Lipid phosphorylation takes place within the chloroplast envelope. In addition to phosphatidic acid, phosphatidylinositol phosphate, and their corresponding lyso-derivatives, we found that two novel lipids underwent phosphorylation in envelopes, particularly in the presence of carrier-free $[\gamma^{32}\text{P}]\text{ATP}$. These two lipids incorporated radioactive phosphate in chloroplasts in the presence of $[\gamma^{32}\text{P}]\text{ATP}$ or $[^{38}\text{P}]\Pi$, and light. Interestingly, these two lipids were preferentially phosphorylated in envelope membranes in the presence $[\gamma^{32}\text{P}]\text{CTP}$, as the phosphoryl donor, or $[\gamma^{32}\text{P}]\text{ATP}$, when supplemented with CDP and nucleoside diphosphate kinase II. The lipid kinase activity involved in this reaction was specifically inhibited in the presence of cytosine 5'-O-(thiotriphosphate) (CTP)S and sensitive to CTP chase, thereby showing that both lipids are phosphorylated by an envelope CTP-dependent lipid kinase. The lipids were identified as phosphorylated galactolipids by using an acid hydrolysis procedure that generated galactose 6-phosphate. CTPS did not affect the import of the small ribulose-bisphosphate carboxylase/oxygenase subunit into chloroplasts, the possible physiological role of this novel CTP-dependent galactolipid kinase activity in the chloroplast envelope is discussed.

One of the main functions assigned to the chloroplast is its ability to assimilate $\text{CO}_2$ under illumination. This key activity must be separated from the rest of the cell by a selective membrane barrier, namely the envelope, which is one of the three main plastid compartments in addition to thylakoids and the stroma. The envelope is constituted of two membranes, the inner and the outer envelopes, each having its own specific characteristic and property (1).

Lipid phosphorylation occurs in chloroplast envelope membranes (2). The first identified lipids incorporating phosphate from $[\gamma^{32}\text{P}]\text{ATP}$ in isolated envelope vesicles were lysophosphatidic acid (LPA), $^1$ phosphatidic acid (PA), phosphatidylinositol phosphate (PIP), and lysophosphatidylinositol phosphates (LPIPs). At the present time, the functions of these phospholipids are not known in chloroplasts, but: (i) they might produce substrates for lipid biosynthesis pathways; for instance, PIP can serve as substrate for a phosphatidylinositol-4,5-phosphate kinase and eventually can be hydrolyzed in second messengers like inositol 1,4,5-trisphosphate and DAG (3); (ii) they could directly interact with intracellular proteins to affect their location and/or activity; (iii) they might also change the local topology of other lipids, thereby modifying electrostatic interactions of membrane components. Indeed, in mammalian systems, LPA and PA, as minor cell lipids, are likely to act as potent activators of plasma membrane tyrosine kinase via $\text{G}$ protein activation and intracellular protein kinases (4, 5). PA is the product of $\text{s}$-diacylglycerol phosphorylation catalyzed by a diacylglycerol kinase (6). In plant signaling pathways, PA is also derived from $\text{PC}$ via phospholipase D (7) and from diacylglycerol pyrophosphatase (8). The production of LPA usually occurs after a first rapid accumulation of DAG, followed by phosphorylation, and activation of a PA-specific phospholipase $\text{A}_2$ within the plasma membrane (9). In plants, a diacylglycerol kinase from Arabidopsis thaliana (10) and LPA, a product of inducible PLA$_2$ (11), have been identified, but their specific role as second messengers has still not been confirmed. The presence of PIP and its lyso-derivative has also been reported in plant plasma membranes (12). Although PIP is likely to be a substrate for PIP kinase (13) and LPIP is possibly generated by an endo-phospholipase $\text{A}_2$, their respective roles have not yet been elucidated in plants.

