

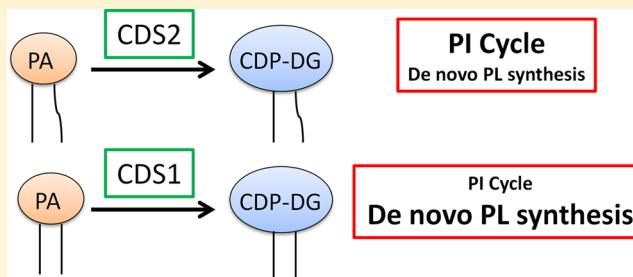
## Distinct Properties of the Two Isoforms of CDP-Diacylglycerol Synthase

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**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 the two isoforms of human CDS, CDS1 and CDS2, show different acyl chain specificities for its lipid substrate. CDS2 is selective for the acyl chains at the *sn*-1 and *sn*-2 positions, the most preferred species being 1-stearoyl-2-arachidonoyl-*sn*-phosphatidic acid. CDS1, conversely, shows no particular substrate specificity, displaying similar activities for almost all substrates tested. Additionally, we show that inhibition of CDS2 by phosphatidylinositol is also acyl chain-dependent, with the strongest inhibition seen with the 1-stearoyl-2-arachidonoyl species. CDS1 shows no acyl chain-dependent inhibition. Both CDS1 and CDS2 are inhibited by their anionic phospholipid end products, with phosphatidylinositol-(4,5)-bisphosphate showing the strongest 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.



CDP-DAG synthases (CDS) are enzymes that catalyze the conversion of phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG).<sup>1</sup> 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.<sup>2–4</sup> In addition, PA is a highly fusogenic lipid involved in the generation of negative curvature in transport complexes, such as vesicles.<sup>5</sup> PA can also be dephosphorylated to diacylglycerol, which is a precursor to phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS).<sup>6,7</sup> Conversely, CDP-DAG is a lipid precursor to several phospholipid classes such as phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL).

Only two CDS isoforms in mammals have been identified and characterized. Both these isoforms are believed to be localized to the endoplasmic reticulum (ER).<sup>8–11</sup> It was believed that CDS1 was present in mitochondria for synthesizing cardiolipin.<sup>12</sup> 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.<sup>13,14</sup> CDS1 and CDS2 are expressed in a variety of tissues. In mice, CDS1 is found in adult brain, eye, smooth muscle, and testis. In the eyes, CDS1 is strongly expressed in the photoreceptor layer of adult retinas, which could suggest a role for CDS1 in phototransduction.<sup>11</sup> CDS2 has a broad expression pattern and was found in virtually every tissue;<sup>10</sup> 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 expressed only in the brain, eye, and testis.<sup>9,10</sup>

The roles of CDS1 and CDS2 have primarily been studied in PI synthesis.<sup>15</sup> Many of the cellular functions attributed to CDS enzymes are believed to result from their role in generating the precursor for phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), a potent signaling molecule. For example, phototransduction signaling in vertebrate and invertebrate systems is believed to proceed, at least partly, via phosphoinositide signaling.<sup>16,17</sup> *cds* mutants in *Drosophila* result in light-induced retinal degradation, and overexpression of a photoreceptor specific CDS increases the amplitude of the light response.<sup>1</sup> Disruption of CDS2 led to a decrease in the level of VEGFA signaling and angiogenesis in zebrafish, primarily through a decreased level of PIP<sub>2</sub> regeneration.<sup>18</sup> A similar but weaker effect was seen when CDS1 was disrupted, whereas knockdown of both isoforms led to embryonic lethality.<sup>18</sup> An arachidonoyl-preferring CDS was also shown to be inhibited by PI species, specifically PIP<sub>2</sub>.<sup>9</sup> This suggests a potential feedback mechanism, in which CDS activity is highest when phosphoinositide [PI(P)<sub>n</sub>] levels are low. Apart from PI signaling, CDS1 was also shown to be involved in the maintenance of phospholipids critical for mitochondrial function and structure.<sup>14</sup>

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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 preferred acyl chain composition is 1-stearoyl-2-arachidonoyl. This is also the acyl chain composition most highly enriched in PI.<sup>19–21</sup> Inhibition of CDS2 is also acyl chain specific, with 1-stearoyl-2-arachidonoyl PI species showing the strongest inhibition. CDS1 in contrast shows virtually no substrate specificity or acyl chain-dependent PI inhibition. Taken together, our results point toward the generation of specific pools of CDP-DAG for phospholipid synthesis by the two different CDS isoforms.

## EXPERIMENTAL PROCEDURES

**Materials.** All lipids were purchased from Avanti Polar Lipids and were stored in a solution containing a 2/1 (v/v) CHCl<sub>3</sub>/CH<sub>3</sub>OH mixture and 0.1% (w/v) butylated hydroxytoluene (BHT). The acyl chain compositions of the preparations of natural lipids purchased from Avanti Polar Lipids are available from the manufacturer. All traces of the solvent phase were evaporated using N<sub>2</sub> gas, and remaining traces of solvent were removed using a vacuum desiccator for 2 h. 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 are summarized in Table 1, together with the abbreviations used.

**Table 1. Lipids Used in This Study**

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-diarchidonoyl-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
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-dilinoleoyl-sn-phosphatidylinositol	18:2/18:2 PI
DLPG	1,2-dilinoleoyl-sn-phosphatidylglycerol	18:2/18:2 PG

The CDS1 and CDS2 DNA constructs used were tagged at their C-termini with a myc epitope tag as described in ref 8. The naturally derived phospholipid species used in this study are listed in Table 2.

