope on DGKε constructs were a mouse anti-FLAGM2 antibody (1:5000) (Sigma) as the primary and horseradish peroxidise (HRP)-conjugated goat anti-mouse antibody (1:5000) as the secondary. The antibodies used to detect actin were the goat anti-actin antibody (1:700) as the primary and an HRP-conjugated donkey anti-goat antibody (1:2000) as the secondary. The immunoblot was detected using ECL Western Blotting Detection Reagents (GE Healthcare) on XAR Biofilm (Kodak). To determine the amount of flag-DGKε in each of the lanes, a known amount of 3× flag-tagged bacterial alkaline phosphatase (BAP) was run along with the samples.

Statistical Analysis- Where necessary, statistical analysis was performed using the one way analysis of variance (ANOVA) tests. Analysis was performed using Origin Pro 8.

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FOOTNOTES

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The abbreviations used are: DGK, diacylglycerol kinase; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4, 5-bisphosphate; LOX, lipoxygenase; DOPC, 1, 2-dioleoyl-sn-glycero-3-phosphocholine; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, dithiothreitol; PVDF, polyvinylidene fluoride; BAP, bacterial alkaline phosphatase; CRAC, cholesterol recognition/interaction amino acid consensus; PKC, protein kinase C. See Table 2 for list of abbreviations of diacylglycerols used in this work.

FIGURE LEGENDS

Figure 1. **Schematic of DGKε structure**. The major domains and motifs are presented. CRD, cysteine-rich domain; LOX, lipoxygenase; CRAC, cholesterol recognition/interaction amino acid consensus sequence.

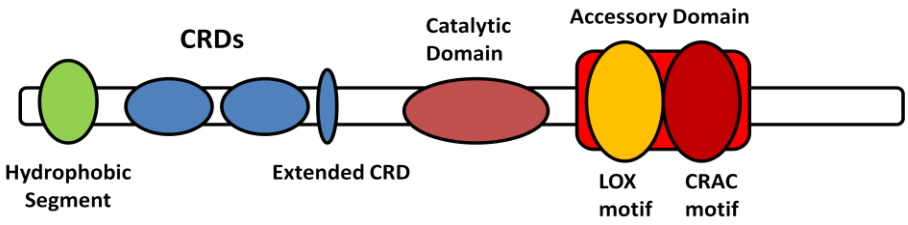


Figure 2. **Comparison of DGKε enzymatic activity.** *A*, Mixed-micelle based enzymatic activity of wild type DGKε and CRAC mutants using SAG and SLG as substrates. Enzymatic activity is adjusted using mock transfected Cos-7 cells (EV control) to account for endogenous DGKε activity. Specific activity is calculated by quantifying the amount of flag-tagged protein on immunoblots. *B*, Enzymatic activity comparing wild type and R457Q mutants in mixed micelles composed of DOPC or DOPC + cholesterol. Data is represented as means ± SD, $n = 9$, $N = 3$.

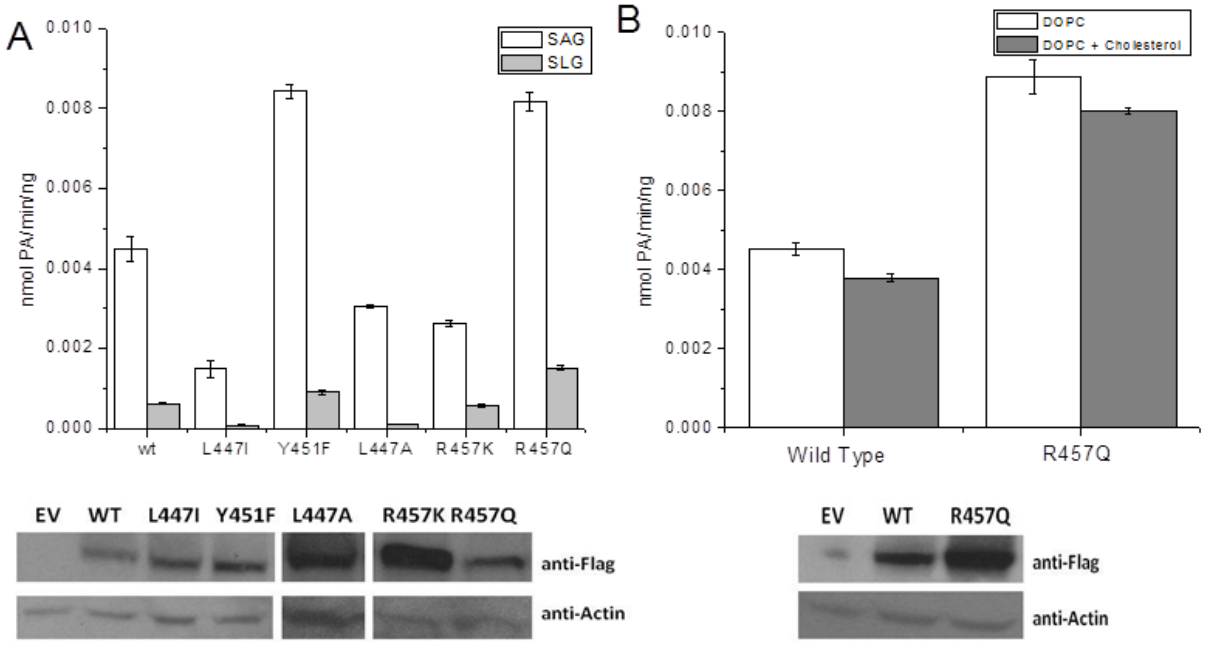


Figure 3. **Kinetic analysis of wild type, Y451F and R457Q mutants.** The mixed-micelle assay was used to test enzymatic activity over a series of substrate concentrations. The total lipid concentrations of DOPC and substrate were adjusted to remain constant at 4.75mM. Substrate concentrations are represented as mol % SAG. Enzymatic activity is adjusted using mock transfected Cos-7 cells (EV control). Two independent experiments are shown, with a nonlinear regression curve fitting experiment 1. Kinetic analysis of *A*, wild type DGKε *B*, Y451F mutant *C*, R457Q mutant. *D*, Immunoblots of lysates of COS7 cells overexpressing DGKε constructs from one experiment. For K_m and V_{max} values, refer to Table 3, $n = 9$, $N = 3$.

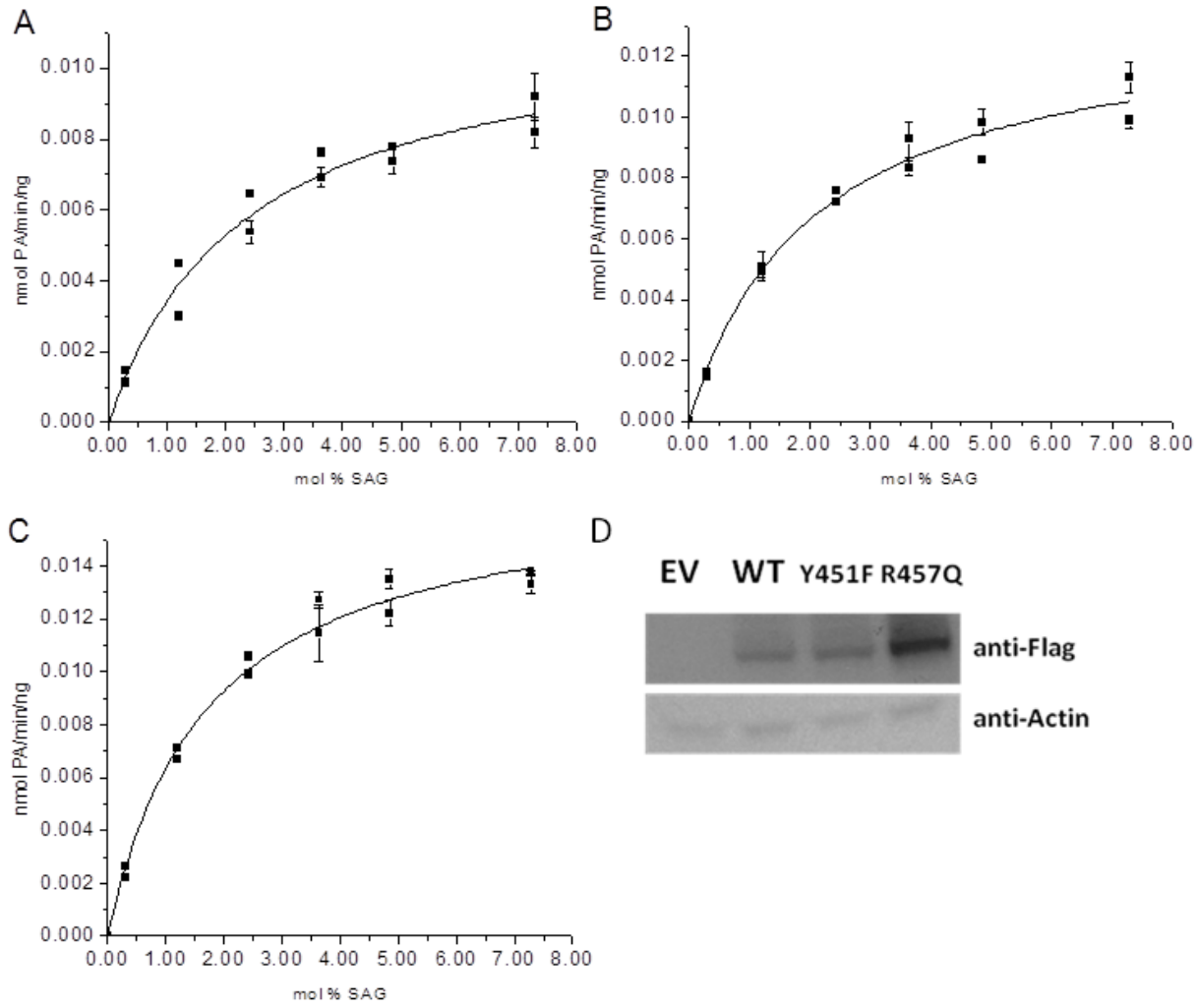


Figure 4. **Substrate specificity assays of mutations involving the CRAC motif.** *A*, Enzymatic activity was tested using six different substrates: SAG, DOG, SLG, AAG, DLG and SDG. *B*, Enzymatic activity when represented as % activity, with activity of DGKε using SAG as a substrate set to 100%. The total lipid concentrations of DOPC and substrate were constant at 4.75mM. For ratios of different substrates to SAG, refer to Table 5. The bars with an * were determined to be statistically different form wild type protein ($P < 0.05$). *C*, Immunoblots of lysates of COS7 cells overexpressing DGKε constructs. Data is represented as means ± SD, $n = 9$, $N = 3$.

