A 25-Amino Acid Sequence of the Arabidopsis TGD2 Protein Is Sufficient for Specific Binding of Phosphatidic Acid*§

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Genetic analysis suggests that the TGD2 protein of Arabidopsis is required for the biosynthesis of endoplasmic reticulum derived thylakoid lipids. TGD2 is proposed to be the substrate-binding protein of a presumed lipid transporter consisting of the TGD1 (permease) and TGD3 (ATPase) proteins. The TGD1, -2, and -3 proteins are localized in the inner chloroplast envelope membrane. TGD2 appears to be anchored with an N-terminal membrane-spanning domain into the inner envelope membrane, whereas the C-terminal domain faces the intermembrane space. It was previously shown that the C-terminal domain of TGD2 binds phosphatidic acid (PtdOH). To investigate the PtdOH binding site of TGD2 in detail, the C-terminal domain of the TGD2 sequence lacking the transit peptide and transmembrane sequences was fused to the C terminus of the Discosoma sp. red fluorescent protein (DR). This greatly improved the solubility of the resulting DR-TGD2C fusion protein following production in Escherichia coli. The DR-TGD2C protein bound PtdOH with high specificity, as demonstrated by membrane lipid-protein overlay and liposome association assays. Internal deletion and truncation mutagenesis identified a previously undescribed minimal 25-amino acid fragment in the C-terminal domain of TGD2 that is sufficient for PtdOH binding. Binding characteristics of this 25-mer were distinctly different from those of TGD2C, suggesting that additional sequences of TGD2 providing the proper context for this 25-mer are needed for wild type-like PtdOH binding.

Many plants, including Arabidopsis, have two parallel pathways of thylakoid lipid biosynthesis involving enzymes at the inner plastid envelope or the endoplasmic reticulum (ER), respectively (1, 2). In Arabidopsis, the two pathways contribute nearly equally to the bulk of galactoglycerolipids (3), which are the predominant lipids in thylakoid membranes. Synthesis by the ER pathway involves the assembly of phosphatidic acid (PtdOH) and phosphatidylcholine (PtdCho) at the ER from fatty acids synthesized in the chloroplast. The current hypothesis suggests that PtdCho returns to the outer plastid envelope, where it is converted to PtdOH by a phospholipase D (4). The TGD1, -2, and -3 proteins are postulated to be involved in the transfer of PtdOH from the outer envelope membrane to the inside of the inner envelope membrane (5–8), where PtdOH is dephosphorylated by a PtdOH phosphatase to diacylglycerol, which is the precursor of galactoglycerolipid biosynthesis at the inner chloroplast envelope membrane. Alternatively, nascent fatty acids are assembled de novo at the chloroplast inner envelope into PtdOH, which then enters galactoglycerolipid biosynthesis, as described above. In either pathway, PtdOH is a key intermediate for thylakoid lipid biosynthesis.

The TGD1, -2, and -3 proteins resemble the permease, substrate binding, and ATPase subunits of a multipartite ABC transporter, which is localized in the inner chloroplast envelope (5–8). Mutants with reduced function of these proteins have complex lipid phenotypes. They accumulate diagnostic oligogalactolipids, such as trigalactosyldiacylglycerol, giving rise to their name, and triacylglycerols in their leaves. Moreover, galactolipid molecular species derived from the ER pathway are underrepresented, and pulse-chase labeling experiments indicate a reduced flux through the ER pathway in the mutants.

TGD2 appears anchored with its N-terminal domain into the inner envelope membrane such that the C terminus faces the intermembrane space (5). The C terminus of TGD2 was shown to specifically bind PtdOH. The TGD2 protein does not share sequence similarity with known PtdOH-binding proteins or domains. However, it does contain a mycobacterial cell entry (MCE) domain required for mycobacterial entry into host cells (9).

For the few cellular protein targets of PtdOH described thus far, no uniform PtdOH binding motif has emerged, making the reliable prediction of protein-PtdOH interactions or PtdOH binding domains difficult. In mammalian cells, protein kinase Raf-1 (10, 11), protein phosphatases SHP-1 (12) and PP1 (13), and protein kinase Ce (14) are among the best studied PtdOH-binding proteins. In yeast, the SNARE protein Spo20p (15) and the inositol-regulated transcriptional repressor Opi1p (16) are recognized as PtdOH-binding proteins.

