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Abstract

Sphinganine-1-phosphate lyase (Dpl1p) is a highly conserved enzyme of sphingolipid metabolism that catalyzes the irreversible degradation of sphingoid base phosphates, which are potent signaling molecules. Sphingoid base phosphates play a vital role in cell survival, proliferation, migration, heat stress, and cell wall integrity pathways. Little is known about the structure and regulation of Dpl1p. In this study, we have undertaken a combined computational modeling and mutagenesis approach for structure-function analysis of Dpl1p to discover possible modes of regulation. Our results identify important residues for catalysis in Dpl1p and confirm it as an integral endoplasmic reticulum-resident protein. Results further indicate that Dpl1p is most likely not regulated spatially. Importantly, we demonstrate that Dpl1p exists as an oligomer and that polar residues in its transmembrane domain are required for its full function in vivo but not for its localization or for its catalytic activity in vitro.
SPHINGANINE-1-PHOSPHATE LYASE: IDENTIFYING KEY RESIDUES FOR FUNCTION IN VIVO AND IN VITRO

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Sphinganine-1-phosphate lyase (Dpl1p) is a highly conserved enzyme of sphingolipid metabolism that catalyzes the irreversible degradation of sphingoid base phosphates which are potent signaling molecules. Sphingoid base phosphates play a vital role in cell survival, proliferation, migration, heat stress and cell wall integrity pathways. Little is known about the structure and regulation of Dpl1p. In this study we have undertaken a combined computational modeling and mutagenesis approach for structure-function analysis of Dpl1p to discover possible modes of regulation. Our results identify important residues for catalysis in Dpl1p and confirm it as an integral ER resident protein. Results further indicate that Dpl1p is most likely not regulated spatially. Importantly, we demonstrate that Dpl1p exists as an oligomer and that polar residues in its transmembrane domain are required for its full function in vivo, but not for its localization or for its catalytic activity in vitro.

INTRODUCTION

Sphingoid long-chain base-1-phosphates (LCBPs) are intermediates in the sphingolipid metabolic pathway and have emerged as key signaling molecules. In mammals, sphingosine-1-phosphate (SIP) mostly through binding to cell surface receptors promotes cell survival, proliferation, migration, angiogenesis, inhibits apoptosis and regulates lymphocyte trafficking (1,2). The involvement of LCBPs in diverse essential cellular processes requires a precise control and regulation of their intracellular and extracellular levels. Moreover, the potential role of LCBPs in disease pathogenesis makes the metabolic enzymes responsible for their regulation attractive targets to study. One such key enzyme is sphinganine-1-phosphate lyase (Dpl1p). This yeast homologue of mammalian sphingosine-1-phosphate lyase 1 (SPL) catabolizes LCBPs to ethanolamine phosphate and an alkyl aldehyde, which is the only irreversible reaction in the sphingolipid pathway. The importance of this particular reaction is further underscored by the fact that it is the only exit point that connects sphingolipids to other metabolic pathways as the products of the reaction can serve as precursors to phospholipid metabolism. Thus like SPL, Dpl1p is in a vital position as it can regulate the flux of precursors between the sphingolipid and the phospholipid metabolic pathways. In fact, flux through sphingolipid pathway and the end products of the lyase reaction serve as a major source of ethanolamine for Leishmania contributing to infectivity and therefore SPL is also a candidate target for treatment of Leishmania diseases (3).

Mammalian SPL has been shown to be involved in tissue integrity, reproduction, normal development, cell survival, immunity and mediates cellular response to stress (4-7). SPL is indicated to be a tumor suppressor and hence is a putative drug target for cancer treatment regime (6). In the yeast Saccharomyces cerevisiae, Dpl1p is instrumental in the heat shock response, diauxic shift and calcium mobilization (8-10).

Little is known about the structural features of SPL or Dpl1p and their regulation in the cell. The high conservation of SPL
throughout evolution makes it reasonable to study Dpl1p, the yeast enzyme, to gain more insights into SPL. Both are predicted to be integral membrane proteins located in the endoplasmic reticulum (11). We constructed a structural model of Dpl1p from which we predicted and tested important features of the protein by site-directed mutagenesis and enzyme characterization. The sequence of Dpl1p encodes 589 amino acid residues and is predicted to possess a single transmembrane segment (spanning residues 59-81 according to TMHMM (12)), followed by a large soluble domain, responsible for the catalytic activity, which was proposed by Van Veldhoven and Mannaerts (1991) to be located in the cytoplasm. This finding was later confirmed by a topology study carried out by Ikeda (13) on mouse SPL. It is therefore reasonable to conclude that residues 1-58 of the Dpl1p sequence reside in the endoplasmic reticulum (ER) lumen. The predicted topology of Dpl1p is shown in Fig. 1A.

Bioinformatic analysis of the cytoplasmic region of Dpl1p shows that a large part of it is distantly related to the sequence of the PLP-dependent enzyme GadB, a bacterial glutamate decarboxylase. In fact, Dpl1p groups with GadB and related enzymes in the PFAM domain database. These facts prompted us to attempt parsed homology modelling of the active site region of Dpl1p. Based on the model and on the biochemistry of PLP-dependent enzymes, we were able to rationally design Dpl1p mutants for functional analysis.

In our study we validated the structural model by experiment. Furthermore, we carried out a mutagenesis study to investigate the role of the luminal and transmembrane domain (TMD) hitherto considered redundant, in Dpl1p function and spatial regulation. Our results show that Dpl1p is statically retained in the ER. Most intriguingly, Dpl1p forms homooligomers whose detection depends upon the presence and sequence features of the transmembrane domain. These features are required for optimal activity in vivo, but not for catalytic activity in vitro even though they have no influence on protein localization to the endoplasmic reticulum. We speculate about possible explanations for this intriguing finding.

