Rapid Triacylglycerol Turnover in Chlamydomonas reinhardtii Requires a Lipase with Broad Substrate Specificity

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When deprived of nitrogen (N), the photosynthetic microalga Chlamydomonas reinhardtii accumulates large quantities of triacylglycerols (TAGs), making it a promising source of biofuel. Prominent transcriptional changes associated with the conditions leading to TAG accumulation have been found, suggesting that the key enzymes for TAG metabolism might be among those that fluctuate in their expression during TAG synthesis and breakdown. Using a Saccharomyces cerevisiae lipase null mutant strain for functional complementation, we identified the CrLIP1 gene from Chlamydomonas based on its ability to suppress the lipase deficiency-related phenotypes of the yeast mutant. In Chlamydomonas, an inverse correlation was found between the CrLIP1 transcript level and TAG abundance when Chlamydomonas cultures were reversibly deprived of N. The CrLIP1 protein expressed and purified from Escherichia coli exhibited lipolytic activity against diacylglycerol (DAG) and polar lipids. The lipase domain of CrLIP1 is most similar to two human DAG lipases, DAGLα and DAGLβ. The involvement of CrLIP1 in Chlamydomonas TAG hydrolysis was corroborated by reducing the abundance of the CrLIP1 transcript with an artificial micro-RNA, which resulted in an apparent delay in TAG lipolysis when N was resupplied. Together, these data suggest that CrLIP1 facilitates TAG turnover in Chlamydomonas primarily by degrading the DAG presumably generated from TAG hydrolysis.

Plants, animals, and fungi accumulate triacylglycerols (TAGs) to store excess carbon and energy. For industrial biofuel production, triacylglycerol can be converted into fatty acid methyl esters (FAMEs), the major components of biodiesel, through transesterification (12). TAGs harvested from cells can thus be a source of biofuel. Thanks to their higher growth rate and higher oil content, microalgae can, in theory, produce TAG at yields surpassing those of plants (29). However, the details of lipid metabolism in algae are poorly understood.

Chlamydomonas reinhardtii has a long history of serving as a model system for studying cell motility and photosynthesis (18, 31, 41). The recent development of genetic and molecular biological tools, such as nuclear genome transformation (21) and gene silencing with artificial micro-RNA (35), has also made Chlamydomonas an excellent model alga for a wider spectrum of research topics, including lipid metabolism and biofuel production. In Chlamydomonas, the glycerolipid composition was explored (16), and the lipid metabolism pathways have been annotated in silico (38). Certain enzymes for TAG metabolism have also been characterized experimentally (7). Like many other algae, Chlamydomonas accumulates TAG under nitrogen deprivation conditions (34, 52).

In plants, TAG biosynthesis starts with the plastid production of fatty acids, which are then converted to glycerol-3-phosphate, forming phosphatidic acid (PtdOH). The endoplasmic reticulum (ER) is another site for lipid assembly and fatty acid modification (44). Diacylglycerol is produced after the removal of the phosphate group by a PtdOH phosphatase. Diacylglycerol acyltransferases (DGATs) and phospholipid:diacylglycerol acyltransferases (PDATs) then transfer an acyl chain from either an acyl coenzyme A or a phospholipid to diacylglycerol (DAG) to synthesize TAG (53), which can be considered to be a vehicle for carbon storage. TAG turnover (lipolysis) is initiated by the action of TAG lipases, which generate DAG and free fatty acids (13). DAG can be hydrolyzed further into monoacylglycerol and glycerol. Certain lipases hydrolyze all acyl chains from the glycerol backbone, while others may act specifically on one kind of glycerolipid (50). To engineer TAG content, both TAG biosynthetic and hydrolytic enzymes have to be considered. For example, overexpressing an Arabidopsis DGAT increases seed oil content (19), whereas deleting the major TAG lipases in the budding yeast Saccharomyces cerevisiae results in enhanced accumulation of TAG in cells (4, 5, 24). In Chlamydomonas, the functions of two DGATs and a PDAT have been described previously (7, 11, 26). The lipases for the TAG turnover chain reactions have yet to be identified.

As with many other microalgae, Chlamydomonas reinhardtii cells do not maintain a large TAG pool during vegetative growth but do so following nutrient deprivation, most notably deprivation of nitrogen (N). Seventy-two hours after N removal, TAG accounts for 50% of all cellular fatty acids, which results from both increased fatty acid biosynthesis and membrane remodeling (33). These metabolic flux changes are accompanied by genome-wide transcription adaptation, including a large number of putative lipase-encoding genes that are up- or downregulated (33). We reasoned that the genes repressed during TAG accumulation may encode lipases that degrade TAG or its initial breakdown products (i.e., diacylglycerol and monoacylglycerol). The N deprivation-induced lipase genes, on the other hand, might be involved in shuffling fatty acids from the membrane lipids to TAG. Here, we describe the identification of a lipase-encoding gene required for rapid TAG turnover in Chlamydomonas.
**TABLE 1 Yeast strains used in this study**

