Target of Rapamycin and LST8 Proteins Associate with Membranes from the Endoplasmic Reticulum in the Unicellular Green Alga *Chlamydomonas reinhardtii*

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The highly conserved target of rapamycin (TOR) kinase is a central controller of cell growth in all eukaryotes. TOR exists in two functionally and structurally distinct complexes, termed TOR complex 1 (TORC1) and TORC2. LST8 is a TOR-interacting protein that is present in both TORC1 and TORC2. Here we report the identification and characterization of TOR and LST8 in large protein complexes in the model photosynthetic green alga *Chlamydomonas reinhardtii*. We demonstrate that *Chlamydomonas* LST8 is part of a rapamycin-sensitive TOR complex in this green alga. Biochemical fractionation and indirect immunofluorescence microscopy studies indicate that TOR and LST8 exist in high-molecular-mass complexes that associate with microsomal membranes and are particularly abundant in the peri-basal body region in *Chlamydomonas* cells. A *Saccharomyces cerevisiae* complementation assay demonstrates that *Chlamydomonas* LST8 is able to functionally and structurally replace endogenous yeast LST8 and allows us to propose that binding of LST8 to TOR is essential for cell growth.

The Ser/Thr kinase target of rapamycin (TOR) is a central regulator of cell growth and metabolism in all eukaryotes (recently reviewed by Wullschleger et al. [54]). Studies of TOR signaling in yeast and mammals have demonstrated that TOR controls cell growth in response to nutrients and different stresses (10, 54). The TOR kinases are large (about 270 kDa) proteins that assemble into two structurally and functionally distinct multisubunit complexes of 1.5 to 2.0 MDa termed TOR complex 1 (TORC1) and TORC2. The similar composition of TORCs in widely divergent kingdoms such as metazoans and fungi suggests that these complexes are broadly conserved across all eukaryotes. The two TORCs were initially identified in yeast (34, 52) and subsequently in mammals (18, 23, 26, 44). In yeast, TORC1 contains either TOR1 or TOR2, KOG1, TCO89, and LST8, whereas TORC2 includes TOR2 (but not TOR1), LST8, BIT61, AVO1, AVO2, and AVO3 (34, 41, 52). Mammalian TOR (mTOR) associates with raptor (homologue of yeast KOG1) and mLST8 to constitute mTORC1, while mTORC2 consists of mLST8, rictor (homologue of yeast AVO3), hSIN1 (homologue of yeast AVO1), and mTOR (15, 18, 22, 23, 27, 34, 44, 57). With the exception of the yeast proteins TCO89, BIT61, and AVO2, all TOR partners are essential for growth of yeast and mammalian cells (16, 34, 42).

Studies performed in yeast and mammalian cells demonstrate that TORC1 mediates the rapamycin-sensitive signaling branch that regulates translation, ribosome biogenesis, autophagy, and nitrogen and carbon degradative pathways (54). Some of these readouts are mediated via the AGC kinases S6K1 (mammals) and Sch9 (yeast), which are phosphorylated and thus activated by TORC1 (4, 21, 50). TORC2, on the other hand, is insensitive to rapamycin and controls actin cytoskeleton organization (23, 34, 44), presumably via its direct AGC kinase substrates Akt/PKB (mammals) and YPK1/2 (yeast) (24, 36, 45). Recently, it has been reported that TORC1 and TORC2 are multimeric supercomplexes in yeast, flies, and mammals, and oligomerization of TORCs has been proposed as an important mechanism for the regulation of TOR activity (49, 55, 58).

Several studies have investigated the cellular localization of mammalian and yeast TORCs by different techniques, including subcellular fractionation, indirect immunofluorescence (IF) microscopy, and immunoelectron microscopy. Albeit with significant discrepancies, these studies all agree that TORCs are primarily membrane associated. Yeast TORC1 has been reported to be associated to the plasma, vacuolar and endosomal membranes, and, more recently, TOR1 has been found to shuttle in and out of the nucleus (3, 5, 30, 32, 52). Localization of TOR in mammalian cells is also controversial since mTOR has been reported to be associated with mitochondrial, endoplasmic reticulum, and Golgi apparatus membranes, as well as in the nucleus (13, 14, 28). The functional significance of TORCs association with multiple membranes is at present unknown.

Despite the relevance of TOR signaling in eukaryotes, little is known about control of cell growth by TOR in photosynthetic organisms. TOR has been identified in *Arabidopsis thaliana* (35, 37) and in the unicellular green alga *Chlamydomonas reinhardtii* (9). Unlike other eukaryotes, plants are insensitive to rapamycin (37), likely due to the inability of plant FKBP12 to bind this drug and to form the active rapamycin-FKBP12 complex that potently inhibits TOR activity (56). In contrast to *Arabidopsis, Chlamydomonas* is sensitive to rapamycin because