At this time, even fewer PtdOH-binding proteins have been identified in plants. The PtdOH-binding proteins ABI1 (ABA-insensitive 1) (17) and PDK1 (phosphoinositide-dependent kinase 1) (18) were only recently reported. ABI1 is a protein phosphatase 2C that negatively regulates signaling of the growth regulator abscisic acid. Arabidopsis PDK1 is a protein kinase that binds both PtdOH and phosphoinositides (19), but...
its kinase is activated only by PtdOH (18). Additional PtdOH targets were isolated from Arabidopsis cell lysates employing a PtdOH analogue affinity matrix followed by mass spectrometry analysis (20). Phosphoenolpyruvate carboxylase was identified and shown to bind preferentially PtdOH over other phospholipids (20). The TGD2 protein contains a new PtdOH binding site, which we characterized and delineated as described below.

EXPERIMENTAL PROCEDURES

Expression and Purification of DR-TGD2 Fusion Proteins—All TGD2 truncated proteins used in this study were derived from the TGD2-dTMD-pQE31 plasmid (also known as TGD2C-pQE31) using a PCR-based strategy (5). Following digestion with Ncol and Xhol, the PCR fragment was ligated into the DsRed-plw01-His plasmid (provided by Dr. Michael Garavito, Michigan State University) to produce Discosoma sp. red fluorescent protein (DR)-TGD2C fusion proteins. Internal deletion mutants and/or point mutants were generated by site-directed mutagenesis applied to TGD2C-DsRed-plw01-His using a PCR-based protocol, with the primers and mutation sites listed in supplemental Table 1. All fusion proteins were expressed in Escherichia coli strain BL21 (DE3) (Novagen, Madison, WI). An overnight preculture of LB medium (5 ml) was used to start a 200-ml culture in LB medium. The protein was induced with 50 μM isopropyl-β-D-thiogalactopyranoside at an A600 of 0.6–0.8 and 16 °C, and growth was continued overnight. Cultures were cooled to 4 °C, washed twice, and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole). The cells were lysed by sonication, followed by centrifugation at 18,000 × g. The resulting supernatant was applied to a Ni2+-nitrilotriacetic acid-agarose column at 37 °C for 1 h, followed by vigorous vortexing for 5 min. The liposomes were precipitated at 20,000 × g and washed twice with ice-cold TBS. Liposomes (250 μg) were mixed with purified DR-TGD2 fusion protein and TBS to make a final 100-μl solution. The mixture was incubated at 30 °C for 30 min and washed twice with ice-cold TBS by centrifugation at 20,000 × g at 4 °C. The liposome pellet mixed with sample buffer was analyzed by SDS-PAGE (22). Immunodetection of the His-tagged protein was accomplished using the above-mentioned penta-His antibody at 1:10,000 and the antimouse antibody at 1:50,000 dilution. For visualization and quantification, a chemiluminescence detection kit from Sigma was used.

Phylogenetic Analysis of TGD2—Full-length TGD2 amino acid sequences were searched against the National Center for Biological Information nonredundant protein database using the BLAST tool (23), and the resulting sequences with high similarities and identities were aligned using ClustalX software (version 2.0.10) (24). Generation of the bootstrapped phylogenetic tree was performed using the PHYLIP software package, as previously described (25).

Protein-lipid Overlay Assay—Membrane lipid strips were purchased from Echelon Biosciences (Salt Lake City, UT). The strips were first blocked with 3% bovine serum albumin in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.25% Tween 20) for 2 h and incubated in 0.5 μg/ml DR-TGD2 fusion protein solution in the blocking buffer at 4 °C overnight. The strips were washed three times for 10 min each with TBST and soaked in 3% bovine serum albumin in TBST with a penta-His mouse monoclonal antibody (Sigma) at a 1:2,000 dilution at 4 °C overnight. The strips were washed twice with TBST and soaked in 3% bovine serum albumin in TBST with horseradish peroxidase-conjugated anti-mouse antibody (Bio-Rad) at 1:20,000 dilution for 1 h at room temperature. Following washing with TBST for 1 h, the protein was detected by using a chemiluminescence detection system (Sigma).