| Strain       | Relevant genotype                  | Source or reference |
|--------------|------------------------------------|---------------------|
| yMK839       | MATα leu2-3 trp1 ura3-52           | This study          |
| yXL001       | MATα leu2-3 trp1 ura3-52g4Δ::K.l. TRP1 | This study          |
| yXL005       | MATα leu2-3 trp1 ura3-52g4Δ::K.l. TRP1g3Δ::KanMX | This study          |
| yXL023       | MATα leu2-3 trp1 ura3-52pMK595 [2μ. URA3] | This study          |
| yXL026       | MATα leu2-3 trp1 ura3-52pMK595ScLIP1 [2μ. URA3] | This study          |
| yXL077       | MATα leu2-3 trp1 ura3-52g4Δ::K.l. TRP1g3Δ::KanMX/pMK595 [2μ. URA3] | This study          |
| yXL080       | MATα leu2-3 trp1 ura3-52g4Δ::K.l. TRP1g3Δ::KanMX/pMK595ScLIP1 [2μ. URA3] | This study          |

(Carnegie Institution, Stanford, CA), was used for all experiments performed on *Chlamydomonas*. The liquid and solid cultures were grown in Tris-acetate-phosphate (TAP) medium as described previously (34). For N deprivation or resupply, the cells were collected by centrifugation at 3,000 × g (4°C, 3 min), washed twice, and resuspended in TAP-N (TAP medium with NH₄Cl omitted) or TAP medium, respectively.

**Quantitative real-time PCR (qRT-PCR) on CrLIP1** was performed using the primers qRT-F and qRT-R on the Applied Biosystems 7500 Fast real-time PCR (RT-PCR) system. The data were normalized to the commonly used RACK1 gene using the primers RACK1-F (receptor for activated C kinase 1 forward) and RACK1 reverse (RACK1-R) (9). The 2⁻ΔΔCT method (28) was employed for data analysis. RT-PCR for the agarose gel electrophoresis was performed using the same primers with the real-time PCR for the RACK1 and CrLIP1 genes. For the DGT1 gene, the primers DGT1T1-F and DGT1T1-R were used.

**Western blotting.** To examine the expression of the CrLIP1 cDNA in yeast, log-phase yeast cells were collected by centrifugation (3,000 × g, 5 min) and then resuspended in 2× SDS-PAGE loading dye (0.12 M Tris-HCl [pH 6.8], 0.04% bromophenol blue, 4% SDS, 20% glycerol, 5.6% β-mercaptoethanol) with the same volume of acid-washed glass beads (0.45 mm; Sigma-Aldrich). The mixture was boiled in a water bath for 5 min followed by 5 min of vortexing. After an additional cycle of boiling and vortexing, the lysates were centrifuged at 21,000 × g for 1 min, and the supernatant was analyzed by SDS-PAGE. To examine the subcellular localization of the recombinant CrLIP1 protein, yeast cell pellets were suspended in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing the Complete protease inhibitor cocktail (Roche) (one tablet for every 10 ml of buffer). Glass beads (0.45 mm) were added, followed by vigorous agitation in a Mini-BeadBeater (BioSpec) for 45 s and a 1-min incubation in ice. The beating was repeated 3 times. After the last bead beating, the lysates were collected by spinning them through a pinhole punched at the bottom of the tube (1,000 × g, 5 min, 4°C). The supernatant of the low-speed spinning was fractionated by 100,000 × g centrifugation (90 min, 4°C). The secondary supernatant was defined as the soluble fraction in Fig. 1. The membrane-enriched pellet was suspended in PBS containing the protease inhibitor cocktail. For immunoblotting, the 12CA5 monoclonal antibody (Roche) was used to probe for proteins with a hemagglutinin (HA) epitope tag, and the histidine (His) tag monoclonal antibody (catalog no. H1029; Sigma-Aldrich) was used for the hexahistidine-tagged protein in *E. coli*.

**Lipid analysis.** To extract lipids, *Chlamydomonas* and *E. coli* cells were harvested and resuspended in the lipid extraction solvent containing methanol, chloroform, and formic acid (88%) (2:1:vol/vol/vol). In addition, the bacterial cells expressing CrLIP1 were shaken at 26°C for 6 h after overnight IPTG (isopropyl-β-D-thiogalactopyranoside) induction before the cells were collected (described below). We found that this extra step allowed for better lipase action in *E. coli*. For lipid extraction, *Chlamydomonas* and *E. coli* cells were vortexed for 30 s in the extraction solvent for cell lysis. For yeast, the cells were resuspended in the solvent, and glass beads (0.45 mm) were added. Yeast lipids were extracted by using a Mini-BeadBeater (BioSpec) as described above for protein preparation, except that 0.4 volume of lipid extraction buffer (1 M KCl, 0.2 M H₃PO₄) was added to the organic phase after its collection by spinning it through a
pinhole at the bottom of the tube. For thin-layer chromatography (TLC), lipids were loaded onto silica gel 60 plates (catalog no. 5721-7; EMD Chemicals) and developed in petroleum ether-diethyl ether-acetic acid (80:20:1 [vol/vol/vol], to separate neutral lipids) or chloroform-methanol-acetic acid-H2O (75:13:9:3 [vol/vol/vol/vol], for the separation of polar lipids). Lipids were visualized by exposure to iodine vapor. For the quantification of fatty acid methyl esters derived from a certain lipid species on the TLC plate, the corresponding spots were scraped and subjected to transesterification and gas-liquid chromatography (GLC) as described previously (43).

