Independent Localization of Plasma Membrane and Chloroplast Components during Eyespot Assembly

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Like many algae, Chlamydomonas reinhardtii is phototactic, using two anterior flagella to swim toward light optimal for photosynthesis. The flagella are responsive to signals initiated at the photosensory eyespot, which comprises photoreceptors in the plasma membrane and layers of pigment granules in the chloroplast. Phototaxis depends on placement of the eyespot at a specific asymmetric location relative to the flagella, basal bodies, and bundles of two or four highly acetylated microtubules, termed rootlets, which extend from the basal bodies toward the posterior of the cell. Previous work has shown that the eyespot is disassembled prior to cell division, and new eyespots are assembled in daughter cells adjacent to the nascent four-membered rootlet associated with the daughter basal body (D4), but the chronology of these assembly events has not been determined. Here we use immunofluorescence microscopy to follow assembly and acetylation of the D4 rootlet, localization of individual eyespot components in the plasma membrane or chloroplast envelope, and flagellar emergence during and immediately following cell division. We find that the D4 rootlet is assembled before the initiation of eyespot assembly, which occurs within the same time frame as rootlet acetylation and flagellar outgrowth. Photoreceptors in the plasma membrane are correctly localized in eyespot mutant cells lacking pigment granule layers, and chloroplast components of the eyespot assemble in mutant cells in which photoreceptor localization is retarded. The data suggest that plasma membrane and chloroplast components of the eyespot are independently responsive to a cytoskeletal positioning cue.

Photosynthetic algae are ubiquitous, morphologically diverse, and important producers of fixed carbon (1–3). Single cells of many algae, both unicellular species and the gametes or spores of multicellular species, assemble flagella, which propel the cells through their aqueous environment, often in response to environmental stimuli, such as chemical concentration gradients (chemotaxis), gravity (gravitaxis), or light (phototaxis) (4–6). Algal flagella are analogous to mammalian cilia, and their outgrowth is organized by cytoplasmic basal bodies which are associated also with the minus ends of cytoplasmic microtubules, including one or more microtubule bundles termed rootlets (7). Flagellate algal species are classified in part based on the characteristic arrangement of the flagella, basal bodies, and associated rootlets, collectively termed the “flagellar apparatus.” One universal feature of the flagellar apparatus is its asymmetry; the morphology and/or function of a single flagellum-basal body-rootlet complex is not the mirror image of the other even though they are often visually indistinguishable (8–11). This asymmetry, believed to derive ultimately from the maturation of cytoskeletal structures over the course of two or more cell cycles, is critical for appropriate directional movement in response to environmental cues (11–15).

In many phototactic algae, light intensity and direction is sensed by a structure called the eyespot, located at a defined asymmetric position relative to the flagellar apparatus, usually at the base of a specific flagellum or associated with a specific rootlet (16). In Chlamydomonas reinhardtii, a phototactic unicellular green alga that is 5 μm to 10 μm in length, the flagellar apparatus comprises two anterior basal bodies and flagella and four highly acetylated rootlets that extend just under the plasma membrane from the basal bodies toward the posterior of the cell (see Fig. 1A) (17–20). The M2 and M4 rootlets, comprising two and four microtubules, respectively, extend from the older or mother basal body, while the D2 and D4 rootlets extend from the younger or daughter basal body (7, 9, 21). The numbering system proposed by Moestrup (11) designates the M2, M4, D2, and D4 rootlets as R1, R2, R3, and R4, respectively, while an alternative nomenclature designates these rootlets as 1d, 1s, 2d, and 2s (22, 23). The ~0.6- to 1.5-μm² Chlamydomonas eyespot is located near the equator of the cell adjacent to the D4 rootlet (see Fig. 1A), placing it closer to the cis flagellum assembled from the daughter basal body than to the trans flagellum assembled from the mother basal body (22, 24–27). The cis flagellum and trans flagellum respond differently to light-evoked signals from the eyespot, causing the cell to turn toward or away from the light source, depending on intensity (28–31). The close physical and functional associations between the algal eyespot and flagellar apparatus have led to the proposal that eyespot position is determined by the geography of the microtubule-based cytoskeleton (22, 32). In Chlamydomonas, the daughter rootlets and the eyespot are assembled de novo in each daughter cell following cell division (see Fig. 1B and C), and the position of the nascent D4 rootlet is hypothesized to determine the asymmetric location of the eyespot (10, 25, 33).