EXPERIMENTAL PROCEDURES

Yeast strains and plasmids- The yeast strains used in this study are JS16, MATα leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3 dpl1Δ::KanMx, SGP3, MATα leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3 (14), RH4863 MATα lcb3Δ::KanMx dpl1Δ::KanMx ura3 leu2 his4 ade2 bar1, pLCB3::URA3 (PCR amplified genomic LCB1 cloned into YCplac33(15)) and RH4564 MATα dpl1Δ::KanMx ura3 leu2 his4 bar1 (our laboratory collection). In JS16 erg6:::hph (hygromycin) was disrupted with gene replacement cassette using standard procedures (16). PEP4 was deleted in JS16 strain by using the plasmid pTS15 (17). pRS415 and pRS416 (New England Biolabs) were used to express DPL1 from its own promoter. pESC-URA (Stratagene) was used to overexpress DPL1 under a galactose-inducible promoter. pESC-Dpl1-Flag was constructed by cloning 1.77-kb EcoRI-SpeI DPL1 PCR amplified fragment in pESC-URA. pRS415 Dpl1-Flag tagged construct was generated by cloning PCR amplified 2.28-kb XbaI-PstI containing the 0.5-kb DPL1 promoter region in XbaI-PstI site in pRS415 and by subsequently subcloning the 0.5-kb StuI-PacI fragment containing FLAG from plasmid pESC-Dpl1-Flag. pRS415 Dpl1-Venus was generated by cloning 0.71-kb SpeI-HindIII PCR amplified fragment in all the Dpl1-Flag plasmids. The plasmids constructed in this study are listed in Table II.

DPL1 mutants were constructed by site directed mutagenesis following standard procedures. All constructs were verified by sequencing.

Phytosphingosine sensitivity Dpl1p functionality test- Cells from overnight culture in SD medium were serially diluted 10-fold (for the last spot 1:5 dilution) and spotted on SD medium containing either ethanol (carrier vehicle) or 50µM phytosphingosine (4-OH sphinganine, PHS). After three days incubation at 30°C plates were imaged.
Pulse chase experiment- Pulse chase analysis was performed essentially as described in (18). Briefly, 10 OD₆₀₀ units of logarithmic phase cells expressing FLAG tagged wild-type or mutant Dpl1p were pulsed with 50µCi/ml of [³⁵S]methionine/[³⁵S]cysteine mix (EasyTag℠ EXPRESS[³⁵S] mix from PerkinElmer Life Sciences) for 6 min at 30°C. The chase was initiated by adding 1/100 volume of a mixture of 0.3% methionine/cysteine in 0.3 M (NH₄)₂SO₄. At specific time intervals aliquots were removed and the reaction terminated by adding NaF/NaN₃ to a 10mM final concentration. Dpl1p was immunoprecipitated using Flag beads. Similarly, the Gas1p control was immunoprecipitated by anti-Gas1p antibody and protein A-sepharose beads. The proteins were eluted into TEPI (100mM Tris-HCl pH 7.5, 10mM EDTA, protease inhibitors) containing 1% SDS buffer, 1% 2-mercaptoethanol by boiling at 95°C for 10 min. Eluates were equally divided into three immunoprecipitation reactions; the first with anti-Flag Ab and protein G-Sepharose beads (GE healthcare biosciences AB); the second with α-1,3-mannose Ab and protein G-Sepharose beads and the third with protein G-Sepharose beads alone. Samples were separated by SDS-PAGE and analyzed by Cyclone Storage Phosphor system (Packard).

Cell extract preparation- Cells were resuspended as 100 OD₆₀₀ units of cells/ml in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 20% glycerol, 1mM PMSF, 1X protease inhibitor cocktail (Sigma)). After glass bead lysis and removal of cell debris by centrifugation, protein concentration was determined by BIO-RAD protein assay.

Endoglycosidase H (EndoH) treatment- Cells grown in selective SD medium were harvested in mid-logarithmic phase. Cells were lysed and processed as previously described (19). 40µl of cell extract was treated with 25mU of EndoH (Roche) for 1 hour at 37°C. Boiled samples were separated by SDS-PAGE and detected by Western blotting(20).

Protein purification and sphinganine-1P lyase assay- FLAG tagged purification was performed as described previously (21) with the following changes. Cells bearing DPL1-FLAG on overexpression plasmids were grown to exponential phase in selective S raffinose 2% (w/v), glycerol 3% (w/v) minimal medium and expression was induced by 2% galactose for 4 hours. Cells were broken in lysis buffer with chilled glass beads. After removal of cell debris, NP-40 was added to a final concentration of 1% to the resulting supernatant. Binding to FLAG beads, washing and elution were performed as described before. [4,5-³H]-sphinganine-1P was prepared from radiolabeled [4,5-³H]-sphinganine (ARC, Saint Louis, MO) by incubation with purified yeast sphingosine kinase, Lcb4p (provided by I. Riezman, Univ of Geneva), separation by TLC and extraction from the plate. The activity of purified wild-type and mutant Dpl1p proteins was assayed by incubating 0.5μg of purified protein with 5μCi [4,5-³H]-sphinganine-1-P at 30°C for 30 min in reaction conditions as previously described (11).

Proteasome inhibition and Cycloheximide (CHX) chase analysis- The experiment was carried out as described in (22) with minor modifications. Cells were grown to 1 OD₆₀₀ and incubated at 30°C with 100µM MG-132 (Sigma) in dimethyl sulfoxide (DMSO). 12 OD₆₀₀ units of cells were removed and added to NaN₃ (final concentration 10mg/ml) at time points 0 and 2 hours. For CHX chase, cells were grown similarly as for the proteasome inhibition assay. 0.5mg/ml final concentration of CHX (Sigma) in DMSO was added to cells. 7 OD₆₀₀ cells were removed per time point. Cell extract preparation was performed as described above.