Yeast metabolic labeling. To measure the rate of lipid synthesis, yeast cells were grown inCAA/H11002 Ura medium for 48 h at 26°C to early stationary phase. To each 5-ml culture, 20 μCi [14C]acetate (specific activity, 45 to 60 mCi/mmol; Perkin Elmer) was added. The cultures were then continually shaken (approximately 150 rpm in a Lab-Line floor incubator) at 26°C and were harvested 25 min, 40 min, 65 min, 100 min, and 140 min
after acetate supplementation. Total lipids were extracted (see above) and resolved by TLC resolution (petroleum ether-diethyl ether-acetic acid, 80:20:1 [vol/vol/vol]) that separated neutral lipids. TAG, DAG, and TLC origins (containing a mixture of polar lipids) were isolated for radioactivity measurement by liquid scintillation counting. For yeast cellular TAG hydrolysis analyses, 50 μCi (0.83 to 1.11 μmol) [14C]acetate was added to 5-ml cultures grown for 48 h at 26°C. After 4 h of labeling, the cells were collected by centrifugation, washed with fresh medium, and diluted 28-fold with freshCAA — Ura medium to an optical density at 600 nm (OD600) of 0.4 to 0.6. The cells were shaken for another 5 h with aliquots frozen at each time point. The growth curves were determined with cells grown exactly under the same conditions except that 1 μmol nonlabeled acetate was used. Lipid analysis was performed as described above for lipid synthesis rate analysis.

**Recombinant protein production.** The CrLIP1 coding sequence was amplified using the primers ligation-independent cloning forward (LICF) and ligation reverse (LICR) and the Phusion polymerase. The PCR product was then integrated into expression vector pMK1006 through ligation-independent cloning (3). *E. coli* strain BL21-CodonPlus (Stratagene) transformed with empty vector pMK1006 or pMK1006/CrLIP1 was grown to the log phase at 37°C, and protein production was induced by the addition of IPTG to a final concentration of 0.5 mM. After 16 h of shaking at 220 rpm at 16°C, the cells were harvested by centrifugation (6,000 × g, 5 min, 4°C). For protein preparation, the cells were collected and suspended in the lysis buffer (20 mM Tris-HCl [pH 7.9], 10% glycerol, 150 mM NaCl, 1 mM dithiothreitol) and subjected to three freeze-thaw cycles, followed by sonication on ice (90 times of alternating 1-s pulses and 0.3-s rests, with 1 min of ice chilling after every 15 cycles; a Branson digital sonicator 250 with 25% energy output was used). The cell extracts were obtained through centrifugation at 21,000 × g for 15 min and applied to a nickel-nitrioltriacetae (Ni-NTA) affinity purification column (Qiagen). The CrLIP1 protein was eluted with the lysis buffer supplemented with 200 mM imidazole. A spectrophotometric assay with the Bio-Rad protein assay dye reagent concentrate (catalog no. 50-006 Edu; Bio-Rad) (8) was used to determine protein concentrations.

**Lipase assay.** For the lipase assay with various substrates, lipids dissolved in organic solvents were dried under a stream of nitrogen gas. The dried lipid was then dissolved in 350 μl of 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing various concentrations of Triton X-100 (see below) by sonication (sonicator 3000 with a Misonix microprobe) 6 times for 10 s each (power setting, 1.5). Purified CrLIP1 protein or the same volume of protein storage buffer was added. Fresh dithiothreitol (DTT) was added to the final concentration of 2 mM. The mixture was then vortexed and incubated at room temperature. The background levels of fatty acids carried over with purified CrLIP1 protein were estimated in a control reaction without a substrate lipid supplied and subtracted from the free fatty acids data obtained with a substrate.

All the lipase reactions were quenched by adding 2 volumes of lipid extraction solvent. The lipid products were then analyzed by TLC as described above. For Rhizopus arrhizus lipase (Sigma-Aldrich), digestion, 200 μg of the enzyme preparation was used, and the incubation time was shortened to 20 min.