Algal eyespots comprise at least two components, a concentration (or “patch”) of photoreceptor molecules and a structure often made up of layers of pigmented lipid granules that intensifies the directional signal by reflecting or focusing light onto and/or shading the photoreceptor patch (4, 16, 34). In the Chlamydomonas eyespot, several layers of orange-red carotenoid pigment gran-
ules, subtended by thylakoid membranes in the chloroplast, lie directly beneath specialized regions of the closely apposed chloroplast envelope and plasma membrane (see Fig. 1B) (22, 35). Channelrhodopsins ChR1 and ChR2, rhodopsin family light-gated cation channels that initiate the phototactic response, are localized in the plasma membrane at the eyespot (36–40). EYE2, an eyespot-specific, thioredoxin superfamily protein of unknown function, is thought to reside in one of the two chloroplast envelope membranes (39, 41, 42). Following cell division, one or more eyespot proteins must respond directly to a positioning cue, such as the D4 rootlet, to initiate de novo eyespot assembly. Assembly might occur in a “cascade,” in which cue-directed localization of one eyespot component leads to localization of the others. Alternatively, plasma membrane and plastid components might localize independently of one another in response to the same cue. Immunofluorescence microscopy (IF) of eye2 (eyeless) mutant cells, which lack the pigment granule layers and the EYE2 protein, showed that the ChR1 photoreceptor can localize independently of the missing plastid components, prompting the hypothesis that D4-directed localization of the photoreceptor(s) is the initial step in eyespot assembly (36, 41, 43). This hypothesis predicts that following cell division, assembly of the D4 rootlet occurs before localization of eyespot components to the position of the nascent eyespot and that photoreceptor localization occurs before that of EYE2 or the pigment granule layers.

Studies of Chlamydomonas cell division using electron, light, and immunofluorescence microscopy form the basis for our understanding of the inherent asymmetry of the flagellar apparatus based on the relative age of its components and the relationship between the flagellar apparatus and the eyespot (25, 44–50). The described changes in these structures during cell division are summarized in Fig. 1C; after mitosis (panel iv), nonacetylated cleavage microtubules assemble in the plane of the metaphase band and the cleavage furrow, and the daughter flagella, acetylated microtubule rootlets, and eyespots assemble de novo in the daughter cells (panel v). Here, we extend the previous work using indirect IF to observe the accumulation and positioning of the photoreceptors and EYE2 relative to assembly of the daughter rootlets and flagella during and following cell division. The data indicate that the D4 rootlet is assembled prior to the initial localization of eyespot proteins, which occurs within the same time frame as rootlet acetylation. Following the initiation of eyespot assembly, the rootlets extend to the posterior end of the cell before shortening to their mature length, which in the case of the D4 rootlet is just beyond the equatorial eyespot (36). In ml1t (multiedey) mutant cells, which have multiple misplaced eyespots and a short D4 (36, 51), photoreceptor localization is retarded but EYE2 localization is not. Together, the data are consistent with a model in which the plasma membrane and plastid components of the eyespot respond independently to the D4 positioning cue and suggest that the anterior-posterior position of the assembled eyespot ultimately determines D4 rootlet length.

MATERIALS AND METHODS

Strains and cell culture. The following Chlamydomonas reinhardtii strains used in this study are available through the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN; chlamycollecction.org): wild type (CC-125, 137c mt+), ml1t mutant (CC-4304, ml1t-1 mt+), eye2 mutant (CC-4302, eye2-1 mt+), eye3 mutant (CC-4302, eye3-1 mt+). The original cop3-null strain (CAL27.01.12) was generated by insertional mutagenesis of the 4A+ wild-type strain (52), with the AphVIII gene conferring resistance to paromomycin. The strain had no ChR1 protein detectable by Western blotting or indirect immunofluorescence using the anti-ChR1 polyclonal antibody (see Fig. S1 in the supplemental material). The cop3Δ strain used here was isolated following two backcrosses of the original null strain to wild-type strain 137c mt+ (CC-124). The ml1t eye3 double mutant was isolated following a cross of an ml1t mt+ strain to strain eye3-3 (CC-4317, eye3-3 mt+). Chlamydomonas cultures were maintained at 25°C on solid Tris-acetate-phosphate (TAP) medium (21) on a 12-12 light-dark cycle to synchronize cell division, which mostly occurred within the first several hours of the dark period (21, 53).

