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The Phosphatidylinositol Synthase-Catalyzed Formation of Phosphatidylinositol Does Not Exhibit Acyl Chain Specificity

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Supporting Information

ABSTRACT: Phosphatidylinositol (PI) and its phosphorylated forms are required for many critical cellular processes. PI is highly enriched at its *sn*-1 and *sn*-2 positions, the major species being 1-stearoyl-2-arachidonoyl PI (18:0/20:4 PI). This enrichment is achieved in part through enzymatic acyl chain specificity in PI synthesis. Here we characterize the acyl chain specificity of phosphatidylinositol synthase (PIS), which is involved in the terminal step of PI synthesis. Unlike several other enzymes involved in PI synthesis, PIS appears to exhibit no acyl chain specificity toward its substrates. Thus, enrichment of newly formed PI occurs prior to the terminal synthesis step.

Phosphatidylinositol (PI) is a minor phospholipid, comprising only 2−10% of phospholipids in eukaryotic membranes.[1] The inositol ring can be phosphorylated at several positions, yielding seven unique phosphoinositide species [PI/$P_{(n)}$]. PI/$P_{(n)}$ have been implicated in fundamental cellular processes, including regulating ion channels, actin−cytoskeletal dynamics, vesicular trafficking, and the generation of lipid secondary messengers.[2] PI/$P_{(n)}$ are tightly regulated both spatially and temporally through the combined actions of phosphatases and kinases. Deregulation of this process can have severe physiological consequences leading to cancer, inflammation, metabolic diseases, and neurodegenerative disorders.[2]

Interaction of PI/$P_{(n)}$ with its downstream targets is primarily mediated through electrostatic and hydrophobic interactions with the headgroup. However, the acyl chain composition also appears to have an important role in cellular processes. For example, phospholipids can serve as sinks of bioactive lipid precursors, such as eicosanoids.[3,4] PI, in particular, is highly enriched with specific acyl chains, the primary species being 1-stearoyl-2-arachidonoyl PI (18:0/20:4 PI). In some organs, such as brain, up to 70% of PI species have this acyl chain composition.[5,6] This enrichment can occur via two different pathways. The first involves acyl chain remodeling (Lands cycle).[7] Lysophosphatidylinositol-acyltransferase 1 (LPIAT1) and lysophosphatidic acid acyltransferase 3 (LPAAT3) have been shown to preferentially transfer the acyl moiety of arachidonoyl-CoA to lysoPI and lysoPA species,[8,9] respectively. The second method involves enzymes involved in PI synthesis.[10] Diacylglycerol kinase ε (DGKε) is well-known to show *sn*-2 arachidonoyl specificity.[11] Additionally, it has recently been shown that CDP-diacylglycerol synthase 2 (CDS2) shows remarkable specificity for *sn*-2 arachidonoyl PA.[12] In both enzymes, there is also some specificity for substrates with an *sn*-1 stearoyl chain. Interestingly, none of the other nine DGK isoforms or the other CDS isoform (CDS1) shows any acyl chain specificity. Additionally, DGKε and CDS2 catalyze two sequential steps in the phosphatidylinositol cycle. The next step in this cycle is catalyzed by PIS.

Phosphatidylinositol synthase (PIS) is involved in the final step of PI synthesis, converting CDP-DAG and *myo*-inositol into PI. PIS also catalyzes a phosphatidylinositol−inositol exchange reaction.[13] PIS is ubiquitously found in all organs, with slightly higher levels in the liver and skeletal muscle.[13] Consistent with a housekeeping function, disruption of PIS in *Saccharomyces cerevisiae* produces nonviable spores.[14] In mammalian cells, enzymatically active PIS is found in the ER and in highly mobile ER-derived membrane compartments called PIPEROsomes.[15] It is not known if PIS exhibits acyl chain specificity for its substrate CDP-DAG. To determine the acyl chain specificity of PIS, we utilized an *in vitro* mixed micelle activity assay using lysates of COS7 cells overexpressing HA-PIS.[16] The rate of formation of PI was determined by the incorporation of [^{3}H]inositol into the lipid fraction. The lipid substrates used in this study are listed in Table 1. However, a major impediment to this method is the lack of commercially available forms of CDP-DAG substrates containing different acyl chains. Thus, we could not directly measure the specificity of PIS using different species of CDP-DAG. Instead, we coupled a reaction of CDS and PIS. CDP-DAG was produced *in situ* starting with different species of PA, which are readily available commercially. Coupling the reaction catalyzed by PIS with that of endogenous CDS resulted in PIS coupling with both CDS1 and CDS2; both isoforms are found in COS7 cells