**amiRNA construct.** The artificial micro-RNA (amiRNA) construct needed to silence CrLIP1 expression was generated according to reference 35. Briefly, four primers were designed using the MicroRNA Designer protocols (http://www3.ncbi.nlm.nih.gov/entrez/query.fcgi?db=miRNA&cmd=blast& TOOL=miRNA &ToolHelp) and amplified by 5 min of boiling and overnight cooling to form a double-stranded fragment with CrLIP1-targeting sequences and overhangs compatible with the SpeI digestion site. This fragment was then integrated into the SpeI-linearized pChlamRNA3int vector (35) through ligation and transformation. To generate CrLIP1 knockdown lines, the artificial micro-RNA construct (pChlamRNA3intCrLIP1) or the empty vector was digested with KpnI (New England BioLabs) and transformed into the dw15.1 Chlamydomonas strain using the glass bead transformation method (21). TAP agar containing 10 μg/ml paromomycin was used for selection. The real-time PCR described above was used to screen for lines with reduced mRNA abundance of CrLIP1.

**Bioinformatics.** A BLAST (1) search of the CrLIP1 protein sequence in the human genome was performed on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/). Protein sequences were aligned with the ClustalW2 program (25).

**RESULTS**

**Heterologous expression of CrLIP1 complemented a yeast mutant deficient in TAG lipase activity.** To identify the Chlamydomonas lipases involved in TAG turnover, we used a yeast mutant deficient in TAG lipase for functional complementation. In yeast, the major TAG lipases are encoded by TGL3 and TGL4 (4, 5, 24). Their deletion results in a hyperaccumulation of TAG in stationary-phase cells. This phenotype can be rescued by an ectopic expression of a mouse adipose triglyceride lipase (24). To choose Chlamydomonas candidate genes for a lipase that suppress the yeast lipid overstocking phenotype, putative lipases based on Gene Ontology analyses of the Joint Genome Institute (JGI) Chlamydomonas 3.0 database were cross-referenced to our transcrip-
Chlamydomonas Acylhydrolase

Heterologous expression of CrLIP1 lowers the rate of TAG biosynthesis in yeast. To explore the role of CrLIP1 in yeast TAG metabolism in a more direct way, metabolic labeling experiments were performed. Stationary-phase cells were chosen, as TAG accumulates when cells enter the stationary phase (45). Additionally, from 48 h to 60 h, the increase in cell numbers was within 4% for all strains tested (Fig. 1F); thus, the cell number during the 2-h labeling period was regarded as constant, permitting us to normalize changes in lipid labeling to cell numbers. The radioactivities incorporated into TAG, DAG, and polar lipids (mainly phospholipids) were measured (Fig. 2A to C). Consistent with the steady-state lipid analysis results (Fig. 1D), the CrLIP1 protein lowered the rate of TAG accumulation (Fig. 2A), while the effects on polar lipids were relatively minor (Fig. 2B). Interestingly, labeling of DAG increased in the first 90 min after the addition of radioactive acetate and dropped afterward. The incorporation of label into DAG (Fig. 2C) was faster than into TAG (Fig. 2A), consistent with DAG being the precursor for TAG biosynthesis. The expression of CrLIP1 did not affect the initial DAG labeling but, rather, accelerated its reduction. As the immediate precursor of TAG, DAG labeling reduction likely reflected its conversion to TAG. The faster appearance of label in DAG than in TAG and the decrease of label in DAG after 90 min are consistent with a substrate-product relationship of DAG and TAG. The faster decline of DAG label in the presence of CrLIP1 would be consistent with CrLIP1 being a DAG lipase, which could affect the synthesis of TAGs, as observed. Indeed, in vitro experiments (see below) confirmed that CrLIP1 has DAG lipase activity.

When growing out of the stationary phase, yeast degrades TAG to produce precursors supporting multiple cellular functions, including membrane polar lipid biosynthesis and energy consumption (45). To shed light on the function of CrLIP1 in yeast, we employed metabolic pulse-chase labeling. To this end, stationary-phase yeast cells were labeled with radioactive acetate and then transferred to fresh medium without the label. The radioactivities in TAG and total polar lipids were quantified. During the course of the labeling period, all strains showed various degrees (up to about 30%) of increases in the optical density (Fig. 2F), a result of cells undergoing mitosis. To accommodate the differences in cell densities, we measured the radioactivities of TAG and polar lipids over a unit volume (per ml) of all strains and normalized them to the starting counts. During the outgrowth, the label in cellular polar lipids increased, whereas TAG labeling remained constant in the wild-type cells (Fig. 2D and E, open squares). Labeling of DAG was very low at the times tested, consistent with very rapid turnover of the DAG pool following the onset of the chase phase of the experiment. The increases of total radioactivities contained in polar lipids likely resulted from the incorporation of persisting radiolabeled acetate and fatty acids. On the other hand, the total amount of TAG remained steady, which we suggest to be a result of balanced TAG synthesis and hydrolysis. Indeed, in the tgl3Δ tgl4Δ double lipase knockout strain (Fig. 2D and E, open triangles), the total TAG amount increased drastically. Importantly, consistent with a role in lipolysis, the expression of CrLIP1 in both the wild-type and yeast lipase null strains caused a reduction in TAG labeling levels when cells emerged out of the stationary phase (Fig. 2D, closed triangles and squares). In addition, the labeling of polar lipids also showed a clear decrease in cells expressing the algal CrLIP1 protein (Fig. 2E). Together, these results strongly suggest a lipolytic function of CrLIP1.