Indirect immunofluorescence labeling and microscopy. Dividing cells were transferred quickly from solid medium to 50 μl phosphate-buffered saline (PBS) (123.0 mM NaCl, 10.4 mM Na2HPO4, 3.2 mM KH2PO4) using a toothpick, harvested by centrifugation at 3,000 × g, and resuspended in 50 μl of autolysin (21). Ten microliters was spotted onto a poly-l-lysine-coated coverslip which was incubated in a humid, dark chamber at room temperature (RT) for 30 min, dipped in ~20°C methanol for 30 s, and air dried. The fixed cells were rehydrated in PBS plus 1% bovine serum albumin (BSA; block) for 30 min at RT and incubated in block containing 0.1% Triton X-100 for 30 min at RT, followed by incubation overnight at 4°C in block containing 1:20 anti-acetylated α-tubulin clone 6-11B-1 (α-Ac-tub) (Sigma-Aldrich, St. Louis, MO) (54) plus one of the following antibodies: 1:20 anti-α-tubulin clone DM1a (α-tub; Sigma-Aldrich, St. Louis, MO), 1:50 polyclonal anti-ChR1 (37), 1:50 polyclonal anti-ChR2 (37) (see Fig. S2B in the supplemental material), 1:50 polyclonal anti-EYE2 (41). Cells were washed with block containing 0.05% Tween 20, incubated for 2 h at room temperature in block containing a 1:1,000 dilution of the appropriate Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, CA), washed thoroughly, and coverslipped with Mowiol mounting medium prepared as follows: 2.4 g Mowiol 4-88 (Calbiochem, EMD Biosciences, Inc., La Jolla, CA) and 6 g of glycerol were combined, 6 ml of water was added, and the solution was stirred at RT for 2 h before the addition of 12 ml of 0.2 M Tris (pH 8.5), being heated to 50°C for 10 min, and being clarified by centrifugation at ~5,000 × g for 15 min. When clone 6-11B-1 was used in conjunction with clone DM1a, Alexa Fluor-conjugated anti-mouse IgG2B and anti-mouse IgG1 secondary antibodies were employed (Invitrogen). When anti-EYE2 was used in combination with anti-ChR1, one of the antibodies was directly conjugated to an Alexa Fluor using the Xenogen IgG labeling kit (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence was viewed using a Deltavision RT (Applied Precision, LLC, Issaquah, WA) with a X-100-magnification, 1.4-numerical-aperture (NA) objective plus a 1.6× optic and appropriate filter sets. Images in Fig. 5A (example 2) and B were captured without the 1.6× opticvar and appropriate filter sets. Images were captured with a CoolSnap HQ charge-coupled-device (CCD) camera (Photometrics, Tucson, AZ). Z-series data sets were collected at a step size of 0.5 μm. Postacquisition deconvolution was performed using SoftWorx software (Applied Precision, LLC, Issaquah, WA).

Light microscopy. A total of 10 μl of cells transferred from solid medium to 50 μl PBS was spotted onto a microscope slide and coverslipped. The cells were viewed with a Leica DMRXA microscope using a Leica PL APO X100-magnification, 1.4-numerical-aperture, oil immersion objective with a 1.6× optic (1 pixel = 0.039 μm) and bright-field or differential interference contrast (DIC) optics. After 5 to 10 min on the slide, the slightly flattened, less motile cells were photographed with a QImaging (Burnaby, BC, Canada) Retiga EX-cooled CCD camera driven by Universal Imaging (Downington, PA) MetaMorph version 6.1.2 software.

Data analysis. Flagellar, rootlet, and cell lengths, and the distances between the basal bodies and the eyespot protein patches, were obtained from the micrographs using either the Softworx “standard two point” or “multiple segment” measuring tool or the ImageJ “lines” tool. For the experiments represented in Fig. 3, 6, and 7D, F, and G, dividing cells were categorized as without flagella or as having flagella that were ≤0.5 μm in length.
length and then scored for the presence or absence of an eyespot protein patch. For the experiments represented in Fig. 4 and 7C, cells were categorized according to flagellar length and then scored for the presence or absence of a pigment granule array. For the experiment represented in Fig. 5, cells were categorized based on rootlet length, and flagellar and cell lengths were determined and compared between groups. For the experiment represented in Fig. 8D, eyespot protein patches in mitotic cells were categorized based on their distance from the basal bodies (the wild-type measurement is diagrammed in Fig. 7A) and scored for the presence or absence of anti-ChR1 and anti-EYE2 fluorescence. For the quantitation described in Fig. 8C, groups of two or four mitotic daughter cells that (i) remained in the mother cell wall and (ii) had at least one pigment granule array in each cell were examined using bright-field microscopy. For each group, the number of daughter cells with two or more pigment granule arrays was determined.

**Western blotting.** Dividing cells were scraped from solid medium and transferred to 500 µl PBS, harvested at 3,000 × g, resuspended in 200 µl of 4× Laemmli buffer (250 mM Tris-Cl [pH = 6.8], 40% glycerol, 20% β-mercaptoethanol, 8% SDS, 0.024% bromophenol blue) with protease inhibitors (5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin A, 1.0 mM phenylmethylsulfonyl fluoride [PMSF]), and heated at 100°C for 5 min. A total of 20 µl to 40 µl of each sample was electrophoresed through 10% polyacrylamide-SDS gels and transferred to BioRad's poly(vinylidene difluoride) (PVDF) membranes (Pall Corp., Ann Arbor, MI) using standard techniques. The blots were blocked in 5% nonfat, dried milk (NFDM) in TBS-T (10 mM Tris-Cl, 150 mM NaCl, 0.05% Tween 20) for 1 h at RT, probed overnight at 4°C with either 1:5,000 anti-ChR1 or 1:10,000 anti-α-tubulin clone B-5-1-2 (Sigma) in 1% NFDM in TBS-T, washed in TBS-T, and probed with 1:10,000 horseradish peroxidase-linked secondary antibody (Pierce, Rockford, IL) in 1% NFDM in TBS-T. Following several washes in TBS-T, the blots were incubated in SuperSignal substrate (Pierce) for 1 min and exposed to ECL Hyperfilm (Amersham Biosciences, Piscataway, NJ).