**Cell Culture and Transfections.** COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) and 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<sub>2</sub>. For transfections, cells were grown to 90% confluence in four plates per assay and transfected with 8 µg of plasmid DNA per plate using lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. After 48 h, cells were harvested by being scraped into a solution of PBS with a 1/100 (v/v) protease inhibitor cocktail. Cells were spun at 1000g for 5 min, and the pellets were flash-frozen at -80 °C.

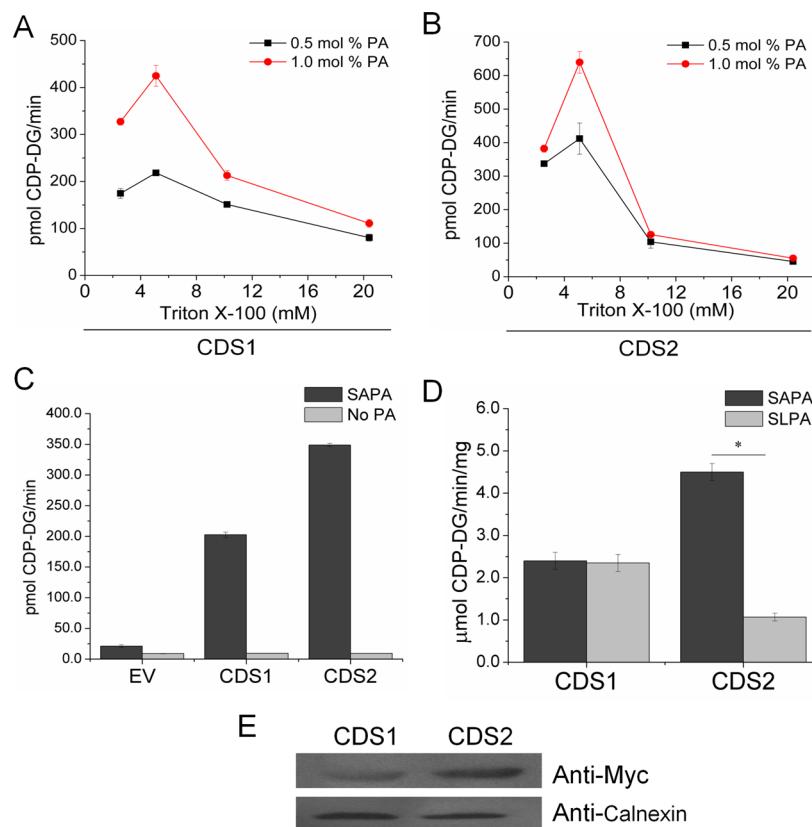
**Table 2. Naturally Derived Phospholipid Species Used in This Study<sup>a</sup>**

abbreviation	full name	predominant acyl chain	ref
soy PI	soy L-α-phosphatidylinositol	18:2 (42%), 16:0 (37%)	35
liver PI	liver L-α-phosphatidylinositol	18:0 (32%), 20:4 (23%)	36
brain PIP	brain L-α-phosphatidylinositol 4-phosphate	18:0 (33%), 20:4 (33%)	37
brain PIP <sub>2</sub>	brain L-α-phosphatidylinositol 4,5-bisphosphate	18:0 (33%), 20:4 (33%)	37
soy PG	soy L-α-phosphatidylglycerol <sup>b</sup>	18:2 (65%)	35
egg PG	egg L-α-phosphatidylglycerol	16:0 (39%), 18:1 (24%)	38

<sup>a</sup>The predominant acyl chain compositions are listed. <sup>b</sup>Soy phosphatidylglycerol prepared by transphosphatidylation of soy phosphatidylcholine. Hence, the acyl chain composition of soy PG reflects that of soy PC.

**Enzyme Preparation for the CDS Activity Assay.** Cell pellets containing transfected constructs were resuspended in an ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.2 mM ethylene glycol tetraacetic 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 passes 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. Resuspended cells were centrifuged at 100000g for 60 min at 4 °C. The microsomal fraction (pellet) was resuspended in lysis buffer and used for the mixed micelle-based enzymatic activity assay. Further attempts to purify the enzyme through binding to affinity resins for the epitope tag resulted in a pure enzyme preparation. However, enzymatic activity was not retained in the mixed micelle assay, regardless of the methods used to obtain an active enzyme.<sup>23</sup>

**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, 10, 25, 75, 100, 150, 200, and 400 µM. For substrate specificity assays, the desired PA species was used at a concentration of 50 µM (~1 mol %). For CDS1 and CDS2 inhibition experiments, SAPA and PI species each at 50 µM 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 guanosine triphosphate (GTP), and 5.10 mM Triton X-100] by vortexing for 2 min. GTP was added because it was found to increase the rate of reaction (see below). We also replaced BSA with the non-lipid binding protein, ovalbumin. This change in protein did not affect CDS activity, indicating that the assay was not perturbed by the lipid binding properties of BSA. Once the films were resuspended, 4 µL of 1 M MgCl<sub>2</sub> 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 20 µL of 0.2 mM CTP (cytidine 5'-[S'-<sup>3</sup>H]triphosphate, 2.5 µCi/sample) and the mixture incubated at room temperature for 5 min. The reaction was terminated by adding 500 µL of stop solution (0.1 N HCl in methanol). The organic phase was washed with 300 µL of 0.6 N HCl and 1 mL of chloroform. The solution was centrifuged at 2000 rpm for 2 min, and the organic phase was transferred to a new tube. The organic phase



