C2 Domain Protein MIN1 Promotes Eyespot Organization in Chlamydomonas reinhardtii

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Assembly and asymmetric localization of the photosensory eyespot in the biflagellate, unicellular green alga Chlamydomonas reinhardtii requires coordinated organization of photoreceptors in the plasma membrane and pigment granule/thylakoid membrane layers in the chloroplast. min1 (mini-eyed) mutant cells contain abnormally small, disorganized eyespots in which the chloroplast envelope and plasma membrane are no longer apposed. The MIN1 gene, identified here by phenotypic rescue, encodes a protein with an N-terminal C2 domain and a C-terminal LysM domain separated by a transmembrane sequence. This novel domain architecture led to the hypothesis that MIN1 is in the plasma membrane or the chloroplast envelope, where membrane association of the C2 domain promotes proper eyespot organization. Mutation of conserved C2 domain loop residues disrupted association of the MIN1 C2 domain with the chloroplast envelope in moss cells but did not abolish eyespot assembly in Chlamydomonas. In min1 null cells, channelrhodopsin-1 (ChR1) photoreceptor levels were reduced, indicating a role for MIN1 in ChR1 expression and/or stability. However, ChR1 localization was only minimally disturbed during photoautotrophic growth of min1 cells, conditions under which the pigment granule layers are disorganized. The data are consistent with the hypothesis that neither MIN1 nor proper organization of the plastidic components of the eyespot is essential for localization of ChR1.

A variety of organelles and multicomponent structures are assembled and maintained within eukaryotic cells. These structures occupy defined, often asymmetric, intracellular locations and must be correctly partitioned or reassembled at every cell division. Elucidation of the mechanisms that govern the assembly and localization of complex structures is critical to our understanding of how basic cellular processes are regulated, how they may have evolved, and the factors driving their ongoing diversification.

Assembly and asymmetric localization of the photosensory eyespot in cells of the biflagellate, unicellular, photosynthetic green alga Chlamydomonas reinhardtii provide useful models for genetic, molecular, and microscopic analyses of organelle biogenesis (11, 14, 23). Chlamydomonas is phototactic, using two anterior flagella to swim toward or away from a light source to locations where light intensity is optimal for photosynthesis but minimally damaging to the photosynthetic membranes (82). The eyespot (Fig. 1A, wild type) is a light-sensing structure positioned near the equator of the cell at an asymmetric location relative to the flagella (25). Stimulation of the rhodopsin family photoreceptors in the eyespot activates a Ca2+-dependent signal transduction pathway(s) that affects flagellar movement and the swimming behavior of the cell (24, 58, 69). Asymmetric localization of the eyespot is required for the transmission of information about the direction of the light source (82).

The sensitivity of the eyespot to light is a result of the properties and organization of the photoreceptors and other eyespot components (Fig. 1B) (18, 30). In the eyespot, the plasma membrane is apposed to the chloroplast envelope and several underlying (plastid-localized) layers of carotenoid-filled granules subtended by thylakoid membranes (44). The pigmented granules give the eyespot its distinctive orange-red color when Chlamydomonas is viewed with a light microscope. The regularly spaced granule/thylakoid membrane layers function as a biological quarter-wave plate, reflecting orthogonal light back toward the rhodopsin photoreceptors in the plasma membrane (alternatively termed Chr1 and Chr2 [51, 52], CSRA and CSRB [74], and Acop1 and Acop2 [78]) and absorbing light from other directions. The defined location of the eyespot within the cell and the layered organization of the plastid and plasma membranes in the eyespot are directly observable examples of coordination that has evolved from association of a historical host with a cyanobacterial endosymbiont (15).

Here we describe the Chlamydomonas MIN1 gene, which is required for proper assembly of the eyespot. MIN1 was identified in a screen for mutant strains that were not phototactic and had missing or abnormal eyespots (33). min1 mutant cells have miniature eyespots located near the equator of the cell (Fig. 1A, min1). In cells grown photoautotrophically (in the light and without a source of reduced carbon, such as acetate), the carotenoid granules of min1 eyespots are disorganized and the chloroplast envelope is no longer apposed to the plasma membrane. Characterization of the MIN1 cDNA predicts a
322-residue protein with a novel domain organization that includes an N-terminal C2 (phospholipid-binding) domain and a C-terminal LysM (peptidoglycan-binding) domain. Similarities between the domains in MIN1 and those in membrane-associated proteins and localization of the C2 domain to the chloroplast envelope in moss cells support the hypothesis that MIN1 promotes membrane apposition in the eyespot via direct interaction with the chloroplast envelope and/or plasma membrane. Analyses of channelrhodopsin-1 (ChR1) photoreceptor interaction with the chloroplast envelope and/or plasma membrane. MIN1 promotes membrane apposition in the eyespot via direct interaction with the chloroplast envelope and/or plasma membrane.

**MATERIALS AND METHODS**

**Strains and media.** Chlamydomonas reinhardtii min1 mutant strain 12-12 (137c min1 mt) (33) was obtained by UV mutagenesis of strain 137c (21), followed by screening for mutants that were not phototactic (ptx-). Strain 12-12 arg2 Δ-1 (min1 arg2 mt) was a spore from a cross of strain 12-12 to strain arg2 Δ-1 (137c arg2 mt) (21).