Immunoprecipitation- Cell extract was prepared as described above and solubilized by incubation at 4°C for 1 hour with digitonin 0.5% (v/v) final concentration. After centrifugation at 14,000 rpm, 4°C for 30 min, the supernatant was collected. 2 mg of total protein were immunoprecipitated with anti-VENUS antibody and Protein A sepharose beads (GE healthcare biosciences AB) or anti-FLAG sepharose beads (Sigma). Beads were washed with 50mM Tris–HCl, pH 7.5, 150mM NaCl, 5mM EDTA 0.1% digitonin. Proteins were eluted by boiling in 2X sample buffer and analyzed by Western blotting(20).
Blue Native PAGE (BN-PAGE)-

Overnight cultures were harvested at 1 OD<sub>600</sub> units and cells were resuspended at 100 OD<sub>600</sub> units/ml in NativePAGE sample buffer (Invitrogen) with 1mM PMSF, protease inhibitor cocktail (Sigma) (NativePAGE-SB). After glass bead lysis, samples were centrifuged at 13,000g for 15 min at 4°C. The resulting pellet was resuspended in NativePAGE-SB with 0.5% digitonin and solubilized for 30 min at 4°C. The solubilized sample was clarified by centrifugation at 14,000 rpm for 30 min at 4°C. The final sample was prepared by adding NativePAGE 5% coomassie G-250 (Invitrogen) to a final concentration of 0.125% to the solubilized clarified extract. Samples were separated on a 4-16% acrylamide gradient NativePAGE Novex Bis-Tris gel (Invitrogen) and Western blotting was performed(20).

Synthetic lethality test- RH4863 cells were transformed with pRS415 plasmids containing wild type or mutant DPL1 genes. Cells were grown in SD medium without leucine, 10 fold serial dilutions were prepared and cells were spotted onto agar plates containing SD without leucine, without uracil, or containing 5-fluoroorotic acid to select for cells that had lost the pLCB3::URA3 CEN plasmid.

Fluorescence microscopy- Cells bearing DPL-VENUS plasmids were grown to exponential phase in minimal selective medium. Fluorescence images were obtained using YFP filter on an Axio Imager Z1 microscope.

Quantification of cellular PHS-1P- JS16 cells carrying pRS415 series plasmids with wild type or mutant DPL1 were grown to late log phase in SD without leucine. 40 OD<sub>600</sub> units of cells were harvested and washed once with SD minus leucine. Cells were resuspended in SD minus leucine containing 20 μM sphinganine (Avanti Polar Lipids) were added before extraction using glass beads and ethanol/ether/pyridine(23) at 60°C, except that the samples were not desalted after extraction. Samples were analysed by HPLC-Tandem Mass Spectrometry as described(24) on a Varian 320 Triple Quadrupole MS. Sphingoid bases were separated on a Uptisphere ODB column (5 μm, 1 mm x 150 mm) at a flow rate of 50 μl/min at 30°C and data acquired using the following optimized transitions (C17 sphingosine, 286>268, -17 V, C17 sphingosine-1P, 366>250, -15 V, sphinganine 302>282, -22V, PHS, 318>282 -24V, sphinganine 1P, 382>282, -18V, PHS-1P, 398>282, -18 V). Two independent experiments were performed, each in duplicate, and each sample was analyzed at least three times by HPLC-MS. Sphinganine was rapidly converted to PHS intracellularly because the former was not detected in most samples. Data for PHS-1P levels was corrected for recovery and detection by reference to the internal standard C17 sphingosine 1P, which allows a reliable relative quantification between samples, but does not permit an absolute quantification.

Parsed homology modeling- A parsed homology model of Dpl1p was created based on the structure of E. coli GadB at low pH (PDB code 1PMM) using the program Modeller (v7) (25). The PLP cofactor was also modeled into the active site and the Schiff base linkage to K380 optimized in a final energy-minimization step with CNS (26). Only the residues in the active site region and the cofactor, which aligned very clearly with the template and other SPL and Gad sequences in multiple sequence alignments, were retained in the final model.

RESULTS

DPL1 parsed homology modeling and mutational testing- According to the PFAM database (27), Dpl1p and related SPLs from different eukaryotes belong to the PLP-dependent aminotransferase superfamily clan. More specifically, their catalytic domain is a pyridoxal-dependent decarboxylase conserved domain (PF00282). A BLAST search of
Dp11p versus the PDB database identifies E. coli GadB (28,29) as the top hit, with a sequence identity of 23% over a 251-residue long aligned region. Based on the two representative structures of PF00282, GadB and Dopa-decarboxylase (30), one can assume that the quaternary assembly of Dpl1p is either dimeric (like Dopa-decarboxylase) or a multiple of dimeric units (like GadB, a hexamer which is trimer of dimers) (Fig. 1B). We analyzed the possibility that Dpl1p is part of a large molecular weight complex by Blue Native PAGE (BN-PAGE) and Western blotting (Fig. 8B). Both Dpl1p-Venus and Dpl1p-FLAG tagged proteins migrated in BN-PAGE gels at an apparent molecular size of over 300,000 daltons confirming that it is part of a large complex. Co-precipitation experiments (see below) prove that Dpl1p subunits self-associate. It is not possible to determine whether the Dpl1p oligomer is a tetramer or a hexamer from our data, a problem that is rendered more difficult to solve by the obligatory presence of detergent to keep the enzyme soluble.

To determine if the luminal domain of Dpl1p has any role in its function, we generated an NΔ57 truncation mutant lacking the entire luminal domain, which as mentioned above, is predicted by TMHMM to span residues 1 to 58. NΔ57 was tested for its ability to complement a dpl1Δ mutant in the presence of phytosphingosine. Under standard laboratory growth conditions, Dpl1p is not essential, but is required for yeast cells to grow on a medium containing an elevated concentration of PHS (50µM was used in this study). PHS is cytotoxic at high concentrations. This mutant was completely inactive in the in vivo complementation assay (Fig. 2A). The steady state level of the NΔ57 mutant protein was nearly undetectable (Fig. 2B). A partial or total absence of the luminal domain could affect the steady state level of Dpl1p by modulating protein stability. We further investigated this possibility by constructing other truncation mutants as discussed later.

One of the major aims of this study was to identify important residues for the structural integrity, catalytic activity and substrate binding of Dpl1p. To this end, a parsed homology model of the Dpl1p active site region was created based on the structure of GadB and on multiple sequence alignments of Dpl1p and SPLs. Also information from PF00282 was taken into account. The model (Fig. 1C) shows that the cofactor, covalently linked to K380, is sandwiched between C344 and H268. The pyridinium nitrogen of the pyridoxal 5'-phosphate ring interacts electrostatically with the side chain carboxylate of D342. The phosphate moiety of the cofactor is held in place by a network of hydrogen bonding interactions with the main chain amides of G235, T236, the side chain imidazole of H379 and the side chain hydroxyl of S422.