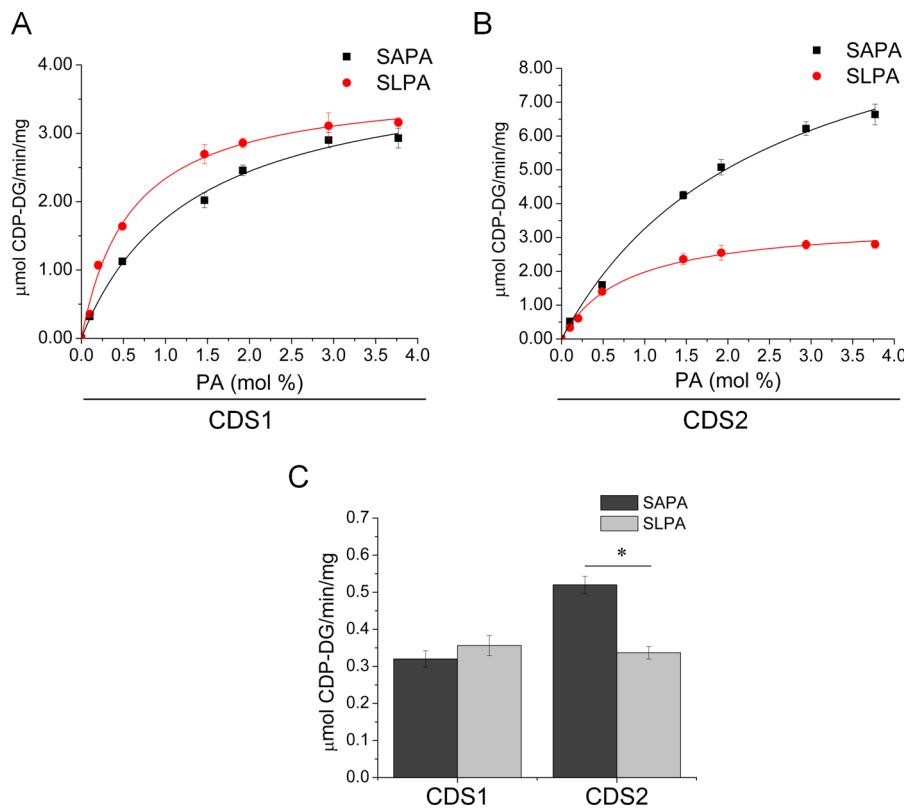
**Figure 1.** Comparison of enzymatic activity for CDS1 and CDS2. Mixed micelle-based enzymatic activity of CDS1 and CDS2 using SAPA and SLPA as substrates. (A) CDS1 and (B) CDS2 both exhibit surface dilution kinetics, with maximal activities seen at 5.1 mM Triton X-100. (C) Crude activity of COS7 lysates containing EV-, CDS1-, or CDS2-transfected cells with and without PA substrates. (D) Specific activity of CDS1 and CDS2. Enzymatic activity is adjusted using EV-myc and normalized using calnexin. The bars with asterisks were determined to be statistically different ( $P < 0.05$ ). Data are represented as the mean  $\pm$  SEM. (E) Immunoblots of lysates of COS7 cells overexpressing either CDS1 or CDS2 constructs carrying a myc tag. Detection of calnexin used as a control.

was washed with 1 mL of an ice-cold “upper phase” solution obtained from a mixture of 0.1 N HCl in a methanol/aqueous 0.6 N HCl/chloroform mixture [10/6/20 (v/v/v)]; 500  $\mu$ L 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 (PerkinElmer). The data from these assays are presented as the mean  $\pm$  the standard deviation (SD). 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 5–15% of the activity, depending on the experiment, that was found with CDS-transfected constructs.

**Kinetic Analysis of CDS Activity.** To determine  $V_{max}$  and  $K_m$  parameters, activity assays were performed using various concentrations of 1-stearoyl-2-arachidonoyl-*sn*-phosphatidic acid (SAPA) and 1-stearoyl-2-linoleoyl-*sn*-phosphatidic acid (SLPA).  $V_{max}$  and  $K_m$  parameters were determined using nonlinear regression analysis by plotting initial enzyme velocity ( $v_o$ ) versus substrate concentration ([S]). Previous studies have shown that the addition of GTP lowered the apparent  $K_m$  of PA.<sup>12</sup> We found a small stimulation of both isoforms with 2 mM GTP (data not shown); thus, GTP was added to all reaction mixtures. Data were 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  $\mu$ L 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  $\mu$ L of 0.5 M HCl was added, the mixture refluxed for 15 min, and 750  $\mu$ L 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  $H_2SO_4$  added. The mixture was incubated at 60 °C for 10 min and 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 an equal volume of lysate and Laemmli sample buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue] and kept at 60 °C for 5 min. The samples were run on a 7.5% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel for 30 min at 200 V. The proteins were then transferred onto a polyvinylidene fluoride membrane (Bio-Rad). The antibody used to detect the myc epitope tag on CDS constructs was a mouse anti-myc antibody (Cell Signaling). The antibody used to detect calnexin was the mouse anti-calnexin antibody (BD Biosciences). The secondary antibody used was the horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma). The immunoblot was detected



**Figure 2.** Kinetics of CDS1 and CDS2. Mixed micelle-based enzymatic assays of CDS1 and CDS2 at various mole percents of SAPA and SLPA. Both (A) CDS1 and (B) CDS2 show typical saturation kinetics. (C) Mixed micelle-based enzymatic activity of CDS1 and CDS2 using SAPA and SLPA at a low level of 0.1 mol %. Enzymatic activity is adjusted using mock-transfected COS7 cells (EV control). The kinetic parameters are summarized in Table 3. Data are represented as the mean  $\pm$  SEM.

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 a recombinant myc-tagged control protein (MYC11-C, Alpha Diagnostics International, San Antonio, TX) was run along with the samples.