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Unlike plant FKBP12, Chlamydomonas FKBP12 is able to bind rapamycin in vivo and to interact, in complex with this drug, with the FKBP12-rapamycin binding (FRB) domain of TOR (9). Treatment of Chlamydomonas cells with rapamycin results in the induction of autophagy-like processes, indicating that CrTOR may control autophagy in photosynthetic organisms (9). Disruption of AtTOR results in premature arrest of endosperm and embryo development, indicating that AtTOR is essential for plant cell growth (37). Two raptor homologues have been identified in Arabidopsis, AtRaptor1 and AtRaptor2 (1, 2, 12, 35), although the essential role of these proteins for cell growth is controversial (2, 12). Direct interaction of AtRaptor1 with the HEAT repeats of ATOR has been demonstrated (35), indicating that at least TORC1 might be conserved in plants. This model is strongly supported by the finding that AtRaptor1 is able to bind and modulate the activity of plant S6K1 homolog (35), a downstream target of mTORC1 (4). It remains to be determined whether LST8 and TORC2-specific members are functionally conserved in plants. Moreover, no studies regarding the cellular localization of TORCs have been reported in photosynthetic organisms.

The present study was undertaken to investigate the localization of TOR complexes in a photosynthetic cell, using Chlamydomonas as a model system. We identified the Chlamydomonas homologue of LST8, which we termed CrLST8. By biochemical fractionation and IF microscopy, we demonstrate that CrTOR and CrLST8 exist in high-molecular-mass complexes that associate with microsomal membranes and are abundant in the peri-basal body region. Pull-down experiments revealed that CrLST8 forms part of a rapamycin-sensitive TOR complex. We also provide evidence that CrLST8 is able to functionally and structurally complement the deficiency of LST8 in yeast. Finally, the identification of three residues that are critical for CrLST8 function and TOR binding led us to propose that binding of LST8 to TOR is essential for cell growth.

Materials and Methods

Strains and growth conditions. Chlamydomonas reinhardtii wild-type strain SC3 was obtained from the laboratory of L-D. Rochaix (University of Geneva). Cells were grown as described by Harris (19) under continuous illumination at 25°C. If required, media (Tri-acetate phosphate medium and high-salt minimal medium) were solidified with 1.2% Bacto agar (Difco). Cells were grown in mx4 medium (9). Treatment of cells with rapamycin results in 3 h at 4°C. The total SE was removed and held on ice, and the pellet (P) was collected. Elution profiles of CrLST8 were analyzed by Western blotting, using monoclonal anti-HA (1:10,000; M Lindahl), 1:10,000; COXIIb (Agrisera), 1:10,000; and FOX1 (Agrisera), 1:10,000. The ECL Plus immunoblotting system (GE Healthcare) was used to detect these proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies.

Gel filtration and fractionation. Total soluble extract (SE) from 5 × 10^8 cells was prepared as indicated above by two cycles of freezing and thawing. Three milligrams of protein (in 200 μl) was loaded onto a Superose 6 HR 10/30 column (Amersham). The flow rate was adjusted to 0.5 ml/min, and 0.5-ml fractions were collected. Elution profiles of CrTOR and CrLST8 were analyzed by Western blotting and compared to the elution profile of known standards (Bio-Rad). For fractionation assays, whole-cell extract was prepared from 10^7 wild-type cells as described above. Microsomal fraction was obtained by centrifuging soluble cell extract in a 90-TI rotor in a Beckman Coulter ultracentrifuge at 120,000 × g for 2 h at 4°C. The total SE was removed and held on ice, and the pellet (P) was resuspended in an adequate volume of cold lysis buffer. For sedimentation analysis, 600 μl of microsomal fraction was loaded onto a 12-ml linear 16 to 55% (wt/wt) sucrose gradient made in lysis buffer and centrifuged in a Beckman Coulter ultracentrifuge at 100,000 × g for 16 h at 4°C. Then, 1-ml fractions were collected from the top of the gradient and processed for western blot analysis.

Cross-linking and pull-downs. GST-CrFKBP12 recombinant protein was obtained and purified from E. coli as described previously (9) and incubated with 4 μM rapamycin or drug vehicle (90% ethanol–10% Tween 20) for 30 min on ice. GST-CrFKBP12 prebound to rapamycin was added to Chlamydomonas total extracts with or without 2 mM DTSSP (Pierce) and incubated for 2 h on ice. Cross-linking reactions were quenched by adding Tris-HCl (pH 7.4) to a final concentration of 100 mM, followed by an additional 30 min of incubation. Fusion protein complexes were immobilized on glutathione-Sepharose 4B beads. After 4 h of incubation at 4°C on a rotary incubator, beads were washed four times with lysis buffer, resuspended in 30 μl of SDS gel-loading buffer, and resolved by SDS-PAGE. To study the interaction of CrLST8 and CrTOR proteins, a cDNA fragment containing the kinase domain (residues 2042 to 2373) of Chlamydomonas TOR was obtained by RT-PCR using the primers kinase-5′ (5′-TGGGAC CTGGTACTACCGTC-3′) and kinase-3′ (5′-GAGCGCCGCAGCTTTGAG-3′). The PCR product was cloned into the pMAL expression vector (New England Biolabs) for expression in bacteria as maltose-binding protein (MBP) fusion. For pull-down assay, 10 μg of the purified fusion protein retained on amylase resin were incubated with 0.5 mg of Chlamydomonas protein extracts in 0.4 ml of PBS. After 4 h of incubation at 4°C on a rotary incubator, beads were washed four times with the loading buffer, resuspended in 30 μl of SDS gel-loading buffer, and resolved by SDS-PAGE.