**Image and figure production.** To produce a final micrographic image, ImageJ was used to collapse 8 to 10 Z-series images from a single channel, add false color, and merge images from different channels. Pixel value histograms were minimally adjusted using either “brightness and contrast” in ImageJ or "levels" in Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Scale bars were added, and the image size was adjusted in Adobe Photoshop. Images, text, and diagrams were assembled in Adobe Illustrator.

**RESULTS**

**D4 rootlet assembly occurs after mitosis but prior to flagellar extension.** During cell division in *Chlamydomonas*, the flagella, daughter microtubule rootlets (D2 and D4), and D4-associated eyespot assemble de novo in daughter cells (Fig. 1C). Our working hypothesis has been that the position of the highly acetylated D4 rootlet directs localization of one or both of the major rhodopsin family photoreceptors in the plasma membrane, ChR1 and ChR2, to the position of the future eyespot, which in turn directs localization and assembly of other eyespot components, such as EYE2, in the chloroplast envelope and the pigment granule layers in the chloroplast. To determine whether photoreceptor localization occurs prior to that of EYE2, dividing cells were analyzed by IF using α-Ac-tub in combination with antibodies specific for ChR1, ChR2, or EYE2 and relating the presence of nascent aggregations or “patches” of each eyespot protein to D4 rootlet assembly and acetylation and flagellar outgrowth in the daughter cells (Fig. 3). In interphase cells, anti-ChR1 and anti-EYE2 polyclonal antibodies specifically label the eyespot (37, 41), with some nonspecific labeling in the region of the basal bodies in cells devoid of these proteins (e.g., see Fig. S2A in the supplemental material). Anti-ChR2 also specifically labeled the eyespot (see Fig. S2B), but a ChR2-null strain was not available as a negative control. Daughter cells which met four criteria were scored for the presence of a patch of eyespot protein that was associated with the D4 and separate from the nonspecific labeling near the basal bodies: (i) the cell was one of a pair of daughters that remained associated with one another, either because cytokinesis was not complete or due to incomplete digestion of the mother cell wall during autolysis treatment, (ii) the two pairs of basal bodies had moved away from one another and from the cleavage plane, (iii) only one pair of basal bodies was present in each cell, indicating that the cell was not entering another round of division (in cells destined to divide again, the new probasal bodies assembled during metaphase [46] had extended, yielding four basal bodies that were strongly labeled with α-Ac-tub), and (iv)
Consistent with our earlier observation that the D4 rootlet was of ChR1 and ChR2, and at least 70% had EYE2 patches (Fig. 3B).

In daughter cells without flagella, eyespot protein patches were never observed. D4 rootlet microtubules were observed in daughter cells without flagella, suggesting that D4 assembly occurs before eyespot assembly. In contrast to daughters without flagella, ≥90% of daughters with flagella of ≤0.5 μm in length had patches of ChR1 and ChR2, and at least 70% had EYE2 patches (Fig. 3B). Consistent with our earlier observation that the D4 rootlet was acetylated within the same time frame as initial flagellar outgrowth, an acetylated D4 was observed in all postmitotic cells with newly emerged flagella but was observed only rarely in daughter cells without flagella. These data indicate that the photoreceptors and EYE2 are localized either simultaneously or in rapid succession and that all three proteins are localized within the same time window as flagellar emergence and D4 acetylation.

To determine when organized pigment granule layers were assembled in the chloroplast, dividing cells were labeled with a poly-
clonal antibody specific for the EYE3 protein, a predicted ABC1 family kinase that is present in the pigment granules (41). However, because the anti-EYE3 background level in dividing cells was too high to discern EYE3 patches, we turned to differential interference contrast (DIC) microscopy, which allows visualization of both the carotenoid-rich eyespot pigment granule layers and flagella. Dividing cells were treated with autolysin for 10 min to "loosen" the mother cell wall so that the flagella were clearly visible with DIC and their length could be measured. Groups of two or four sister cells were categorized according to their average flagellar length and the presence (or absence) of a visible pigment granule array in at least one of the sister cells (Fig. 4). Pigment granule arrays were observed in only 8% of the sister groups, with an average flagellar length of \( \approx 1.0 \mu m \). As photoreceptor and EYE2 patches were observed in the majority of cells with flagella of \( \approx 0.5 \mu m \) in length, the data suggest that the photoreceptor and EYE2 proteins are localized prior to assembly of the pigment granule layers. However, the sensitivity of pigment granule array visualization by DIC microscopy may be lower than that of detection of eyespot proteins by immunofluorescence (24). As the average flagellar length increased, the number of sister groups with pigment granule arrays also increased, indicating that the eyespot pigment granule layers and flagella are assembled within the same time frame.