Chlamydomonas cultures were maintained on solid TAP medium (21) or on TAP medium plus 0.2 mg/ml arginine. For phototaxis assays, Western blot analyses of total cellular protein, or microscopy, liquid cultures were grown phototrophically in modified Sager and Granick medium I with Hutner’s trace elements (M medium) (22) or mixotrophically in the same medium containing 0.1% sodium acetate (R medium). For isolation of genomic DNA, liquid cultures were grown in R medium. For transformation of strain 12-12 arg2 Δ-1, liquid cultures were grown in R medium limited for NH4NO3 (0.125 mM) and supplemented with 0.2 mg/ml arginine (RNA medium). All cultures were grown at 25°C under continuous light.

**Phototaxis assays.** Following overnight growth in liquid M medium, a simple assay in which phototactic cells in a test tube swim toward a lighted slit at the bottom of an otherwise dark box (33) was used to determine whether cells were phototactic (ptx- ) or not (ptx+).

**Identification of the MIN1 gene.** The min1 mutant strain H6-2 (137c min1::ARG7 mt-) was isolated from an ARG7 (arginosuccinate lyase) insertion library (2, 66) and crossed to strain arg7 Δ-1 (137c arg7 mt-) (21). Phenotypic analysis of 20 tetrads and 21 random spores showed that the ARG7 insertion in H6-2 was linked to min1 (data not shown). Plasmid rescue was used to recover genomic sequence neighboring the ARG7 insertion in H6-2 (66, 79). Probes derived from the recovered sequence identified a restriction fragment length polymorphism that segregated with the min1 phenotype (data not shown), confirming linkage to min1.

Oligonucleotides 5B-22 and 5B-783 (see Table S1 in the supplemental material), derived from the recovered genomic sequence, identified cosmid H5b from a pARG7.8cos cosmid library (63). Following transformation of strain 12-12 arg2 Δ-1 with linearized H5b, 7% of the Arg+ transformants had normal eyespots when viewed by light microscopy (data not shown), a 5.0-kb BamHI-HpaI fragment from H5b was ligated to BamHI- and EcoRV-digested pARG7.8 to construct pARG7.8-MIN1BH. Following transformation of strain 12-12 arg2 Δ-1 with pARG7.8-MIN1BH (pMINI in Table 1), a pARG7.8 vector (containing the Chlamydomonas ARG7 gene), and Arg+ transformants were assayed for their phototaxis ability (ptx-).

![FIG. 1. Eyespots in wild-type and min1 mutant cells. (A) As previously described (33), a light micrograph of a min1 mutant cell (strain 12-12) reveals a miniature, equatorially localized eyespot (arrow). (B) Diagram of a wild-type eyespot showing layers of carotenoid pigment granules (dark gray circles) and thylakoid membrane (TM) immediately apposed to the inner and outer membranes of the chloroplast envelope (CE) (arrows) and the plasma membrane (PM). The eyespot photoreceptors (light gray ovals) are presumably in the plasma membrane.](http://ec.asm.org/)

**TABLE 1. MIN1 constructs and phototaxis rescue**

| MIN1 construct | Description | No. of transformants | % Rescue |
|---------------|-------------|---------------------|---------|
| pMINI         | 5.0-kb BamHI-to-EcoRV genomic fragment ligated to pARG7.8 | 22/132 | 17 |
| pMINI-Δ3′ATG  | ATG-to-CTG mutation of 3′ UTR ORF start codon in pMINI | 16/103 | 16 |
| pR-MINI       | RbcS2 promoter plus intron 1 fused to MIN1 cDNA plus intron 7 | 192/362 | 53 |
| pR-D19A,D77A  | Mutations in PR-MINI/codons for conserved loop aspartate residues | 40/72 | 56 |
| pR-K37A,K42A  | Mutations in PR-MINI codons for lysine residues in strands B3 and B4 | 34/48 | 74 |
| pR-T38A       | Mutation in PR-MINI codon for conserved threonine residue in B3 | 49/81 | 61 |
| pMINI-HA      | pMINI with the triple-HA tag fused in frame at codon 249 of MINI | 21/304 | 7 |
| pR-MINI-HA    | pR-MINI with the triple-HA tag fused in frame at codon 249 of MINI | 98/202 | 49 |
| pR-T38A-HA    | pR-T38A with the triple-HA tag fused in frame at codon 249 of MINI | 0/168 | |

*Strain 12-12 arg2Δ-1 (min1 arg2 mt- ) was transformed with the indicated MIN1 constructs (described in Materials and Methods and diagrammed in Fig. 2) ligated to the pARG7.8 vector (containing the Chlamydomonas ARG7 gene), and Arg+ transformants were assayed for their phototaxis ability (ptx-).*
12.5% of Arg^+ transformants (25/200) were phototactic and had normal eye spots. The sequence of both strands of the 5.0-kb insert in pMINI was determined by progressive design of oligonucleotide primers (see Table S1 in the supplemental material). Comparison of the 5.0-kb sequence to version 3.0 of the Chlamydomonas genome sequence (48) at the DOE Joint Genome Institute (JGI) yielded a match on scaffold 11 (model 11000167).