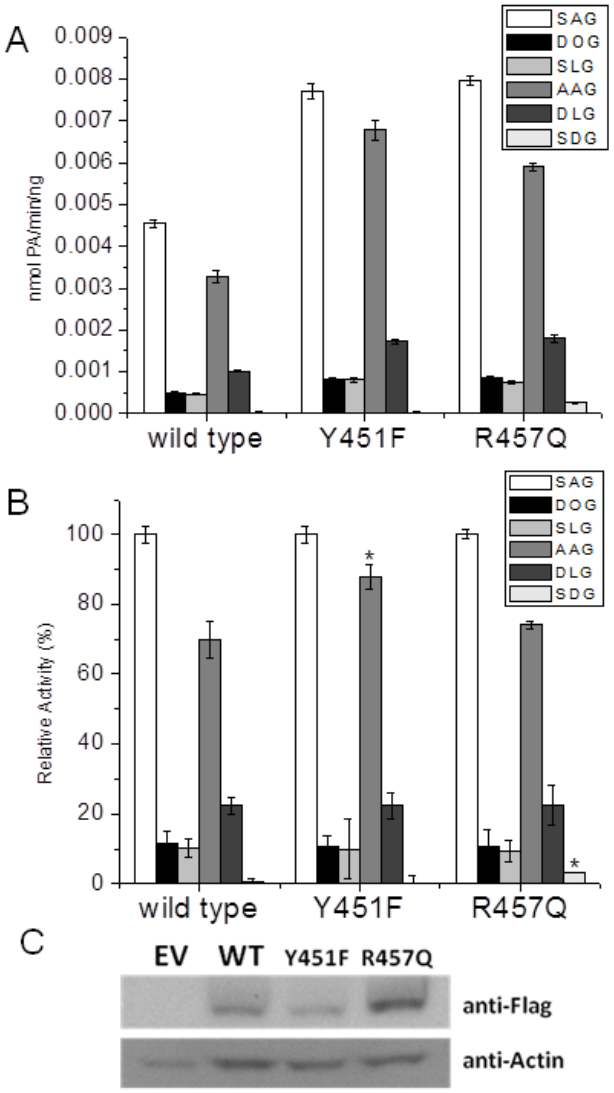
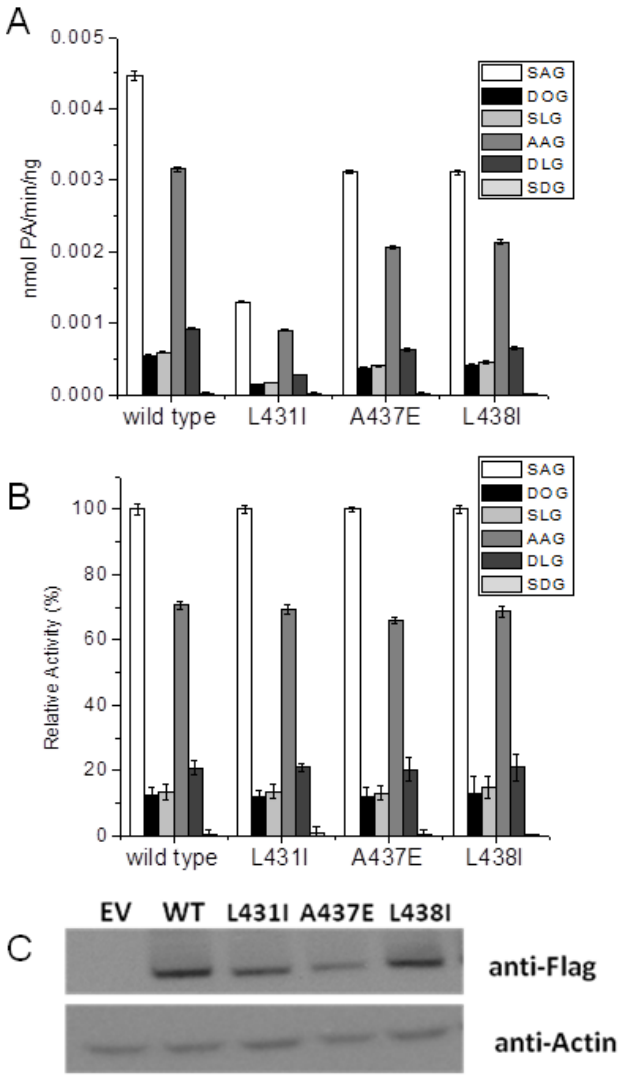


Figure 5. **Comparison of DGKε enzymatic activity and substrate specificity assays of mutations involving the LOX motif.** *A*, Substrate specificity assay of wild type DGKε and LOX mutants using six different substrates. Enzymatic activity is adjusted using mock transfected Cos-7 cells. *B*, Enzymatic activity when represented as % activity, with activity of protein with SAG as a substrate set to 100%. The total lipid concentrations of DOPC and substrate were constant at 4.75mM. *C*, Immunoblots of lysates of COS7 cells overexpressing DGKε constructs. Data is represented as means ± SD, n = 9, N = 3.



TABLES

Table 1. **Partial sequence alignment of DGKε of multiple species.** Conserved residues in the LOX motif are highlighted in red whereas conserved residues in the CRAC motif are highlighted in blue. The A/E437 residue is highlighted in green.

GENE ID	Species		Sequence	
GENE ID:8526	[*Homo sapien*]	425	KKVELELDGERVALPSLEGIIVLNIGYWGGGCRLWEGM	462
GENE ID:468414	[*Pan troglodytes*]	425	KKVELELDGERVALPSLEGIIVLNIGYWGGGCRLWEGM	462
GENE ID:100395461	[*Callithrix jacchus*]	425	KKVELELDGERVALPNLEGIIVLNIGYWGGGCRLWEGM	462
GENE ID: 538147	[*Bos taurus*]	422	KKVELELDGERVELPNLEGIIVLNIGYWGGGCRLWEGM	459
GENE ID: 100056676	[*Equus caballus*]	422	KKVELELDGERVELPNLEGIIVLNIGYWGGGCRLWEGM	459
GENE ID:100093422	[*Ornithorhynchus anatinus*]	418	KKVELELDGERVELPNLEGIIVLNIGYWGGGCRLWEGM	455
GENE ID: 770911	[*Gallus gallus*]	425	KKVELELDGERIELPNLEGIIVLNIGYWGGGCRLWEGM	462
GENE ID: 56077	[*Mus musculus*]	429	KKIELELDGERVELPNLEGIIVLNIGYWGGGCRLWEGM	466

Table 2. **Diacylglyceorols used in this study**

Abbreviation	**Full Name**	***sn-1*/*sn-2* notation**
SAG	1-Stearoyl-2-arachidonoyl-*sn*-glycerol	18:0/20:4 DG
SLG	1-Stearoyl-2-linoleoyl-*sn*-glycerol	18:0/18:2 DG
DOG	1, 2-Dioleoyl-*sn*-glycerol	18:1/18:1 DG
AAG	1, 2-Diarachidonoyl-*sn*-glycerol	20:4/20:4 DG
DLG	1, 2-Dilinoleoyl-*sn*-glycerol	18:2/18:2 DG
SDG	1-Stearoyl-2-Docosahexaenoyl-*sn*-glycerol	18:0/22:6 DG

Table 3. **Summary of Vmax and Km parameters for wild type DGKε, Y451F and R457Q mutants**. The results show that both Y451F and R457Q mutants have a higher V_{max} value than wild type DGKε, which explain why these mutants have higher activity than wild type protein. K_m values are within error of each other. Graphs showing the activities of enzyme at different SAG concentrations are shown in Figure 3, $n = 9$, $N = 3$.

Construct	**V_{max} (nmol PA/min/ng)**	**K_m (mol % SAG)**
Wild type	0.0103 ± 0.0004	1.45 ± 0.20
Y451F	0.0122 ± 0.0003	1.78 ± 0.15
R457Q	0.0168 ± 0.0004	1.78 ± 0.12

Table 4. **SLG/SAG ratio of wild type DGKε and mutants in the CRAC motif.** The ratio of enzymatic activities of constructs with SAG and SLG used as substrates were compared. The R457K mutant resulted in a higher SLG/SAG ratio than wild type protein or other mutants, but R457K had a much lower enzymatic activity than wild type protein. The enzymatic data of constructs are represented in Figures 2 and 4, $n = 9$, $N = 3$.