**Daughter cells pass through a “long rootlet” stage.** During IF examination of dividing cells labeled with \( \alpha \)-Ac-tub, we observed flagellated daughter cells that had completed cytokinesis in which the acetylated rootlets extended to the posterior end of the cell (Fig. 5). Many of the cells with long rootlets also had more than four acetylated microtubules or microtubule bundles. The length of the rootlets contrasted with that in interphase cells, in which the D4 rootlet, typically the longest, extends just beyond the equatorial eyespot such that the distance from the basal bodies to the end of the rootlet (R) relative to the length of the cell (L) is 0.68 \( \pm 0.12 \mu m \) (36). To further characterize the cells with long rootlets, flagellated cells from a dividing culture were randomly chosen, and distances R and L and the length of a flagellum (F) were measured (Fig. 5C, inset). The cells were categorized as having either an R/L value of \( \leq 0.8 \) for all rootlets (as for interphase cells) or as having long rootlets, with an R/L value of \( > 0.8 \) for two or more rootlets. Cells without long rootlets \( (n = 52) \) averaged 5.83 \( \pm 0.52 \mu m \) in length, with an F/L value of 1.34 \( \pm 0.19 \). Cells in the long rootlet category \( (n = 54) \) were only slightly smaller, with an L value of 5.36 \( \pm 0.52 \mu m \), but had significantly shorter flagella, with an F/L value of 0.97 \( \pm 0.14 \), most likely because they had recently divided and had not yet assembled full-length flagella. These data are indicative of a post-cell division stage during which the acetylated rootlet microtubules are longer than those in the fully mature cell.

**Photoreceptor localization and EYE2 localization in postmitotic cells are independent of one another and of EYE3 localization.** Consistent with a cascade model of eyespot assembly in which localization of the photoreceptors directs assembly of the chloroplast components of the eyespot, ChR1 patches are correctly localized in interphase eye2 or eye3 (eyeless) mutant cells, which lack organized pigment granule arrays (41, 51). To determine whether the absence of pigment granule layers affected the
timing of photoreceptor localization, the presence or absence of photoreceptor patches in dividing eye2 or eye3 daughter cells was analyzed (Fig. 6A, D, and F). Similar to dividing wild-type cells, photoreceptor patches were present in the majority of eye2 or eye3 daughter cells with flagella of /H11349 0.5 /H9262 m in length but were not observed in eyeless daughter cells without flagella. The percentage of daughter cells (no.) that contained a patch of accumulated eyespot protein associated with the D4 rootlet is indicated below each image. The lower percentage of cells scored as having EYE2 patches was most likely due to the higher background produced by anti-EYE2 in some experiments, making small patches of EYE2 associated with the D4 rootlet more difficult to discern than similarly sized photoreceptor patches. Patches of fluorescence associated with the D4 rootlet were not observed when cop3Δ or eye2 mutant cells were labeled with anti-ChR1 or anti-EYE2, respectively (e.g., see Fig. S2A in the supplemental material). Interpretive diagrams summarizing the data are to the left of the micrographs: dark blue, mother basal bodies and rootlets and the metaphase band; light blue, daughter basal bodies and rootlets; black lines, flagella; red circle, eyespot protein patch. Scale bars = 1 /H10005 m.

FIG 3 Eyespot photoreceptors and EYE2 localize to patches in cells with newly emerged flagella. IF images of dividing cells that had completed mitosis but either had not extended flagella (A) or had flagella that were ≤0.5 /H10005 m in length (B): green, α-Ac-tub; red, anti-ChR1 or anti-ChR2; blue, anti-EYE2. The percentage of daughter cells (no.) that contained a patch of accumulated eyespot protein associated with the D4 rootlet is indicated below each image. The lower percentage of cells scored as having EYE2 patches was most likely due to the higher background produced by anti-EYE2 in some experiments, making small patches of EYE2 associated with the D4 rootlet more difficult to discern than similarly sized photoreceptor patches. Patches of fluorescence associated with the D4 rootlet were not observed when cop3Δ or eye2 mutant cells were labeled with anti-ChR1 or anti-EYE2, respectively (e.g., see Fig. S2A in the supplemental material). Interpretive diagrams summarizing the data are to the left of the micrographs: dark blue, mother basal bodies and rootlets and the metaphase band; light blue, daughter basal bodies and rootlets; black lines, flagella; red circle, eyespot protein patch. Scale bars = 1 /H10005 m.

