Supplemental material for

THE ACTIVE SITE OF YEAST PHOSPHATIDYLINOSITOL SYNTHASE PIS1 IS FACING THE CYTOSOL

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Supplemental materials and methods

Materials

Anti-V5-HRP antibody was from Invitrogen, anti-FLAG, anti-HA and anti-mouse IgG antibodies were from Sigma, anti-Kar2 from Santa Cruz Biotech. HRP-labeled antibodies were visualized with Advanstar WesternBright ECL (K-12045-D50).

Western blotting

Samples were separated on 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) in transfer buffer (10 mM Tris, 100 mM glycine, 10% methanol )[35] and Western blotting was performed using first anti-FLAG antibodies (1/2000 in TBS with 1% milk powder) to detect the luminal control Gpi8. After having dried on the bench overnight, blots (without stripping) were probed by anti-V5-HRP antibodies (1/10'000 in PBS with 5% milk powder) to detect Pis1. Similarly, for Pis1 quantification, blots were first probed by anti-Kar2, dried and then probed with anti-V5-HRP.

Calculation of PI synthase activity of microsomes.

For all enzyme assays, routinely done with freshly prepared microsomes, the amount of Pis1-V5-His6 present in these microsomes was quantitated on a frozen sample in a semi-quantitative way by Western blotting (Fig. 3C). For this quantification, a large batch of microsomes from pis1Δ cells containing wt Pis1-V5-His6 on a plasmid, induced for 30 min, was prepared and identical aliquots thereof were added as “the” reference sample in all SDS-PAGE gels to be used for blots. All extracts and the reference sample were run in duplicate on SDS-PAGE gels. The Western blots were probed with anti-V5-HRP to detect Pis1 and by anti-Kar2. The western blot signals were normalized, first by averaging the duplicate samples, then by normalizing signals for loading by taking the Kar2 signal of the reference sample as the reference. Once the averaged signals of mutant microsomes were corrected for loading errors, the Pis1 signals of mutants were compared to the Pis1 signal of the reference sample and a correction factor calculated. E.g., if the Pis1 signal of a mutant was 80% of the Pis1 signal in the reference sample, that factor was set as 1.25. This correction factor was used to correct the result of the enzyme assay (nmol of Ino...
incorporated into lipids/min). These correction factors were always between 1 and 2. Enzyme assays on a given microsomal preparation were done in duplicate, and standard deviations of these duplicate assays are routinely reported.

Thus the reported activities ought to be comparable in the sense that they all correspond to roughly the same but unknown amount of Pis1 protein. Thus, for brevity figures report Pis1 activity as nmol of PI formed per minute and per mg of microsomal protein, but for normalized samples the term nmol Ino/min/mg is slightly incorrect and represents an abbreviation for what more precisely is described as nmol of Ino incorporated per minute per an amount of microsomal protein containing the same amount of Pis1 as 1 mg of our reference sample. As for the empty vector control sample, it was not normalized and added just to show that the 30 min induction leads to a higher than physiological level of activity.

Estimation of detergent:lipid ratios and mol% values of lipid components
We arbitrarily assumed that the protein:lipid ratio (w/w) in washed microsomes is 1. Molecular weights of yeast type phospholipids PC, PE, PI and PS are 760, 718, 854 and 783 Da. Given their relative abundances, we estimate the average MW of phospholipids to be 770 Da. DDM has a molecular mass of 510 Da. Based on these estimates we calculate the assays to contain 130 nmol of membrane lipids, 196 nmol of DDM and from 1.92 to 61.5 nmol CDP-DAG resulting in DDM:lipid ratios of 1.5 – 1 (CDP-DAG calculated as a lipid). Assuming a 1:1 ratio of protein:lipids in membranes enabled us also to calculate the mol% of CDP-DAG in assays. The 4-64 µg CDP-DAG added depending on assay correspond to 1.2 – 16 % of the sum of [nmol of microsomal lipids + nmol of detergent + nmol of CDP-DAG]. The exact mol% of CDP-DAG 18:1 thus were 1.19%, 4.59 and 16.2 mol% which in figures, for the sake of gaining space, were indicated as 1.2, 4.5 and 16 mol%.