**Identification of the min1 mutation in strain 12-12.** Genomic DNA isolated from the min1 mutant strain 12-12 was used as the template in PCRs using overlapping oligonucleotide primer pairs (see Table S1 in the supplemental material), and the PCR products were ligated to the pGEM-T Easy plasmid (Promega, Madison WI) and sequenced. Repeated amplification with primer pair A7 plus B7 verified the G-to-T transversion at codon E61 of the predicted MIN1 protein, which changed a GAG codon to a premature TAG termination codon. The deduced exon/intron structure of the coding sequence containing only intron 7. In the HA-tagged constructs (Table 1), sequence encoding the triple HA epitope was ligated in frame at codon 249, just 5' of sequence encoding the predicted transmembrane domain. (C) Light micrographs of a wild-type cell and of min1 cells transformed with pMIN1-HA, pR-MIN1, or pR-MIN1-HA (see Materials and Methods and Table 1). The cultures were grown in M medium, and the single cells shown were typical of the majority of cells in each culture. (D) Western blot of total cellular protein isolated from M medium-grown cultures of untransformed strain 12-12 (min1) or of transformant strains containing the indicated constructs. The blot was probed with anti-HA (MIN-HA) (clone 12CA5; Sigma, St. Louis, MO), followed by antitubulin (clone B-5-1-2; Sigma). Shorter (top) and longer (middle) exposures of the blot probed with anti-HA are shown. pR-MIN1-HA strains 68 and 70 were obtained following three rounds of enrichment for ptx^+ cells.
that replaced wild-type sequence with sequence that altered a single codon and created a unique restriction site, which was used to assemble the fragments, and wild-type sequence in either pMIN1 (for pMIN1-Δ3′ATG) or pR-MIN1 (for pR-D19A,D77A, pR-K37A,K42A, or pR-T38A) was replaced with the mutant MIN1 sequence.

Plasmids ΔLMTM-YFP and ΔLMTMΔmo (used for transient transfection of moss cells) were constructed by in-frame fusion of PCR-amplified (oligonucleotide primers are listed in Table S1 in the supplemental material) MIN1 cDNA sequence encoding residues 1 through 180 to PCR-amplified yellow fluorescent protein (YFP). The fusion was ligated to a moss expression vector containing the actin promoter and transcription termination sequences (36).

**Chlamydomonas transformation.** Chlamydomonas strain 12-12 arg2 was transformed using silicon carbide whiskers (13) as previously described (66) with the following modification: following growth in liquid RNA medium to approximately 2 × 10^6 cells/ml, the cells were harvested by centrifugation, resuspended in 200 ml of low-nitrogen medium, and grown overnight at 25°C under continuous light. Cells were harvested from 0.5 ml of culture at 2,700 g for 10 min. Thirty microliters of each sample was electrophoresed through a Leica DMRXA microscope using a Leica PL APO 100×/1.4-numerical-aperture oil immersion objective with a 1.6× 0.15 μm (1 pixel = 0.039 μm) and bright-field optics. Images were captured with a QImaging (Burnaby, British Columbia, Canada) Retiga EX-cooled charge-coupled device camera driven by Universal Imaging (Downington, PA) MetaMorph v.6.1.2 software. The images shown are summed maxima of Z-series (each Z-series contained 6 to 10 images, captured at 0.5-μm intervals) that were adjusted for brightness and contrast.

**Figure preparation.** Figures were produced using Microsoft Word, Adobe Photoshop, Adobe Illustrator, or a combination of these programs. Micrograph or immunoblot images were minimally adjusted for grayscale levels or brightness and contrast, cropped, and reduced from the original size.

**Nucleotide sequence accession number.** The sequence of the 5.0-kb insert in pMIN1 is available from GenBank/EMBL/DDJB under accession number AY45207.

**RESULTS**

The MIN1 gene. MIN1 was originally identified by genetic analysis of a phototaxis-deficient strain with miniature, disorganized eyespots in which the plasma membrane and chloroplast envelope are not apposed (Fig. 1) (33). Measurement of eyespot area (in pixels; see Materials and Methods) in digital light micrographs indicated that min1 eyespots (39 ± 11 pixels, n = 33) cover approximately 25% of the area covered by wild-type eyespots (145 ± 53 pixels, n = 46). To identify MIN1, Chlamydomonas genomic sequence neighboring the ARG7 insertion in strain H6-2 (137c arg1 min1:ARG7) was isolated and used to identify a 5.0-kb BamHI-HpaI fragment of the genome that, following ligation to the pARG7.8 vector, restored phototaxis (ptx+) in 17% of the Arg- transformants (Table 1). Light microscopy confirmed that the ptx+ transformants had eyespots of normal size (data not shown). The sequence of the MIN1-containing fragment (GenBank accession AY452057) matched sequence on scaffold 11 of the DOE Joint Genome Institute Chlamydomonas reinhardtii genome sequence (JGI version 3.0, http://genome.jgi-psf.org/Chlr3/Chlr3.home .html) (48) approximately 913,000 bp from MLT1 (multied) (33; T. M. Mittelmeier and C. L. Dieckmann, unpublished data) and 958,000 bp from EYE2 (eyeless) (66). The 7% or 9% recombination observed between MIN1 and the MLT1 or EYE2 locus, respectively (33), is consistent with the recombination rate of 100 kb per cM estimated for the Chlamydomonas genome (48).