Several mutants were designed based on the active site homology model: H268A was aimed at impairing PLP binding via removal of the stacking aromatic ring of H268. Two Lys to Ala mutants, K380A and K386A, were assumed, respectively, not to be able to form an internal aldimine link with the cofactor (removal of the catalytic lysine side chain) and to be impaired in substrate binding (removal of a Lys side chain which is near the region where the phosphate moiety of LCBP is supposed to bind). All of the above mutants were created and tested for their ability to complement the dpl1Δ mutant in presence of PHS. As predicted, the mutants did not complement the dpl1Δ deletion mutant and therefore we conclude that they exhibit very low or no activity (Fig. 2A). The K380 and K386 mutants exhibit normal steady-state protein levels, but they lack function (Fig. 2B). We confirmed that the absence of function in the K380 and K386 mutants is due to a lack of enzymatic activity by using an in vitro enzyme activity assay (Fig. 2C). Another residue, C344, is a PLP-stacking residue and may be the nucleophilic Cys that was proposed to attack the C3 carbon of the substrate (31). The mutant Dpl1p-C344A was partially active in the in vivo complementation assay (Fig. 2A) and present at almost normal levels (Fig. 2B). Interestingly, the residue corresponding to C344 in mammalian SPL, C317, has been
mutated to Ser and exhibits a severe loss of activity \textit{in vitro} (32).

An additional histidine mutant, H340 was designed based on the consideration that a fully conserved residue in members of the PF00282 family, H241 of GadB, has been proposed to play a critical structural role for the stability of this group of proteins (33). We created this mutant and it was also inactive for complementation (Fig. 2A). Steady state levels of this mutant protein were substantially lower than the wild type, suggesting that the mutant protein is indeed unstable, which is consistent with the proposed function of this residue (Fig. 2B).

Two additional mutants, Y554A and Y554F, belonging to the C-terminal region of Dpl1p, were designed by analogy with GadB. Namely, the C-terminal region of GadB, which encompasses residues 452-466, does not belong to the PF00282 domain core and is flexibly disordered at low pH. This region can take up an ordered conformation and insert into the active site at neutral pH, thus inhibiting the enzyme. The inhibition has been recently shown to take place \textit{via} a covalent linkage between the distal side chain nitrogen of H465 (the penultimate residue of the protein) and the C4` atom of the cofactor (29). A $Ka/Ks$ ratio analysis performed with SELECTON (34) on 44 nucleic acid sequences coding for GadB proteins (G. Capitani, unpublished) revealed that the C-terminal region of GadB (residues 452-466), which is flexibly disordered at low pH, possesses only one residue under strong purifying selection and this is H465, followed by a small residue, T466.

A similar $Ka/Ks$ ratio analysis was carried out on 10 coding sequences of Dpl1p and related SPLs. The C-terminal region of Dpl1p, encompassing 46 residues if one uses the GadB domain boundaries, was inspected to see if it exhibited a feature similar to the H465-T466 region of GadB. Thus, the search aimed at His, Tyr or Lys residues (capable of acting as nucleophiles and of exchanging protons) which were both under strong purifying selection and were followed by a small residue like Gly, Ser, Ala or Thr. One such residue was Y554 (followed by G555). We constructed two mutants, Y554A and Y554F, which showed partial activity in the \textit{in vivo} complementation tests, even though the protein is present in normal amounts at steady-state (Fig. 2B), suggesting that this residue is important, but not absolutely required (Fig. 2A). It is quite possible that this residue plays a regulatory role.

\textbf{Dpl1p is statically retained in the ER-} Dpl1p is reported to localize to ER (35,36). We observed Dpl1p tagged with the yellow fluorescent protein variant Venus (functional in the \textit{in vivo} complementation test – data not shown) by direct fluorescence microscopy. Dpl1p-Venus displayed cortical and perinuclear ER localization consistent with previous studies (Fig. 3A). ER retention can be mediated by static retention or continuous retrieval from an early Golgi compartment. Dpl1p is glycosylated (see below) and to measure whether Dpl1p reaches the Golgi compartment we tested if its N-linked glycan could be modified in the \textit{cis}-Golgi compartment by $\alpha$-1,6 mannosylation. By pulse chase analysis, no $\alpha$-1,6 mannosylation could be detected on Dpl1p after 60 minutes suggesting that the enzyme is not transported to the \textit{cis}-Golgi within this time frame (Fig. 3B). Under the same conditions we were able to detect $\alpha$-1,6 mannosylation of a control protein, Gas1p. These results indicate that Dpl1p is retained statically in the ER (Fig. 3B).

\textbf{Dpl1p is glycosylated and the luminal domain is essential for enzyme stability-} We observed that Dpl1p migrates as a doublet upon overexpression on SDS-PAGE (our unpublished data) and the Dpl1p sequence has a classical N-linked glycosylation motif Asn-X-Thr(Ser) in the N-terminus. To determine if the protein is glycosylated, we created a mutant protein (Dpl1N6Ap), treated the wild type protein with endoglycosidase H (EndoH) and analyzed their migration by SDS-PAGE. Both mutation and EndoH treatment caused an increase in protein mobility demonstrating that Dpl1p undergoes N-linked glycosylation (Fig. 4B). Glycosylation can modulate protein function/activity by affecting conformation, stability and targeting (37). We checked the role of Dpl1p glycosylation by \textit{in vivo}...
complementation and results show that glycosylation is dispensable for Dpl1p function (Fig. 4C).