Recombinant CrLIP1 hydrolyzes diacylglycerol, but not triacylglycerol, in vitro. To examine the lipase activity of CrLIP1 biochemically, the cDNA of CrLIP1 was cloned into an E. coli expression vector. A hexahistidine-tagged CrLIP1 protein was produced, purified (Fig. 3A), and subjected to TAG hydrolysis assays (Fig. 3B). Purified CrLIP1 ran as a doublet on SDS-PAGE (Fig. 3A). Although the underlying reason for this phenomenon remains unclear, the fact that both bands reacted with the anti-His antibody (see Fig. S1 in the supplemental material) indicates that these two bands are isoforms of CrLIP1, not copurified contaminants. Radioactive triolein supplemented with olive oil was incubated with the purified CrLIP1 protein before TLC analysis of the reaction products. Neither iodine vapor stain (not shown) nor autoradiography showed a detectable production of free fatty acids by CrLIP1 (Fig. 3B). Multiple attempts under various reaction conditions all failed to detect the TAG lipase activity of CrLIP1. We also tried another neutral lipid-steryl ester extracted from...
yeast cells as the substrate and detected no lipolytic activity (see Fig. S2 in the supplemental material).

We had noted that BLAST searches (1) against nonredundant databases revealed considerable similarity between CrLIP1 and the human sn-1-specific diacylglycerol lipases DAGL/H9251 and DAGL/H9252 (6) (identity scores of 22 with DAGL/H9251 and 26 with DAGL/H9252) (see Fig. S3 in the supplemental material). This prompted us to test the activity of CrLIP1 against DAG.

Commercial radiolabeled DAG was not available. To choose a DAG substrate capable of differentiating the reaction products from bacterial fatty acids copurified with the recombinant CrLIP1 protein, we took advantage of the fact that E. coli does not synthesize oleic acids (18:1/9) (22); thus, we used diolein [1,2-di(cis-9-octadecenoyl)glycerol] as the substrate for in vitro lipolysis assays. Reaction mixtures were resolved by TLC, followed by gas-liquid chromatography (GLC) of FAMEs, to identify the fatty acid products. At the position of free fatty acids, there was no visible staining in the absence of CrLIP1 (Fig. 4A), demonstrating that diolein by itself remained intact throughout the entire reaction. In the presence of CrLIP1, on the other hand, iodine staining clearly revealed the production of free fatty acids. Indeed, GLC

FIG 2 Metabolic labeling and chase studies of TAG and phospholipid synthesis in yeast strains overexpressing CrLIP1. [14C]acetate was added to stationary-phase yeast cultures. The samples were taken at the time points indicated, with TAG (A), polar lipids (B), and DAG (C) extracted and quantified. The radioactivities were normalized to the cell number. Data are represented as averages ± SDs (n = 3). (D and E) Chase experiments. Stationary-phase yeast cells were labeled, briefly washed, and then diluted in fresh nonlabeled medium. The cells were collected at four time points, with the radioactivities of TAG (D) and polar lipids (E) quantified and normalized to the starting counts. (F) Quantification of cell numbers over the course of the chase experiments. The optical density at 600 nm was measured. For panels D, E, and F, the data are presented as the averages of two technical replicates, with the error bars indicating SDs.

FIG 3 Recombinant CrLIP1 fails to hydrolyze triacylglycerol in vitro. (A) SDS-PAGE of bacterial whole-cell lysates (WCL) (6 μg) and purified CrLIP1 protein. Arrows point to two potential isoforms of recombinant CrLIP1. The unlabeled lane shows molecular mass markers. (B) Thin-layer chromatograph of lipids extracted from triacylglycerol lipase assay mixtures with radioactive triolein and olive oil as the substrate. An autoradiograph of TLC is shown. For the buffer-only control lane, the same volume of a CrLIP1 storage buffer was used instead of the purified CrLIP1 protein. Rhizopus lipase (RL)-digested triolein was loaded as the marker for free fatty acids.
analysis of the free fatty acids isolated from the TLC plate determined unequivocally the presence of oleic acid when CrLIP1 was present in the reaction mixture (Fig. 4C). We thus conclude that CrLIP1 possesses DAG lipase activity.

Recombinant CrLIP1 degrades membrane lipids of *E. coli*. Using *E. coli* crude lysates for the initial testing of the CrLIP1-dependent lipase activity, we noticed a prominent signal of free fatty acids prior to the addition of an exogenous substrate, suggesting that CrLIP1 may have already acted on an abundant bacterial lipid species. To explore this possibility further, we isolated bacterial lipids for TLC analysis and observed the presence of substantial amounts of free fatty acids in the CrLIP1-containing extracts (Fig. 5A). In *E. coli*, phosphatidylethanolamine (PtdEtn) is the most abundant glycerolipid (39), and we suspected that the free fatty acids were at least partly derived from PtdEtn hydrolysis. If so, one of the PtdEtn hydrolysis intermediates, lyso-PtdEtn, might be detectable in the bacterial lipid extracts when CrLIP1 is overproduced. Indeed, when the sn-1-specific *Rhizopus arrhizus* lipase (14) was used to generate a standard for lyso-PtdEtn, lyso-PtdEtn increased along with the free fatty acids (Fig. 5B). GLC analyses provided the quantitative support for increases in both free fatty acids and lyso-PtdEtn in the CrLIP1-overexpressing sample (Fig. 5C).