The presence of lipid kinase activities in chloroplast envelope membranes suggests that signal transduction pathways and/or membrane protein regulation occur in envelopes. This possibility is supported by the fact that small $\text{G}$ proteins are present in envelope membranes (14), as well as specific protein kinase activities (15). Such response pathways should therefore occur for cross-talk between stroma and cytosol. Envelopes are also known to be a major site for lipid biogenesis, translocation of nuclear encoded proteins and metabolites, and coordination of the expression of nuclear and plastid genomes (1). Therefore, all these activities have to be controlled by fine tuning such as membrane topology and posttranslational modification that may involve lipid phosphorylation activities.

cycliglycerol; DQpDG, digalactosyldiacylglycerol; LMGpDG, lysomonomogalactosyldiacylglycerol; MGDG, monogalactosyl diacylglycerol; MGDG, monogalactosyldiacylglycerol; NDPK, nucleoside diphosphate kinase; PEI, polyethyleneimine; PLA$_2$, phospholipase $\text{A}_2$; PSSu, precursor protein of the ribulose-bisphosphate carboxylase/oxygenase small subunit; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; Mops, 4-morpholinopropanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; ATP$^\ast$, adenosine 5'-O-(thio-triphosphate); CTPS, cytosine 5'-O-(thiotriphosphate).

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* This work was supported in part by Swiss National Science Foundation Grant 3100.043297.95. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ This work was performed in partial fulfillment of the requirements for the doctoral program, Université de Neuchâtel, Neuchâtel Switzerland.

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$^*$ The abbreviations are: LPA, lysophosphatidic acid; PA, phosphatidic acid; PIP, phosphatidylinositol phosphate; LPIP, lysophosphatidylinositol phosphate; Chl, chlorophyll; DAG, diacylglycerol; DGDG, digalactosyl diacylglycerol; DQpDG, digalactosyldiacylglycerol; LMGpDG, lysomonomogalactosyldiacylglycerol; MGDG, monogalactosyl diacylglycerol; MGDG, monogalactosyldiacylglycerol; NDPK, nucleoside diphosphate kinase; PEI, polyethyleneimine; PLA$_2$, phospholipase $\text{A}_2$; PSSu, precursor protein of the ribulose-bisphosphate carboxylase/oxygenase small subunit; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; Mops, 4-morpholinopropanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; ATP$^\ast$, adenosine 5'-O-(thiotriphosphate); CTPS, cytosine 5'-O-(thiotriphosphate).
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CTP-dependent Lipid Phosphorylation in Chloroplast Envelopes

Interestingly, other envelope membrane lipids such as galactolipids (MGDG, DGDG, and trigalactosyldiacylglycerol) and phosphatidylglycerol molecular species (16, 17) are also putative substrates for phosphorylation, because both lipid classes have at least one free hydroxyl group that is able to receive phosphate from a phosphodiester high energy bond. The present report documents for the first time a CTP-dependent galactolipid kinase activity in chloroplast envelope membranes.

EXPERIMENTAL PROCEDURES

Preparation of Plasma Membranes, Envelope, and Thylakoid Membranes—Spinach plasma membranes were a kind gift of Dr. C. Larsson (Department of Plant Biochemistry, Lund University, Lund, Sweden). Spinach (Spinacia oleracea L.) leaves were purchased from the local market and intact chloroplasts purified according to Mouririou and Douce (18). Thylakoid membranes were exempted from envelope membrane contamination by using a 5% Percoll gradient as described previously (19). Envelope membranes were isolated from intact chloroplasts according to the method of Douce and Joyard (20). Inner and outer envelope membranes were prepared according to the method of Keegstra and Youssif (21), modified by Siegenthaler and Dumont (22). The protein concentrations of plasma and envelope membranes were determined using the methods described previously (23, 24).