Construct	**SLG/SAG ratio**
Wild type	0.109 ± 0.009
L447A	0.034 ± 0.004
L447I	0.054 ± 0.013
Y451F	0.107 ± 0.010
R457K	0.217 ± 0.022
R457Q	0.099 ± 0.007

Table 5. **Affinities for different substrates for wild type DGKε and CRAC motif mutants.** From Figure 4, the ratio of mean enzymatic activity of DOG, SLG, AAG, DLG and SDG were compared to the mean enzymatic activity of SAG. Activity with SAG is assigned a value of 1. The cases for which the mutant shows a significantly different ratio compared with the wild type is indicated in bold type ($P < 0.05$), $n = 9$, $N = 3$.

DGKε Construct	**DOG**	**SLG**	**AAG**	**DLG**	**SDG**
Wild type	0.112 ± 0.006	0.103 ± 0.005	**0.722 ± 0.050**	0.222 ± 0.010	**0.005 ± 0.005**
Y451F	0.107 ± 0.006	0.105 ± 0.011	**0.880 ± 0.052**	0.224 ± 0.013	0.003 ± 0.005
R457Q	0.108 ± 0.007	0.095 ± 0.004	0.741± 0.022	0.225 ± 0.016	**0.033 ± 0.002**

CHAPTER 4:

Distinct Properties of the two isoforms of CDP-diacylglycerol Synthase

Kenneth D'Souza, Tamas Balla and Richard M. Epand

The paper characterizes two CDP-DAG synthase (CDS) isoforms, both of which provide substrates for PI synthesis. We determined that CDS1 and CDS2 show markedly different substrate specificities. Both CDS1 and CDS2 are also inhibited by PI is a charge dependant manner, but are differentially affected by the acyl chain composition of these PI species. My contribution to this paper was in performing all the experiments (Figures 1-6), preparing the tables, the writing of the manuscript and was involved in the editing process.

Distinct Properties of the two isoforms of CDP-diacylglycerol Synthase

Kenneth D'Souza, Tamas Balla and Richard M. Epand

Abstract

CDP-diacylglycerol Synthases (CDS) are critical enzymes that catalyze the formation of CDP-diacylglycerol (CDP-DAG) from phosphatidic acid (PA). Here we show *in vitro* that both isoforms of human CDS, CDS1 and CDS2, shows different acyl chain specificities for its substrate. CDS2 is selective at both the *sn-1* and *sn-2* acyl chain positions, the most preferred species being 1-stearoyl-2-arachidonoyl-*sn*-phosphatidic acid. CDS1, conversely, has almost no substrate specificity, showing similar activities for almost all substrates tested. Additionally, we show that inhibition of CDS2 by phosphatidylinositol is also acyl chain dependant, with the greatest inhibition seen with the 1-stearoyl-2-arachidonoyl species. CDS1 shows no acyl chain dependant inhibition. Both CDS1 and CDS2 are inhibited in a charge dependant manner, with phosphatidylinositol-(4,5)-bisphosphate showing the greatest inhibition. Our results indicate that CDS1 and CDS2 could create different CDP-DAG pools that may serve to enrich different phospholipid species with specific acyl chains.

Introduction

CDP-DAG synthases (CDS) are enzymes that catalyze the conversion of phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG) (1). Both PA and CDP-DAG serve critical roles in cellular functions. PA is involved in several signal transduction pathways and also plays structural and biosynthetic roles. For example, PA has been shown to modulate several proteins including phosphatidylinositol-4-phosphate-5-kinase, protein phosphatase 1 and mammalian target of rapamycin (2-4). In addition, PA is a highly fusogenic lipid involved in the generation of negative curvature in transport complexes, such as vesicles (5). PA can also be dephosphorylated to diacylglycerol, which is a precursor to phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) (6-7). Conversely CDP-DAG is a lipid precursor to several phospholipid classes such as phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) (6).

Only two CDS isoforms in mammals have been identified and characterized. Both these isoforms are believed to localize to the endoplasmic reticulum (ER) (8-11). It was believed that CDS1 was present in mitochondria and is responsible for synthesizing cardiolipin (12). However, a recent study using yeast has shown that the enzyme Tam 41 is responsible for this activity and that CDS1 does not reside in the mitochondria, although its presence may affect mitochondrial lipid composition (13-14). CDS1 and CDS2 are expressed in a variety of tissues. In mice, CDS1 is found in adult brain, eye, smooth muscle and testes. In the eyes, CDS1 is highly expressed in the photoreceptor layer of adult retinas, which could suggest a role for CDS1 in phototransduction (11). CDS2 has a broad expression pattern and was found in virtually every tissue (10); however, some discrepancies exist in the tissue localization of CDS2. For example, another study showed that an arachidonoyl preferring CDS (CDS2 based on our results) was only expressed in

the brain, eye and testis (9-10). Further work is needed to accurately determine the tissue distributions of these isoforms.

The roles of CDS1 and CDS2 have primarily been studied in PI synthesis (15). Many of the cellular functions attributed to CDS enzymes are believed to results due to its role in generating phosphatidylinositol-(4,5)-bisphosphate (PIP_2), a potent signalling molecule. For example, phototransduction signalling in vertebrate and invertebrate systems are believed to proceed at least partly via phosphoinositide signalling (16-17). *cds* mutants in *Drosophila* result in light induced retinal degradation and over-expression of a photoreceptor specific CDS increases the amplitude of the light response (1). Disruption of CDS2 led to a decrease in VEGFA signalling and angiogenesis in zebrafish, primarily though decreased PIP_2 regeneration (18). A similar, yet lesser effect was seen when CDS1 was disrupted, whereas knockdown of both isoforms led to embryonic lethality (18). An arachidonoyl preferring CDS was also shown to be inhibited by PI species, specifically by PIP_2 (9). This suggests a potential feedback mechanism, in which CDS activity is highest when phosphoinositide ($PI(P)_n$) levels are low. Apart from PI signalling, CDS1 was also shown to be involved in the maintenance of PLs critical for mitochondrial function and structure (14).

In this study, we use *in vitro* assays to characterize both human isoforms of CDS, *i.e.* CDS1 and CDS2. CDS2 exhibits specificity for the nature of the acyl chains in the substrate, PA. The prefered acyl chain composition is 1-stearoyl-2-arachidonoyl. This is also the acyl chain composition most highly enriched in PI (19-21). Inhibition of CDS2 is also acyl chain specific, with 1-stearoyl-2-arachidonoyl PI species showing the greatest inhibition. CDS1 in contrast shows virtually no substrate specificity or acyl chain dependant PI inhibition. Taken together,

our results point towards the generation of specific pools of CDP-DAG for phospholipid synthesis by different CDS isoforms.

Abbreviations

The abbreviations used are: ATP, adenosine triphosphate; BHT, butylated hydroxytoluene; CDP-DAG, Cytidine diphosphate-diacylgylcerol; CDS, CDP-DAG synthase; CL, cardiolipin; CTP, cytidine tri-phosphate; DGKε, diacylglycerol kinase epsilon; EGTA, ethyleneglycoltetraacetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GTP, guanine triphosphate; PA, phosphatidic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, L-α-phosphatidylinositol-4-phosphate; $PI(P)_n$, phosphoinositides; PIP_2, L-α-phosphatidylinositol-4,5-bisphosphate; PM, plasma membrane; PS, phosphatidylserine; UTP, uridine triphosphate; WT, wild type. Refer to Table 1 for full list of lipid abbreviations.

Materials and Methods

Materials- All lipids were purchased from Avanti Polar Lipids and were stored in a solution containing 2:1 (v/v) $CHCl_3/CH_3OH$ and 0.1% (w/v) butylated hydroxytoluene (BHT). All traces of the solvent phase were evaporated using N_2 gas and remaining traces of solvent were removed using a vacuum desiccators for two hours. The lipid films were then stored under argon gas for stability. Most other chemicals and reagents were purchased from Sigma unless otherwise noted. All lipids used in this study and summarized in Table 1, together with the abbreviations used.

Cell Culture and Tranfections- COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) and were supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO) and 1:100 (v/v) Pen/Strep (GIBCO). Cells were maintained at 37°C with 5% CO_2. For

transfections, cells were grown to 90% confluency and tranfected using lipofectamine 2000 reagent (Invitrogen) as per the manfacturer's instructions. After 48 hours, cells were harvested by scraping into a solution of PBS with a 1:100 (v/v) protease inhibitor cocktail. Cells were spun at 1000× g for 5 min and the pellets were flash-frozen at -80°C.

Enzyme preparation for CDS activity assay- Cells pellets containing transfected constructs were re-suspended in an ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 0.2 mM ethyleneglycoltetraacetic acid (EGTA) and 1:100 (v/v) protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich). The cells were broken by 30 passages through a 25-gauge needle syringe. Unbroken cells and nuclei were removed from the cell homogenate by centrifugation at 1000g for 10 min at 4°C. Re-suspended cells were centrifuged at 100000× g for 60 min at 4°C. The microsomal fraction (pellet) was re-suspended in lysis buffer and were used for the mixed micelle-based enzymatic activity assay.