FIG 4 Pigment granule arrays assemble after the emergence of flagella. Differential interference contrast (DIC) images of groups of two or four sister cells: black arrows, newly emerged flagella; white arrows, eyespot pigment granule arrays. (A) Sister cells with a mean flagellar length of ≤1.0 /H10005 m. (B) Sister cells with a mean flagellar length between 1.1 /H10005 m and 2.0 /H10005 m; (C) sister cells with a mean flagellar length between 2.1 /H10005 m and 3.0 /H10005 m. For each flagellar-length category, the percentage of sister cell groups (no.) that included at least one cell that contained an organized pigment granule array is given below the image. Scale bars = 1 /H10005 m.

suggest that either eyespot assembly is independent of photoreceptor localization or that assembly of the chloroplast components of the eyespot is dependent on the very similar ChR2 photoreceptor (40). Therefore, EYE2 localization and ChR2 localization were analyzed in dividing cop3Δ mutant cells (Fig. 6B and C). Similar to the wild type, both proteins formed patches associated with the D4 rootlet in daughter cells with newly emerged flagella, but not in nonflagellated daughters. Together, these data suggest that the photoreceptors and EYE2 localize independently of one another and of pigment granule layer assembly.

Photoreceptor but not EYE2 localization is retarded in mlt1 mutant cells. The majority of mlt1 (multiple eyespot) mutant cells
in interphase have two or more eyespots, and the “extra” eyespots are often found associated with a rootlet other than D4 (36, 51). The most posterior eyespot (closest to the equatorial position of the wild-type eyespot) is usually associated with D4. We wondered whether this asymmetry in eyespot position along the anterior-posterior axis is the result of differences in the assembly of individual \textit{mlt1} eyespots (e.g., the components assemble in a different order) or in the timing of eyespot formation (e.g., the more posterior eyespots associated with D4 are assembled prior to the others) and whether potential differences might yield clues to the requirements for eyespot assembly. To explore these questions, dividing \textit{mlt1} cells harvested between 1 and 2 h after the start of the dark period were examined by IF using \textit{α}-Ac-tub and anti-ChR1, anti-ChR2, or anti-EYE2 (Fig. 7). In wild-type cells, photoreceptor and EYE2 patches were observed in the majority of postmitotic cells with flagella that were \( 0.5 \) µm in length (Fig. 3). By the time the flagella had reached \( 3.0 \) µm in length, colocalized photoreceptor and EYE2 patches were invariably present in wild-type cells at a distance of \( 2.28 \) µm from the anterior end of the cell (Fig. 7A, \( n = 72 \) cells). In contrast, photoreceptor patches were not

\textbf{FIG 5} Acetylated rootlets transiently extend to the posterior end of daughter cells. (A) Two examples of IF images (example 1 was captured with the use of a 1.6× optivar, while example 2 was captured without the optivar) of cells that had recently completed cytokinesis (flagella were not yet full length) in which the acetylated microtubules extended to the posterior ends of the cells (light-blue arrows). The recently divided cells are among more mature cells (larger, with full-length flagella) with shorter acetylated rootlets (green arrows): green, \textit{α}-Ac-tub; red, \textit{α}-tub. (B) IF image (captured without the 1.6× optivar) of recently divided cells with long acetylated rootlets (light-blue arrows) associated with patches of ChR1 (red arrows) compared to more mature cells with shorter rootlets (green arrows). The inset illustrates measurements R, L, and F (dashed yellow lines): green, \textit{α}-Ac-tub; red, anti-ChR1. Scale bars = 1 µm.

\textbf{FIG 6} Localization of the photoreceptors and of EYE2 do not depend on each other or on EYE3. IF images of dividing mutant cells with flagella that were \( 0.5 \) µm in length: green, \textit{α}-Ac-tub; red, anti-ChR1 or anti-ChR2; blue, anti-EYE2. The percentage of mutant daughter cells (no.) with flagella that were \( 0.5 \) µm in length that contained a patch of the labeled protein is given below each image. (A) ChR1 in \textit{eye2} mutant cells; (B) EYE2 in \textit{cop3Δ} mutant cells; (C) ChR2 in \textit{cop3Δ} mutant cells; (D) ChR1 in \textit{eye3} mutant cells; (E) EYE2 in \textit{eye3} mutant cells; (F) ChR2 in \textit{eye3} mutant cells. Scale bars = 1 µm.
observed in postmitotic mlt1 cells (Fig. 7B), even after cytokinesis was complete and the flagella approached full length; only 15% or 12% of mlt1 daughter cells with flagella of ≥3.0 µm in length had patches of ChR1 or ChR2, respectively (n = 86 for ChR1, n = 65 for ChR2), and none of the patches were ≥2.0 µm from the anterior end of the cell. ChR1 (Fig. 7B and D) and ChR2 fluorescence was concentrated at the anterior end of recently divided mlt1 cells, near the basal bodies. In mlt1 cop3Δ double mutant cells, the anterior anti-ChR1 fluorescence was greatly diminished (Fig. 7E), indicating that the overabundant anterior fluorescence in mlt1 cells was due to accumulated ChR1 rather than nonspecific binding of the antibody. Surprisingly, bright-field microscopy showed that localization of the pigment granule layers in dividing mlt1 cells was similar to that observed in wild-type cells (Fig. 7C), with a single eyespot orthogonal to the cleavage plane in daughter cells with nascent flagella. EYE2 patches were also localized normally in both mlt1 and mlt1 cop3Δ cells, even as the photoreceptor(s) accumulated at the anterior ends of the cells (Fig. 7D and F); 95% of mlt1 cells with flagella of ≥3.0 µm in length had EYE2 patches (n = 62), and 68% of the EYE2 patches were ≥2.0 µm from the anterior end of the cell. The data suggest that the MLT1 gene product promotes postmitotic photoreceptor localization and that the photoreceptor proteins are trafficked to the anterior end of the cell prior to localization at the developing eyespot.