Like SPL, Dpl1p is predicted to be a type I membrane protein (Fig. 1A). It has been shown that the TMD of recombinant SPL is important for membrane association but not for in vitro activity (32). A requirement for the TMD of SPL in vivo has not been reported in the literature. We investigated the in vivo requirement of the TMD and the luminal domain (as mentioned earlier). To this end, we generated several truncations in the N-terminal domain of Dpl1p schematically represented in Fig. 4A. An NΔ54 truncation mutant bearing some additional residues after the TMD of Dpl1p to facilitate insertion into the ER membrane was constructed. This mutant also demonstrated no complementation on PHS plates and a lower steady state level, with the mutant protein being barely detectable (Figs. 4C and 4D). On the other hand, the NΔ31 truncation mutant was functional and stable. This suggests that the 32-54 amino acid region might play an important role in the stability of Dpl1p in the ER membrane. As Dpl1p does not contain a cleavable signal sequence for ER insertion, it might be that this mutation affects membrane insertion or the subsequent topology of the inserted protein. Improper insertion would most likely lead to instability.

Dpl1p has an atypical transmembrane domain with many polar residues- The TMD of Dpl1p is predicted to encompass amino acid residues from 59 to 81 (Fig. 5A). Deletion of the entire luminal domain and TMD, NΔ81, resulted in lack of function in vivo (Fig. 5B). Like the NΔ54 and NΔ57 mutants, nearly undetectable steady state levels are likely to be the cause of lack of function (Fig. 5C). Interestingly, the predicted TMD of Dpl1p possesses three polar and one charged residue (Fig. 5A). To identify the possible function of these residues, we mutated C65, K67, S70 and N71 individually, in pairs and all of them together to leucine, a more common component of TMDs. K67L complemented the dpl1Δ although not quite as efficiently as the wild-type enzyme, while the double mutants K67LS70L, S70LN71L both showed similar partial complementation on PHS plates (Fig. 5B). Strikingly, the quadruple mutant did not complement at all (Fig. 5B). The lack of complementation on PHS plates cannot be explained by protein instability in this case because the quadruple mutant was expressed at the same level as the wild-type protein (Fig. 5C). Therefore, the polar residues in the transmembrane domain are required for Dpl1p function in vivo. This mutant is recessive as it did not confer a phenotype when expressed in the presence of the wild-type protein (Fig. 5D).

In order to provide additional assays for the in vivo function of the mutants affecting the luminal and transmembrane regions we performed two assays. Dpl1p is one of the two major enzymes that degrade PHS-1P, which is apparently toxic to cells in high amounts(38). The other enzyme is the sphingoid base phosphate phosphatase, for which the major activity is encoded by LCB3. Mutation of DPL1 and LCB3 is synthetically lethal, but cells can be maintained alive by expressing LCB3 from a URA3 containing plasmid. We introduced our constructs into a dpl1 lcb3 double mutant containing pLCB3::URA3 and screened for the ability of the cells to lose the LCB3 plasmid on 5-fluoroorotic acid plates, a measure of the ability of the Dpl1p mutants to degrade sphingoid base phosphates. These assays (Fig 6A) agree well with the PHS plate assay results.

We also assessed the in vivo activity of the Dpl1p constructs more quantitatively by measuring the intracellular PHS-1P concentrations relative to wild type cells by HPLC tandem mass spectrometry. To increase cellular levels of sphingoid bases we added external sphinganine for 10 min then stopped all reactions with TCA. Sphingoid bases were extracted and quantified by mass spectrometry. The amount of sphingoid bases phosphates in the mutant strains is expressed as a fold increase over wild type cells. As can be seen (Fig 6B) cells carrying the plasmid without insert showed a 4 fold rise in PHS-1P, whereas the Δ31dpl1 mutant behaved like wild type cells. The Δ81dpl1 mutant cells behaved like the dpl1 null. The point mutants in the TMD showed intermediate results, which are
consistent with the plate assays if one assumes that there is a threshold level of activity that is required in the presence of excess PHS or when LCB3 is absent. This threshold would be situated in between the activities of the K67L and CSKN mutant levels. In any event, the MS results show that the activity in vivo of the CSKN and K67L mutants are compromised.

It has been previously reported that charged residues in TMDs are important for ER retention (39,40). The defect in the quadruple mutant could be due to loss of ER retention and subsequent mislocalization. To determine if the quadruple mutant exits the ER and reaches the cis-Golgi where its N-linked glycan would be remodeled by α-1,6 mannosylation, we performed pulse chase analysis. No α-1,6 mannosylation could be detected within the 60 minutes time frame of the experiment (Fig. 3B). Hence, it does not appear that the lack of complementation is due to a defect in ER retention. We further checked the subcellular localization of Dpl1p mutants tagged with Venus by direct fluorescence microscopy. All mutants except NΔ54 showed a cortical and perinuclear ER localization like wild-type Dpl1p (Fig. 6A). The mutant NΔ54, detected only at increased camera sensitivity because of its low steady state level, does not show the characteristic ER pattern and seems to form aggregates of unknown localization. To test if the punctate structure observed for NΔ54 represents molecules transported from the ER, we performed a localization study in sec18-ts cells which are defective in ER to Golgi transport at 37°C (restrictive temperature). Localization of NΔ54 in the sec18-ts was not altered after 1 hour incubation at the restrictive temperature indicating that the punctate structure observed is not a product of transport from the ER (our unpublished data). This indicates that the luminal domain of Dpl1p is also important for its proper localization to the ER. The complete lack of function displayed by the NΔ54 mutant in vivo is probably due to a combined effect of its low steady state levels and its mislocalization, since another mutant (K67S70 LL) with lower steady state levels, but localized to ER, is partially functional.

There is no obvious ER retention signal in the Dpl1p sequence and the quadruple mutant comprising all of the polar residues in the TMD does not leave the ER, so there could be redundant retention signals; one in the TMD and one in the luminal domain. To test this idea we combined the NA31 truncation mutant with the quadruple mutant and checked functionality, stability and localization. This mutant did not complement, but was fairly stable and remained localized in the ER (Figs. 6C and D). In all of our constructs where Dpl1p was stable we found it localized in the ER and never observed it in other localizations, even at various stages of growth (our unpublished data). In yeast it is evident that the major sphingosine kinase Lcb4p is regulated spatially in the cell (41-43) as is mammalian sphingosine kinase 1 (44). But the two yeast enzymes that dephosphorylate LCBPs, Lcb3p and Ysr3p, both have also been shown to be localized to the ER membrane like Dpl1p (45,46). Therefore, it is clearly important to understand how Dpl1p and the LCBP phosphatases can act on LCBPs that have been generated elsewhere than in the ER.