Immunoprecipitation. For immunoprecipitation, yeast extracts from cells expressing hemagglutinin (HA)-TOR2 were prepared as indicated above. A total of 20 μl of monoclonal anti-HA immobilized on agarose (Sigma) was added to the extracts, and the tubes were rotated for 3 h at 4°C. Beads were collected by

Antibody production. Antibodies were raised against the CrFKBP12 domain of CrTOR. A 282-bp DNA fragment encoding the entire FRB domain of CrTOR (residues 1961 to 2055) was obtained by reverse transcription-PCR (RT-PCR) using the primers FRB-BamHI (5′-CTGGTGGACCGACGATGGGCAC-3′) and FRB-XhoI (5′-TACGCGCTGTCGTTGCGC-3′), TTA was added to the 5′ end of the reverse primer to create in-frame stop codon. The RT-PCR product was cloned into the pGEX-4T-1 plasmid (Amersham) and introduced into Escherichia coli DH5α. Expression of the glutathione S-transferase (GST) fusion protein was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. The recombinant protein was further purified on glutathione-Sepharose 4B (Amersham), eluted by glutathione incubation, and resuspended in 50 mM Tris-HCl (pH 8.0) buffer. The purified recombinant protein was used to immunize two rabbits according to standard immunization protocols. A His6-CrLST8 fusion protein was generated by cloning the full-length cDNA of CrLST8 gene into pET3a (Qiagen). Soluble sites were added to forward and reverse primers. The insoluble, His6-CrLST8 fusion protein was solubilized from inclusion bodies by incubation with 8 M urea, purified on a nickel column, and injected into two rabbits. CrTOR and CrLST8 antibodies were purified on a protein A-Sepharose column (Millipore).

Protein preparation and Western blotting. Chlamydomonas cells from liquid cultures in log phase were collected by centrifugation (5,000 × g, 5 min), washed once in 10 mM sodium phosphate (pH 7.0), and resuspended in a minimal volume of the same solution supplemented with 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by two cycles of slow freezing to −80°C, followed by thawing to room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15,700 × g, 15 min) at 4°C, and stored at −80°C until they were analyzed. Proteins were quantitated with the Coomassie blue dye binding method (Bio-Rad). Yeast extracts from logarithmically growing cells were prepared in lysis buffer (1× phosphate-buffered saline [PBS], 10% [wt/vol] glycerol, 0.5% Tween 20, 1 mM phenylmethylsulfonyl fluoride) by vortexing them 10 times for 30 s each time with glass beads. Cr TOR and CrLST8 antibodies were purified on a protein A-Sepharose column (Millipore).
centrifugation, washed three times with 1 ml of lysis buffer, and resuspended in 2× SDS-PAGE sample buffer for electrophoresis. After SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes and immunoblotted with monoclonal anti-HA antibody (Sigma). Proteins were detected with horseradish peroxidase-conjugated anti-mouse secondary antibody and ECL Plus reagents (GE Healthcare).

**Yeast complementation.** For expression of CrLST8 in yeast cells, the cDNA of the CrLST8 gene was cloned into the Smal site of the p425GPD vector under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (39). Wild-type and lst8 strains were transformed with empty vector or the p425GPD-CrLST8 plasmid. For depletion of endogenous pGAL1-driven LST8, cells were grown in SD medium for 15 h. Cultures were normalized and subjected to 10-fold serial dilutions, and spotted onto SD or SGal-Leu plates. Cells were incubated at 30°C for 3 days.

**Fluorescence microscopy.** Wild-type Chlamydomonas cells were fixed and stained for IF microscopy by the alternate protocol of Cole et al. (7). The primary antibodies used were rabbit polyclonal anti-CrTOR, rabbit polyclonal anti-CrLST8, and a mouse monoclonal anti-acetylated tubulin (Sigma). For signal detection, a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit (FITC) and a Texas Red-labeled goat anti-mouse antibody were used. Preparations were photographed on a DM6000 microscope (Leica) with a DFC350FX camera (Leica) and processed with the LasAF software package (Leica).

**RNA isolation and Northern blot analysis.** Chlamydomonas total RNA was isolated as previously described (9). For the analysis of yeast genes, yeast cells growing exponentially in SGal medium were shifted to glucose medium for 15 h. Yeast RNA preparation and Northern blot analysis were performed as reported (8). DNA probes were obtained by PCR using the primers 5'-TCTGGTAGGCGTGGTGATCTG-3' and 5'-TCATGCCTAGTTTCAACAGGAAC-3' (PCY1), 5'-ATGGTGCTTATGATGC-3' and 5'-ATGGTGGTGGTTGAGATGC-3' (DAL80), and 5'-TGGAAGGAGGACACTTTGG-3' and 5'-CAGGATCGTGATTTGC-3' (25S rDNA).