Measuring hydrophobicity of PEG-mal
Duplicate samples of 5 - 10 mg of PEG-mal was diluted in 500 µl water, 500 µl of solvent was added and mixed by vortexing. The samples were incubated 30 min at RT, then centrifuged for 5 min at 16'000 x g. The two phases were transferred to new, weighed tubes and dried in a rotary evaporator. The samples from the organic phases were re-suspended in 500 µl of water and dried again, to obtain samples with a similar residual hydration levels. The presence of PEG-mal in each phase was determined by weighing the tubes.

Building of heatmap with R 3.1.0
The derivatized fraction measured from Western blots were exported from a spreadsheet to a text file with tab-separated values. The file was uploaded to R Studio 3.1.0 and the heatmap was build with the following commands:
#load pheatmap package; the data frame must be changed to a numeric matrix.
data.frame<-read.table("data.txt", sep= "\t", header=TRUE,row.names=1)
Mat<-as.matrix(data.frame)  #to turn it into a matrix
mode(Mat)  #to check if the matrix is numeric
pheatmap(Mat,
color = colorRampPalette(c("#fcff", "#0452bf"))(20), #(20) to get a 20 steps color scale
kmeans_k = NA, breaks = NA, border_color = "#281d1df",
cellwidth = 15, cellheight = 15, scale = "none",
cluster_rows = FALSE, cluster_cols = FALSE,
legend = TRUE, legend_breaks = c(0,20,40,60,80,100), legend_labels = c(0,20,40,60,80,100),
annotation = NA, annotation_colors = NA,
annotation_legend = TRUE, drop_levels = FALSE,
show_rownames = TRUE, show_colnames = TRUE, main = NA,
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number_format = "%.0f",
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lwd = 0.8, lineheight = 0.8)
Supplemental Tables S1-S3

**Table S1. Yeast strains**

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<th>Genotype</th>
<th>Reference</th>
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<td>Euroscarf</td>
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<td>this study</td>
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<tr>
<td>Construct</td>
<td>Name</td>
<td>Description</td>
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<td>Empty pYES2NT/B</td>
<td>pYES2NT/B</td>
<td>pYES2NT/B vector from Invitrogen (V8252-20) 2µ, GAL1 promoter, URA3 and ampr, C-terminal V5 epitope and poly-His tags.</td>
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<td>As 855 but all five native Cys of <em>PIS1</em> changed to alanine</td>
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<td>C154A in 855</td>
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<td>pBF459</td>
<td>C100A and C154A in 855</td>
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<td>Q68C in Pis1-K, construct having a total of 6 Cys</td>
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<tr>
<td>PIS1-K-V69C</td>
<td>pBF468</td>
<td>V69C in Pis1-K, construct having a total of 6 Cys</td>
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<td>S71C in Pis1-K, construct having a total of 6 Cys</td>
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<td>PIS1-V69S</td>
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<td>V69S in Pis1-V5-H6</td>
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<td>pBF452</td>
<td>V69N Pis1-V5-H6</td>
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<tr>
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<td>G133N and G135S mutations in Pis1-V5-H6</td>
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<td>PIS1-G135N</td>
<td>pBF456</td>
<td>G135N in Pis1-V5-H6</td>
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<tr>
<td>PIS1-134-6/NES</td>
<td>pBF457</td>
<td>E134N, G135E and E136S mutations in Pis1-V5-H6</td>
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Table S3. Primers

Underlined: restriction sites; bold: mutation; s: sense; a: antisense.

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<th>No</th>
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<td>1082</td>
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<td>AAAAGCTTATGAGTTCTGAATTCAACACC</td>
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<td>AATCTAGAGTATTCTTTTCTTTTC</td>
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<td>CATGGTTGTATAGCATACGAGACTACTGGATAGCG</td>
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<td>1085</td>
<td>PIS1 C90A C93A C100A (substitution of native Cys 90, 93, 100)</td>
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<td>1086</td>
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<td>MATout (mating type determination)</td>
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<td>aacggccacagctggacactgtggtcttccggc</td>
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<td>cataaagttgggctgagtctggctgagttgatagttac</td>
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<td>PIS1 R1-loop, to amplify the CAPT loop, after C53, before the KXXXX signal</td>
<td>GTAACCATGTCCTCAGAACGCC</td>
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<td>PIS1 F2-plasmid, to amplify the end of PIS1, after the CAPT loop</td>
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<td>PIS1-corr-R2, to amplify the end of PIS1 and add the KXXXX signal at the end</td>
<td>GCCGGGTTTAAACTCAgaagtctttctctttATGGTATGG TGATGATGACC</td>
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References in supplemental material