Liposome Association Assay—The liposome association assay was performed according to Ref. 26. Lipids (dioleylphosphatidylcholine or dioleyl-phosphatidic acid) were dried under a stream of nitrogen and incubated in TBS (50 mM Tris-HCl, pH 7, 0.1 M NaCl) at 37 °C for 1 h, followed by vigorous vortexing for 5 min. The liposomes were precipitated at 20,000 × g and washed twice with ice-cold TBS. Liposomes (250 μg) were mixed with purified DR-TGD2 fusion protein and TBS to make a final 100-μl solution. The mixture was incubated at 30 °C for 30 min and washed twice with ice-cold TBS by centrifugation at 20,000 × g at 4 °C. The liposome pellet mixed with sample buffer was analyzed by SDS-PAGE (22). Immunodetection of the His-tagged protein was accomplished using the above-mentioned penta-His antibody at 1:10,000 and the antimouse antibody at 1:50,000 dilution. For visualization and quantification, a chemiluminescence detection kit from Sigma was used.

Quantitative Analysis of Immunoblots—Quantification and detection of signals on protein immunoblots were done by two methods: 1) by film-based autoradiography followed by densitometry scanning, in which distinct protein bands were quantified using the computer software Multi Gauge version 3.0 (Fuji Film USA, Valhalla, NY), and 2) using a camera-based gel documentation system (LAS-3000; Fuji Film Life Science USA, Stamford, CT). The linear range for detection extended over 4 orders of magnitude for the camera-based system as compared with 1.5 orders of magnitude for the film-based system. Linearity of the response of the respective detection system was established using a serial dilution of a test protein. Under the conditions used, both detection systems were in the linear range of detection and gave similar results during quantification of signals and were used interchangeably as indicated. The data obtained by both methods were fit to a Hill equation modified for ligand binding data (10, 27), using OriginPro8 (Origin Lab Corp., Northampton, MA). Relevant variables calculated by this method were k (indicating the PtdOH lipid fraction at half-maximal binding) and the Hill coefficient n (indicating cooperativity).
Phosphatidic Acid Binding to TGD2

Specific Binding of the DR-TGD2C WT Fusion Protein to PtdOH—A His-tagged version of the C-terminal domain of TGD2, TGD2C-His, was found to specifically bind PtdOH (5). This truncated TGD2 protein was difficult to solubilize, and mutant forms were completely insoluble when the respective constructs were expressed in E. coli. To overcome this problem, we fused the C-terminal TGD2 domain to the C terminus of an engineered monomeric DR (28). Contrary to maltose-binding protein and glutathione S-transferase that were initially considered as fusion partners for TGD2C, DR did not show lipid binding by itself in the protein-lipid overlay or liposome binding assays that were employed in this study (see supplemental Figs. 1 and 2). However, the soluble DR-TGD2C fusion protein specifically bound to PtdOH, as demonstrated in the lipid overlay assay shown in supplemental Fig. 1, confirming previous results on the TGD2C-His-tagged protein (5). Moreover, deletion and point mutant variants of the DR-TGD2C protein encoding constructs generally expressed well in E. coli, and the resulting proteins were soluble.