To identify the MIN1 coding sequence, oligonucleotide primers (see Table S1 in the supplemental material) based on open reading frame (ORF) predictions made by the GeneMark algorithm (39) were used to amplify MIN1 cDNAs by PCR (see Materials and Methods). The MIN1 cDNA is ap-
proximately 2.1 kb, comprising eight exons and seven introns (Fig. 2A), and includes a 322-codon ORF. In the original mini1 mutant strain 12-12, the 322-codon ORF is interrupted by a termination codon resulting from a GTG-to-AGT transversion at codon 61 (data not shown), consistent with the prediction that this ORF encodes MIN1. The 5′ splice junctions have the sequence R/G (R = A or G), while the 3′ splice junctions have the sequence CGN, which conform to proposed consensus splice sequence (63). The approximate 5′ and 3′ ends of the mRNA were defined by oligonucleotide primer pairs that did or did not yield amplified cDNAs (see Materials and Methods). The 5′ untranslated region (UTR) of the cDNA is less than 150 nt long, while the 3′ UTR is over 1 kb with the consensus polyadenylation sequence TGTTAA (47, 73) 1,122 nt downstream of the stop codon. The 3′ UTR also contains two short overlapping ORFs (84 and 74 codons), but the rate of phenotypic escape was unaffected by an GTG-to-CTG mutation in the start codon of the longer ORF (Table 1, pMIN1Δ3′-ATG), indicating that this ORF, if expressed, is not required for eyespot assembly or function. A construct in which the MIN1 coding sequence, containing only the final intron, was fused to the RbcS2 promoter and first intron (Fig. 2B) (20, 40) consistently yielded a rescue rate of nearly 50% (Table 1, pR-MIN1). Thus, the genomic MIN1 promoter and 5′ UTR are not required for functional expression of the MIN1 protein.

The size of a MIN1-HA protein is consistent with the predicted MIN1 ORF. In an attempt to localize the MIN1 protein in Chlamydomonas, epitope-tagged MIN1 constructs were assessed for phenotypic rescue. Constructs expressing the MIN1-coding sequence fused to C-terminal tags did not rescue the mini1 phenotype (data not shown). Integration of the triple-HA tag (17) just N terminal of the predicted MIN1 transmembrane sequence in the context of either the genomic MIN1 clone (pMIN1-HA) or the RbcS2-MIN17 fusion construct (pR-MIN1-HA) yielded ptx− transformants (Table 1). However, the rate of rescue by pMIN1-HA was relatively low, pMIN1-HA transformant cultures displayed weak phototaxis and had miniature eyespots following overnight growth (Fig. 2C), and both pMIN1-HA and pR-MIN1-HA transformants eventually lost the ptx− phenotype. These data suggest that the HA tag negatively affected the phenotypic expression of the MIN1 gene, either by reducing expression of the transgene, perhaps by increasing silencing (8), and/or by compromising function of the MIN1 protein.

To allow further analysis of the HA-tagged MIN1 protein, two pR-MIN1-HA transformants were subjected to three rounds of selection for ptx− cells following sequential phototaxis assays (see Materials and Methods). The enrichment yielded the ptx−-stable strains pR-MIN1-HA-68 and -70 (Fig. 2C); the enrichment may have favored cells in which the transgene was not silenced. The anti-HA monoclonal antibody 12CA5 detected a 35-kDa protein on Western blots of total cellular protein from strains pR-MIN1-HA-68 and -70 and from a pMIN1-HA transformant following overexposure of the blot. The 35-kDa protein was close in size to the 37.7 kDa predicted for MIN1-HA (34.2 kDa for MIN1 plus 3.5 kDa for the triple-HA tag) and was not detected in transformants containing the untagged construct, confirming its identity as the MIN1-HA fusion protein. These data are consistent with the conclusion that the 322-codon ORF within the MIN1 cDNA encodes the MIN1 protein.

While the MIN1-HA protein was detectable by Western blotting in the enriched strains, repeated attempts to localize the MIN1-HA protein in Chlamydomonas by immunofluorescence using several anti-HA monoclonal antibodies (12CA5 from Boehringer Mannheim, 3F10 from Roche, or HA.C5 from AbCam) were not successful (data not shown). As the usefulness of the HA-tagged construct was limited, alternative approaches will be necessary to characterize the expression and localization of the MIN1 protein.

The MIN1 protein contains three conserved domains. Queries of the NCBI databases using blastp (1, 41) identified two conserved domains within the predicted MIN1 protein (Fig. 3A). The N-terminal 121 residues had significant similarity to Ca2+/phospholipid-binding C2 domains SMART00239.7 (35, 72) and pfam 01686 (4), while the C-terminal 46 residues were similar to LysM domain sequences (pfam 01476) that bind peptidoglycan components of the bacterial cell wall (3, 6, 77). The secondary-structure prediction program TMHMM (transmembrane helix prediction using the hidden Markov model) (31) identified residues 251 through 270 as a probably membrane-spanning α-helix. Residues 122 through 234 of MIN1 comprise an alanine-rich region (26% alanine), a common feature of Chlamydomonas proteins due to the high GC content of the genome (48). To date, the domain architecture of MIN1 is unique; no other proteins containing both a C2 domain and a LysM domain were identified by searches of pfam or the Conserved Domain Database (43).