**Figure S1.** Topology prediction for PIs1 by Phobius (http://phobius.sbc.su.se).

Positional probabilities for TMs are represented by grey vertical lines. Only the region between 150 and 170, which corresponds to TM5, has a high probability of being a TM, with a concomitant low probability for being a cytosolic (red) or luminal (blue) loop. In Phoebius, the TOPCONS TMs 2, 4 and 6 appear as being part of loops rather than as TMs. The N-terminus is considered to be rather luminal. The orientation of the CAPT region (between amino acids 56 - 81) and the C-terminus are not clearly predicted.
Figure S2. TOPCONS predictions for the pfam01066 members using CDP-DAG as a substrate (A – C), for Cpt1 (D) and Ept1 (E), which use CDP-activated choline and ethanolamine, and Cds1 (F), the CDP-DAG synthase, which does not have a CAPT motif, respectively. Schematic representations of final topology are shown below each TOPCONS prediction. Red: cytosolic; blue: luminal; grey and white boxes: TMs. Lines a – e represent topology predicted by SCAMP1-seq, SCAMP1-msa, PRODIV, PRO and OCTOPUS algorithms. Line f represents final TOPCONS prediction taking into account a – e plus two further algorithms. Orange rectangles contain the CAPT motif. Strongly conserved residues of Cds1 are dispersed over the entire protein (orange lines). In the schemes at the bottom of each panel, continuous lines indicate unanimous predictions, dashed lines contradictory predictions. Note that CAPT motifs usually encompass the end and the beginning of two vicinal predicted TMs.
Figure S3. Cysteine reaction with maleimide. A, maleimide reacts with thiols at pH 6.5-7.5 to form a stable thioether bond. In this pH range maleimides, when added to microsomes, react specifically with the thiol group of Cys of proteins. B, heterobifunctional Cys and Lys specific crosslinkers with spacers of various lengths ranging from 7.3 Å to 24.6 Å were utilized. C, conjugation of crosslinkers to ubiquitin. The crosslinker (L) was conjugated to ubiquitin (Ub) by the N-hydroxysuccinimide ester group (NHS-ester), since ubiquitin does not contain any Cys residues. Sulfonated groups increase the solubility of linkers without interfering with the reaction. D, structure of PEG-mal (methoxy poly(ethylene glycol) maleimide). PEG-mal is a mixture of variable number of ethyleneglycol units. The lot used had an average of 5 kDa, with a polydispersity of 1.4 (Sigma).
Figure S4. Partitioning of PEG-mal in various water-organic solvent (1:1) systems.

Percentage of PEG-mal recovered after phase separation in aqueous phase (top, hatched) and various organic phase (bottom, black) for solvents with dielectric constants from 1.9 to 10.3 determined as described in supplemental materials and methods. Total recovery of PEG-mal at the end was >85% of initial amount.
Figure S5. Preliminary PI synthase in vitro assays. A, enzymatic activity of pis1Δ + Pis1 microsomes, prepared from spheroplasts and used immediately (yellow) or after one freeze/thaw cycle (red), or obtained by glass bead disruption of cells (grey) with various substrate concentrations. The reactions were run at RT for 25 min. B, activity of microsomes freshly prepared from spheroplasts in presence of Triton X-100 (TX-100), DDM or octyl-glucopyranoside (OGP) (0.1 and 2%), with 0.6 mol% CDP-DAG 18:1 (10 μM) and 1 μCi of [3H]Ino (0.25 μM). The reactions were run at RT for 25 min. C, activity of microsomes prepared as in B, with increasing Ino concentration and only endogenous CDP-DAG in 0.05% DDM. The reactions were run at RT, for 5, 10, 20 or 30 min.