To obtain corroborating and more quantitative evidence for PtdOH binding, a liposome binding assay was used. Purified recombinant proteins were incubated as described under “Experimental Procedures” with liposomes of different lipid composition. Following centrifugation, proteins bound to the liposomes were associated with the lipid pellet, whereas non-binding proteins remained in the supernatant (Fig. 2A). Because lipids are not soluble in water, PtdOH was mixed at different w/w ratios with PtdCho to determine the weight % fraction of PtdOH required for binding to DR-TGD2C. This fraction provided an indication of the affinity of the DR-TGD2C wild-type protein to PtdOH and allowed a quantitative comparison with mutant versions of the TGD2C protein.
The DR-TGD2C wild-type fusion protein was found to bind liposomes consisting of PtdCho, PtdOH lipid mixtures, depending on the fraction of PtdOH (Fig. 2A, left). On the contrary, DR alone was exclusively present in the supernatant and did not bind to the liposomes (Fig. 2A, right). At the protein concentration tested (1 μg of total protein), a significant increase in binding between 30 and 40% PtdOH was observed. In Fig. 2B, the blot was analyzed using a digital camera system that shows linear responses over 4 orders of magnitude. The individual signals were quantified, and the resulting data were plotted and fit to the Hill equation modified for receptor-ligand binding (10, 27). Because we established that the detection system itself was not saturated under the conditions used (see “Experimental Procedures”), we interpreted the observed saturation as true saturation of binding of the protein to the liposomes. When the data were fit to the modified Hill plot, half maximal binding at 39.6% of PtdOH was apparent. Using the film-based detection methods, a similar value was obtained (see below). This value is comparable with the results obtained for RafC-PtdOH association (20 mol % PtdOH) (10). From the binding plot, a Hill number of 5.7 was calculated, suggesting positive cooperativity (Fig. 2B). Again, this value is similar to that obtained for RafC-PtdOH interaction (Hill number between 3.3 and 6.2) (10). It seems likely that TGD2 is forming a homomultimer, showing cooperativity of PtdOH binding among the involved C-terminal TGD2 domains.

Identification of TGD2C Amino Acid Sequences Necessary for PtdOH Binding—Because most previously described amino acid sequences involved in PtdOH binding lack recognizable similarities at the primary sequence level (29), a deletion and truncation approach was used to systematically identify sequences necessary for PtdOH binding by TGD2C. The described liposome binding assay was sufficiently quantitative to compare PtdOH binding with different versions of DR-TGD2C. Pure PtdOH liposomes were used which gave maximal binding of DR-TGD2C (see Fig. 2B). In parallel, a negative control was included using pure PtdCho liposomes. To test for specificity of binding, liposomes containing 50% (w/w) PtdOH and PtdCho were included as well. All DR-TGD2C mutant proteins described here were soluble and more than 90% pure based on Coomassie Brilliant Blue protein gel staining (see supplemental Fig. 2). Schematic representations of the mutant proteins and the respective binding data are shown in Fig. 3. In an initial set, five truncated mutant versions ranging in length from 130 to 180 amino acids across the length of the TGD2C sequence (Fig. 3, B and C) were tested for binding. All of the truncated proteins (DR-TGD2C T1–T5; Fig. 3, B and C) showed significant PtdOH binding to pure PtdOH liposomes, suggesting that a fragment from residues 221–250 common to these fragments might be involved in PtdOH binding.

In a second set of experiments, two internal deletion mutants probing the 221–250 sequence were generated (DR-TGD2C D1 and D2; Fig. 3C) and tested for PtdOH binding. Surprisingly, deleting the entire segment (residues 221–250, DR-TGD2C D1; Fig. 3C) did not affect binding, whereas deleting a smaller frag-
ment within this segment (residues 221–225, DR-TGD2C D2; Fig. 3C) diminished binding of the fusion protein to PtdOH. It seems possible that DR-TGD2C D1 with the larger deletion folds just right to still reconstitute a nearby site, whereas the smaller deletion in DR-TGD2C D2 does not allow reconstitution.

A TGD2 Minimal Domain Sufficient for PtdOH Binding—Coarse mapping described above suggested peptide sequences overlapping or surrounding residues 221–225 as a major contributing factor to PtdOH binding by TGD2. Fine mapping was initiated from residue 225 in both directions, starting with the fragment containing residues 119–225, which fused to DR (DR-TGD2C T6; Fig. 4A). This fragment tested positive for PtdOH binding with liposomes. Truncations were made from the N terminus of DR-TGD2C T6 to narrow down this PtdOH binding site in the TGD2 protein. The two truncation clones DR-TGD2C T7 and T8 still showed binding to PtdOH (Fig. 4A). Apparently, the clones DR-TGD2C T6–T8 have a 25-amino acid peptide (residues 201–225) in common that is sufficient to

FIGURE 3. Identification of a PtdOH binding domain in TGD2C. A, primary structure of TGD2C, indicating a predicted transit peptide (TP), transmembrane domain (TMD), and a conservative MCE domain. B and C, series of deletion and truncation mutants were generated for TGD2C and fused to the C terminus of the DR open reading frame. The black oval represents the DR carrier protein; gray bars represent deletions. Liposome association assays were performed to assess binding of various mutants to PtdCho and PtdOH/PtdCho mixtures or PtdOH liposomes. Fluorograms of Western blots are shown. PtdOH-specific binding data are summarized on the right based on visually examined signal intensity (+ + + + +, ++++, ++++, ++, +, and −, diminishing signal intensity; −, no binding).