Lipid Kinase Assay and Two-phase Extraction of Membrane Lipids—Envelope membranes (200 μg) were incubated in the presence of 10 μCi of [γ-32P]ATP (3000 Ci/mmol), 5 mM MgCl2, and 50 mM Mops (pH 7.6) at 25 °C for 5 min in a final volume of 200 μl. The lipid kinase reaction was stopped by mixing the samples with 1.5 ml of cold chloroform/methanol (1:2). After adding the chloroform/methanol to phosphorylated envelopes, 100 nmol of PA (Sigma), 100 nmol of LPA (Sigma), and 600 nmol (1:2) of phospholipid mixture containing PIPE2, PIIP, phosphatidylinositol, and phosphatidylserine (Sigma) were added as carriers. Lipid extraction was carried out by immediately adding 0.8 ml of HCl/EDTA-Na2 (1.25 N/0.5 mM) and 0.5 ml of cold chloroform and vortexing thoroughly. The two-phase separation was achieved by centrifugation at 3000 × g for 30 min. The upper phase containing the labeled lipids was frozen in liquid nitrogen and dissolved in 100 μl of chloroform/methanol (3:1). Phospholipid Separation by TLC—Envelope membranes using a mixture of carrier-free [γ-32P]CTP (50 mM Mops-NaOH (pH 7.6), 5 mM MgCl2 and 1 unit of yeast hexokinase (EC 2.7.1.1, Sigma)) at 25 °C for 10 min and spotted on plates.

Chloroplast Protein Import—[35S]-Met-labeled pSSu was synthesized from pSSu/pET8c using the TNT quick coupled transcription/translation system (Promega). Chloroplasts were isolated from spinach leaves and used for the import of the labeled translation products under standard conditions (29). The quantitative determination of chloroplast membranes using a mixture of carrier-free [γ-32P]ATP, 50 mM Mops-NaOH (pH 7.6), 5 mM MgCl2 and 1 unit of yeast hexokinase (EC 2.7.1.1, Sigma) at 25 °C for 10 min and spotted on plates.

RESULTS

Lipid Phosphorylation in Chloroplast Envelope Membranes—We have previously identified four phosphorylated lipids in envelope membranes, namely PA, LPA, PIP, and LPIP (2). They were originally found after 32P-labeling of envelope membranes using [γ-32P]ATP in the presence of 0.05 mM ATP (Fig. 1A, lane 2). As shown in Fig. 1A (lane 1), the labeling of these four lipids was also found in the presence of carrier-free [γ-32P]ATP alone, but to a lesser extent for PA, LPA, and PIP. Interestingly, three other lipids underwent strong labeling under this phosphorylation condition, namely X, Y, and Z. The lipid X comigrates with the LIPID

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marker, but there is still no conclusive proof that this lipid corresponds to LPIP (Fig. 1A, compare lanes 1 and 2). As shown in Fig. 1B, the lipid phosphorylation took place in envelope membranes exclusively. Nevertheless, a slight labeling in PA and LPA was found in thylakoid membranes which likely resulted from a small contamination with envelope membranes (19). In chloroplasts, the labeling of lipids occurred in the presence of [γ-32P]ATP and was not dependent on the plastid integrity (Fig. 1C). Indeed, the disruption of envelope membranes by osmotic shock had no effect on lipid phosphorylation. This observation is partially and indirectly confirmed by comparing lipid phosphorylation patterns after incubation of intact chloroplasts with [γ-32P]P, and light (Fig. 1D, left) or with [γ-32P]ATP (Fig. 1D, right). Indeed, under both conditions, PA, LPA, X, and Y were labeled, therefore indicating that both chloroplastic and exogenous sources of ATP can generate phosphorylated lipids. In contrast, labeled phosphatidylinositol derivatives comigrating with LPIP, PIP, and PIP₂ used [γ-32P]ATP exclusively.