**CDS Immunostaining.** For immunostaining, cells were plated on 25 mm glass coverslips for 24 h and cotransfected with 0.3  $\mu$ g of CDS1-GFP and CDS2-myc (or CDS2-GFP and CDS1-myc), each for 24 h. Cells were then washed with PBS and fixed with 4% paraformaldehyde and 0.4% glutaraldehyde for 10 min. After being washed with PBS three times, cells were quenched with 1% freshly made sodium borohydride (5 min) and washed thoroughly four times. The first antibody (1/300 of mouse anti-myc or rabbit anti-GFP) incubations were then performed in PBS containing 0.4% BSA and 0.2% saponin (1 h) followed by washing and incubation with Alexa488 or Alexa568 second antibodies (1/1000) for 30 min. After several washes, coverslips were mounted using Fluoromount-G and the cells observed with a Zeiss LSM510 confocal microscope. Note that this procedure eliminates GFP fluorescence, so it was detected by immunostaining and in some cases visualized with a red-colored secondary antibody.

**Statistical Analysis.** Where necessary, statistical analysis was performed using the one-way analysis of variance (ANOVA) tests followed by Tukey's test. Analysis was performed using Origin Pro 8. Experiments were repeated at least three times independently in triplicate. Data are presented as the mean  $\pm$  the standard error of the mean (SEM).

## RESULTS

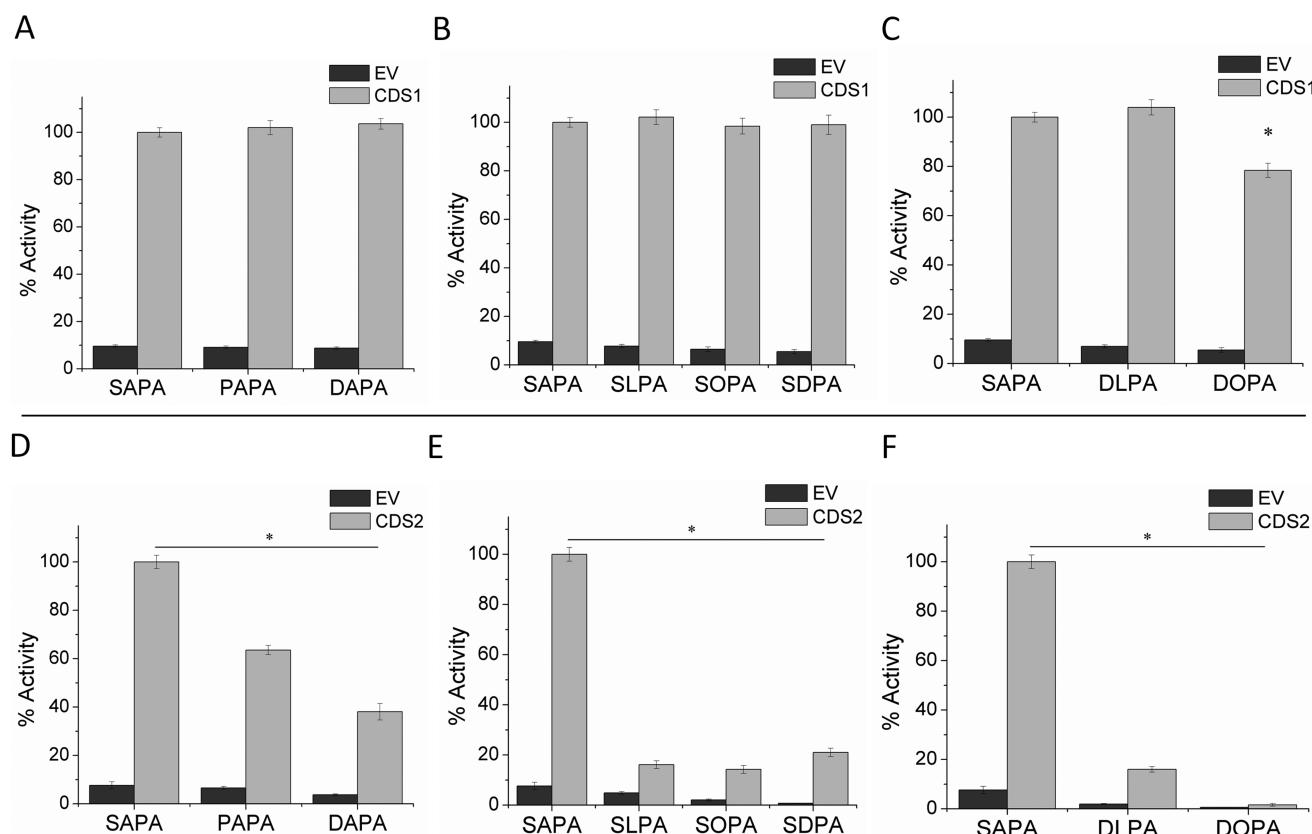
**Validation of the Assay.** Because both the substrate PA and the enzyme, CDS, are membrane-bound, we expect the enzyme to follow surface dilution kinetics.<sup>22,23</sup> We indeed find that both CDS1 and CDS2 follow surface dilution kinetics at higher concentrations of Triton. Maximal activity is found at 5.1 mM Triton X-100 (Figure 1A,B). Both CDS1 and CDS2 are expressed well and show a significant increase in the level of incorporation of  $^3$ H over empty vector (EV)-transfected mock controls (Figure 1C). These results demonstrate that although we were unable to purify the enzyme in its active form, the enzymatic activity we measure is a property of the overexpressed CDS isoform with little contribution from endogenous enzyme activity. Additionally, both isoforms require exogenously added PA to show any significant activities over EV samples (Figure 1C). Furthermore, we also show (see Figure 3F) that even adding a PA that is a poor substrate for CDS2 does not result in  $^3$ H incorporation. The initial rate of formation of product is linear with time over 10 min, and an increase in the rate of CDP-DG formation is seen after 60 min (not shown). For comparison of reactions, we use a standard incubation time of 5 min. Hence, the observed reaction is dependent on excess CDS expression, and in comparison, the endogenous activity is negligible. In addition, there are no other reactions causing the incorporation of cytidine into a lipid-soluble product because the reaction requires the presence of a substrate PA and the product formed is stable over a longer period of time.