ClustalW (80) alignment of the MIN1 predicted transmembrane sequence or LysM domain sequence to protein sequence identified by blastp searches of GenBank are shown in Fig. 3B and C. The transmembrane sequence was similar to sequences in eubacterial hypothetical plasma membrane proteins, including probable sulfate and phosphate transporters from Nocardiooides and Dinoroseobacter and an Na+/H+ antiporter from Stigmatella. The MIN1 LysM domain was most similar to the LysM domain in a hypothetical protein from the photosynthetic (but eyespot-less) alga Ostreococcus tauri (50% identity), the smallest known autotrophic eukaryote (59), and to those in hypothetical proteins from the eubacteria Mooreella, Roseovarius, and Ralstonia (Fig. 3C). The aligned Ostreococcus and Mooreella proteins each contain a predicted transmembrane helix, while the proteins from Roseovarius and Ralstonia contain BON (bacterial OsmY and nodulation; pfam04972) domains, which are hypothesized to bind phospholipids (83). Of interest is the observation that the LysM domain sequences most similar to that of MIN1 are in proteins predicted to be associated with membranes.

The MIN1 N terminus is a C2 domain capable of membrane association. C2 domains are found in proteins from a wide variety of eukaryotic organisms (and in the alpha-toxin of Clostridium perfringens) (54) that are either located in, or transiently associated with, cellular membranes (9, 65). C2 domains bind phospholipids, often in a Ca2+-dependent manner (34, 53). The MIN1 C2 domain sequence is most similar to hypothetical proteins from the red flour beetle (30% identity with the MIN1 C2 domain), the chicken (23%), the nematode Caenorhabditis (26%), the protozoan Leishmania (26%), the parasitic protozoan Trypanosoma (24%), and plants (Fig. 4A).
With the exception of the Leishmania and Trypanosoma proteins, each of the aligned proteins was predicted by TMHMM to contain a transmembrane sequence. The Arabidopsis (25% identity) and rice (Oryza sativa, 25% and 26% identity) proteins also contain GRAM domains (named after the glycosyltransferases, Rab-like GTPase activators, and myotubularins that contain the domain), which were originally identified in eukaryotic proteins that function in membrane-associated processes (12). As was the case with the LysM domain, the MIN1 C2 domain sequence is most similar to that of C2 domains in eukaryotic proteins that are predicted to be associated with membranes.

C2 domain sequences form a sandwich of two β-sheets, each comprised of four β-strands arranged in one of two distinct topological folds (26). In topology I structures, such as those in synaptotagmins (16), the most N-terminal β-strand (β1) occupies the same position in space as the most C-terminal strand (β8) in topology II structures, such as that of phospholipase A2 (60). In many C2 domains, three loops on one end of the sandwich contain five conserved aspartate residues that coordinate Ca\(^{2+}\) and are important for phospholipid binding (42, 70). Other C2 domains associate with phospholipid membranes in a Ca\(^{2+}\)-independent manner, and nonacidal residues substitute for one or more of the loop aspartates (38, 55, 75). In protein kinase C, a cluster of lysine residues in strands β3 and β4 (of a topology I domain) are also important for membrane binding (67).

The 3D-PSSM (28) and LOOPP (45, 81) threading algorithms predicted that the three-dimensional structure of the MIN1 C2 domain could be comprised of four β-sheets, each containing five conserved aspartate residues. Dots denote conservation of residues in ≥50% of the sequences. (B) ClustalW alignment of the MIN1 transmembrane domain with predicted membrane-spanning sequences identified using blastp. The GenBank accession number follows each sequence: Nocardioides sp. strain JS614 sulfate transporter, Natronomonas pharaonis membrane-spanning sequences identified using blastp. The GenBank accession number follows each sequence: Chlamydomonas reinhardtii predicted protein Ot12g0040, Roseovarius rubifluvius hypothetical protein RSc2148.

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With the exception of the Leishmania and Trypanosoma proteins, each of the aligned proteins was predicted by TMHMM to contain a transmembrane sequence. The Arabidopsis (25% identity) and rice (Oryza sativa, 25% and 26% identity) proteins also contain GRAM domains (named after the glycosyltransferases, Rab-like GTPase activators, and myotubularins that contain the domain), which were originally identified in eukaryotic proteins that function in membrane-associated processes (12). As was the case with the LysM domain, the MIN1 C2 domain sequence is most similar to that of C2 domains in eukaryotic proteins that are predicted to be associated with membranes.

C2 domain sequences form a sandwich of two β-sheets, each comprised of four β-strands arranged in one of two distinct topological folds (26). In topology I structures, such as those in synaptotagmins (16), the most N-terminal β-strand (β1) occupies the same position in space as the most C-terminal strand (β8) in topology II structures, such as that of phospholipase A2 (60). In many C2 domains, three loops on one end of the sandwich contain five conserved aspartate residues that coordinate Ca\(^{2+}\) and are important for phospholipid binding (42, 70). Other C2 domains associate with phospholipid membranes in a Ca\(^{2+}\)-independent manner, and nonacidic residues substitute for one or more of the loop aspartates (38, 55, 75). In protein kinase C, a cluster of lysine residues in strands β3 and β4 (of a topology I domain) are also important for membrane binding (67).