FIGURE 4. Fine mapping of a TGD2 minimal PtdOH binding sequence. A, truncation mutants fused to DR focusing on the PtdOH binding domain. PtdOH binding activity was assessed by a liposome association assay. B, specificity of the minimal PtdOH binding domain fused to DR was verified by a protein-lipid overlay assay using a phospholipid-containing membrane strip. LPtdOH, lysophosphatidic acid; LPtdCho, lysophosphatidylcholine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; S1P, sphingosine 1-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine.

C, PtdOH binding of point mutations of the minimal PtdOH binding domain fused to DR shown by a liposome association assay with 100% PtdOH liposomes. A section (residues 201–225) of the same sequence alignment as in Fig. 1 is shown. Point mutations in the sequence are indicated by arrows, P1, DR-TGD2C T8 R204A; P2, DR-TGD2C T8 N205A; P3, DR-TGD2C T8 E209A; P4, DR-TGD2C T8 H216A; P5, DR-TGD2C T8 E218A; P6, DR-TGD2C T8 E219A; P7, DR-TGD2C T8 K221A; P8, DR-TGD2C T8 E222A.
mediate binding to PtdOH. Specificity of PtdOH binding by the minimal fragment DR-TGD2C T8 was confirmed using the protein-lipid overlay assay applied to a membrane containing multiple lipids (Fig. 4B).

Probing from TGD2 amino acid 221 toward the C terminus, a fragment consisting of residues 221–250 (DR-TGD2C T9; Fig. 4A) did not show binding of PtdOH when fused to DR, indicating that this sequence might not play a direct role in PtdOH binding. This result was consistent with the previous observation that DR-TGD2C D1 lacking this specific fragment still binds PtdOH (Fig. 3C).

In the few cases in which PtdOH binding sites have been analyzed in detail, basic amino acids or tryptophan were found to be critical for PtdOH binding (29). In particular, a recent study suggested that electrostatic interactions of PtdOH with basic amino acids (i.e. lysine and arginine), along with hydrogen bond interactions, form the basis of the specific binding of PtdOH to its cognate targets (30). Similarly, we set out to map PtdOH to its cognate targets (30). Similarly, we set out to map

FIGURE 5. Reduced affinity of the minimal PtdOH binding domain for PtdOH. A, PtdOH binding for DR-TGD2C (wild type). B, PtdOH binding for DR-TGD2C T8 carrying the 201–225 residue minimal domain. C, quantification of relative binding of PtdOH for DR-TGD2C and DR-TGD2C T8. D, PtdOH binding for DR-TGD2C T8, E, PtdOH binding for DR-TGD2C D3 (DR-TGD2C with deletion of minimal PtdOH binding domain residues 201–225). F, quantification of relative binding of PtdOH for DR-TGD2C T8 and DR-TGD2C D3. Liposome association assays were performed. Data shown in A–C were obtained in the same representative experiment; likewise, data obtained in D–F were obtained in the same representative experiment.