The presence of 2 mM GTP resulted in a small stimulation of both CDS1 and CDS2 and was therefore added as a

Table 3. Summary of Kinetic Parameters for CDS1 and CDS2<sup>a</sup>

	lipid species	$V_{max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	$K_m$ (mol %)	$k_{cat}$ ( $\text{s}^{-1}$ )	$k_{cat}/K_m$ ( $\text{s}^{-1}/\text{mol \%}$ )
CDS1	SAPA	$3.3 \pm 0.3$	$0.8 \pm 0.2$	$2.9 \pm 0.3$	$3.6 \pm 1.4$
	SLPA	$3.6 \pm 0.1$	$0.6 \pm 0.1$	$3.2 \pm 0.2$	$5.3 \pm 1.0$
CDS2	SAPA	$9.3 \pm 0.4$	$1.4 \pm 0.2$	$8.0 \pm 0.3$	$5.7 \pm 1.0$
	SLPA	$3.5 \pm 0.1^b$	$0.9 \pm 0.1^b$	$2.9 \pm 0.1^b$	$3.2 \pm 0.5^b$

<sup>a</sup>A mixed micelle assay was used to test enzymatic activity over a series of substrate concentrations. Substrate concentrations are represented as mole percent SAPA. The low enzymatic activity from EV-myc is subtracted, and the activity is then normalized using calnexin. Data are represented as the mean  $\pm$  SEM. The  $k_{cat}/K_m$  of CDS2 for SAPA is significantly greater than that of SLPA, whereas the  $k_{cat}/K_m$  values of CDS1 for SAPA and SLPA are not. <sup>b</sup>Determined to be statistically different from the value for SAPA ( $P < 0.05$ ).



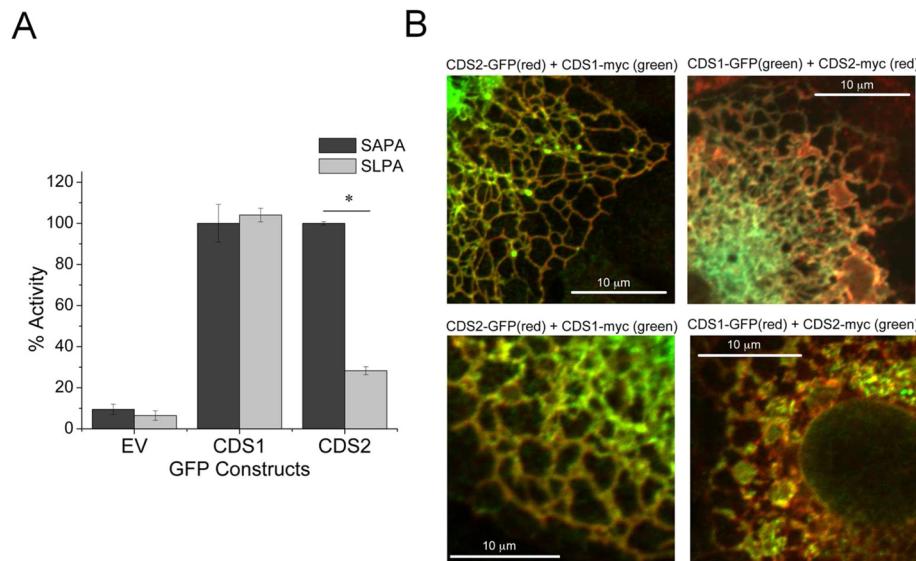
**Figure 3.** Substrate specificity of CDS1 and CDS2. A mixed micelle assay was used to test enzymatic activity using a variety of substrates. PA concentrations were quantified prior to use, and 1 mol % substrates (50  $\mu\text{M}$ ) were used for each assay. The substrate specificity of CDS1 was measured by varying the (A) *sn*-1, (B) *sn*-2, and (C) *sn*-1/*sn*-2 acyl chains. The substrate specificity of CDS2 was measured by varying the (D) *sn*-1, (E) *sn*-2, and (F) *sn*-1/*sn*-2 acyl chains. The bars with asterisks were determined to be statistically different ( $P < 0.05$ ). Data are represented as the mean  $\pm$  SEM. The activities of CDS1 and CDS2 are  $164.4 \pm 4.6$  and  $289.8 \pm 12.8$  pmol of CDP-DG/min, respectively, and are defined as 100% for SAPA.