**RESULTS**

**TOR-interacting proteins in Chlamydomonas: identification of CrLST8.** A survey of the Chlamydomonas draft nuclear genome for orthologs to the conserved TOR partner proteins raptor/KOG1, LST8, hSIN1/AVO1, and rictor/AVO3 revealed the presence of genes encoding at least one LST8 homologue and one raptor/KOG1 homologue. TBLASTN analysis using yeast or mammalian LST8 as the query identified a gene product encoded by a genomic locus consisting of six exons and five introns. The complete CrLST8 cDNA was cloned from a Chlamydomonas cDNA library (see Materials and Methods). The CrLST8 gene encodes 315 amino acids with a predicted molecular mass of 35 kDa. An evolutionary study of CrLST8 evidenced that this protein is clustered with plant LST8s (Fig. 1C), being 65 and 54% identical to AtLST8.1 and AtLST8.2, respectively, and 52% identical to yeast LST8. The CrLST8 protein is composed entirely of seven WD-40 repeats (Fig. 1A) and, based on a modeling study of the protein (Fig. 1B), the predicted structure of CrLST8 is very similar to the beta-propeller fold of WD-repeat proteins (48). We have also identified a putative homologue to raptor/KOG1 on scaffold 64 of the Chlamydomonas nuclear genome, although the DNA sequence of this gene is still incomplete, and the available sequence only covers ca. 35% of the gene.

**CrTOR localizes in high-molecular-mass complexes.** The presence of TOR kinases in high-molecular-mass complexes has been reported in yeast and mammalian cells. To investigate whether TOR may localize in large complexes in a photosynthetic organism, we examined the elution profile of CrTOR by gel filtration chromatography using a specific antibody generated against the FRB domain of CrTOR (see Materials and Methods). Western blots of whole-cell protein demonstrated that the anti-CrTOR antibody recognizes a protein of about 270 kDa (Fig. 2A), a finding in agreement with the predicted molecular mass of CrTOR (9). Gel filtration chromatography assays revealed that CrTOR has an apparent molecular mass of approximately 2 MDa (Fig. 2B), indicating that CrTOR is in large complexes. Moreover, the absence of a CrTOR signal in fractions of lower size strongly suggests that almost the entire amount of CrTOR present in the total SE is forming part of these high-molecular-mass complexes. To estimate the percentage of CrTOR present in the SE from the total amount of CrTOR in the cell, we treated the insoluble fraction resulting after cell disruption with a high concentration of salt and/or a nonionic detergent and examined the presence of CrTOR in the released material. Our results indicated that most CrTOR was extracted in the initial soluble fraction, whereas no signal was detected in the cellular debris (Fig. 2C).

TOR has been reported to be partially associated with internal membranes such as the endoplasmic reticulum (ER), Golgi, or plasma membranes in yeasts and mammals (14, 30, 52). To examine whether CrTOR associates with membranes, we performed biochemical fractionation of Chlamydomonas lysates. An SE from Chlamydomonas cells was subjected to ultracentrifugation to separate soluble and microsomal fractions. We found that about half of CrTOR is pelleted with the membranous microsomal fraction (Fig. 2D), indicating that at least part of CrTOR is membrane associated. The presence of microsomes in the insoluble fraction after ultracentrifugation was confirmed by immunodetection of the luminal binding protein BiP, an established ER marker, in this fraction (Fig. 2D). Vacuolar and plasma membranes were also detected in the microsomal fraction by using antibodies generated against a plant vacuolar membrane ATPase or the plasma membrane ferroxidase FOX1 (31), respectively (Fig. 2D). Parallel immunoblot assays with antisera specific to D1, a chloroplast membrane protein, to COXIIb, a mitochondrial membrane protein, or to CrFKBP12, a cytoplasmic soluble protein (9, 51), showed that the membrane-containing fraction is not significantly contaminated with thylakoidal and mitochondrial membranes or soluble cytoplasmic proteins (Fig. 2D). To determine the nature of CrTOR association with internal membranes, we treated the SE prior to ultracentrifugation with agents that disrupt membranes or membrane-protein interactions, as previously reported for yeast TOR complexes (6, 52). High concentration of salt in combination or not with a nonionic detergent significantly, but not completely, converted CrTOR into a soluble form, whereas treatment with an ionic detergent or with sodium carbonate had only a minor effect (Fig. 2D). These results suggest that CrTOR is tightly associated to internal membranes.
CrLST8 associates with CrTOR and membranes. LST8 was originally identified in yeast as a component of the exocytic secretory pathway (42) and later on as a negative regulator of the RTG1/3 transcription factors (33). To our knowledge, no published studies have reported the characterization of LST8 from photosynthetic organisms, prompting us to initiate a detailed analysis of CrLST8. To characterize CrLST8, we generated a polyclonal antibody against the recombinant CrLST8 protein, which recognizes a protein of 35 kDa from a Chlamydomonas SE (Fig. 2A). We also observed that CrLST8 is more abundant than CrTOR in Chlamydomonas cells (Fig. 2B and unpublished data). To explore the possible association of CrLST8 to CrTOR, we first examined whether, similar to CrTOR, CrLST8 localizes in high-molecular-mass complexes. We found that a fraction of CrLST8 elutes from a gel filtration column in the same high-molecular-mass fractions than CrTOR, but most CrLST8 eluted separately from CrTOR (Fig. 2B). Biochemical fractionation of Chlamydomonas lysates evidenced that part of CrLST8 is, like CrTOR, present in the microsomal fraction, suggesting that CrLST8 is membrane associated (Fig. 2D). Treatment of cell extracts with detergent and/or salts resulted in the complete extraction of CrLST8 to the soluble fraction, indicating that CrLST8 was peripherally associated with membranes (Fig. 2D). These results also indicate that the binding of CrLST8 to membranes is qualitatively weaker than the one observed for CrTOR. To further investigate the cofractionation of CrLST8 and CrTOR with internal membranes, we performed sucrose gradient sedimentation of the microsomal fraction. Membrane-associated CrTOR co-sedimented with the ER marker BiP and part of CrLST8; however, no significant cofractionation of CrTOR was detected with vacuolar or plasma membrane markers (Fig. 2E). Therefore, our results are consistent with the notion that CrLST8 and CrTOR are components of an ER-associated complex(es).