Unstable Dpl1p mutant proteins are degraded in the ER. Several of the mutations/truncations we created seem to render Dpl1p unstable. To verify this we performed a cycloheximide (CHX) chase assay with the NΔ81 truncation mutant, which displays a low steady state level. The amount of protein for NΔ81 was much lower both at time point 0 and after 60 min of CHX treatment, NΔ81 showed a 50% decrease in amount while wild-type Dpl1p amount was altered only by 20% (Fig. 7A). This instability may be either due to transport through the secretory pathway and degradation in the vacuole or degradation directly via the proteasome. Unstable sphingoid base kinase seems to be degraded in the vacuole (42,47).

To resolve the above issue, we created a dpl1Δpep4Δ mutant strain that lacks vacuolar protease activity. Reducing vacuolar protease activity did not render the mutants functional nor were they stabilized (Figs 7B and C). This suggests that the Dpl1p mutants might be degraded by the proteasome. To test this, we
DISCUSSION

Our experiments clearly demonstrate that Dp1p forms higher order complexes and Dp1CKSN_LLLL seems to do so less efficiently. We speculate that the failure to self-associate into a large quaternary assembly may be important for Dp1p function in vivo. Higher order assembly is not absolutely mandatory for enzymatic activity as the protein remains active in the in vitro assay, even though its activity is compromised in vivo. This rather surprising finding suggests that the TMD of Dp1p plays a role in function that might not be directly required for catalytic activity. The role of the TMD in oligomerization does not seem to be important for stabilizing the enzyme structure, since the TMD quadruple mutant protein appears to be quite stable.

The precise role of the TMD is not clear from our data but one highly interesting suggestion would be that the TMD itself and/or enzyme oligomerization is important for presentation of the substrate to the enzyme in the membrane. The properties of the Dp1p substrate are rather particular comprising a large hydrophobic domain that should deeply penetrate the lipid bilayer and a very hydrophilic ethanolamine phosphate group. One could also speculate that the lysine residue near to the cytoplasmic side of the predicted TMD might form an ion pair with the phosphate of the LCBP helping to position the substrate for the enzymatic reaction, which would take place at the hydrophobic-hydrophilic interface of the membrane. This rather precise positioning information would be less important for the in vitro activity of the enzyme which takes place in the presence of detergent.

We have also shown that membrane association of the enzyme is not required for catalytic activity in vitro, but is absolutely required for activity in vivo. It is also possible to imagine that a particular as yet undefined property of the membrane interaction is required for optimal activity in vivo.
In addition to the surprising role of the TMD, we have identified by parsed homology modeling critical residues in the Dpl1p active site, which we have subsequently validated by experiments. Our results show that Dpl1p is glycosylated, but glycosylation does not seem to play an important functional role. The luminal domain of Dpl1p is important for protein stability. Our results also indicate that Dpl1p is not likely to be regulated spatially and its ER localization is important for its function, even though it is likely that its substrate can be generated elsewhere in the cell. Interestingly, it seems that delivery of sphingoid base phosphates to the phosphatase, Lcb3p, requires formation of the sphingoid base phosphates on the ER membrane (48), at least for productive dihydroceramide formation. If the same is true for Dpl1p, then sphingoid bases phosphates formed at other locations than on the ER membrane would constitute a distinct intracellular pool of these molecules not accessible to the major activities that degrade them.

Our structural modeling and mutagenesis study might be useful for designing inhibitors that affect the enzyme active site. FTY720, a known immunosuppressant which has already been shown to be pharmaceutically useful has also been found to inhibit SPL (49). The results from this study might also help to identify genetic alterations that lead to loss of enzyme function without affecting protein stability or activity and in the future may serve as genetic markers for predisposition to disease.
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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Dpl1p is an oligomer. (A) Schematic representation of Dpl1p predicted topology. Dpl1p is predicted to be a type 1 membrane protein with a short luminal domain and a large cytoplasmic domain containing the active site. The basic unit of Dpl1p is a dimer. Proteins of the PF00282 PFAM family are constitutive homodimers (like Dopa-decarboxylase, top panel) or higher oligomers resulting from the assembly of homodimers (like GadB, a trimer of dimers, bottom panel). Both Dopa-decarboxylase (1JS6) and GadB (1PMM) are shown in stereo cartoon representation and colored by chain. Their PLP cofactors appear in spacefill representation (yellow). (C) Stereo view of the modeled Dpl1p active site.

Fig. 2. Analysis of predicted important residues for structure and activity of Dpl1p. (A) In vivo complementation of DPL1-FLAG mutants constructed as predicted by the model. (B) Stability test. JS16 (dpl1Δ) cells carrying plasmids expressing FLAG tagged Dpl1p or Dpl1p mutants under own promoter 1: vector; 2: DPL1; 3: NΔ57DPL1; 4: H268A; 5: H340A; 6: C344A; 7: K380A; 8: K386A; 9: Y554A; 10: Y554F were analyzed. Results are shown from one experiment, but are representative of three separate experiments. (C) Activity test of Dpl1p active site mutants. The average and standard deviation for three independent determinations is shown.

Fig. 3. Dpl1p shows a static localization to ER. (A) Localization of Dpl1p-Venus. (B) Dpl1p and CKSN_LLLL mutants possible recycling between ER and Golgi was assessed by [35S] Met/Cys pulse labeling and chase analysis.

Fig. 4. Role of glycosylation and luminal domain of Dpl1p. (A) Schematic representation of Dpl1p and various truncation constructs generated in this study. (B) Dpl1p is
glycosylated. Glycosylation status of Dpl1p was determined by treatment with endoglycosidase H treatment and compared with the glycosylation mutant N6A. (C) In vivo complementation of Dpl1p glycosylation and luminal domain truncation mutants. (D) Stability test. JS16 (dpl1Δ) cells carrying plasmids expressing FLAG tagged Dpl1p or Dpl1p mutants under own promoter 1: vector; 2: DPL1; 3: NΔ31DPL1; 4: NΔ54DPL1 were analyzed.