To determine whether, in the absence of ChR1 and ChR2 patches, EYE2 localization required the presence of pigment granule layers, EYE2 localization in dividing mlt1 eye3 double mutant cells was analyzed (Fig. 7G). EYE2 patches were present in all mlt1

FIG 7 Photoreceptor localization is retarded in mlt1 mutant cells but EYE2 localization is not. IF or DIC images of recently divided cells harvested after 1 h to 2 h in the dark: green, anti-acetylated tubulin; red, anti-ChR1 or anti-ChR2; blue, anti-EYE2. (A) Recently divided wild-type cells with localized ChR1 (yellow arrows) and EYE2 (light-blue arrows); the distance from the anterior end of the cell to the anterior edge of the patch of colocalized eyespot protein (dashed yellow line) was 2.3 ± 0.5 µm (n = 72). (B) ChR1 (yellow arrows) remained at the anterior end of mlt1 mutant cells; ChR1 fluorescence only is shown in the image on the right. (C) DIC image of mlt1 sister cells containing single arrays of pigment granules (black arrows) distributed similarly to those in wild-type cells: 13% (n = 23) of sister cell groups with a mean flagellar length of ≤1.0 µm, 44% (n = 23) of groups with a mean flagellar length between 1.1 µm and 2.0 µm, and 100% (n = 15) of groups with a mean flagellar length between 2.1 µm and 3.0 µm had at least one pigment granule array. (D) In mlt1 cells, ChR1 (yellow arrows in the image to the right) remained at the anterior end of each cell, but EYE2 patches (blue arrows in the image to the left) were localized similarly to those in the wild type (see the text). (E) Anti-ChR1 fluorescence due to nonspecific binding of the antibody in mlt1 cop3Δ double mutant cells (red arrows) was significantly lower than the fluorescence resulting from specific binding of the antibody to ChR1 in mlt1 single mutant cells (see the description for panel B, above). (F) EYE2 patches associated with the D4 rootlet (blue arrows) were observed in 97% (n = 36) of mlt1 cop3Δ double mutant cells with flagella of ≤0.5 µm in length. (G) In mlt1 eye3 double mutant cells, EYE2 patches (blue arrows) were localized similarly to those in wild-type and mlt1 cells (see the text). Scale bars = 1 µm.
eye3 cells with flagella of ≥3.0 μm in length (n = 22), and the majority of these patches (86%) were ≥2.0 μm from the anterior end of the cell. Therefore, EYE2 is able to accumulate at the developing eyespot in the absence of both the pigment granules and stoichiometric levels of the photoreceptors. Also, the presence of only one EYE2 patch opposite the cleavage plane in recently divided ml1 daughter cells suggests that the extra eyespots observed in interphase ml1 cells form after the assembly of EYE2 and the pigment granules at an initial or “primary” eyespot.

The multieyed phenotype arises after cytokinesis and flagellar outgrowth. We observed by IF that as ml1 cells proceeded through cytokinesis and flagellar outgrowth, anti-ChR1 fluorescence at the anterior end of the cells increased (Fig. 8A), as did the size of ChR1 eyespot patches in wild-type cells. To verify that the observed increases in fluorescence were the result of increased levels of ChR1 protein, whole-cell protein samples isolated from wild-type or ml1 cells after 1.0 h or 8.0 h in the dark were analyzed by Western blotting using anti-ChR1 and α-tubulin (Fig. 8B). The level of ChR1 protein relative to that of tubulin increased during the dark period in both wild-type and ml1 cells, presumably due to both an increase in the percentage of cells that had completed mitosis and an increase in relative eyespot size (24, 53). We also note that while pigment granule layers of the mother cell eyespot were observed at the cleavage plane of some dividing cells (25, 58), patches of ChR1 at the metaphase band in premitotic cells were not observed by IF (see Fig. S3 in the supplemental material). In wild-type cells on a 14-10 light-dark schedule, COP3 RNA levels were low just prior to mitosis and then increased during mitosis and the first half of the dark period (Jim Umen, personal communication). Together, the data suggest that the ChR1 protein is degraded prior to cell division and resynthesized during or shortly after mitosis.