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

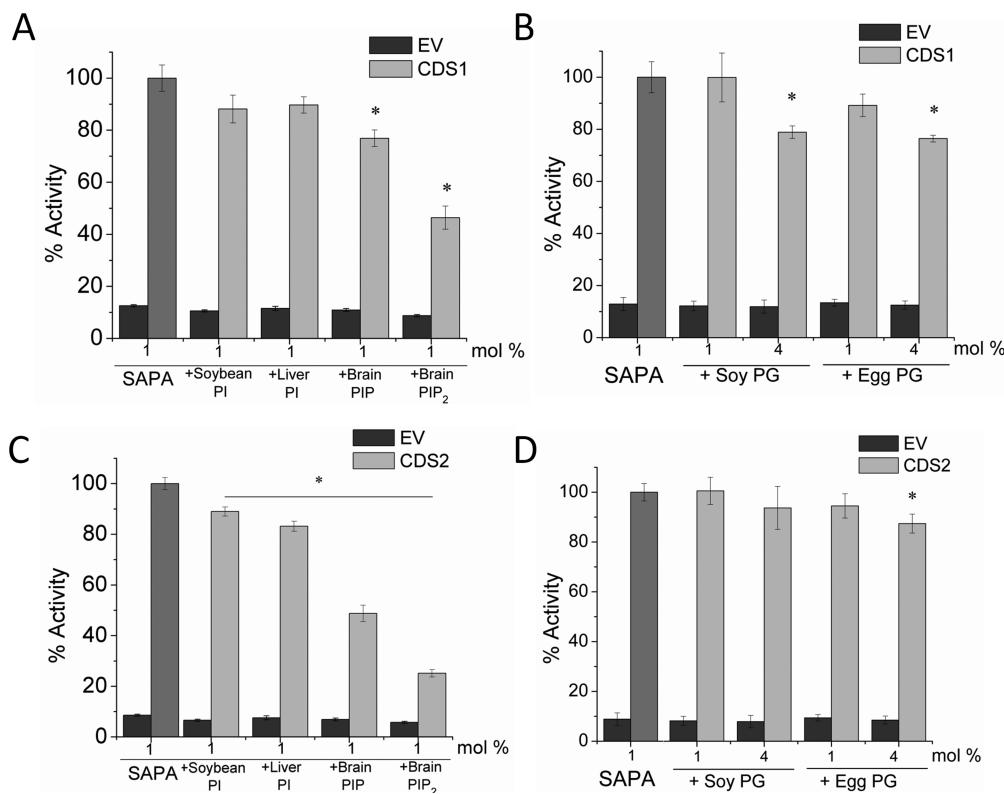
**CDS1 and CDS2 Show Different Substrate Specificities.** We first determined the specific activities and kinetic parameters of CDS1 and CDS2. The specific activities of CDS1 and CDS2 were determined to be  $2.4 \pm 0.2$  and  $4.5 \pm 0.2$   $\mu\text{mol}$  of CDP-DAG  $\text{min}^{-1} \text{mg}^{-1}$ , respectively, for the substrate SAPA at 1 mol % (Figure 1D). The activities were normalized to both the amount of CDS1/CDS2-myc and calnexin levels (Figure 1E). Interestingly, there is a large difference in substrate acyl chain specificity between CDS1 and CDS2. Whereas CDS1

shows no significant preference between substrates SAPA and SLPA, CDS2 shows a roughly ~4-fold increase in its preference for SAPA over SLPA (Figure 1D).

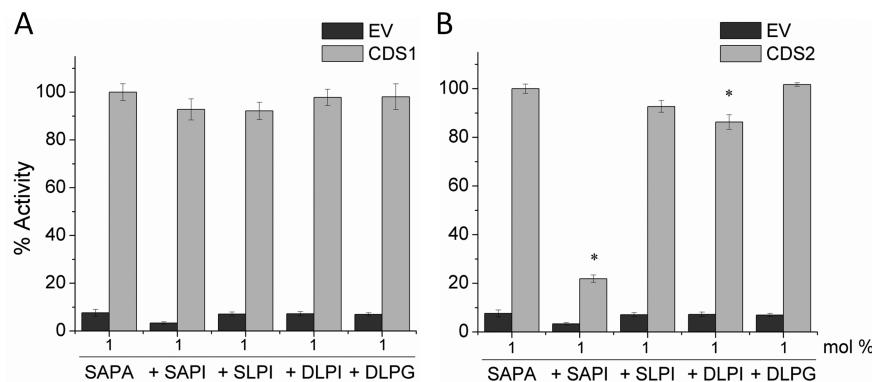
We then determined the kinetic parameters of CDS1 and CDS2 for SAPA and SLPA. CDS1 and CDS2 show typical saturation kinetics (Figure 2A,B), and the kinetic parameters are summarized in Table 3. CDS1 has  $V_{max}$  values of  $3.3 \pm 0.3$  and  $3.6 \pm 0.1$   $\mu\text{mol}$  of CDP-DG  $\text{min}^{-1} \text{mg}^{-1}$  for SAPA and SLPA, respectively. Conversely, CDS2 has  $V_{max}$  values of  $9.3 \pm 0.4$  and  $3.5 \pm 0.1$   $\mu\text{mol}$  of CDP-DG  $\text{min}^{-1} \text{mg}^{-1}$  for SAPA and SLPA, respectively. For comparison, these are very similar values compared to those obtained using a purified, yeast CDS enzyme.<sup>23</sup> CDS1 shows no significant changes in  $k_{cat}/K_m$  for SAPA and SLPA. Conversely, CDS2 has a significantly higher  $k_{cat}/K_m$  for SAPA than for SLPA (Table 3). Because there is an intrinsically high error in  $K_m$ , to further confirm differences in



**Figure 4.** Activity and subcellular localization of labeled CDS1 and CDS2. (A) Mixed micelle activity assay used to determine the substrate specificity of CDS1-GFP and CDS2-GFP. (B) ER localization of CDS1 and CDS2 enzymes. Cells were transfected with the indicated constructs and immunostained as detailed in the Experimental Procedures. Note the color conversion in the top left and bottom two panels where the GFP is detected with a red secondary antibody (the GFP fluorescence is quenched by our fixation procedure that preserves the ER architecture). Both CDS forms are ER-localized with only subtle differences: CDS1 being more enriched in the central ER and CDS2 being slightly more prevalent in peripheral ER sheets. The activities of CDS1 and CDS2 are  $156.9 \pm 15.7$  and  $202.6 \pm 4.2$  pmol of CDP-DG/min, respectively, and are defined as 100% for SAPA.