Interpretation of data in panel B: Taking into account the molar amounts CDP-DAG, the variable amounts of detergent and of membrane lipids (assuming the lipid:protein ratio in microsomal membranes to be 1:1), we calculate the mol% of CDP-DAG in the assays with 0.1 and 0.5 % of DDM to be 0.382 mol% and 0.18 mol%, respectively. Thus, surface dilution of the CDP-DAG substrate should not reduce activity at 0.5% DDM to less than 47% of the activity at 0.1% DDM. Yet, going from 0.1 to 0.5 % DDM or Triton X-100 totally abolishes activity. Thus, we feel that the data clearly indicate that detergent changes the structure of Pis1 or acts as an inhibitor.
**Figure S6. CDP-DAG 18:1 vs. CDP-DAG 16:0.** Microsomal activity of wt cells (+ empty vector), *pis1Δ + Pis1, pis1Δ + Pis1ΦC* and Pis1ΦC-variants in presence of CDP-DAG 18:1 or CDP-DAG 16:0 as indicated at the bottom. **A**, assays run at constant Ino concentration (240 μM) and **B**, at constant CDP-DAG concentration (16 mol% = 307 μM). Black bars: boiled controls, containing the highest concentrations of both substrates.
**Figure S7. Quantification of SCAMs of Pis1ΦC variants.** A, TOPCONS PRODIV topology prediction for Pis1 as in Fig. 1C. Absolutely conserved residues are represented by orange lines and dashed lines link relative positions in the sequence to the substituted residues in the Pis1ΦC variants. B, degree of derivatization in % of different alleles in different conditions. Top panel: PEG-mal/NEM method. Lower Panels, Ub-mal/DDM method. The squares color shaded in blue represent the derivatizations in absence of detergent, squares shaded in red show derivatizations in presence of DDM, those shaded in purple derivatizations in presence of SDS (1%). Grey cells: not tested or result discarded because microsomes were not tight. The experiments, in which 1% instead of 0.05% DDM was used are framed in red. Percentages were calculated according to \((\text{Pis1}_{\text{deriv.}} / [\text{Pis1}_{\text{deriv.}} + \text{Pis1}_{\text{non-deriv.}}]) \times 100\) (see materials and methods) counting as derivatized only monosubstituted forms of Pis1, except for the sample of wt Pis1 derivatized in SDS: there, all derivatized forms were counted. NA, not applicable.
## Table A

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Figure S8. Western blots of Ub-mal/DDM and PEG-mal/NEM SCAMs for Gpi8. Gpi8 control Western blots of the SCAMs assays presented in Figure 4. From left to right: smallest linker (GMBS, 7 Å) to the longest (PEG-mal, 460 Å), with the linker length given in Å next to the name of the linker. Color code is the one used in Fig. 4. Ub-mal/DDM method: Lanes 1: control, lanes 2: Ub-mal, lanes 3: Ub-mal/DDM, lanes 4: Ub-mal/SDS. PEG-mal/NEM method: Lanes 5: control, lanes 6: PEG-mal, lanes 7: PEG-mal in presence of SDS, lanes 8: 0.3 mM NEM, then PEG-mal/SDS, lanes 9: 5 mM NEM, then PEG-mal/SDS. In these last two lines, the microsomes were first derivatized by NEM (0.3 mM and 5 mM) without detergent to block surface Cys; subsequent treatment with PEG-mal in SDS derivatized buried Cys. Cropping of plots of Pis1-K and its variants removed the principle species, which contained 5 or 6 PEG-mals. A, FLAG-Gpi8 Western blots for the Pis1ΦC variants having a Cys substitution outside of the CAPT motif. B, FLAG-Gpi8 Western blots for the Pis1ΦC and Pis1-K variants having a Cys substitution within the CAPT motif.
**Figure S9. N-glycosylation sites.** Microsomes of cells harboring Pis1 constructs with N-glycosylation sites introduced by site-directed mutagenesis in non-conserved residues were solubilized in SDS and treated with endoglycosidase H (endo H, +) or with endo H buffer (-). Western blots were probed with anti-V5-HRP (top) and anti-Gas1 antibodies (bottom). The constructs as well as the wt Pis1 (last two lanes) were not glycosylated and insensitive to endo H treatment, contrary to Gas1, which is a well known N-glycosylated GPI protein.
AfDIPPS, (PDB structure 4MND), homology region with Pis1 (amino acids 270-469)

Af2299, (PDB structure 4O6N), homology region with Pis1 (amino acids 196-372)

Figure S10. TOPCONS predictions for archeal CAPTs. The homology regions of the two crystallized *A. fulgidus* CAPTs (gi|661918374 and gi|635575527) with Pis1 were analyzed by TOPCONS. Annotation as in Fig. 1C. The integrated final predictions are shown in line f. The same topology is obtained when the entire sequences including the N-terminal hydrophilic regions are included in the TOPCONS query (not shown).