The 3D-PSSM (28) and LOOPP (45, 81) threading algorithms predicted that the three-dimensional structure of the MIN1 C2 domain could be comprised of four β-sheets, each containing five conserved aspartate residues. Dots denote conservation of residues in ≥50% of the sequences. (B) ClustalW alignment of the MIN1 transmembrane domain with predicted membrane-spanning sequences identified using blastp. The GenBank accession number follows each sequence: Nocardioides sp. strain JS614 sulfate transporter, Natronomonas pharaonis membrane-spanning sequences identified using blastp. The GenBank accession number follows each sequence: Chlamydomonas reinhardtii predicted protein Ot12g0040, Roseovarius rubifluvius hypothetical protein RSc2148.
cells is required for eyespot assembly, the D19A and D77A mutations were introduced into the RbcS2-MIN1 fusion construct in pR-MIN1 (see Materials and Methods) and the phenotypic consequences of the changes were assessed in Chlamydomonas. The aspartate residue mutations had no effect on the rate of ptx−H/1100 rescue (Table 1, pR-D19A,D77A), and eyespots in the transformed cells had a size similar to that of eyespots in wild-type cells (Fig. 6). Likewise, changing conserved lysine residues D19, K37, T38, K42, and D77 to alanine had no effect on the rate of ptx−H/1100 rescue (Table 2, pR-D19A,K37A,T38A,K42A,D77A). The aspartate residue mutations had no effect on the ptx−H/1100 rescue (Table 1, pR-D19A,D77A), and eyespots in the transformed cells had a size similar to that of eyespots in wild-type cells (Fig. 6). Likewise, changing conserved lysine residues D19, K37, T38, K42, and D77 to alanine had no effect on the rate of ptx−H/1100 rescue (Table 2, pR-D19A,K37A,T38A,K42A,D77A).
residues K37 and K42 to alanines or changing residue T38 to alanine had little to no effect on phenotypic rescue (Table 1 and Fig. 6, pR-K37A,K42A and pR-T38A). These data suggest that in *Chlamydomonas*, any interaction between the MIN1 C2 domain and eyespot membranes either has atypical requirements or is not essential for eyespot assembly, possibly because the function is redundant with that of another eyespot protein.

**ChR1 photoreceptor levels are dependent on MIN1.** To determine whether the loss of MIN1 affects levels or localization of ChR1, one of the major photoreceptors in the eyespot (5, 51, 74), polyclonal anti-ChR1 (5) was used to analyze ChR1 levels and localization in wild-type or *min1* mutant cells. On Western blots of *Chlamydomonas* whole-cell extracts from cells grown photoautotrophically (in the light in acetate-free me-

![Fig. 5](image1.png)

**FIG. 5.** The MIN1 C2 domain is associated with the chloroplast envelope in moss cells. (A) *Physcomitrella patens* (moss) cells were transiently transfected with plasmid DNA encoding a MIN1 C2 domain-YFP fusion protein containing either wild-type sequence (ΔLMTM:YFP) or sequence encoding a C2 domain in which conserved aspartic acid residues D19 and D77 were changed to alanines (ΔLMTMΔa:YFP). Cells expressing the YFP constructs were analyzed using an Olympus Fluoview FV500 laser confocal microscope. Green indicates YFP fluorescence, and red indicates chlorophyll autofluorescence. (B) High-magnification view of cells transformed with the MIN C2 domain-YFP constructs and plasmid DNA encoding an *Arabidopsis* outer chloroplast envelope protein (GenBank accession number 18419973)-CFP fusion protein. Green indicates YFP fluorescence, red indicates chlorophyll autofluorescence, and blue indicates CFP fluorescence.

![Fig. 6](image2.png)

**FIG. 6.** Conserved residues in the MIN1 C2 domain are not essential for eyespot assembly. Light micrographs of a wild-type cell or of *min1* cells transformed with pR-D19A,D77A or pR-T38A (see Materials and Methods and Table 1) are shown. The cultures were grown in M medium, and the single cells shown were typical of the majority of cells in each culture.
dium, conditions under which the min1 eyespot is disorganized), the level of ChR1 was reduced to 65% ± 15% in the min1 nonsense mutant (strain 12-12) compared to the wild type (Fig. 7A) (see Materials and Methods and Table 1). The blot was probed with a polyclonal antibody against the ChR1 photoreceptor (5), followed by antitubulin (clone B-5-1-2; Sigma, St. Louis, MO). ChR1 levels were unaffected by either the RbcS2-MIN1 fusion or the mutation of conserved residues within the MIN1 C2 domain. These data indicate that MIN1 promotes photoreceptor expression and/or stabilization and that the conserved residues analyzed are not essential for this function.

Immunofluorescence with anti-ChR1 supported and extended these data (Fig. 7B). As observed previously, ChR1 was localized to a single equatorial spot in wild-type cells (5). Anti-ChR1 staining of the basal bodies observed in both wild-type and min1 cells. In the photograph of min1 cells, the arrow points to a cell containing two roughly equatorial “spots” of anti-ChR1 signal. The signal at the basal bodies is most likely nonspecific binding of anti-ChR1 (5).