To investigate whether CrLST8 and CrTOR are in the same complex, we performed pull-down assays from Chlamydomonas total extract using purified Chlamydomonas FKBP12 (CrFKBP12) prebound to rapamycin, based on the finding that the CrFKBP12-rapamycin complex interacts with the FRB domain of CrTOR (9). As expected, a weak, but reproducible interaction was observed between CrTOR and CrFKBP12 in
FIG. 2. Localization of CrTOR and CrLST8 in large, membrane-associated complexes. (A) Whole-cell extracts were made from wild-type cells and probed with CrTOR and CrLST8 antibodies or preimmune antisera (pre). Arrows indicate single bands at the predicted molecular masses of ~280 and ~35 kDa recognized by CrTOR and CrLST8 antibodies, respectively. (B) Gel filtration elution profiles of CrTOR and CrLST8. Whole-cell lysates prepared from wild-type cells were loaded onto a Superose 6 sizing column. Fractions (0.5 ml) were collected and processed for immunodetection of CrTOR and CrLST8. The elution patterns of known molecular mass standards are indicated. (C) Fraction of CrTOR present in an SE versus total CrTOR in the cell. SE was prepared as described in Materials and Methods by freezing and thawing cells. Intact cells were removed from the cellular debris pellet by centrifugation at 400 × g for 1 min. The remaining insoluble membranous fraction was washed three times with PBS buffer and then treated or not (control) with salt and/or a nonionic detergent for 30 min on ice. The amount of CrTOR present in the SE, in the solubilized material, and in the remaining pellet (P) from the untreated control sample was monitored by Western blotting.
FIG. 3. CrLST8 interacts with CrTOR through its kinase domain. (A) A 25-μg portion of purified GST-CrFKBP12 was bound to rapamycin and incubated with 1 mg of Chlamydomonas total extracts in the presence or absence of the reversible cross-linker DTSSP (for details, see Materials and Methods). Fusion protein complexes were immobilized on glutathione-Sepharose 4B beads and resolved by SDS-PAGE. CrTOR and CrLST8 were detected by Western blotting. (B) Pull-down assays were performed with the kinase domain of CrTOR fused to MBP (MBP-CrTORkinase) or MBP alone and total SE from Chlamydomonas cells. About 10 μg of MBP or MBP fusion protein were incubated with 4 mg of Chlamydomonas total extract. Endogenous CrLST8 bound to MBP-kin was detected by Western blotting with CrLST8 antibody.

the presence of rapamycin, whereas no binding was detected in the absence of the drug (Fig. 3A). Similarly, we found that CrLST8 copurified with CrFKBP12 and CrTOR only when CrFKBP12-rapamycin was used in the pull-down assay (Fig. 3A), indicating that CrLST8 forms part of a rapamycin-sensitive TOR complex in Chlamydomonas. Given the weakness detected in the interaction of CrTOR and CrLST8 to CrFKBP12, we performed similar pull-down assays with the reversible cross-linker DTSSP, which significantly enhanced the interaction between these proteins and confirmed the presence of CrLST8 in a rapamycin-sensitive TOR complex (Fig. 3A). It has been reported that LST8 binds to the kinase domain of TOR in yeast and mammalian cells (27, 55). To further characterize the interaction of CrLST8 with CrTOR, we sought to determine whether the kinase domain of CrTOR, when immobilized, could precipitate CrLST8 from Chlamydomonas total extracts. Total SEs from Chlamydomonas cells were incubated with recombinant MBP or MBP-CrTORkinase purified from E. coli. Amylose resin was then added to immobilize the MBP fusion protein. After several washes, the bound fractions were analyzed by immunoblotting using anti-CrLST8 antibody (Fig. 3B). We observed that CrLST8 interacts with the kinase domain of CrTOR, whereas no binding to the MBP control was detected.