**Figure 5.** Inhibition of CDS1 and CDS2 by natural phospholipid species. A mixed micelle assay was used to determine the inhibition of CDS1 and CDS2 with naturally derived PI and PG species. An equal mole percent of phospholipids was used as the substrate PA, unless otherwise noted. The concentration of all lipid species was quantified prior to use. Black bars represent the presence of the substrate, SAPA, only. Other bars represent the same concentration of SAPA together with a second lipid of equimolar or 4-fold higher concentration, as indicated on the graph: (A) PI inhibition of CDS1, (B) PG inhibition of CDS1, (C) PI inhibition of CDS2, and (D) PG inhibition of CDS2. The bars with asterisks were determined to be statistically different ( $P < 0.05$ ). Data are represented as the mean  $\pm$  SEM. The activities of CDS1 and CDS2 are  $176.9 \pm 22.6$  and  $231.6 \pm 6.7$  pmol of CDP-DG/min, respectively, and are defined as 100% for SAPA.



**Figure 6.** Inhibition of CDS1 and CDS2 by PI and PG species. A mixed micelle assay was used to determine the inhibition of CDS1 and CDS2 with select PI and PG species. The concentrations of all species were quantified prior to use. The activity as compared to EV controls: (A) CDS1 and (B) CDS2. The bars with asterisks were determined to be statistically different ( $P < 0.05$ ). Data are represented as the mean  $\pm$  SEM. The activities of CDS1 and CDS2 are  $88.9 \pm 3.7$  and  $160.4 \pm 10.8$  pmol of CDP-DG/min, respectively, and are defined as 100% for SAPA.

substrate specificities, we determined the activities of both enzymes at 0.1 mol % SAPA and SLPA. For enzymes that follow Michaelis–Menten kinetics, at  $[S] \ll K_m$ , the rate should follow first-order kinetics with activities being proportional to  $k_{cat}/K_m$ . As shown in Figure 2C, whereas CDS1 shows no significant differences in substrate specificity, CDS2 shows significantly higher activities for SAPA.

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 3). CDS1 appears to have no substrate preference for SAPA, with variations of the *sn*-1 and *sn*-2 acyl chains resulting in no significant changes in preference compared with that for SAPA. CDS1 showed decreased specificity only toward DOPA over the other substrates tested. However, CDS1 showed similar activity toward another PA with variations at the *sn*-1 and *sn*-2 acyl chains (SAPA vs DLPA) (Figure 3C).

Conversely, CDS2 showed 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 a >50% loss of activity (Figure 3D). Even similar substitutions at the *sn*-1 position [stearoyl (18:0) vs palmitoyl (16:0)] resulted in a significant loss of activity (Figure 3D). PA with differing acyl chains at the *sn*-2 position or both *sn*-1 and *sn*-2 positions showed an even greater loss of activity than at the *sn*-1 position alone. Any substitution at the *sn*-2 arachidonoyl position resulted in activities that were approximately 10–20% of the activity against SAPA (Figure 3E). DLPA, which varies at both acyl chain positions, was the only PA in this group with activity (Figure 3F). DOPA showed virtually no activity with CDS2 (Figure 3F). In contrast, DOPA had roughly 75–80% of the activity of SAPA for CDS1, which shows the striking difference in substrate specificities between these two isoforms.

**Subcellular Localization of CDS1 and CDS2.** Because CDS1 and CDS2 show substantially different substrate specificities, we decided to see whether there are differences in the localizations of the two isoforms. First, we tested whether the GFP tag affects substrate specificity. Both CDS1-GFP and CDS2-GFP show substrate specificities similar to those of their myc-tagged counterparts. CDS1-GFP shows similar activities for both SAPA and SLPA, whereas CDS2-GFP shows a marked preference for SAPA (Figure 4A). Previous studies showed that GFP-tagged CDS1 and CDS2 showed a typical ER tubular localization when they were expressed in COS7 cells.<sup>8</sup> To make

a more thorough comparison between the localization of the two proteins, we co-expressed the GFP-tagged and myc-tagged forms of the two enzymes and studied their colocalization after fixation and immunostaining. We used cells expressing the smallest detectable amounts of the proteins to best approximate the endogenous distribution. We also conducted these experiments in two ways, using CDS1-GFP together with CDS2-myc and CDS2-GFP with CDS1-myc to rule out the potential effect of GFP on the localization. These studies confirmed that both CDS forms localized to the ER. There were only subtle but notable differences between the distributions of the two proteins. These included a more pronounced enrichment of the CDS1 enzyme in the pericentriolar central ER and the enrichment of the CDS2 enzyme in ER sheets relative to the tubular ER (Figure 4B). However, these differences were only relative, as both enzymes could be found in all ER areas. Because of the lack of antibodies suitable for the detection of the endogenous proteins, these are the best data available as yet for the relative localizations of these proteins.

**CDS1 and CDS2 Show Inhibition by Natural Phospholipid Species.** We next decided to test whether CDS1 and CDS2 show inhibition by physiologically relevant PI species (Figure 5). An equimolar amount of these lipids (1 mol %) was chosen as PA. Both CDS1 and CDS2 show inhibition by PI species, with PIP<sub>2</sub> showing the strongest inhibition. CDS1 shows no significant inhibition in activity when either soybean or liver PI is used at equimolar concentrations with respect to SAPA (Figure 5A). In the endoplasmic reticulum of resting macrophages, there is roughly 5 times more PI than PA.<sup>24</sup> CDS1 is significantly inhibited by PIP (25% inhibition) and even more by PIP<sub>2</sub> (40% inhibition) (Figure 5A). CDS2 also shows inhibition by PI species, but to an extent greater than that of CDS1 (Figure 5C). There is a significant inhibition of CDS2 by both soybean and liver PI (15–20% activity). Addition of 1 mol % PIP and PIP<sub>2</sub> to the reaction mixture resulted in 55 and 80% inhibition of CDS2, as compared to the case without PI species (Figure 5C).