Fig. 5. Role of transmembrane domain of Dpl1p (A) Schematic representation of Dpl1p structure including the sequence of the transmembrane domain with polar residues in bold. (B) In vivo complementation of Dpl1p transmembrane domain mutants. (C) Stability test. JS16 (dpl1Δ) cells carrying plasmids expressing FLAG tagged Dpl1p or Dpl1p mutants under own promoter 1: vector; 2: DPL1; 3: NΔ81DPL1; 4: K67L; 5: CKSN_LLLL; 6: K67S70_LL; 7: S70N71_LL were analyzed. (D) Dominance test of CKSN_LLLL mutant.

Fig. 6. Function of Dpl1p mutants in vivo. (A) Strain RH4863 carrying the designated wild type DPL1, mutant dpl1 alleles or empty vector were 10 fold serially diluted and spotted onto SD plates without leucine or on SD plates with uracil and 5-fluororotic acid (5-FOA). Only strains with a functional Dpl1p can lose the plasmid and grow on 5-FOA plates. The colonies were allowed to grow for 2 days and then photographed. (B) PHS-1P was quantified in wild type and mutant cells (plasmid borne DPL1 genes) by HPLC tandem mass spectrometry and the amount was compared to wild type cells (set to 1). The error bars, determined from 4 independent determinations in duplicate represent one standard deviation. If one assumes that recovery and detection of PHS-1P and the internal standard C17 sphingosine-1P is identical, then the wild type had 230 ng PHS-1P per 40 OD600 units of cells.

Fig. 7. Dpl1p is an ER resident protein and mutants remain in ER. (A) Localization of Dpl1p and mutants. (B) NΔ31CKSN_LLLL is non-functional like CKSN_LLLL in the in vivo complementation test. (C) Stability test. JS16 (dpl1Δ) cells carrying plasmids expressing FLAG tagged Dpl1p or Dpl1p mutants under own promoter 1: vector; 2: DPL1; 3: NΔ31DPL1; 4: NΔ31CKSN_LLLL; 5: CKSN_LLLL were analyzed. Results shown are from one experiment representative of three separate experiments.

Fig. 8. Dpl1p unstable mutants are degraded in ER by proteasome. (A) Cycloheximide chase analysis of wild-type Dpl1p and NΔ81 mutant. Densitometric measurements of Western blots in top and bottom panel were made with the OptiQuant 0.400 program. Dpl1p signals (top panel) were normalized to the Dpm1p signals (bottom panel) for each time point. Results are shown from one experiment. (B) In vivo complementation test for dpl1Δpep4Δ strain bearing DPL1 and unstable mutants on plasmid. (C) Stability test in dpl1Δpep4Δ. Cells carrying FLAG tagged DPL1 or DPL1 mutants under own promoter on plasmid 1: DPL1; 2: NΔ54DPL1; 3: NΔ81DPL1; 4: H340A; 5: vector were analyzed. Results shown are from one experiment representative of three separate experiments (D) Stabilization of FLAG tagged Dpl1p unstable mutants by proteasome inhibition. Results shown are from one experiment and represent three independent experiments.
Fig. 9. Transmembrane domain of Dpl1p is important for activity and oligomerization. (A) Activity test. Results are averages ± s.d. of three independent experiments. (B) Blue Native PAGE shows Dpl1p as an oligomer. 1 (left panel): dplΔ DPL1-FLAG DPL1-VENUS. Dpl1p detected by anti-VENUS antibody, 2: dplΔ DPL1-FLAG DPL1-VENUS. Dpl1p is visualized by anti-FLAG antibody, 3 (right panel): dplΔ DPL1-FLAG. Dpl1p is detected by anti-FLAG antibody, 4: dplΔ DPL1CKSN_LLLL-FLAG. The protein is detected by anti-FLAG antibody, 5: dplΔ + empty vector. (C) Quadruple mutant of Dpl1p does not co-immunoprecipitate with wild-type Venus tagged Dpl1p. Protein immunoprecipitation by FLAG beads and immunoblotting with either anti-FLAG antibody (top) or anti-Venus antibody (bottom panel) (D) Protein immunoprecipitation with anti-Venus antibody, followed by immunoblotting with either anti-Venus antibody (top) or anti-FLAG antibody (bottom panel). Results shown are from one experiment representative of three independent experiments.

Table I: Important DPL1 residues from parsed homology modelling based on the GadB structure

| DPL1 residue | GadB residue | putative function                                      |
|--------------|--------------|-------------------------------------------------------|
| Gly235       | Ser126       | binding of PLP phosphate group                         |
| Thr236       | Ser127       | binding of PLP phosphate group                         |
| His268       | Gln163       | stacking of PLP ring                                  |
| His340       | His341       | structurally critical residue for the PF00282 family   |
| Asp342       | Asp243       | salt bridge with pyridinium nitrogen of PLP ring       |
| Asp377       | Ser273       | binding of PLP phosphate group                         |
| Cys344       | Ala245       | Van der Waals interaction with PLP ring                |
| His379       | His275       | binding of PLP phosphate group                         |
| Lys380       | Lys276       | covalent binding of PLP (internal aldimine)            |
| Lys386       | Lys282       | providing a positive charge for binding of             |
| Ser422 | Ser318 | binding of PLP phosphate group |
|--------|--------|-------------------------------|