Localization of CrTOR and CrLST8 by indirect IF microscopy. Biochemical fractionation of CrTOR and CrLST8 indicated that part of these proteins may associate with membranes in Chlamydomonas (see Fig. 2). We used rabbit polyclonal anti-CrTOR and anti-CrLST8 purified antibodies to further examine the subcellular localization of these proteins by IF microscopy. CrTOR was localized to discrete punctae that are more abundant in regions adjacent to the plasma membrane and the apical end of the cell, close to the basal bodies (Fig. 4A). The signal of CrLST8 by IF microscopy was more diffused than the one observed for CrTOR, although punctate staining was also visible (Fig. 4A). Similar to CrTOR, CrLST8 was concentrated in the proximity of basal bodies, but, unlike CrTOR, CrLST8 was more abundant around the nucleus (Fig. 4A). No significant signal was detected with secondary antibody alone or in combination with preimmune sera (data not shown). Double staining with a mouse anti-tubulin antibody revealed that the signal we observed of CrTOR and CrLST8 at the flagella base corresponds to the peri-basal body region (Fig. 4B). To determine whether internal membranes are abundant in this part of the cell, we also examined the cellular localization of BiP using a rabbit polyclonal antibody raised against plant BiP that specifically recognized the Chlamydomonas BiP protein. As expected for an ER protein, BiP was detected all over the cell by IF microscopy, although the signal was more intense in the surroundings of basal bodies, indicating that indeed this region is membrane enriched (Fig. 4A). Thus, in agreement with the membrane association observed for CrTOR and CrLST8 by biochemical fractionation, the staining pattern of CrTOR and CrLST8 strongly suggest that these proteins might localize in membranous...
FIG. 4. Immunofluorescence localization of CrTOR, CrLST8, and BiP proteins in Chlamydomonas cells. (A) Exponentially growing wild-type cells were collected and processed for IF microscopy analysis as described in Materials and Methods. The antigens of interest are shown in green (FITC), whereas DNA staining is shown in blue (DAPI). Merge images show a merge of the green and blue channels. (B) Double staining of α-tubulin and CrTOR (top) or CrLST8 (down). CrTOR and CrLST8 are shown in green (FITC), α-tubulin in red (tubulin), and DNA in blue (DAPI). Merge images show a merge of the green, red, and blue channels.
structures such as the ER system, which is abundant near the nucleus and the peri-basal body region.

CrLST8 is able to functionally and structurally replace yeast LST8. To investigate the possible role of CrLST8 protein identified in *Chlamydomonas* in TORC signaling, we studied the functionality of CrLST8 expressed in yeast mutant cells lacking endogenous LST8. The *LST8* gene is essential in yeast and mammalian cells (16, 42), and depletion of LST8 in yeast cells results in a rapid arrest of cell growth (34, 42). We used a yeast mutant strain (*lst8*) with the *LST8* gene under control of the glucose-repressible and galactose-inducible *GAL1* promoter. This strain allows disruption of TORC1 and TORC2 simply by shifting cells from galactose- to glucose-containing medium (34). Therefore, *lst8* cells cannot grow on glucose medium (Fig. 5A and 34). We found that in contrast to *lst8* mutant cells transformed with an empty vector, *lst8* cells expressing CrLST8 are able to grow on glucose medium, indicating that CrLST8 functionally substitutes for yeast LST8 (Fig. 5A).

To explore the functionality of CrLST8 in yeast cells, we decided to examine the ability of this protein to mediate LST8-controlled processes with regards to TOR signaling. LST8 has been implicated in the regulation of the GLN3 and RTG1/3 controlled processes with regards to TOR signaling. LST8 has been used as a yeast complementation assay of yeast LST8. To investigate the possible role of the CrLST8 protein identified in *Chlamydomonas* in TORC signaling, we studied the functionality of CrLST8 expressed in yeast mutant cells lacking endogenous LST8. The *LST8* gene is essential in yeast and mammalian cells (16, 42), and depletion of LST8 in yeast cells results in a rapid arrest of cell growth (34, 42). We used a yeast mutant strain (*lst8*) with the *LST8* gene under control of the glucose-repressible and galactose-inducible *GAL1* promoter. This strain allows disruption of TORC1 and TORC2 simply by shifting cells from galactose- to glucose-containing medium (34). Therefore, *lst8* cells cannot grow on glucose medium (Fig. 5A and 34). We found that in contrast to *lst8* mutant cells transformed with an empty vector, *lst8* cells expressing CrLST8 are able to grow on glucose medium, indicating that CrLST8 functionally substitutes for yeast LST8 (Fig. 5A).

Our results indicate that CrLST8 is able to replace endogenous yeast LST8, and strongly suggest that the *Chlamydomonas* protein must integrate into TOR complexes to perform its regulatory functions. To explore this hypothesis, we performed coimmunoprecipitation assays with yeast HA-tagged TOR2, which is present in TORC1 and TORC2, and the precipitates were then examined for the presence of CrLST8. We found that TOR2 coimmunoprecipitated with CrLST8 (Fig. 5C), demonstrating that the *Chlamydomonas* protein is able to integrate into yeast TOR complexes.