Like PI(P)<sub>n</sub>, PG is also a phospholipid end product of CDS; therefore, we decided to examine the effect of PG on both CDS isoforms. Neither soy nor egg PG had any significant effect on either CDS1 (Figure 5B) or CDS2 (Figure 5D) when an equal mole percent of these species to PA was used. However, when a higher concentration of PG was added, significant inhibition of both CDS isoforms was observed. The PG/PA ratio in the

endoplasmic reticulum of macrophages is ~30.<sup>24</sup> Therefore, inhibition by PG would be physiologically significant.

**Inhibition of CDS2 Is Acyl Chain-Dependent, but That of CDS1 Is Not.** 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 because of the limited variability of the acyl chain composition of commercially available synthetic, more inhibitory PIP and PIP<sub>2</sub> species. CDS1 shows no acyl chain-dependent inhibition for SAPI, SLPI, or DLPI (Figure 6A). The lack of acyl chain-dependent inhibition is similar to its substrate specificity (Figure 3A–C). CDS2, meanwhile, does show acyl chain-dependent inhibition, with SAPI showing the strongest 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-dependent inhibition of CDS2 is also reflective of the enzyme's substrate specificity (Figure 3D–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.<sup>19,20</sup> 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 chains.<sup>25,26</sup> 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 acyl chain preference for PA, showing no discrimination for the *sn*-1/*sn*-2 acyl chain composition of PAs. CDS1 shows a weaker preference for only DOPA; however, this PA species is not physiologically abundant. Conversely, CDS2 shows substrate specificity at both the *sn*-1 and *sn*-2 positions. The most preferred substrate is SAPA, a form of PA having the acyl composition most enriched in PI species. Variations at the *sn*-1 position, even to similar acyl chains (e.g., stearoyl vs palmitoyl), resulted in a roughly 40% loss of activity (Figure 3D). CDS2 showed even less preference for substrates with an *sn*-1 stearoyl acyl chain but a different acyl chain at the *sn*-2 position (Figure 3E). 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, DGKE.<sup>27–29</sup> Like DGKE, the arachidonoyl acyl chain at the *sn*-2 position is critical for the enzyme's substrate specificity.<sup>28</sup> Changes at the *sn*-1 position also play a role in the enzyme's preference for its substrate.<sup>29</sup> However, CDS2 appears to be even more selective for its substrates than DGKE. For example, CDS2 shows 60 and 30% activity for PAPA and DAPA, respectively, whereas DGKE shows roughly 90 and 70% activity for 1-palmitoyl-2-arachidonoyl glycerol and diarachidonoyl glycerol, respectively.

While CDS1 and CDS2 show inhibition by PI species, the extent of inhibition and acyl chain dependence differ between these two isoforms. Inhibition of both isoforms is seen by its lipid end product, with PIP<sub>2</sub> species showing the strongest inhibition. However, CDS2 shows different extents of inhibition by different PI species. This suggests that the acyl chain composition of the PI species plays a role in the 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 strongest inhibition for SAPI.

If either CDS1 or CDS2 can be used for PI synthesis, how could they contribute to 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.<sup>30</sup> The *de novo* synthesis of PI involves only the ER and generates mainly saturated and monounsaturated acyl chains.<sup>20,31,32</sup> The PI cycle is a cyclical pathway that involves the breakdown and regeneration of PIP<sub>2</sub>.<sup>33</sup> The PI cycle involves both the ER and PM and results in the enrichment of 1-stearoyl-2-arachidonoyl species.<sup>28,34</sup> Both pathways involve common features, one of which is the conversion of PA species to CDP-DAG by CDS enzymes. It appears likely that CDS2 would be involved in the PI cycle.

The acyl chain selectivity of CDS2 is similar to that of DGKE, 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 an arachidonoyl chain. CDP-DAG produced by CDS2 can be used only for the synthesis of phospholipids. Conversely, PA synthesized by DGKE can be used for signal transduction pathways and, structurally, for phospholipid synthesis and can be dephosphorylated back to diacylglycerol by phosphatidic acid phosphatase.<sup>6</sup> In contrast, the step in the PI cycle catalyzed by CDS is essentially irreversible because one of the products is pyrophosphate that is rapidly hydrolyzed by pyrophosphatase, preventing the reversal of this step. Both DGKE and CDS2 are needed for the first steps of PIP<sub>2</sub> synthesis, and 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 with an arachidonoyl chain. Both DGKE and CDS2 are strongly expressed in certain organs, such as brain, and so could contribute to particularly high levels of 1-stearoyl-2-arachidonoyl species in these organs.

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## ■ ABBREVIATIONS

BHT, butylated hydroxytoluene; CDP-DAG, cytidine diphosphate-diacylglycerol; CDS, CDP-DAG synthase; CL, cardiolipin; CTP, cytidine triphosphate; DGKE, diacylglycerol kinase  $\epsilon$ ; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; EV, empty vector, mock transfection; FBS, fetal bovine serum; GFP, green fluorescent protein; GTP, guanosine triphosphate; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, L- $\alpha$ -phosphatidylinositol 4-phosphate; PI(P)<sub>n</sub>, phosphoinositides; PIP<sub>2</sub>, L- $\alpha$ -phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; PS, phosphatidylserine; SAPA, 1-stearoyl-2-arachidonoyl-sn-phosphatidic acid; WT, wild type. Refer to Table 1 for a full list of lipid abbreviations.

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