(γ-32P)CTP as the Main Phosphoryl Donor in the Labeling of X and Y—Recent experiments have shown that the main nucleotides formed after incubation of intact chloroplasts with [γ-32P]ATP as well as with a ribonucleoside diphosphate mixture were [γ-32P]CTP and [γ-32P]UTP (26). To determine whether CDP and UDP were labeled in intact chloroplasts in the absence of exogenous NDPs, the experiments were repeated using intact chloroplasts incubated in the presence of [γ-32P]P, and light or [γ-32P]ATP (Fig. 2A, right and left). The data show that, in both cases, [γ-32P]CTP was formed. Apparently, no exogenous CDP is required to synthesize labeled CTP in the presence of [γ-32P]ATP. In addition, Fig. 2A (right) shows that the chlorolastic ATP rapidly and continuously phosphorylated GDP and CDP, but added [γ-32P]ATP had a preference for CDP (left). In lysed chloroplasts (data not shown), the synthesis of [γ-32P]CTP was strongly reduced compared with intact organelles, suggesting that the integrity of the plastid is necessary for this nucleotide phosphotransferase activity. These experiments and the knowledge that CTP is also a specific substrate for farnesol and dolichol phosphorylation (32, 33) encouraged us to investigate whether CTP also plays a role in the envelope lipid phosphorylation. The incubation of envelope membranes in the presence of purified NDPK-I (34) (as the phosphotransferase), [γ-32P]ATP, and CDP was performed (Fig. 2B). The data show that the presence of NDPK-II itself did not significantly modify the lipid phosphorylation pattern compared with control conditions (compare lanes 1 and 2). However, the addition of NDPK-II in the presence of CDP markedly stimulated the specific incorporation of labeled phosphate into X and Y (lane 4). This observation suggests that [γ-32P]CTP, resulting from the phosphotransfer of the terminal phosphate of [γ-32P]ATP to CDP, is the preferential substrate for the lipid kinase(s) in this phosphorylation process. The addition of CDP alone also stimulated, although to a lesser extent, the labeling of X and Y (lane 3), thus indicating that phosphotransferase activities are present in envelope membranes.

In the next step, we wished to demonstrate that lipids X and Y specifically incorporated [32P]P from [γ-32P]CTP. As [γ-32P]CTP is not commercially available, it was enzymatically produced by phosphotransfer from [γ-32P]ATP to CDP in the presence of NDPK-II (26). After separation on PEI-cellulose TLC (Fig. 3A), [γ-32P]CTP was scrapped from the plate and the TLC matrix carefully removed by filtration and sedimentation. [γ-32P]CTP was then used as potential phosphorylation substrate for envelope lipids (Fig. 3B). Minute amounts (corresponding to 3 nCi, about 1 fmol) of carrier-free [γ-32P]CTP (lane 1) and [γ-32P]ATP (lane 2) were used as labeled nucleotides. The data show that labeling in X and Y was stronger with the pyrimidine nucleotide than with the purine one. The CTP-dependent lipid kinase is apparently localized exclusively in envelope membranes, because X and Y were not found to be labeled when plasma membrane preparations (compare Fig. 3C, lanes 1 and 2) were subjected to phosphorylation with [γ-32P]CTP. These results indicate that the unlabeled membrane lipid molecules leading to X and Y were phosphorylated by an envelope lipid kinase(s) specific for [γ-32P]CTP.

Labeling of X and Y in the Presence of CTPγS—As far as we know, although some phosphotransferase activities can utilize CTP as the phosphate donor (35, 36), plant lipid phosphorylation activities using CTP as the preferential substrate have not yet been reported in the literature. In order to have a further confirmation that CTP is indeed the substrate, we have enzymatically synthesized CTPγS from ATPγS, CDP, and NDPK in vitro (for details, see “Experimental Procedures”). To demonstrate the specificity of CTP for the lipid kinase(s) involved in the emergence of labeled lipids X and Y, we analyzed the inhibitory effect of CTPγS compared with ATPγS on the lipid phosphorylation in the presence of [γ-32P]CTP (Fig. 4). The results show that 10 μM CTPγS reduced the phosphorylation to 20% of the control activity and 0.1 mM CTPγS almost completely inhibited the labeling of X and Y. Interestingly, identi-
CAL CONCENTRATIONS OF ATPγS DID NOT PREVENT THE LABELING BUT EVEN HAD A STIMULATORY EFFECT IN BOTH LIPIDS. A QUANTITATIVE ANALYSIS REVEALED THAT THIS INDUCTION RESULTED IN AN APPROXIMATELY 25% LABELING INCREASE IN THE TOTAL INCORPORATION OBSERVED UNDER CONTROL CONDITIONS. A SMALL STIMULATORY EFFECT WAS ALSO OBSERVED IN THE PRESENCE OF VERY LOW CONCENTRATION OF CTPγS, SUCH AS 1 AND 0.1 μM CTPγS FOR X AND Y, RESPECTIVELY.