Identification of residues critical for CrLST8 function. Taking advantage of the simple yeast complementation assay of *lst8* mutant cells with the CrLST8 gene, we screened for residues that are critical for CrLST8 function. A number of residues required for the normal function of the protein have been identified in yeast and mammalian LST8s (27, 33) (Fig. 1A). By
PCR random mutagenesis of the CrLST8 cDNA, we identified three residues whose substitution results in the complete loss of CrLST8 function: Asp106, Thr228, and Ser230. CrLST8 alleles bearing mutations D106G, T228A, or S230N were unable to promote growth of lst8 cells on glucose medium (Fig. 5A), indicating that these residues are essential for CrLST8 activity. Mutation D106G was localized in strand C of WD-repeat 3, whereas mutations T228A and S230N were localized in strand B of WD-repeat 6 (Fig. 1A). Mutation D106G affects the WD dipeptide characteristic of WD-repeats, and mutations T228A and S230N might influence on the proper folding of the WD-repeat, since a correct orientation of strands B and C is important for Gβ propeller blade structure (48). Structural modeling of CrLST8 suggests that all mutations localize on the surface of the protein (Fig. 1B).

To further investigate the effects of these mutations on CrLST8 function, we examined GLN3 and RTG1/3 regulation in LST8-depleted cells expressing the different CrLST8 mutant alleles. In agreement with the inability of CrLST8 mutants to functionally complement lst8 cells, we observed that GLN3 and RTG1/3 were similarly deregulated in cells lacking LST8 or in cells expressing any one of the mutant alleles (Fig. 5B).

Binding of LST8 to TOR has been demonstrated to be required for TOR function in yeasts and mammals (27, 55). Mutations on mLST8 that strongly reduce its association to mTOR also affect the kinase activity of mTOR (27). However, no direct correlation has been established between the capacity of LST8 to bind TOR and the requirement of a functional LST8 to promote cell growth. To answer this question, we decided to examine whether mutations identified in CrLST8 may affect the interaction of CrLST8 with yeast TOR2. Unlike wild-type CrLST8, none of the CrLST8 mutants were able to coimmunoprecipitate with TOR2 (Fig. 5C). Thus, our results strongly suggest that CrLST8 mutants cannot functionally replace endogenous yeast LST8 due to the inability of these mutants to bind TOR and directly link cell growth to LST8-TOR interaction.

**DISCUSSION**

In this study we report the identification of CrTOR and CrLST8 proteins in membrane-associated, high-molecular-mass complexes in the photosynthetic model organism *Chlamydomonas*. TORC1 and TORC2 complexes have been described in animal and yeast cells, and most TOR partners seem to be evolutionarily conserved. TORC1 might also be conserved in photosynthetic organisms since a raptor homologue has been recently described in *Arabidopsis* (1, 2, 12, 35), and the *Chlamydomonas* genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) seems to contain a single gene with high identity to yeast and plant raptor (our unpublished observation). No obvious homologues exist for the TOR2-specific proteins AVO1/hsSin1 and AVO3/victor in plants and algae (54; our unpublished observations), raising the question of whether TORC2 is structurally conserved in photosynthetic organisms. It is possible, however, that plants and algae functionally maintain a TORC2 complex, although the proteins that constitute this putative complex may substantially differ from their yeast and mammalian counterparts. The identification in *Chlamydomonas* of a homologue to the TORC1- and TORC2-shared protein LST8 suggests that TORC2 might be conserved in photosynthetic organisms. We found that CrLST8 is part of, at least, a possible TORC1 complex sensitive to rapamycin in *Chlamydomonas*, since CrLST8 copurifies with CrTOR and CrFKBP12 in the presence of rapamycin (Fig. 3A). LST8 binds to the kinase domain of yeast and mammalian TORs, and this interaction is required for full catalytic activity of TOR (27, 55). We found that CrLST8 is able to bind to purified kinase domain of CrTOR (Fig. 3B), suggesting that LST8 may perform a similar function in CrTOR. However, we have been unable to detect interaction of CrLST8 and the kinase domain of CrTOR by yeast two-hybrid assays (unpublished data), indicating that additional factors may be required for the stable association of CrLST8 to the kinase domain of CrTOR in vivo, as previously proposed for other components of yeast TORC2 (55).