FIG. 7. Photoreceptor levels are low in min1 mutant cells. (A) Western blot of total cellular protein from M medium-grown cultures of wild-type (wt) or min1 mutant (strain 12-12) cells or of min1 cells transformed with pR-MIN1, pR-D19A,D77A, pR-K37A,K42A, or pR-T38A (see Materials and Methods and Table 1). The blot was probed with a polyclonal antibody against the ChR1 photoreceptor (5), followed by antitubulin (clone B-5-1-2; Sigma, St. Louis, MO). (B) Immunofluorescence of M medium-grown wild-type cells or min1 mutant cells (strain 12-12) probed with anti-ChR1. In the photographs of wild-type cells, the arrows point to an eyespot-associated “stripe” of immunofluorescence regularly observed in both wild-type and min1 cells. In the photograph of min1 cells, the arrow points to a cell containing two roughly equatorial “spots” of anti-ChR1 signal. The signal at the basal bodies is most likely nonspecific binding of anti-ChR1 (5).
ing of the anti-ChR1 polyclonal antibody, as it was observed in
cells in which ChR1 expression had been silenced (5). In the
min1 nonsense mutant, ChR1 spots were significantly smaller
than those in the wild type, and in approximately one-fourth of
the cells the fluorescence was distributed among two or more
spots. However, the anti-ChR1 staining remained localized
near the equator and to a single side of the cells. Given the
disorganization of the pigment granule layers in min1 cells
grown without acetate (33), these observations suggest that
approximate localization of ChR1 does not require proper
organization of the plastid components of the eyespot.

In every staining of either wild-type or min1 cells, we also
observed cells with a longitudinal “stripe” of anti-ChR1 signal
extending from the anterior end of the cell toward, or just next
to, the eyespot (Fig. 7B) and sometimes beyond. The percentage
of cells in which this stripe was observed varied between
experiments, and additional data are required to determine
whether this staining indicates specific or nonspecific binding
of the anti-ChR1 polyclonal antiserum.

**Growth in acetate affects ChR1 in min1 mutant cells.**
*Chlamydomonas* can grow photoautotrophically in the
presence of light and CO₂, mixotrophically in the presence of both
acetate and light, and heterotrophically in the dark, utilizing
acetate as a carbon source. In min1 nonsense mutant cells
grown photoautotrophically (in acetate-free M medium), the
eyespot pigment granule layers are relatively disorganized and
are not apposed to the plasma membrane (33). Surprisingly, in
min1 cells grown mixotrophically (in acetate-containing R me-
dium), the pigment granule layers are more organized and are
apposed to the plasma membrane (33). To determine whether
increased organization of the granule layers is correlated with
increased levels of ChR1, Western blots of total cellular pro-
tein from min1 nonsense mutant cells (strain 12-12) grown in
the light, with or without acetate, were probed with anti-ChR1
(Fig. 8A). Contrary to what was expected in min1 cells grown
with acetate (pigment granules more organized), the level of
ChR1 was only 70% ± 10% of that in min1 cells grown without
acetate (pigment granules disorganized). This difference was
not observed in wild-type cells. Immunofluorescence analyses
were consistent with the Western blot data (Fig. 8B); equato-
rial spots of ChR1 fluorescence were less frequent and notice-
able smaller in min1 cells grown mixotrophically than in those
grown photoautotrophically. Again, this difference was not
observed in wild-type cells. These data suggest that ChR1 expres-

![FIG. 8. ChR1 levels are very low in min1 cells grown in acetate-containing medium. (A) Western blot of total cellular protein from wild-type (wt) or min1 mutant (strain 12-12) cells grown in medium either lacking (M) or containing (R) acetate. The blot was probed with a polyclonal antibody against the photoreceptor ChR1 (5) followed by antitubulin (clone B-5-1-2; Sigma, St. Louis, MO). (B) Anti-ChR1 immunofluorescence of wild-type or min1 (strain 12-12) cells grown in either the absence (panels M) or presence (panels R) of acetate. For the min1 cells grown in M or R medium, both a field of cells and representative individual cells are shown. The arrows point to equatorial anti-ChR1; fluorescence at the anterior ends of the cells is most likely the result of nonspecific binding of anti-ChR1 in the region of the basal bodies (5).](http://ec.asm.org/Downloaded from)
sion and/or stability is more sensitive to the absence of MIN1 in cells grown mixotrophically than in cells grown photoautotrophically.

**DISCUSSION**

The eyespot of the green alga *Chlamydomonas* is a photosensory structure required for phototaxis. In *min1* (mini-eyed) mutants, eyespot pigment granule numbers are reduced, and apposition of the plastid and plasma membrane components of the eyespot is disrupted (33). Here we describe the identification and initial characterization of the MIN1 gene and the encoded protein. The data highlight the novel domain composition of MIN1 and suggest that MIN1 promotes both apposition between the plastid and plasma membranes and expression and/or stability of the ChR1 photoreceptor.

The MIN1 protein contains N-terminal C2 (phospholipid membrane-binding) and C-terminal LysM (peptidoglycan-binding) domains, separated by a membrane-spanning α-helix. The LysM domain was originally found in bacterial cell wall-degrading enzymes that bind peptidoglycans (3). More recently the domain has been identified in plant proteins, specifically plasma membrane-localized receptors that interact with NOD (nodulation) factors in the cell walls of rhyzobial bacteria (37, 64, 76) and membrane proteins that trigger the defense response to chitin oligosaccharides in the cell walls of pathogenic fungi (27, 29). A LysM domain is also present in the *Chlamydomonas* eyespot protein, EYE2 (66, 71), and in a number of predicted *Chlamydomonas* proteins (unpublished observation), but potential interacting partners for these domains remain unknown. The MIN1 LysM domain is most similar to the same domain in the green alga *Ostreococcus tauri* and a variety of eubacteria. Each of these proteins also contains either a predicted membrane-spanning helix or a BON domain, which is hypothesized to bind to membranes (83). Together with proteomics data identifying MIN1 in eyespots (71) and the observation that MIN1 does not contain a chloroplast-targeting sequence identifiable by homology, the data suggest that MIN1 is embedded in the chloroplast envelope or plasma membrane in the eyespot.