PULSE-CHASE EXPERIMENT ON X AND Y LABELING AND TIME-COURSE PHOSPHORUS WITH CHLOROPHIAL ATTP—The effect of an addition of CTP following envelope labeling in the presence of [γ-32P]CTP is presented in Fig. 5. After 5 min of incubation, the addition of 1 μM CTP to the phosphorylation medium induced a time-dependent change in the labeling of X, while displaying a rapid [32P]incorporation pulse in Y. It should be noted that PA and LPA labeling were only weakly affected by the addition of CTP, thereby supporting the data presented in Figs. 2B and 3B, showing that [32P] incorporation in PA and LPA was ATP-dependent, but not CTP-dependent. An identical pulse-chase experiment, using [γ-32P]ATP as the phosphoryl donor and 1 mM ATP as the chase nucleotide, did not show any similar inverse relationship in the labeling of X and Y (data not shown), confirming that labeling of X and Y is closely related to CTP.

Identification of Lipids X and Y—The identification of the phosphorylated lipids X and Y was performed using acid hydrolysis. The hydrolysate products (labeled heads) were separated either on silica (Fig. 6A) or on cellulose (Fig. 6B) plates. Under different migration systems, X and Y hydrolysis was identified as phosphatidylinositol (PI) and phosphatidylglycerol (PG) respectively.
products (Fig. 6, A (lanes 2 and 1) and B (lanes 2 and 4)) comigrated with galactose-6-P, but not with glucose-6-P (Fig. 6, A (right) and B (lanes 1 and 3)), galactose-1-P, and glucose-1-P (data not shown). The second smaller labeled spot on lane 3 of Fig. 6A revealed that some glucose molecules were present in the galactose preparation (Sigma). These data strongly suggest that labeled lipids X and Y are galactolipid phosphorylated at the C₆-OH position.

To confirm that galactolipids are indeed a site of lipid phosphorylation in chloroplast envelopes, some additional biochemical experiments were performed. Fig. 7A shows that labeling of X was strongly increased when MGDG was added to envelope membranes solubilized with 0.06% Triton X-100 (compare lanes - and +). This observation was not found in the absence of detergent (data not shown), indicating that solubilization of lipids is necessary for enzyme-substrate interactions. Minor new lipid products were also found to be phosphorylated (including Y) in the presence of MGDG (see Fig. 7A, lane +). Another experiment shows that ³²P incorporation in X and Y was strongly reduced in the presence of increasing amounts of galactose (Fig. 7B). This competitive inhibitory effect is likely to be very specific for X and Y compared with the constant phosphorylation profile of PA and LPA (Fig. 7B).

Protease activities are known to affect the balance of galactolipids within the chloroplast envelope (16). Indeed, galactosyltransferases, generating DGDG and DAG from 2 molecules of MGDG, can be removed from outer envelope membranes by a thermolysin treatment, thereby increasing the ratio of MGDG versus DGDG in thermolysin-treated chloroplasts (37). Fig. 7C shows that isolated envelope membranes from intact chloroplasts treated with thermolysin (MGDG-enriched envelope membranes) and untreated membranes underwent a quantitatively different lipid phosphorylation pattern. Interestingly, the labeling of X and Y was largely stimulated in treated envelopes. Increased phosphorylation was not observed for PA and LPA, but their labeling was even strongly (PA) or slightly (LPA) reduced compared with the control. The lipid X was also enzymatically hydrolyzed when phosphorylated envelope membranes (100 μg) were incubated in the presence of 0.05 unit of β-galactosidase in 10 mM citrate (pH 4.4) for 25 min at 25 °C (data not shown). Although the galactose-P was removed only partially (~30% from the control), this result further confirms that MGDG is a phosphorylation substrate (MGpDG) in envelope membranes.