We found that only a portion of CrLST8 coelutes with CrTOR from a gel filtration column (Fig. 2B), in agreement with previous studies with yeast LST8 (34). Interestingly, most of CrLST8 that elutes separately from CrTOR is still in complex with other proteins, and we detect a peak of CrLST8 with an apparent molecular mass of ~160 kDa (Fig. 2B). These observations might be explained by partial disruption of the CrTOR-CrLST8 complex in gel filtration assays or by TOR-independent functions of CrLST8. Our results strongly suggest that CrLST8 is more abundant than CrTOR (Fig. 2B and unpublished data), and therefore CrLST8 may likely perform other functions independently to TOR signaling. Studies in fission yeast indicate that LST8 is likely to participate in signaling pathways other than TOR. Mutations in the LST8 ortholog Wat1p result in multiple phenotypes not necessarily associated with defects in the TOR pathway (25, 40). Other evidence that might indicate additional functions of CrLST8 is the limited colocalization found between this protein and the ER marker BiP (Fig. 2 and 4), which suggests that, unlike its yeast homologue (42), CrLST8 might be involved in other cellular processes besides the exocytic secretory pathway in *Chlamydomonas*. Accordingly, our IF microscopy studies indicated that the cellular distribution of CrLST8 significantly differs from the plasma and endosomal membrane association previously reported for yeast LST8 (6, 52).

Biochemical fractionation of *Chlamydomonas* cell extracts revealed that CrTOR and CrLST8 are peripherally associated to internal membranes (Fig. 2) and therefore suggests that TOR complexes might localize on these membranous sites. Attachment to membrane seems to be stronger for CrTOR than for CrLST8, as revealed by treatment of *Chlamydomonas* SEs with salt and/or detergents (Fig. 2C). This finding indicates that TOR complexes are not anchored to membranes through CrLST8. Accordingly, it has been reported in yeast that TOR1 is still associated with internal membranes in a lst8 mutant (6). Mammalian and yeast TORs have been detected in different cellular compartments, including plasma, vacuolar, and ER membranes, as well as the nucleus, the mitochondria, and the Golgi apparatus (5, 13, 14, 28, 30, 52). Therefore, the site(s) of action of TOR complexes still remains controversial, although recent studies in yeast and flies have revealed a close correlation between TOR function and the endocytic pathway (3, 20). Our study of CrTOR and CrLST8 localization in *Chlamydomonas* cells strongly suggests that TOR complexes might as-
associate with membranes from the ER system. However, we cannot exclude other locations for TOR complexes such as endosomal membranes, since no components of the endocytic pathway have been described in *Chlamydomonas* thus far. As would be expected for membrane-bound proteins, the cellular distribution of CrTOR was coincident in some aspects with the staining pattern of the ER marker Bip, such as localization to discrete bodies and accumulation in the peri-basal body region. Flagellar growth is conducted from the region surrounding the basal body through the transport of building blocks to the distal end of flagella in a process known as intrflagellar transport (for a recent review, see reference 43). Flagellar proteins are synthesized on the rough ER and carried by vesicles from the Golgi apparatus to the base of the flagellum, where they fuse with the plasma membrane of the cell (43). The peri-basal body region is therefore membrane enriched and might also constitute an important place for regulation of polarized growth in the cell. Why do TOR complexes concentrate in the peri-basal body region in *Chlamydomonas*? A high demand of protein synthesis at the flagella base may explain localization of TOR complexes at this site since TOR promotes cell growth by positively regulating translation in all eukaryotes (54). Another possibility is that CrTOR performs more specific function(s) in the flagellum or in the peri-basal body region. A link has been recently established between mTOR and proteins that localize to primary cilia or to the basal bodies in epithelial cells from kidney cysts. Defects in the ciliary protein polycystin-1 (PC1) cause autosomal-dominant polycystic kidney disease (reviewed in reference 53). Interestingly, it has been found that mTOR activity is upregulated in renal epithelial cells where PC1 or other proteins from the basal body are inactive (47). Moreover, it has been proposed that PC1 inhibits mTOR activity by interacting with tuberin (47), an mTOR regulatory protein (54). It remains to be determined whether a functional relationship exists between CrTOR and flagella, as proposed for mTOR and cilia function (38, 47).

Most of the components of TORC1 and TORC2 are evolutionarily conserved from yeast to humans. Our yeast complementation assays demonstrate that CrLST8 is able to functionally and structurally replace yeast LST8 (Fig. 5), indicating that LST8 functions must be conserved in photosynthetic organisms as well. LST8 is an essential protein in yeast and mammalian cells (16, 42), and it is likely that CrLST8 might be also required for cell growth in *Chlamydomonas*; accordingly, we have not been able to reduce expression of this gene by RNA interference (data not shown). Physical interaction of CrLST8 with CrTOR and possibly with other proteins must play important roles in regulating the CrTOR pathway since CrLST8 consists entirely of seven WD-40 repeats (Fig. 1), and this domain mediates protein-protein interactions (48). In agreement with this hypothesis, we have identified three residues that are critical for CrLST8 function. Mutations at positions Asp106, Thr228, or Ser230 of CrLST8 resulted in the complete inactivation of the protein as revealed by yeast complementation assays (Fig. 5A), and a failure to interact with yeast TOR2 (Fig. 5C). Our results also demonstrate that inactive CrLST8 mutants are unable to properly regulate TOR-controlled functions in yeast cells (Fig. 5B) and directly link cell growth to the interaction of LST8 and TOR proteins. In animals, the analysis of mutations in mLST8 that reduce or abolish interaction of this protein with mTOR have correlated the ability of mLST8 to bind and activate mTOR (27), although it has not been determined whether the same mutations affect cell growth.

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