Detergent-phospholipid-mixed micelle-based enzymatic activity assay- Lipid films were prepared by solvent evaporation from a chloroform-methanol solution of the lipids. For kinetic experiments, SAPA was used at concentrations of 0, 25, 100, 200 and 400μM. For substrate specificity assay, 50μM of the desired PA species was used. For CDS1 and CDS2 inhibition experiments, 50μM of both SAPA and PI species were used. Lipid films were suspended in 166 μl of Assay buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mg/ml bovine serum albumin (BSA), 100 mM guanine triphosphate (GTP) and 5.10 mM Triton X-100) by vortexing for 2 minutes. GTP was added because it was found to increase the rate of reaction (see below). Once the films were re-suspended, 4μl of 1M $MgCl_2$ was added to reach a final volume of 170 μl. Supernatants from CDS transfected cells were added to the mixed micelles to a final volume of 180 μl. The reaction was initiated by adding 20μl of 0.2 mM [^{3}H-CTP, 2.5 μCi/sample] CTP and

incubated at room temperature for 5 min. The reaction was terminated by adding 500 µl of stop solution (Methanol/0.1N HCl). The organic phase was washed with 300 µl of 0.6 N HCl and 1ml of chloroform. The solution was centrifuged at 2000× rpm for 2 minutes and the organic phase was transferred to a new tube. The organic phase was washed with 1 ml of an ice cold 'upper phase' solution (the aqueous phase of a solution of methanol/0.1N HCl, 0.6N HCl and chloroform (10/6/20 v/v/v)). 500µl of the organic phase was used to determine the level of incorporation of ^{3}H into PA to form CDP-DAG. The organic phase was evaporated and the samples were read using UltimaGoldF (Perkin Elmer). The data from these assays are presented as the mean ± S. D. To account for endogenous levels of CDS1 and CDS2 and background activity, lysates with mock transfected COS7 cells (empty vector) were used as a negative control, after normalizing for the calnexin content. The mock transfected lysates were included with each activity assay and had roughly 10-15% activity as CDS transfected constructs.

Kinetic Analysis of DGKε activity-To determine V_{max} and K_m parameters, activity assays were performed using various concentrations of SAPA. V_{max} and K_m parameters were determined using nonlinear regression analysis by plotting initial enzyme velocity (v_o) against substrate concentration ([S]). Data was analyzed using Origin8.

Quantification of phosphatidic acid- The concentration of all PA stocks used in this study was determined using their phosphate content. Briefly, 30 µl of 10% (w/v) $Mg(NO_3)_2$ in 95% (v/v) ethanol was added to PA samples or KH_2PO_4 standards (up to 80 nmol) in acid-washed Pyrex tubes. The solution was flamed until the organic phosphate was completely ashed. 350 µl of 0.5 M HCl was added, the mixture was refluxed for 15 min and 750 µl of a 1:6 (v/v) mixture of 10% (w/v) L-ascorbic acid and 0.42% (w/v) ammonium molybdate tetrahydrate in 0.5 M H_2SO_4 was

added. The mixture was incubated at 60 °C for 10 min, allowed to cool to room temperature and the absorbance at 820 nm was measured.

Immunoblot Analysis- Immunoblotting was used to quantify the amount of protein from transfected COS7 cells. Samples were prepared by mixing equal volume of lysate and Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) and were boiled at 60°C for 5 min. The samples were run on a 7.5% Tris-glycine SDS-PAGE gel for 30 min at 200V. The proteins were then transferred onto a polyvinylidene fluoride membrane (Bio-Rad). The antibodies used to detect the myc epitope tag on CDS constructs were a mouse anti-myc antibody (Cell Signalling). The antibodies used to detect calnexin were the mouse anti-calnexin antibody (BD Biosciences). The secondary antibodies used were the horseradish peroxidise (HRP)-conjugated goat anti-mouse antibody (Sigma). The immunoblot was detected using ECL Western Blotting Detection Reagents (GE Healthcare) on XAR Biofilm (Kodak). To determine the amount of CDS1/2-myc in each of the lanes, a known amount of myc-tagged control protein was run along with the samples.

Statistical Analysis- Where necessary, statistical analysis was performed using the one way analysis of variance (ANOVA) tests followed by a Tukey's test. Analysis was performed using Origin Pro 8. Experiments were repeated at least three times independently in triplicates. Data is presented as mean ± SEM.

Results

CDS1 and CDS2 are mildly stimulated by specific nucleotides

Previous studies on CDS enzymes show that the enzymes are stimulated in the presence of nucleotides (12, 15). We incubated both CDS1 and CDS2 in the presence of ATP, GTP or UTP

and compared the activity without any additional nucleotides, besides the substrate CTP (Figure 1). The presence of nucleotides results in a small stimulation of both CDS1 and CDS2. CDS1 is only significantly stimulated by GTP, though the activity is only ~120% of enzymes with no nucleotides. CDS2 conversely is significantly stimulated by not only GTP, but UTP as well. However, as with CDS1, this stimulation is minor and the activity is roughly ~115-120% compared with conditions without nucleotides.

The addition of inorganic pyrophosphate, one of the products of the CDS reaction, could slow the rate by shifting the position of equilibrium. However, we found no significant effect on the activity (data not shown), suggesting that the position of equilibrium was far toward the production of the product CDP-DAG.

CDS1 and CDS2 show different substrate specificities

We determined the specific activities and kinetic parameters of CDS1 and CDS2. Both CDS1 and CDS2 express well and show a roughly ~4× and ~8× fold increase in ^{3}H incorporation than mock transfected cells (EV), respectively (Figure 2A). The specific activities of CDS1 and CDS2 were determined to be 3.52 ± 0.09 and 6.6 ± 0.25 pmol CDP-DAG/min/ng respectively, for the substrate SAPA. Interestingly, there is a large difference in substrate acyl chain specificity between CDS1 and CDS2. Whereas CDS1 shows no significant preference between the substrates SAPA and SLPA, CDS2 shows a roughly ~5× increase in preference for SAPA over SLPA (Figure 2B).

We next decided to determine the kinetic parameters of CDS1 and CDS2 for SAPA (Figure 3). We used a wide range of substrate concentrations (25-400μM). Concentrations above 400 μM resulted in a significant inhibition of CDS1 and CDS2, and so were not used in the calculation of

kinetic constants. CDS1 and CDS2 show typical saturation kinetics (Figure 2) and calculation of kinetic parameters show that CDS2 has a significantly higher maximal activity (V_{max}) value than CDS1. The K_m values of both enzymes were determined to not be statistically significantly different, though the inherent errors in these values prevent a more precise conclusion.

We further tested the substrate specificities of CDS1 and CDS2 for PA substrates with a variety of acyl chains (Table 1). CDS1 and CDS2 show very different substrate specificities (Figure 4). CDS1 appears to have no substrate specificity for SAPA. Variations of the *sn-1* and *sn-2* acyl chains resulted in no significant changes in preference compared with SAPA. CDS1 only showed a decrease specificity towards DOPA over the other substrates tested. However, CDS1 showed similar activity towards another PA with variations at the *sn-1* and *sn-2* acyl chains (SAPA vs. DLPA) (Figure 4C).

Conversely, CDS2 shows substrate specificity at both the *sn-1* and *sn-2* acyl chain positions. The most preferred substrate is SAPA. Substitutions of the *sn-1* stearoyl group with an arachidonoyl acyl chain resulted in over a 50% loss in activity (Figure 4D). Even similar substitutions at the *sn-1* position (stearoyl (18:0) vs. palmitoyl (16:0)) resulted in a significant loss in activity (Fig. 4D). PA with differing acyl chains at the *sn-2* position or both *sn-1*/*sn-2* positions showed an even greatest loss in preference than at the *sn-1* position. Any substitution at the *sn-2* arachidonoyl position resulted in activities about 10-20% as SAPA (Figure 3E). DLPA, which varies at both acyl chain positions, was the only PA in this group with activity. DOPA and DDPA showed virtually no activity with CDS2 (Figure 4F). In contrast, DOPA had roughly 75-80% activity as SAPA for CDS1, which shows the striking difference in substrate specificities between these two isoforms.

CDS1 and CDS2 shows inhibition by natural PI species

We next decided to test whether CDS1 and CDS2 show inhibition by physiologically relevant PI species (Figure 5). The acyl chain profile of these lipids are available in Supplemental Figure 1. Both CDS1 and CDS2 show inhibition by PI species in a charge dependant manner. CDS1 shows a non-significant inhibition in activity when both soybean and liver PI are used. CDS1 is significantly inhibited by PIP (25% inhibition) and even a greater inhibition with PIP_2 (40% inhibition) (Figure 5A).

CDS2 also shows inhibition by PI species, but to a greater extent than CDS1 (Figure 5B). There is a significant inhibition of CDS2 by both soybean and liver PI (15-20% activity). Addition of PIP and PIP_2 to the reaction resulted in a 55% and 80% inhibition of CDS2, as compared to no PI species.