**FIG 4** Recombinant CrLIP1 degrades diacylglycerol in vitro. (A) Thin-layer chromatograph of lipids extracted from diacylglycerol lipase assay mixtures with diolein as the substrate for CrLIP1. Lane 1, control reaction with diolein and CrLIP1 storage buffer; lane 2, reaction with diolein and purified CrLIP1 protein; lane 3, control reaction with purified CrLIP1 protein without diolein to show fatty acids contained in the protein preparation. Diolein and oleic acid (18:1Δ9) were loaded as markers. The deep staining at the origins might be from ingredients of the reaction buffer, such as dithiothreitol or glycerol. (B to D) Gas-liquid chromatographs of fatty acid methyl esters (FAMEs) derived from free fatty acids scraped from the TLC plate shown in panel A. Arrows point to the location or expected location of oleic FAME.

**FIG 5** Recombinant CrLIP1 hydrolyzes phosphatidylethanolamine, the major membrane lipid of *E. coli*. (A) Thin-layer chromatographs of total lipids extracted from *E. coli* cells. Note the CrLIP1-dependent production of a significant increase of free fatty acids in the *E. coli* lysate. The arrow points to an unidentified lipid. PL indicates polar lipids. (B) TLC of PtdEtn hydrolysis reactions. The CrLIP1 protein causes the reduction of the PtdEtn substrate and produces free fatty acids and lyso-PtdEtn. The markers include PtdEtn and oleic acid from a commercial source and lyso-PtdEtn generated by the *Rhizopus* lipase (RL). (C) Quantitative analysis of CrLIP1-dependent PtdEtn hydrolysis in *E. coli*. Data are presented as the ratio of fatty acids in each lipid to fatty acids in the total lipid extract. Averages and SDs are shown by the ranges at the tops of the bars in the graph (n = 3).
origin of this species. While this phenomenon is likely related to the biochemical activity of CrLIP1, a definitive identification of this bacterial lipid species will require additional efforts that are beyond the scope of the current work.

Recombinant CrLIP1 hydrolyzes polar lipids. Many lipases, such as the human adipose triglyceride lipase (ATGL) (20) and yeast Tg4, (37) act on a broad spectrum of glycerolipid substrates. The apparent CrLIP1-dependent lipolysis of PtdEttn in E. coli prompted us to explore what other membrane lipids might be substrates for CrLIP1. We focused on abundant Chlamydomonas polar lipids that each constituted more than 10% of the membrane lipids: MGDG (monogalactosyldiacylglycerol), DGTs (diacylglycerol-\(\text{N},\text{N},\text{N}\)-trimethylhomoserine), PtdEttn, PtdGro (phosphatidylglycerol), and DGDG (digalactosyldiacylglycerol). Radiolabeled polar lipids were prepared by growing cells in \([^{14}\text{C}]\)acetate-containing medium, since acetate predominantly labels acyl chains rather than the glycerol backbone (46). These substrates would therefore allow us to follow the lipase activity by tracking the production of free fatty acids. The lipolysis reaction products were resolved by TLC and detected by autoradiography.

For the five substrates used, lipolysis was documented by the apparent production of radioactive free fatty acids in the presence of CrLIP1 (Fig. 6A and B). While CrLIP1 appeared to be more active on phospholipids (i.e., PtdEttn and PtdGro) under the assay conditions, it was difficult to quantitatively determine the substrate preference for these polar lipids, because they were mixtures of molecular species with various specific activities due to the preparation of the substrates by pulse labeling of Chlamydomonas cells. Nonetheless, we were able to demonstrate the lipase activity of CrLIP1 against DAG and polar lipids (as shown in Fig. 4 to 6).