The MIN1 C2 domain is also most similar to C2 domains in predicted membrane-associated proteins. C2 domains fold into an eight-stranded β-sheet “sandwich,” and membrane association often requires Ca\(^{2+}\) coordination by aspartate residues in loops connecting individual β-strands. Membrane association of some C2 domains requires lysine residues on one side of the sandwich. A MIN1 C2 domain-YFP fusion protein localized to the chloroplast envelope in moss cells, and mutation of the two loop aspartate residues conserved in MIN1 abolished this association. These data indicate that the MIN1 C2 domain is capable of membrane association in a manner similar to that of more well-characterized C2 domains (34). However, *Chlamydomonas* transformants containing full-length MIN1 with the same aspartate residue mutations were phototactic and had wild-type eyespots. Similarly, transformants with mutations in conserved lysine residues in the MIN1 C2 domain were phototactic and had normal eyespots. One explanation for these data is that properties of the full-length MIN1 protein and/or the *Chlamydomonas* cellular environment minimize the requirement for these conserved residues. In previous studies (46, 75), the phenotypic consequences of C2 domain mutations were affected by the protein and cellular context, consistent with the hypothesis that interaction of C2 domains with phospholipid membranes is dependent on the distribution of electrostatic potential on the surface of the binding site rather than the presence of specific residues or Ca\(^{2+}\) coordination (49, 55).

A second possibility is that the membrane-binding potential of the MIN1 C2 domain is not required for eyespot assembly. Either the C2 domain does not interact with membranes in *Chlamydomonas*, or the function of the domain in eyespot assembly is redundant. The existence of another protein that promotes eyespot membrane apposition could also explain the increased organization of the pigment granule/thylakoid membrane layers in *min1* mutants grown mixotrophically. Further analyses are required to determine whether the MIN1 C2 domain associates with a *Chlamydomonas* membrane and, if so, whether the association is Ca\(^{2+}\) dependent and/or essential for eyespot assembly.

To date, the C2-plus-LysM domain composition is unique to MIN1, perhaps reflecting the fact that *Chlamydomonas* is the only eyespot-containing organism for which the complete genome sequence is available (48). The similarity of the C2 domain to eukaryotic sequences and of the LysM domain to eubacterial sequences prompts the speculation that MIN1 is the result of domain shuffling between genes encoding membrane-associated proteins in the original eukaryotic host and the endosymbiotic cyanobacterium and reflects the symbiotic origins of the eyespot (7, 19). Eyespot assembly requires that organization of the plastid pigment granule/thylakoid membrane layers, derived from an endosymbiotic cyanobacterium, is coordinated with localization of photoreceptors and other plasma membrane components, some of which are presumably derived from the host. How did this intricate coordination evolve? Insight may come from analyses of the function of a MIN1-like protein in modern symbiotic relationships such as the developing symbiosis between a green alga in the genus *Nephrosemis* and the flagellate *Hatena arenicola* (56, 57). *Hatena* cells containing an engulfed *Nephrosemis* cell have an apical eyespot in which *Nephrosemis* plastid and plasma membranes are apposed to the *Hatena* plasma membrane. At cell division, the *Nephrosemis* cell and the eyespot are inherited by one of the daughters, while the other daughter develops an apical feeding apparatus and resumes a phagocytic lifestyle. Are MIN1-like proteins encoded by the *Hatena* and/or *Nephrosemis* genome? If so, do they function in eyespot assembly in symbiotic cells and/or assembly of the feeding apparatus in phagocytic *Hatena* cells? Future molecular and cell biological studies should provide answers to these questions.

In *min1* mutants grown photoautotrophically, the eyespot pigment granule/thylakoid membrane layers are disorganized and the overlying chloroplast envelope is no longer apposed to the plasma membrane (33). In cells grown mixotrophically in the light with acetate, *min1* eyespots are more ordered and the chloroplast envelope and plasma membrane remain apposed. Do MIN1 and/or the physiological state of the cell also affect the plasma membrane components of the eyespot, specifically the photoreceptors? In *min1* cells grown photoautotrophically, the level of ChR1, a rhodopsin family eyespot photoreceptor, is lower than that in wild-type cells, indicating that MIN1 promotes ChR1 expression and/or stability. ChR1
was even more reduced in min1 mutant cells, but not wild-type cells, grown mixotrophically, which suggests that under mixotrophic conditions, the requirement for MIN1 is more stringent despite the apparent increased organization of the plastid components of the eyespot.

In approximately 25% of min1 cells grown photoautotrophically, ChR1 was found in two or more roughly equatorial aggregations in the plasma membrane. This pattern is notably different from that of the pigment granules in photoautotrophically grown cells, which occur as a single aggregation that is disorganized and no longer apposed to the plasma membrane (33). This observation is consistent with the hypothesis that photoreceptor localization is not dependent solely on proper organization of the underlying pigment granule layers. We propose a testable model in which MIN1 is embedded in the plasma membrane or chloroplast envelope in the eyespot. The MIN1 C2 domain is predicted to promote membrane apposition, perhaps in combination with another eyespot protein. MIN1 also promotes expression and/or stabilization of ChR1; however, neither MIN1 nor proper organization of the pigment granule/thylakoid membrane layers is required for proper localization of the ChR1 photoreceptor.

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