the substrate phosphate
Table II Plasmids constructed in this study

| Plasmid name                        | Yeast marker | Replication origin | Source   |
|------------------------------------|--------------|--------------------|----------|
| pRS415 Dpl1-Flag                   | LEU2         | CEN                | This study |
| pRS415 Dpl1N6A-Flag                | LEU2         | CEN                | This study |
| pRS415 NΔ16Dpl1-Flag               | LEU2         | CEN                | This study |
| pRS415 NΔ31Dpl1-Flag               | LEU2         | CEN                | This study |
| pRS415 NΔ54Dpl1-Flag               | LEU2         | CEN                | This study |
| pRS415 NΔ57Dpl1-Flag               | LEU2         | CEN                | This study |
| pRS415 NΔ81Dpl1-Flag               | LEU2         | CEN                | This study |
| pRS415 Dpl1C65L-Flag               | LEU2         | CEN                | This study |
| pRS415 Dpl1K67L-Flag               | LEU2         | CEN                | This study |
| pRS415 Dpl1S70L-Flag               | LEU2         | CEN                | This study |
| pRS415 Dpl1N71L-Flag               | LEU2         | CEN                | This study |
| pRS415 Dpl1C65K67_LL-Flag          | LEU2         | CEN                | This study |
| pRS415 Dpl1K67S70_LL-Flag          | LEU2         | CEN                | This study |
| pRS415 Dpl1S70N71_LL-Flag          | LEU2         | CEN                | This study |
| pRS415 Dpl1CKSN_LLLL-Flag          | LEU2         | CEN                | This study |
| pRS415 NΔ31Dpl1CKSN_LLLL-Flag      | LEU2         | CEN                | This study |
| pRS415 Dpl1H268A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1H340A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1C344A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1K380A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1K386A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1Y554A-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1Y554F-Flag              | LEU2         | CEN                | This study |
| pRS415 Dpl1-Venus                  | LEU2         | CEN                | This study |
| pRS415 NΔ31Dpl1-Venus              | LEU2         | CEN                | This study |
| pRS415 NΔ54Dpl1-Venus              | LEU2         | CEN                | This study |
| pRS415 Dpl1K67L-Venus              | LEU2         | CEN                | This study |
| pRS415 Dpl1CKSN_LLLL-Venus         | LEU2         | CEN                | This study |
| pRS415 Dpl1K67S70_LL-Venus         | LEU2         | CEN                | This study |
| pRS415 Dpl1S70N71_LL-Venus         | LEU2         | CEN                | This study |
| pRS415 NΔ31Dpl1CKSN_LLLL-Venus     | LEU2         | CEN                | This study |
| pRS416 Dpl1-Venus                  | URA3         | CEN                | This study |
| pESC-Dpl1-Flag                     | URA3         | 2μ                 | This study |
| pESC-Dpl1K380A-Flag                | URA3         | 2μ                 | This study |
| pESC-Dpl1K386A-Flag                | URA3         | 2μ                 | This study |
| pESC-NΔ81Dpl1-Flag                 | URA3         | 2μ                 | This study |
| pESC-Dpl1K67L-Flag                 | URA3         | 2μ                 | This study |
| pESC-Dpl1CKSN_LLLL-Flag            | URA3         | 2μ                 | This study |
**Figure 2**

A) Wild-type, K380A, K386A, DPL1, ΔDPL1, Δ57dpl1, dpl1H268A, dpl1H340A, dpl1C344A, dpl1K380A, dpl1K386A, dpl1Y554A, dpl1Y554F

B) Anti-Flag and Anti-Hexokinase

C) Graph showing arbitrary units for Wild-type, K380A, and K386A
Figure 3

A

Dpl1p

FL

DIC

B

| chase | 0’ | 15’ | 30’ | 60’ |
|-------|----|-----|-----|-----|
| Dpl1p |    |     |     |     |
|       |    |     |     |     |
|       |    |     |     |     |
|       |    |     |     |     |
|       |    |     |     |     |
|       |    |     |     |     |
|       |    |     |     |     |

anti-Flag

anti-α 1,6

beads only

| chase | 0’ | 15’ |
|-------|----|-----|
| CKSN  |    |     |
|       |    |     |
|       |    |     |
|       |    |     |
|       |    |     |
|       |    |     |
|       |    |     |

anti-Gas1

antigli 1,6

beads only

Figure 3
Figure 4

(A) 

|    | N-terminal (lumen) | Transmembrane domain | C-terminal (cytoplasm) |
|----|--------------------|-----------------------|------------------------|
| wt |                    |                       |                        |
| NΔ16 |                    |                       |                        |
| NΔ31 |                    |                       |                        |
| NΔ54 |                    |                       |                        |
| NΔ81 |                    |                       |                        |

(B) 

EndoH
- + - -

Dpl1p  Dpl1p  Dpl1N6Ap

(C) 

SD-leu+EtOH  SD-leu+50 μM PHS

wt  dpl1Δ  DPL1  NΔ16dpl1  NΔ31dpl1  NΔ54dpl1  dpl1N6A

(D) 

1 2 3 4

anti-Flag

anti-Hexokinase
A

N-terminal  Transmembrane C-terminal

1  

59 Y L F V I F C Y K L I S N F F Y L L K V Y G P  81

59

B

|               | SD-leu+EtoH | SD-leu+50µM |
|---------------|-------------|-------------|
| wt            |             |             |
| dpl1∆        |             |             |
| DPL1         |             |             |
| N81∆dpl1     |             |             |
| dpl1C65L     |             |             |
| dpl1K67L     |             |             |
| dpl1S70L     |             |             |
| dpl1N71L     |             |             |
| dpl1CKSN_LLLL|             |             |
| dpl1C65K67LL |             |             |
| dpl1K67S70LL |             |             |
| dpl1S70N71LL |             |             |

C

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |

anti-Flag

anti-Hexokinase

D

|               | SD-leu+EtoH | SD-leu+50µM |
|---------------|-------------|-------------|
| wt            |             |             |
| dpl1∆        |             |             |
| DPL1         |             |             |
| dpl1CKSN_LLLL|             |             |
| wt+dpl1CKSN_LLLL|         |             |
Figure 7

Panel A: Fluorescence (FL) and differential interference contrast (DIC) images of cells expressing different Dpl1 variants. 

Panel B: Yeast colony assays showing growth on SD-leu+EtOH and SD-leu+50 µMPHS media for wild type (wt), dpl1Δ, DPL1, NΔ31dpl1, dpl1CKSN_LLLL, and NΔ31dpl1CKSN_LLLL mutants.

Panel C: Western blots using anti-Flag and anti-Dpm1 antibodies for samples 1 to 5.
Figure 8
Figure 9