Inhibition of CDS1 and CDS2 is also acyl chain dependant

The difference in inhibition of CDS1 and CDS2 suggested that the acyl chain composition of the PI species plays a role in the inhibition of these enzymes. We chose PI species, primarily due to the limited variability of acyl chain composition of commercially available synthetic PIP and PIP_2 species. CDS1 shows no acyl chain dependant inhibition for either SAPI, SLPI and DLPI (Figure 6A). The lack of acyl chain dependant inhibition is similar to its substrate specificity (Figure 4a-c). CDS2, meanwhile, does show acyl chain dependant inhibition, with SAPI showing the greatest inhibition (80% inhibition). DLPI also showed a statistically significant inhibition (20%), whereas the inhibition by SLPI was not significant (Figure 6B). As with CDS1, this acyl chain dependant inhibition is also reflective of the enzymes substrate specificity (Figure 4d-f)

Discussion

Several phospholipid classes show enrichment with specific acyl chains. PI for example, has been shown to have 40-70% of species with 1-stearoyl-2-arachidonoyl acyl chains (Supplementary Figure 1) (19-20). Conversely, PG is enriched with oleoyl (18:1) and linoleoyl (18:2) acyl chains and cardiolipin in heart mitochondria is enriched predominantly with linoleoyl acyl chain (22-23). CDS1 and CDS2 supply precursors to several phospholipids, such as PI, PG and cardiolipin. As such, these enzymes can contribute to the enrichment of phospholipids with specific acyl chains by showing substrate specificity.

We have shown that CDS1 and CDS2 show very different substrate specificities. CDS1 exhibits almost no substrate specificity for PA, showing no discrimination for the *sn-1/sn-2* acyl chain composition of PAs. CDS1 only shows a lower preference for DOPA; however, this PA species is not physiologically relevant. Conversely, CDS2 shows substrate specificity at both the *sn-1* and *sn-2* positions. The most preferred substrate is SAPA, the acyl composition most enriched in PI species. Variations at the *sn-1* position, even to similar acyl chains (stearoyl vs. palmitoyl), resulted in a loss of activity of roughly 40%. CDS2 showed even less preference for substrates with a *sn-1* stearoyl acyl chain but a different acyl chain at the *sn-2* position. CDS2 shows only 10-20% activity for these PA's, when compared to SAPA. Our results suggest that CDS2 is selective for both acyl chains of PA, which is similar to the acyl chain selectivity of another enzyme involved in PI synthesis, DGKε (24-26). Like DGKε, the arachidonoyl acyl chain at the *sn-2* position is critical for the enzymes substrate specificity (25). Changes at the *sn-1* position also play a role in the enzyme's preference for its substrate (26). However, CDS2 appears to be more selective for its substrates than DGKε. For example, CDS2 shows 60% and 30% activity for PAPA and DAPA, whereas DGKε shows roughly 90% and 70% activity for PAG and DAG,

respectively.

While CDS1 and CDS2 show inhibition by PI species, the extent of inhibition and acyl chain dependence differs among isoforms. Inhibition of both isoforms is charge dependent, with PIP_2 species showing the greatest inhibition. However, CDS2 shows a greater inhibition by the PI species chosen. This suggests that the acyl chain composition of the PI species plays a role in its inhibition. As shown in Figure 6, this idea is supported; whereas CDS1 shows no acyl chain dependence among the three PI species, CDS2 shows the greatest inhibition for SAPI.

If both CDS1 and CDS2 are required for PI metabolism, how could they contribute the PI synthesis and acyl chain enrichment? Studies have shown that PI synthesis can occur through two pathways, both of which generate different species: the *de novo* synthesis pathway and the PI cycle. The *de novo* synthesis of PI involves only the ER and generate mainly saturated and monounsaturated acyl chains (20, 27-28). The PI cycle is a cyclical pathway which involves the breakdown and generate of PIP_2. The PI cycle involves both the ER and PM and results in the enrichment of 1-stearoyl-2-arachidonoyl species (25, 29). Both pathways involve common features, one of which is the conversion of PA species to CDP-DAG by CDS enzymes. It is conceivable that CDS2 would be involved in the PI cycle, whereas CDS1 would be involved in the *de novo* synthesis of PI.

The acyl chain selectivity of CDS2 is similar to that of DGKε, which was shown to be required for the arachidonoyl enrichment of PI species. CDS2 could play a similar, yet greater role in the enrichment of PI with arachidonoyl. CDP-DAG produced by CDS2 can only be used for the synthesis of phospholipids; Conversely, PA created by DGKε can be used for signal transduction pathways, structurally, for phospholipid synthesis and can be dephosphorylated back to

diacylglycerol by phosphatidic acid phosphatase (PAP) (6). However, as DGKε and CDS2 are needed for the first steps of PIP_2 synthesis, both of these enzymes can supply precursors enriched with arachidonoyl acyl chains. The cyclical nature of the PI cycle suggests a progressive enrichment of PI species. Both enzymes are also highly expressed in certain organs, such as brain, and so could contribute to particularly high levels of 1-steareoyl-2-arachidonoyl species in these organs (Supplementary Figure 1).

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Table 1: Lipids used in this study. For the natural PIs species, the acyl chain compositions are found in Supplementary Figure 1.

Abbreviation	Full Name	*sn*1/*sn*2 Notation
SAPA	1-Stearoyl-2-arachidonoyl-*sn*-phosphatidic acid	18:0/20:4 PA
PAPA	1-Palmitoyl-2-arachidonoyl-*sn*-phosphatidic acid	16:0/20:4 PA
DAPA	1, 2-Diarachidonoyl-*sn*-phosphatidic acid	20:4/20:4 PA
SLPA	1-Stearoyl-2-linoleoyl-*sn*-phosphatidic acid	18:0/18:2 PA
SOPA	1-Stearoyl-2-oleoyl-*sn*-phosphatidic acid	18:0/18:1 PA
SDPA	1-Stearoyl-2-docosahexaenoyl-*sn*-phosphatidic acid	18:0/22:6 PA
DLPA	1, 2-Dilinoleoyl-*sn*-phosphatidic acid	18:2/18:2 PA
DOPA	1, 2-Dioleoyl-*sn*-phosphatidic acid	18:1/18:1 PA
DDPA	1,2-Didocosahexaenoyl-*sn*-phosphatidic acid	22:6/22:6 PA
SAPI	1-Stearoyl-2-arachidonoyl-*sn*-phosphatidylinositol	18:0/20:4 PI
SLPI	1-Stearoyl-2-linoleoyl- *sn*-phosphatidylinositol	18:0/18:2 PI
DLPI	1, 2-Diolinoleoyl-*sn*-phosphatidylinositol	18:2/18:2 PI
PI	L-α-Phosphatidylinositol	Varies
PIP	L-α-phosphatidylinositol-4-phosphate	Varies
PIP_2	L-α-phosphatidylinositol-4,5-bisphosphate	Varies

Table 2: Summary of kinetic parameters for CDS1 and CDS2. That data is shown as mean ± SEM and is represented graphically in Figure 3, $n = 9$, $N = 3$.

	$\mathbf{V_{max}}$ (pmol/min/ng)	$\mathbf{K_m}$ (mol %)	$\mathbf{K_{cat}}$ (pmol/s)
CDS1	4.7 ± 0.6	0.8 ± 0.4	4150 ± 500
CDS2	13.3 ± 0.9	1.6 ± 0.4	11400 ± 800

Figure 1: **Stimulation of CDS1 and CDS2 by nucleotides**. Mixed-micelle based enzymatic activity of CDS1 and CDS2 using SAPA as substrates. Enzymes were incubated with either ATP, GTP and UTP. The bars with an * were determined to be statistically different ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.