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
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
DLPA	1,2-dilinoleoyl-*sn*-phosphatidic acid	18:2/18:2 PA
DOPA	1,2-dioleoyl-*sn*-phosphatidic acid	18:1/18:1 PA
CDP-DOG	1,2-dioleoyl-*sn*-cytidine diphosphate	18:1/18:1 CDP-DAG

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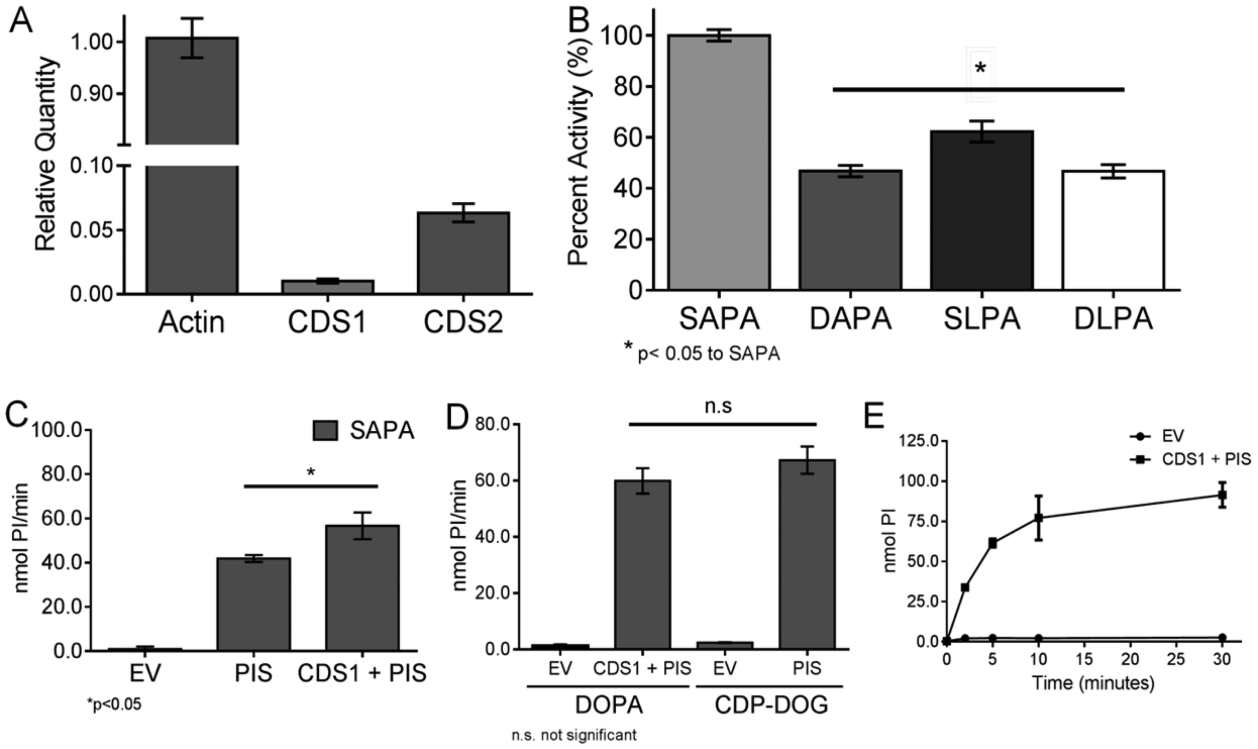

Figure 1. Validation of experimental procedures. (A) qPCR of CDS1 and CDS2, showing that both isoforms are expressed in COS7 cells. Expression has been normalized to actin, which is set to 1. (B) Crude activity of untransfected COS7 lysates with various PA substrates. The activity is 20 ± 2 pmol/min and is defined as 100%. (C) Enzymatic activity of COS7 lysates overexpressing PIS or CDS1 + PIS with SAPA used as a substrate. (D) Comparison of enzymatic activity of CDS1 + PIS and PIS with DOPA and CDP-DOG used as substrates, respectively. (E) Formation of [^{3}H]PI over 30 min in CDS1 + PIS- vs EV-transfected COS7 cells. The bars with an asterisk were determined to be statistically different ($p < 0.05$). Data are represented as means ± the standard error of the mean.