shown in Fig. 4C and were tested for PtdOH binding using pure PtdOH liposomes. Of all of the point mutants analyzed, only the substitution in DR-TGD2C T8 K221A drastically reduced PtdOH binding. No detectable binding to pure PtdCho liposomes was observed for any of the point mutants (data not shown). Taken together, these data suggest that TGD2 residues 201–225 represent a previously undescribed PtdOH binding domain, which is localized near but not in the MCE domain. Lysine 221, a positively charged amino acid at physiological conditions, seems to be a critical residue within this PtdOH binding domain that might be required for electrostatic interactions with the negatively charged phosphoryl group of PtdOH. PtdOH Binding Properties of the Minimal PtdOH Binding Domain—Positive cooperativity of ligand binding to its target, as observed for PtdOH binding by DR-TGD2C (Fig. 2B), usually requires the interaction of multiple protein subunits or protein domains. On the other hand, the minimal 25-amino acid PtdOH binding domain represented by DR-TGD2C T8 (residues 201–225) was hardly expected to show this cooperativity. To test this hypothesis, we measured PtdOH binding to DR-TGD2C T8 (see Fig. 5, B and C) using PtdCho/PtdOH mixed liposomes and pure lipid liposomes. For consistency, we analyzed DR-TGD2C in parallel as well (Fig. 5, A and C). In this experiment, we used film-based detection and quantification of the Western signal, as described under "Experimental Procedures," and obtained results for DR-TGD2C (Fig. 5, A and C; k = 37.7%, n = 2.5) very similar to results observed for camera-based detection (Fig. 2B; k = 39.6%, n = 5.7). Binding of DR-TGD2C T8 to PtdOH was much weaker compared with DR-TGD2C to the extent that interpretations regarding cooperativity were inconclusive (Fig. 5C), and a fit to a Hill plot was not attempted.

We also constructed an additional DR-TGD2C D3 deletion mutant lacking only residues 201–225 and compared it directly with DR-TGD2C T8 carrying only residues 201–225 (Fig. 5, D–F). The deletion mutant DR-TGD2C D3 still had residual binding activity but less than observed for DR-TGD2C T8 carrying the identified 25-amino acid minimal PtdOH binding domain. When the data were fit to a Hill plot, positive cooperativity was indicated (Fig. 5F; k = 53%, n = 8.2). Again, the analysis of DR-TGD2C T8 gave data too noisy to draw conclusions regarding cooperativity (Fig. 5F). Taken together, these data suggest that TGD2C residues 201–225 are important to PtdOH binding and sufficient by themselves. They may repre-
Phosphatidic Acid Binding to TGD2

A crystal structure for TGD2 binding to its ligand PtdOH will ultimately be required to map the three-dimensional relationships between TGD2 amino acid residues and the ligand. In the absence of such a structure, systematic functional dissection of the primary amino acid sequence offers a valuable opportunity to learn more about this interaction. Previous genetic evidence has provided a solid foundation for the next logical step to learn more about this interaction. The primary amino acid sequence offers a valuable opportunity to learn more about this interaction.

To test the possibility of secondary PtdOH binding sites or residues participating in PtdOH binding by TGD2, an additional series of mutants with truncated sequences starting either from the C terminus (residue 381) or from the center of the protein (residue 204) were generated, fused to DR, and tested in pure PtdOH liposome binding assays as shown in Fig. 6. Comparing the PtdOH binding activity of the different truncation clones, three regions were identified between residues 161 and 204, 251 and 300, or 291 and 340 that could contribute to binding of PtdOH to TGD2 outside of the core segment represented by residues 201–225.

DISCUSSION

A crystal structure for TGD2 binding to its ligand PtdOH will ultimately be required to map the three-dimensional relationships between TGD2 amino acid residues and the ligand. In the absence of such a structure, systematic functional dissection of the primary amino acid sequence offers a valuable opportunity and the next logical step to learn more about this interaction. Previous genetic evidence has provided a solid in vivo framework for the function of TGD2 as the possible substrate-binding protein of an ABC-type transporter hypothesized to be involved in ER-to-plastid lipid trafficking and required for chloroplast development (5). Other proteins involved in this process constitute the permease (TGD1) and ATPase (TGD3) of this presumed lipid ABC-type transporter in the inner plastid envelope (4). The evidence to date that this system transports PtdOH is still indirect and is based on the observations that the tgd1 mutant of Arabidopsis accumulates PtdOH and that isolated tgd1 plastids show reduced incorporation of PtdOH into plastid lipids (8) and, most convincingly, that TGD2 specifically binds PtdOH (5). TGD2 appears anchored with a transmembrane domain into the inner envelope membrane, but a major C-terminal domain presumably faces the intermembrane space (5). The fact that the TGD2 wild-type protein is resistant to the protease trypsin, which penetrates the outer envelope but not the inner envelope membrane of chloroplasts, but that a mutant TGD2 protein and a green fluorescent protein fused to TGD2 are sensitive to the protease suggests that the TGD2 protein is in a trypsin-inaccessible location, possibly in a junction between the inner and outer envelope membrane. Taken together, these observations allow us to hypothesize that TGD2 might provide the substrate PtdOH to the TGD1-TGD3 complex in the inner envelope membrane by extracting PtdOH from the intermembrane leaflet of the outer plastid envelope membrane. Although this hypothesis is difficult to prove at this time, one step toward a better understanding of TGD2 function is a delineation of its actual PtdOH binding site and its mode of PtdOH binding. Important questions are whether the PtdOH binding site of TGD2 is localized in the intermembrane space, whether it is close to the predicted transmembrane domain and, therefore, close to the inner plastid envelope membrane, and whether it is synonymous to the MCE domain, for which a molecular function is not yet known but which appears to be involved in membrane penetration by mycobacterial pathogens (9).