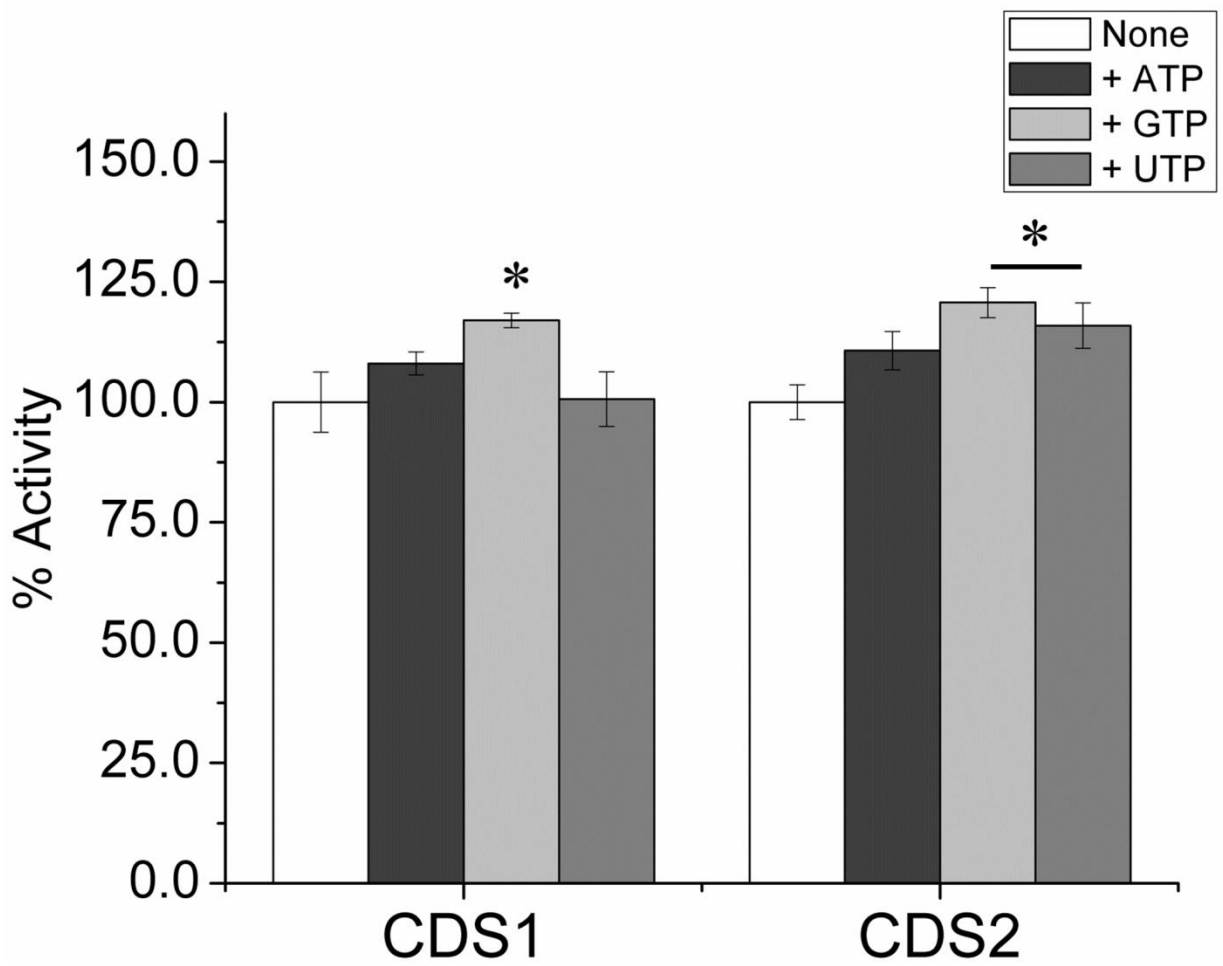


Figure 2: **Comparison of enzymatic activity for CDS1 and CDS2.** Mixed-micelle based enzymatic activity of CDS1 and CDS2 using SAPA and SLPA as substrates. *A.* Comparison of enzymatic activity of CDS1 and CDS2 versus mock transfected cells (EV-myc). *B.* Specific activity of CDS1 and CDS2. Enzymatic activity is adjusted using EV-myc and normalized using calnexin. The bars with an * were determined to be statistically different ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.

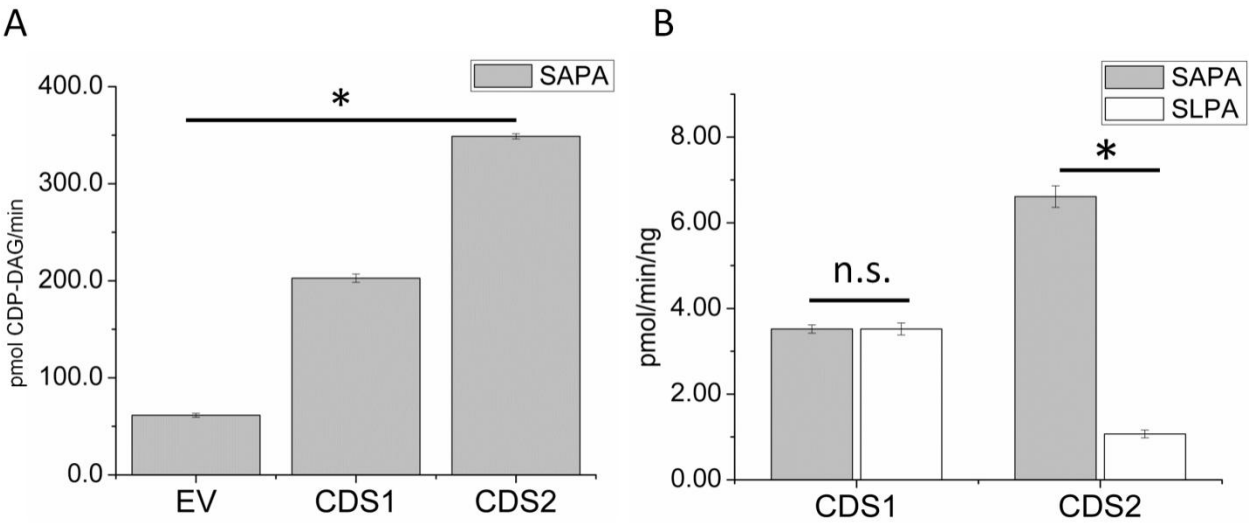


Figure 3: **Kinetic analysis of CDS1 and CDS2.** A mixed-micelle assay was used to test enzymatic activity over a series of substrate concentrations. Substrate concentrations are represented as mol % SAPA. Enzymatic activity is adjusted using EV-myc and normalized using calnexin. For K_m, V_{max} and K_{cat} values, refer to Table 2. Data is represented as means ± SEM, *n* = 9, *N* = 3.

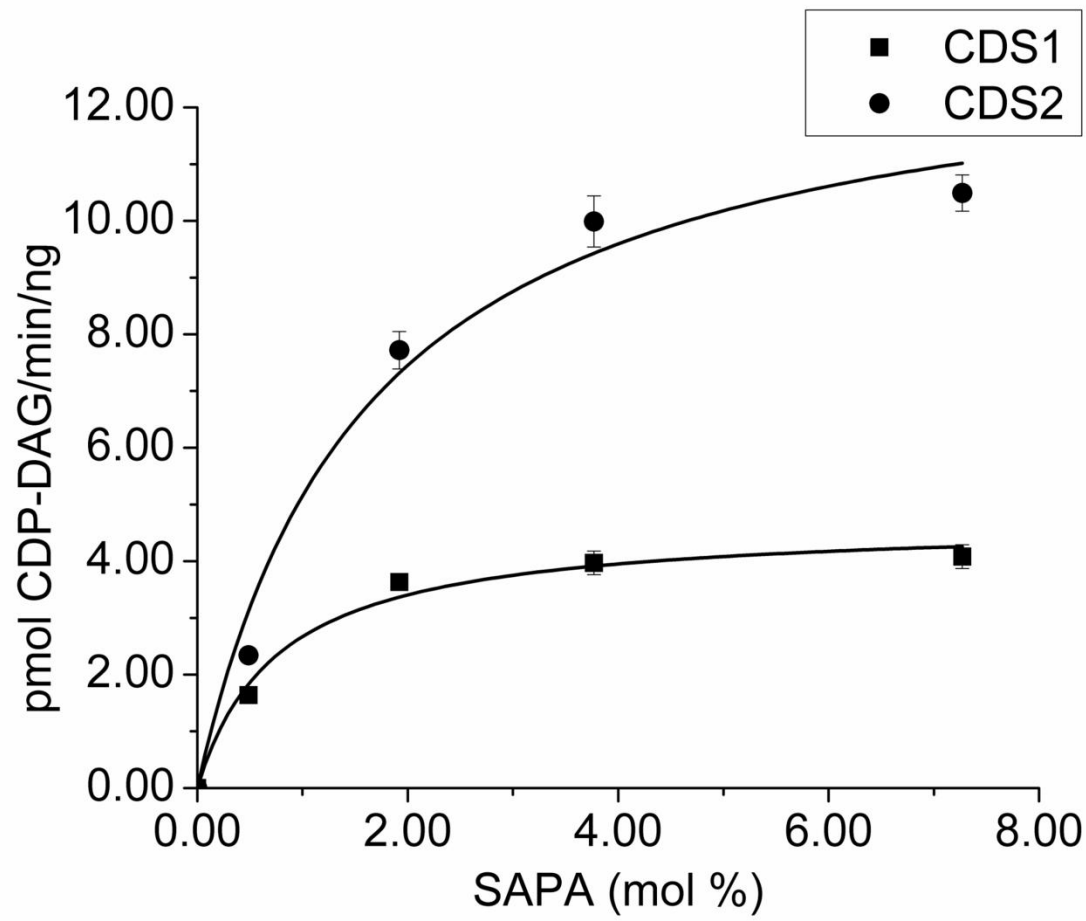


Figure 4: **Substrate specificity of CDS1 and CDS2**. A mixed-micelle assay was used to test enzymatic activity for a variety of substrates. PAs were quantified prior to use and 50μM of substrates were used for each assay. The substrate specificity of CDS1 was measured by varying the *A. sn1 B. sn2* and *C. sn1/sn2* acyl chains. The substrate specificity of CDS2 was measured by varying the *D. sn1 E. sn2* and *F. sn1/sn2*. The bars with an * were determined to be statistically different ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.