Role and Function of Phosphorylated Galactolipids within the Envelope Membranes—MGDG is a non-bilayer lipid (38) and therefore plays a fundamental role in the structure of envelope membranes. In this respect, it has been suggested that MGDG microenvironments in envelope membranes may favor interactions with protein complexes responsible for protein import (39, 40). As indicated in Figs. 6 and 7, MGpDG (X) seems to be one of the main (galacto)lipids phosphorylated in envelope membranes. Thus, it was tempting to postulate that MGpDG may affect the protein import process into chloroplasts. In order to test this hypothesis, protein import experiments were performed using pSSu as the precursor protein, 1 mM ATP and 0.1 mM CTP as (as inhibitor for X and Y, see Fig. 4), or 0.1 mM ATP/S as a nonhydrolyzable ATP analogue, already reported to prevent protein import; Ref. 41). The results show that such concentrations of CTP/S did not affect protein import as ATP/S did. Furthermore, no marked effect compared with the control conditions were found on the binding of pSSu (P) to envelopes (data not shown). In conclusion, these experiments suggest that galactolipid phosphorylation apparently does not control the protein import into the chloroplast stroma.

**DISCUSSION**

In a first report we have demonstrated the presence of four phosphorylated lipids in chloroplast envelope membranes, namely PA, LPA, PIP, and LFIP (2). In the present contribution, we show that two additional lipids (X and Y) undergo phosphate incorporation in envelope membranes when carrier-free γ-³²P|ATP is used as the sole phosphoryl donor (Fig. 1). This lipid kinase activity is associated with envelope membranes, but not with plasma and thylakoid membranes (Fig. 1). Several data argue in favor of a novel CTP-dependent lipid kinase that catalyzes phosphorylation of X and Y in envelope membranes. (i) γ-³²P|CTP is the preferential phosphoryl donor compared with γ-³²P|ATP (Fig. 3) and γ-³²P|UTP (not shown); (ii) the addition of γ-³²P|ATP, CDP, and NDPK-II to envelope membrane vesicles is comparable to the addition of γ-³²P|CTP in terms of X and Y labeling (Fig. 2); (iii) CTP/S, but not ATP/S, prevents ³²P incorporation in X and Y (Fig. 4); (iv) after γ-³²P|CTP-dependent labeling, CTP chases and pulses the radioactive phosphate from X and Y, respectively (Fig. 5).
Concerning the effect of CTPγS and ATPγS on lipid phosphorylation, CTPγS acts as a competitor for X and Y labeling at concentration higher than 1 μM. The slight stimulation observed at very low CTPγS concentration could possibly result from an inhibition of envelope endogenous phosphotransferase activities (see Fig. 2B, lane 3), which would inhibit the formation of labeled ATP by transfer of Pi to endogenous ADP. Consequently, the CTP pool would be available only for lipid phosphorylation. On the other hand, the stimulating effect by CTPγS at concentration higher than 1 μM could possibly result from an inhibition of envelope endogenous phosphotransferase activities (see Fig. 2B, lane 3), which would inhibit the formation of labeled ATP by transfer of Pi to endogenous ADP. Consequently, the CTP pool would be available only for lipid phosphorylation. On the other hand, the stimulating effect by CTPγS could be due to a saturating effect on other potential fixation sites for [γ-32P]CTP, leading to a specific stimulation in the [32P]incorporation in X and Y, which is in fact independent of ATP. To demonstrate that the CTP-dependent labeling is restricted to the lipid compounds X and Y, envelope protein phosphorylation experiments showed that no polypeptides were specifically labeled with [γ-32P]CTP and [γ-32P]UTP, in contrast to [γ-32P]ATP (data not shown).

The identification of phosphorylated lipids X and Y has been performed by acid hydrolysis (Fig. 6). The results show that, in both cases, the labeled product generated by acid hydrolysis comigrated with galactose-6-P, indicating that envelope galactolipids are the substrates for CTP-dependent lipid kinase(s). These data are confirmed by two different biochemical approaches. First, the addition of galactose to the phosphorylation medium (Fig. 7) led to a strong reduction of galactolipid phosphorylation. In this case, galactose likely competes with the active site of the CTP-dependent galactolipid kinase(s) and, therefore, limits the labeling intensities. Second, the thermolysin treatment of envelopes, which prevents the consumption of MGDG by the galactolipid:galactolipid galactosyltransferase (37), increases galactolipid phosphorylation.