Recombinant CrLIP1 hydrolyzes both acyl chains of PtdCho, with a preference for the \(sn\)-1 position. Diacylglycerol and polar lipids harbor two acyl chains at the \(sn\)-1 and \(sn\)-2 positions of the glycerol backbone. Many lipases prefer one position over the other. For example, the lipase from Rhizopus arrhizus strongly prefers the \(sn\)-1 ester bond (14). The human DAGLs also prefer \(sn\)-1 over \(sn\)-2, although activities at both positions were detectable (6). To explore the position preference of CrLIP1, we chose phosphatidylcholine (PtdCho) as the substrate. PtdCho is not present in Chlamydomonas, but it gave us an opportunity to test a number of commercially available molecular species. Specifically, we used 18:1\(\Delta^7\)/16:0 (\(sn\)-1/\(sn\)-2) and 16:0/18:1\(\Delta^9\) PtdCho (Fig. 7). By following the time-dependent liberation of fatty acids from each substrate, the preference (or the lack of preference) for position and/or saturation was observable. Lipase reactions were sampled at 0, 6, and 16 h, and PtdCho, lyso-PtdCho, and free fatty acids were extracted from the corresponding spots on TLC plates for GLC analysis. Only negligible amounts of free fatty acids and lyso-PtdCho were present at 0 h (Fig. 7C and D). After 6 h of incubation, both free fatty acids and lyso-PtdCho emerged, along with a decrease in PtdCho (Fig. 7E and F). For both PtdCho species, fatty acids were mainly, but not exclusively, released from the \(sn\)-1 position, regardless of the acyl chain. From 6 to 16 h (Fig. 7G and H), the amount of PtdCho remained largely unchanged, whereas the level of lyso-PtdCho decreased, suggesting that CrLIP1 was also able to hydrolyze lyso-PtdCho. Based on these results, we conclude that CrLIP1 prefers the \(sn\)-1 position but can also hydrolyze the acyl chain at the \(sn\)-2 position. Moreover, the \(sn\)-1 oleate and palmitate were mobilized equally well by CrLIP1, suggesting that both oleate and palmitate are comparable substrates for CrLIP1.

Involvement of CrLIP1 in Chlamydomonas TAG metabolism. Although we have yet to directly observe TAG lipolysis by using a purified recombinant CrLIP1 protein, it is possible that CrLIP1 is indirectly involved in cellular TAG metabolism. This is because the remodeling of lipids, membrane, and storage is one of the responses to N deprivation that leads to TAG accumulation (33). In addition, DAG is both a precursor and a product (intermediate) for TAG and membrane lipid metabolism (32). The lipase action of CrLIP1 (as shown in Fig. 4 to 7) may impact the flux of DAG and polar lipids, which could eventually affect TAG biosynthesis and breakdown. We took several approaches to test this hypothesis. First, we examined the relationship between changes in the CrLIP1 transcript and TAG abundance following N deprivation and N resupply. If CrLIP1 plays a role in storage lipid metabolism in Chlamydomonas cells, it was expected that the ex-
pression of CrLIP1 gene would also respond to N fluctuation. Chlamydomonas cells started to accumulate TAG shortly after N removal and continued to increase their TAG content (Fig. 8A) (34, 49, 52). N resupply triggered fast TAG mobilization. Consistent with a role in lipolysis, the transcript of CrLIP1 declined steadily when TAG was accumulating (Fig. 8B). Notably, there was a fast surge of CrLIP1 transcript that coincided with the N resupply-triggered TAG mobilization. The obvious inverse correlation between the CrLIP1 transcript level and TAG abundance strongly suggests that CrLIP1 exerts a catabolic function in Chlamydomonas TAG metabolism.

To further link CrLIP1 expression to TAG metabolism, we used an artificial micro-RNA (amiRNA) (35) to reduce the expression of CrLIP1. Sixty independent transformants were screened for reductions of CrLIP1 mRNA levels, and two lines (1-44 and 1-56) exhibited 70 to 80% reductions (Fig. 9A). These two lines and a vector-only control were subjected to N deprivation and TAG quantification. We only observed a slight increase in the TAG levels for the 1-56 line 48 and 72 h into N deprivation (Fig. 9B). The lack of a significant change in the TAG pool size in CrLIP1 knockdown lines seemed to be in agreement with the N deprivation-dependent decline of CrLIP1 mRNA; that is, the CrLIP1 lipase activity was likely to be downregulated when TAG synthesis prevailed in the overall energy flux. Knockdown of the CrLIP1 transcript level thus caused a minimal effect on the total TAG level. On the other hand, when ammonium was added to N-deprived cultures to trigger TAG breakdown, knocking down CrLIP1 expression resulted in a delay of TAG mobilization.