(Figure 1A). This resulted in a slight enrichment of PI containing arachidonoyl chains because of the presence of CDS2 (Figure 1B). However, as CDS1 appears to have no substrate specificity,[12] it can be used to provide similar levels of different CDP-DAGs for PIS. Both CDS1 and PIS express well, and lysates from cells transfected with these two enzymes show a significant increase in the level of incorporation of [^{3}H]inositol over cells transfected with empty vector (EV) or with only PIS (Figure 1C). The use of exogenous PA is required for CDS1 and PIS to show any significant increases in activity over EV controls (Figure 1A,B of the Supporting Information). Additionally, adding the CTP and *myo*-inositol together rather than individually resulted in higher activities in CDS1 + PIS reactions (Figure 1C of the Supporting Information). This indicates that the greatest amount of PI is formed when inositol is continually present in the reaction mixture. Importantly, similar levels of [^{3}H]PI are seen with the use of DOPA + CDS1/PIS versus CDP-DOG + PIS (Figure 1D). Production of PI remained linear for up to 5 min, and the amount of PI formed did not decrease over 30 min, indicating a stable product is formed. For our activity assays, a 5 min incubation was used (Figure 1E). We next tested whether PIS shows acyl chain specificity to different CDP-DAGs. We used four different precursor PAs (Table 1) with different acyl chain compositions. Concurrently run samples showed that CDS1 produces similar levels of CDP-DAG, independent of the starting PA (Figure 2A). (For detailed experimental procedures, see the Supporting Information.) We also confirmed that the production of CDP-DG by CDS1 was not affected by the assay conditions optimized for PIS assays (data not shown). Like CDS1, however, PIS shows no specificity toward different CDP-DAGs (Figure 2B), which contrasts the substrate specificity seen with DGKε and CDS2. The results suggest that acyl chain enrichment of *de novo*-synthesized PI occurs during the initial steps of PI synthesis, i.e., during steps catalyzed by DGKε and CDS2. CDS2, in particular, may serve as a critical enzyme in acyl chain enrichment; the CDS-catalyzed reaction is likely irreversible because of the formation of pyrophosphate that can be subsequently hydrolyzed to inorganic phosphate. Together, our results provide insight into which steps newly synthesized PI is enriched with specific acyl chains.

On the basis of previous studies describing the subcellular localization of enzymes involved in *de novo* PI synthesis, steps leading to the enrichment of PI are likely spatially linked within the ER. DGKε is predominantly localized in ER, although low levels are also found in the plasma membrane.[17] Both CDS1 and CDS2 are also found in the ER, although with some localization differences within the ER.[12] PIS was determined to be localized in both the ER and PIPEROsomes.[15] However, enzymatically active PIS (the focus of our study) appears to be found only within PIPEROsomes. Both CDS isoforms are not found within these organelles.[15] These PIPEROsomes make multiple contacts with the ER and are ER-derived; therefore, it is conceivable that CDP-DAG (including arachidonoyl-enriched CDP-DAG) produced in the ER is presented to PIS during formation of PIPEROsomes or by lipid transfer from the ER.

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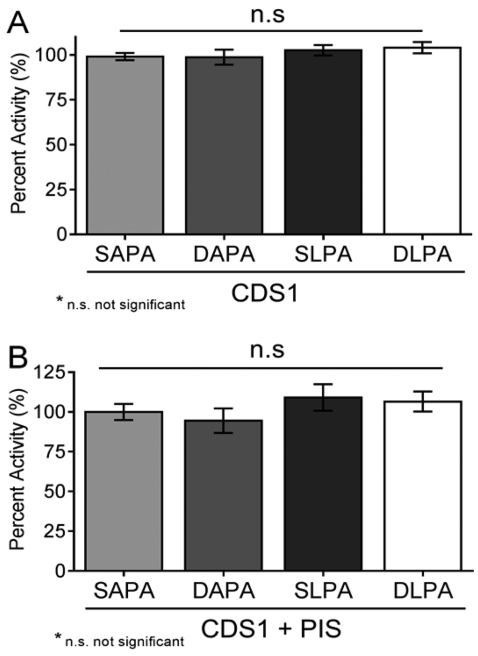

Figure 2. Substrate specificity of PIS. A mixed-micelle assay was used to test the enzymatic activity for a variety of substrates. PAs were quantified prior to use, and 50 μM substrates were used for each assay. The substrate specificities of (A) CDS1 and (B) PIS were measured by varying the *sn*-1, *sn*-2, and *sn*-1/*sn*-2 acyl chains. Data have been normalized to EV activities. Data are represented as means ± the standard error of the mean. The activities of CDS1 and PIS are 204 ± 3 pmol/min and 57 ± 6 nmol/min, respectively, and are defined as 100%.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed methods and one supplementary figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

CDP-DAG, cytidine diphosphate-diacylglycerol; CDS, CDP-DAG synthase; DGKε, diacylglycerol kinase ε; EV, empty vector; PA, phosphatidic acid; PI, phosphatidylinositol; PI/$P_{(n)}$, phosphoinositides.

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