Concerning the precise molecular identification of the phosphorylated galactolipids (X and Y), two pieces of evidence show that X correspond to phosphorylated MGDG (MGpDG). First, the phosphorylation of X was strongly stimulated by an addition of pure MGDG (1 μM) to envelope membranes solubilized with TX-100 (Fig. 7); second, the RP of MGpDG (0.74), due to its negative charge, was, under our TLC conditions, smaller than that of MGDG (0.86). On the other hand, Y is likely to be lyso-MGpDG (LMGpDG), although one cannot completely exclude, due to its RP position, that Y might be DGpDG or another labeled galactolipid form. However, other biochemical observations suggest that Y is LMGpDG. Indeed, X is possibly converted to Y by the action of an endogenous PLA2 under the following conditions: (i) after CTP chase (Fig. 5), (ii) after phosphorylation of pure MGDG in envelope vesicles (Fig. 7A), (iii) and during the time-course lipid phosphorylation in intact chloroplasts using [32P]Pi, as phosphoryl donor (data not shown). The very low amount of phosphorylated lipids formed during these experiments (in the range of fmol mg⁻¹ of proteins) did not allow to purify sufficient amounts to verify the data by NMR.

Concerning the specificity of the galactolipid kinase toward CTP, it is the first time that such an enzymatic activity is described in chloroplast envelope membranes. However, kinase(s) using CTP as a specific substrate has already been reported for other eukaryotic systems in two cases. Farnesol, when incubated with microsomal fractions of Nicotiana tabacum cell cultures or rat liver, is specifically labeled with CTP (33, 35). However, this activity is very different from the one documented in this report regarding the location of the phosphorylated products, since phosphorylated farnesol has been found to be located in mitochondrial and peroxisomal fractions. In yeast and Artemia larvae, phosphorylation of dolichols by CTP has also been reported (42, 43). This dolichol kinase activity leads to the formation of active dolichyl phosphate available for mannosylation and glucosylation, which are the first steps in protein glycosylation. In the chloroplast, the origin of
the CTP pool leading to the phosphorylation of galactolipids likely results from active CTP phosphotransferase activities within the chloroplast (26). As shown in Fig. 2A, such a high endogenous CTP production compared with other NTPs could be specific for galactolipid phosphorylation, but also for the synthesis of CDP-DAG as the precursor of PG (16).

In plant cells, phosphorylated lipids can act as precursors of second messengers or recruit specific signaling proteins to the membrane (44, 45), but, to date, the involvement of such envelope lipid phosphorylation in signaling pathways between stroma and cytosol has not been reported. Interestingly, some of the phosphorylated lipids of the envelope (PIP, PA, LPA), also present in other eukaryotic membranes, have already been reported to be involved in such transfer of information and cross-talk between cell compartments (3, 46). In this investigation, we describe for the first time the existence of a CTP-dependent galactolipid kinase activity in a photosynthetic organism, we describe for the first time the existence of a CTP-dependent galactolipid kinase activity in a photosynthetic organism. Our first attempt to demonstrate the role of galactolipid phosphorylation within the envelope was unsuccessful. Protein import in the presence of CTP-γS, an inhibitor of the galactolipid phosphorylation, was not inhibited. Several other roles can be considered. (i) MGpDG may exhibit different biological properties compared with the non-bilayer MGDG and generates new membrane microdomains, which may be involved in the interactions between the two envelope membranes or the regulation of transport processes; (ii) MGpDG present in the envelope membranes could be, in itself, a source of energy, if one consider the hydrolysis of the polar head (galactose-6-P) occurring within the envelopes; (iii) the implication of MGpDG in signaling and cross-talk. As general processes, it is also relevant to consider that environmental, mechanical, and biotic stress, which can affect leaf development, could modulate pathways of the envelope lipid kinase activities. The purification of the CTP-dependent lipid kinase and the identification of a knock-out mutant should allow us to obtain more insights in the physiological role of this novel lipid kinase.

Acknowledgments—We are very grateful to U. Kutzke for expert technical assistance, to Dr. F. Kessler for providing us with pSSu, and to Dr. T. Chuard for the acid hydrolysis procedure, which allowed us to identify lipids X and Y.

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