The initially encountered protein solubility issue was solved by fusing the TGD2C protein lacking the predicted N-terminal membrane-spanning domain to an engineered highly soluble monomeric DR carrier protein (28). It should be noted that neither glutathione S-transferase nor maltose-binding protein were useful for this purpose, because in our hands both carrier proteins showed significant lipid binding above background on their own. However, DR does not bind lipids by itself. We generally tested binding of PtdOH to the fusion proteins using two assays, a protein-lipid overlay assay providing a measure for the specificity of binding and a liposome binding assay providing a semi-quantitative assessment of the affinity of TGD2 or its mutant derivatives for PtdOH. To avoid misinterpretation of the binding data, care was taken to stay within the linear range of the assays. Bearing the technical limitations of the available approaches in mind, we were able to delineate a 25-amino acid stretch in the TGD2 protein that was sufficient for PtdOH binding although with reduced affinity. Upon generation of a point mutant (K221A) within the minimal domain, PtdOH binding was diminished, identifying lysine 221 as a critical residue involved in this process. This finding is consistent with other work proposing basic amino acids and/or tryptophan as critical residues for PtdOH binding (29, 30).

In the reverse experiment, deleting residues 201–225 from TGD2C did not fully abolish PtdOH binding activity (Fig. 5, E and F), suggesting the presence of other, secondary amino acids involved in PtdOH binding. The presence of six proline residues in the identified primary binding site represented by TGD2 residues 201–225 suggests that this minimal region forms a loop-strand fold lacking helical or β-strand structure,
as indicated by secondary structure prediction (Fig. 1B). Additional elements are clearly required to hold this loop in place and enable the TGD2 protein to bind PtdOH in a cooperative manner, as suggested by the DR-TGD2C binding data.

In general, PtdOH binding sites of different proteins lack conservation in their primary structures (29). Likewise, the observed TGD2 PtdOH binding site does not follow an established pattern. Presumed orthologs of TGD2 (Fig. 1) show more similarity in sequences outside the identified minimal PtdOH binding site than within it. N-terminally adjacent to the minimal PtdOH binding is the MCE domain, a highly conserved feature initially found in mycobacterial cell surface proteins required for entry of the pathogen into the host cell (9). This study clearly shows that the MCE domain is not required for PtdOH binding and must have other functionalities that nevertheless could lead to cell membrane fusion or penetration, as would be necessary for mycobacterial entry into mammalian host cells. Whether the close proximity of the two sequence features in TGD2 is necessary for the function of the protein can only be speculated upon at this time. The central location of the identified binding domain ~100 residues C-terminal of the predicted membrane-spanning domain does not rule out the possibility that TGD2 could extract PtdOH out of the outer envelope membrane assuming that it is anchored with its N terminus into the inner envelope membrane. How PtdOH would be formed at the outer envelope remains unclear. However, the recent identification of the TGD4 protein involved in ER-to-plastid lipid trafficking, which is the first TGD protein associated with the ER (31), provides new avenues toward an understanding of PtdOH formation from ER-derived precursors in predicted contact zones between the ER and the outer plastid envelope membrane.

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