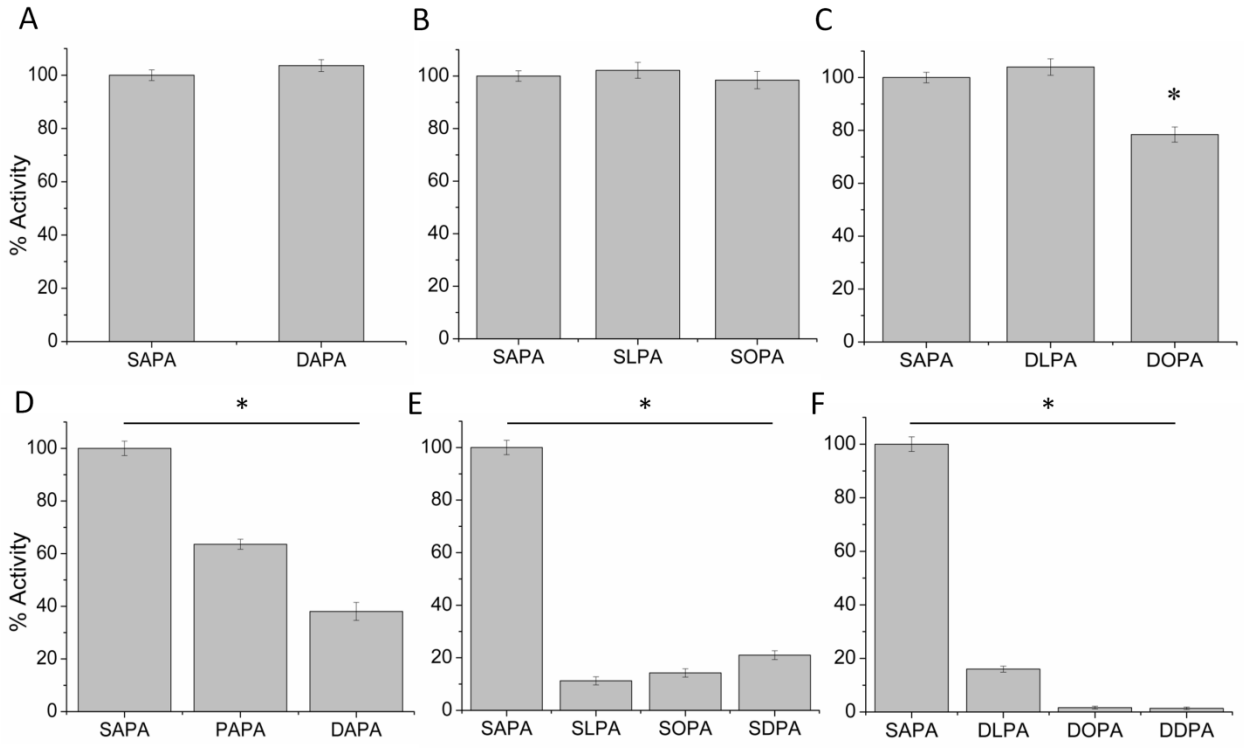


Figure 5: Inhibition of CDS1 and CDS2 by natural PI species. A mixed-micelle assay was used to the inhibition of CDS1 and CDS2 with natural PI species. An equal concentration of PI species as substrate (SAPA). All species were quantified prior to use. *A.* CDS1 *B.* CDS2. The bars with an * were determined to be statistically different ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.

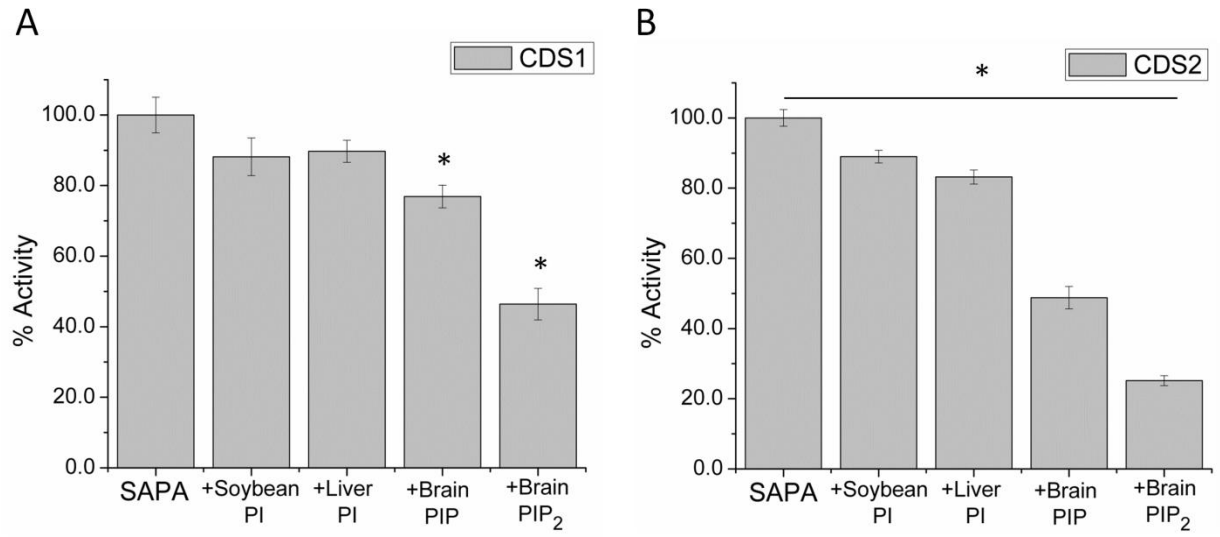
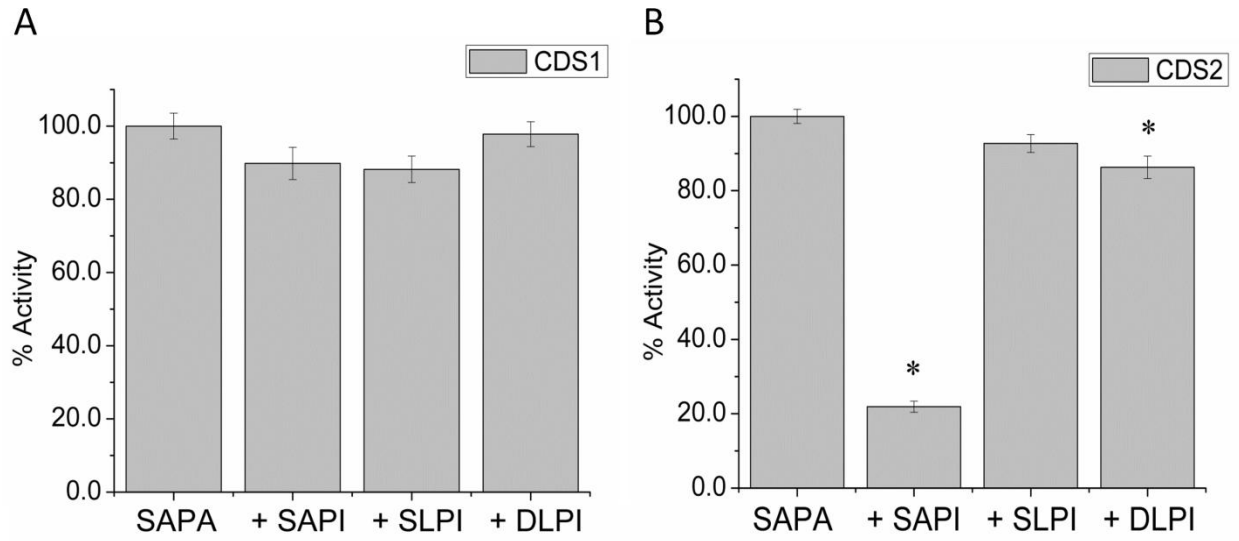
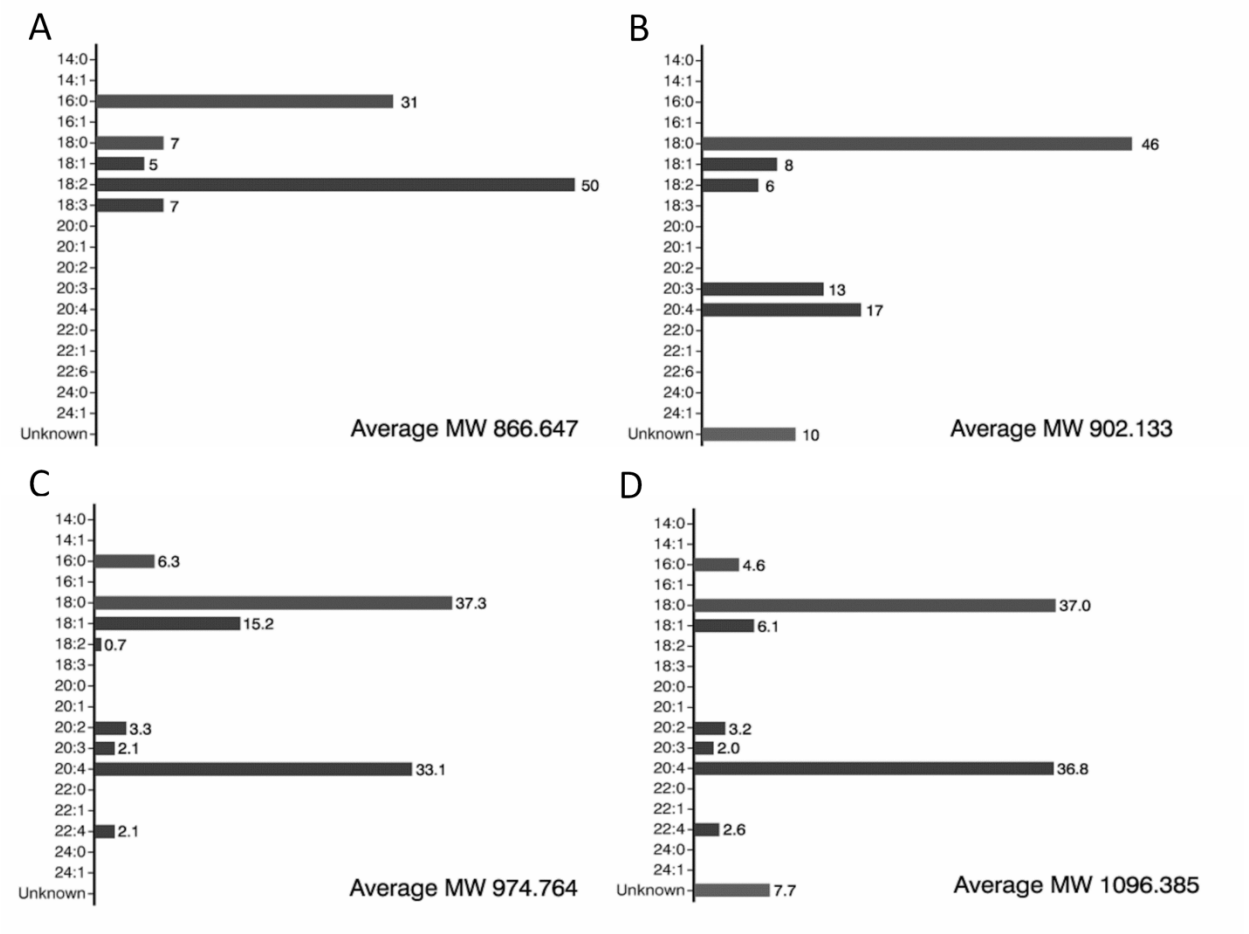


Figure 6: Inhibition of CDS1 and CDS2 by PI species. A mixed-micelle assay was used to the inhibition of CDS1 and CDS2 with PI species. An equal concentration of PI species as substrate (SAPA). All species were quantified prior to use. *A.* CDS1 *B.* CDS2. The bars with an * were determined to be statistically different ($P < 0.05$). Data is represented as means ± SEM, $n = 9$, $N = 3$.