DISCUSSION

CrLIP1 affects TAG content possibly by degrading DAG. From the data presented above, we suggest that CrLIP1 affects Chlamydomonas and yeast TAG metabolism in different ways. In vitro, the recombinant CrLIP1 protein clearly can hydrolyze DAG and phospholipids but not TAG under the standard test conditions. On the other hand, the metabolism of TAG in yeast and in Chlamydomonas is affected by changes in the amounts of CrLIP1. Given that DAG is an immediate precursor of TAG synthesis and...
an intermediate of TAG degradation, changes in DAG pool sizes will presumably impact TAG abundance in different directions. Accordingly, as shown in Fig. 10, we propose that CrLIP1 participates in TAG turnover in *Chlamydomonas* by facilitating DAG removal during TAG mobilization (Fig. 10, reaction 1), thus contributing to expedient conversion of TAG into cell building blocks and metabolic energy following the reintroduction of N. The repression of CrLIP1 expression by artificial micro-RNA may transiently increase the DAG pool, which then delays TAG turnover by-product inhibition through feedback control. The overall rate of TAG hydrolysis is thus reduced. However, possibly due to functional redundancy, or as a result of metabolism of DAG through other routes such as conversion to phosphatidic acid (2), *TAGL* that accumulates following N deprivation eventually will be turned over quantitatively (Fig. 8B). In yeast, the *tgl3Δ tgl4Δ* double mutant has no detectable TAG lipase activity (Fig. 10, reaction 2) (24). This argues against the notion that CrLIP1 suppresses the TAG accumulation phenotype solely by expediting DAG removal following the first TAG hydrolysis step, as the lipolytic route of DAG production is blocked by the deletion of TAG lipases. Rather, we suggest two hypotheses. First, yeast gradually accumulates TAG as cells enter the stationary phase (10, 27). The introduction of a heterologous DAG lipase CrLIP1 may result in a shortage of DAG supply for TAG biosynthesis (Fig. 10, reaction 3), partially preventing the TAG-accumulating phenotype seen in the lipase knockout strains. Second, due to the pleiotropic metabolic, as well as the signaling, functions of DAG, the steady-state DAG amount has to be tightly controlled (47). The action of CrLIP1 in yeast may cause alterations in TAG metabolism by an indirect route involving DAG signaling. Nevertheless, we cannot exclude the possibility that CrLIP1 is indeed a TAG lipase *in vivo* but requires an unknown cofactor that was absent in our assay mixtures or a posttranslational modification on the protein that could not be performed in *E. coli* cells.

In addition to phenotypes in TAG metabolism, overexpressing CrLIP1 in yeast also affected the rate of polar lipid accumulation in cells emerging from the stationary phase. The observed phospholipid lipase activity (Fig. 5 to 7) can account for the phenotype discussed above, although the stationary-phase cells appear to be relatively insensitive to CrLIP1 overexpression (Fig. 1D and 2B). Alternatively, as DAG can be converted to phosphatidic acid and other phospholipids, including PtdEtn and PtdCho (17), CrLIP1 may simply hydrolyze and remove DAG from the pool for polar lipid biosynthesis, thus creating a futile cycle that hampers the rate of polar lipid accumulation.

Is there another function of CrLIP1? In *Chlamydomonas*, CrLIP1 appears to be actively expressed during vegetative growth when cells maintain a negligible amount of steady-state TAG (Fig. 8, time 0). The qRT-PCR results shown in Fig. 8 are consistent with our earlier work, in which global whole-transcriptome shotgun sequencing (RNA-seq) analysis was employed to examine the transcriptomic changes during N deprivation. The number of *CrLIP1* transcript hits was $2,921 \pm 687$ under N-replete conditions and were down to $1,335 \pm 149$ following 48 h of N depriva-
tion (33). Comparatively, the average number of transcript hits for all genes was only 570 during vegetative growth. qRT-PCR verified the abundant CrLIP1 mRNA when compared with a constitutively expressed RACK1 gene (see Fig. S4 in the supplemental material). These data demonstrate the active expression of CrLIP1 during vegetative growth. Assuming that transcript abundance correlates with the protein level, these expression data suggest that CrLIP1 may be a relatively abundant protein during vegetative growth. Given that many Chlamydomonas major membrane lipids, such as MGDG and DGDG, are derived from DAG (33) and that CrLIP1 is capable of hydrolyzing DAG and these major Chlamydomonas polar lipids, it seems possible that CrLIP1 may be involved in DAG and membrane lipid turnover during mitotic growth when active membrane synthesis and remodeling take place. In contrast, following N deprivation, DAG is synthesized but then channeled into TAG production by DGATs (7). A DAG lipase activity of CrLIP1 would be at odds with the need for cells to accumulate TAG, hence the observed decline of CrLIP1 transcript following N deprivation. To delineate the potential additional function of CrLIP1 in vegetative cells, the localization of CrLIP1 may have provided some useful information. Unfortunately, our attempts to express a CrLIP1-green fluorescent protein (GFP) fusion protein have not been successful. Intriguingly, CrLIP1 was detected in a flagellum proteomic survey and was designated FAP12 (flagellum-associated protein 12) (36). Thus far, we have not observed a discernible flagellar phenotype associated with either of the CrLIP1 knockdown lines characterized above (data not shown). One possibility is that CrLIP1 repression in the amrRNA lines was not sufficient to bring about an apparent flagellar phenotype. On the other hand, given the similarity between CrLIP1 and the human DAG lipases DAGLa and DAGLb that are localized in the plasma membrane (6), it is possible that part of the flagellum-associated CrLIP1 population may have derived from the plasma membrane, as the flagellar membrane is confluent with the plasma membrane (42). Thus, the presence of CrLIP1 in flagella does not contradict our hypothesis of CrLIP1 involvement in TAG metabolism.

CrLIP1 did not exhibit TAG lipase activity under our test conditions, and repressing CrLIP1 expression delayed, but did not prevent, TAG turnover. Therefore, other TAG lipases in Chlamydomonas reinhardtii remain to be identified.

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