Supplementary Figure 1: The acyl chain profile of natural PI species. The acyl chain profiles of the various PI species. *A.* soybean PI *B.* liver PI *C.* brain PIP *D.* brain PIP_2. Adapted from Avanti Polar Lipids <http://avantilipids.com/index.php?option=com_content&view=article&id=128&Itemid=136>



CHAPTER 5: Conclusions and Future Directions

Phosphatidylinositol is a critical phospholipid implicated in several cellular processes, including signal transduction and trafficking. Deregulation of this phospholipid and its derivatives have been implicated in a host of different physiological conditions[1]. PI is highly enriched in acyl chains at both the *sn-1* and *sn-2* positions, the major species being *sn-1*-stearoyl-*sn-2*-arachidonoyl[2-4]. The acyl chain composition is critical to several of the cellular and physiological functions of PI. For example, as explored in the introduction, the *sn-2* arachidonoyl acyl chain is a precursor to a group of pro- and anti-inflammatory class of lipids called eisocanoids[5-6]. As such, understanding how PI is enriched with specific acyl chain has been a focus of this thesis. Our aim was to characterize two enzymes believed to contributed to the enrichment of PI with stearoyl and arachidonoyl acyl chains; DGKε and CDS1 and 2.

DGKε

Several conclusions can be drawn on our work on DGKε. First, we have determined that several additional motifs play a role in the enzymatic activity and substrate specificity of DGKε. We characterized a proposed ATP-binding site, defined by the consensus sequence *GGDG*, that is conserved among all DGKs and from vertebrate to invertebrates within DGKε (Chapter 2). Not surprisingly, mutations in this phosphate binding site (P-motif), specifically G278D and D280A, destroys its enzymatic activity. DGKε's substrate specificity is also affected by mutating the ATP-binding site. Whereas the wild type enzyme shows substrate specificity for SAG, our ATP-binding mutants do not show significant preference for SAG over other substrates. The inhibition of DGKε by its product is also acyl chain dependant. However, our P-motif mutants also lose this acyl chain dependant product inhibition. These results are a confirmation that DGKε's

enzymatic activity and substrate specificity are closely linked. Mutations in the P-motif are also likely to affect the positioning of preferred substrates within the catalytic site.

We believe that although DGKε primarily supplies arachidonoyl-containing PA substrate to the PI cycle, not all PA is used just to regenerate PI. Several additional roles of PA are discussed, one of which is as a fusogenic lipid in transport[7]. We provided some preliminary evidence that the PA generated from DGKε may also affect its own transport. A loss of PA, such as with our P-motif mutants, could restrict DGKε to the ER (Chapter 2).

There has been some inconsistency in the literature as to what the ATP-binding site of DGK is. Interestingly, there are two different ATP-binding motifs in DGK's; the *GGDG* motif and the *GxGxxG* motif. We believe that the *GGDG* motif is likely the ATP-binding site of DGK's as this motif is universally conserved, unlike the *GxGxxG* motif. Additionally, similar mutations in the *GGDG* motif have a larger impact on enzymatic activity that the *GxGxxG* motif (Chapter 2).

Interestingly, mutations in the catalytic domain of DGKε have been shown to present with atypical hemolytic-uremic syndrome (aHUS)[8]. Although these mutations do not occur in the P-motif, it would be interestingly for us to determine how these specific mutations affect the enzymes catalytic activity and substrate specificity.

There are several additional studies that need to be done in order to verify our results and conclusions. For one, we were able to detect changes in the localization of DGKε primarily through fractionation experiments. There are several caveats to using this technique. The cells must be lysed, our results rely on transfected-DGKε samples and finally there could be spillover when collecting fractions. One additional experiment to do would be to supplement these studies using fluorescence microscopy. We have attempted some microscropy with limited success (data

not shown). We utilized the smaller fusion protein, miniSOG[9], but were only able to see weak fluoresence with a DGKε-miniSOG construct. Adding a linker also had no effect (data not shown). Future experiments would involve optimizing and troubleshooting a DGKε fluorescent construct.

It is important to determine if DGKε's localization changes in response to PI-mediated signalling pathways. Some considerations would be to determine the localization patterns of DGKε in the presence/absence of stimulating conditions. Furthermore, how DGKε's moves between membranes in unknown. DGKε is membrane bound and cannot easily travel from the ER to the PM and vice versa[10]. Our experiments point to a potential PA-dependant mechanism though several other mechanisms exist. For example, a recent study identified the presence of highly dynamic PI-synthesizing platforms that supply PI to several membranes[11]. Conceivably, DGKε could travel within these organelles. Additionally, DGKε could be found at membrane contact site and could travel via lipid tethers[12].

As a minor study, we also looked at the CRAC motif in DGKε. Like our work on the ATP-binding motif, mutations in the CRAC motif affected enzymatic activity and substrate specificity. While most mutations that we made resulted in a loss of enzymatic activity, surprisingly, we saw two mutants that increased the activity (Chapter 3). Both Y451F and R457Q significantly increased the enzymatic activity, while the substrate specificity of SAG vs. SLG was unaffected. These mutants also affected the enzyme's substrate specificity, with Y451F showing greater activity for AAG and R457Q showing greater activity for SDG.

Adding cholesterol to our lipid films had no significant effects on the enzymatic activity of DGKε; therefore, whether cholesterol plays a role in determining the enzymatic activity and/or

substrate specificity *in vivo* is unknown. A better assay to determine the effect of cholesterol would be a bilayer-based assay, though we have not had success in transferring DGKε from micelles into bilayers.

CDS1/2

CDP-DAG synthase is a critical enzyme that supplies pre-cursors to several phospholipids. We characterized both known isoforms of CDS (CDS1/2), though a mitochondrial specific CDS enzyme was recently found[13]. We found that CDS1 and CDS2 show different substrate specificities (Chapter 4). CDS1 shows almost no substrate specificity, whereas CDS2 is highly specific for 1-stearoyl-2-arachidonoyl PA. The acyl chain composition most preferred by CDS2 is one most enriched in PI. Both isoforms are inhibited by PI in a charge dependent manner, though the extent of inhibition is greater for CDS2 than CDS1. The greater inhibition is likely due to the acyl chain composition of PI species; CDS2 also shows acyl chain dependent inhibition, whereas CDS1 does not.

We believe that CDS1 and CDS2 may serve to enrich different pools of PI. As mentioned, PI is synthesized via two different routes, each of which generate PI species with different acyl chain compositions. PI synthesized via the *de novo* pathway generate mainly saturated and monounsaturated acyl chains, where PI synthesized via the PI cycle may serve to enrich 1-stearoyl-2-arachidonoyl species[14-15]. Both pathways share the common enzymes CDS and PI synthase (PIS). CDS1 appears to be a non-specific enzyme and can supply CDP-DAG towards the *de novo* synthesis of PI and additionally towards PG and CL synthesis. Conversely, CDS2 shows a strong preference for SAPA and could likely supply CDP-DAG for the enrichment of PI.

Our work primarily relied on *in vitro* mixed micelle assays to characterize CDS1 and CDS2. However, these results need to be supplemented and confirmed with *in vivo* experiments. To determine whether CDS1/2 create different phospholipid pools, we plan to create stable knock-downs of each isoforms using shRNAs. Using lipidomics, we can determine whether there are any changes in the levels and acyl chain compositions of several phospholipid classes.

Additionally, we have been interested in determining motifs responsible for arachidonoyl specificity. Previous work has identified and characterized a U shaped channel, known as the lipoxygenase (LOX) motif that is critical for arachidonoyl specificity[16]. CDS2 has a LOX motif whereas CDS1 does not; CDS2 shows remarkable specificity for arachidonoyl containing substrates, whereas CDS1 shows no substrate specificity (Chapter 4). We have done some preliminary experiments with testing to see whether the LOX motif determines arachidonoyl specificity in CDS2. Conservative mutations do not appear to critically affect the enzymes activity or its substrate specificity (data not shown). However, we have not yet created or tested more drastic mutations. Additionally, while CDS1 does not have a LOX motif, mutation of only one of its residues (K446) will 'create' a LOX motif. We are currently determining whether this CDS1 construct has increased arachidonoyl specificity.

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