As described above, shortly after ml1 cell division, localization of a single pigment granule array and EYE2 patch opposite the cleavage plane was similar to that observed in wild-type cells. To determine when the multieyed phenotype arises, bright-field microscopy throughout the dark period was used to determine the number of pigment granule arrays in ml1 cells that had recently divided (two or four sister cells remained together in the mother cell wall) and had already assembled at least one pigment granule array in each of the sister cells (e.g., after formation of the primary array). Halfway through the dark period, fewer than 25% of daughter ml1 cells had multiple pigment granule arrays, but by the end of the dark period, over 50% of the cells had multiple arrays of pigment granules (Fig. 8C). This was true for each of three cultures that were followed for 48 h.

The presence of secondary or ectopic eyespots in ml1 cells offered the possibility of determining whether aggregation of any one eyespot protein is sufficient to prompt assembly of a complete eyespot. To analyze the secondary eyespots, dividing ml1 cells harvested after 10 h to 12 h in the dark were triple labeled with α-Ac-tub, anti-ChR1, and anti-EYE2 (Fig. 8D, examples 1 and 2). Eyespot protein patches in daughter cells were then characterized as being <2.0 μm or ≥2.0 μm from the anterior end of the cell.
and as including either ChR1 or EYE2 or both. Even at the end of the dark period, only 20% of patches of ≥2.0 μm from the anterior \((n = 108\) patches) had both ChR1 and EYE2, and the other 80% contained EYE2 only. In contrast, 86% of patches of <2.0 μm from the anterior \((n = 105\) patches) had both ChR1 and EYE2, with the remaining patches having either ChR1 or EYE2 only. As ChR1 and EYE2 were present to the same degree in the more anterior, secondary patches, we were unable to conclude which component might be sufficient for directing ectopic eyespot assembly.

**DISCUSSION**

During each cell division in the unicellular green alga *Chlamydomonas reinhardtii*, the single photoreceptive eyespot is assembled *de novo* in the daughter cells at a unique position relative to the microtubule-based flagellar apparatus. The flagellar apparatus comprises two anterior flagella and the associated mother (older) and daughter (younger) basal bodies and four highly acetylated microtubule rootlets. Two rootlets, one with two microtubules and another with four, extend from each basal body toward the posterior end of the cell; the eyespot is found at the equator of the cell associated with the four-membered rootlet that extends from the daughter basal body (D4). The *Chlamydomonas* eyespot, like the eyespots of many green algal species, is built of photoreceptors in the plasma membrane and organized layers of pigment granules in the chloroplast (4, 22, 35). Here, we used immunofluorescence and DIC microscopy to determine the timing of eyespot assembly relative to that of the D4 rootlet and to flagellar outgrowth in postmitotic daughter cells.

During cell division, the D4 rootlet was assembled prior to the eyespot, consistent with the long-standing hypothesis that the position of the nascent D4 dictates the position of the eyespot. Wild-type daughter cells that had not yet assembled flagella did not contain pigment granule layers or discernible patches of the eyespot photoreceptors ChR1 and ChR2 or of the chloroplast envelope protein EYE2. Nonacetylated microtubules in the expected position of the nascent D4 rootlet were, however, observed in nonflagellated daughter cells (Fig. 2). In these daughter cells, the mitotic spindle had been disassembled but cytokinesis was not complete, as evidenced by the presence of cleavage microtubules at the division plane. As rootlet microtubules are acetylated to a much greater degree than nonrootlet cytoplasmic microtubules, partial acetylation of these microtubules in some nonflagellated daughters supports their identification as the D4 rootlet (20). Previous electron microscopic data are suggestive of daughter rootlet assembly prior to flagellar outgrowth (46, 49), and Lechtrock and Silflow (59) also noted the presence of potential D4 rootlets in daughter cells in late mitosis/early cytokinesis when striated fibers of SF-assemblin had formed in association with the D2 rootlet. During cytokinesis, the SF-assemblin fibers form a cross associated with the anterior ends of the rootlets, leading to the authors’ proposal that the striated fibers play a role in organizing microtubule rootlet assembly.

In daughter cells with newly emerged flagella (less than 0.5 μm in length), the D4 rootlet was invariably acetylated, suggesting that acetylation of the rootlet microtubules occurs after their polymerization. Acetylation of α-tubulin after microtubule assembly has been observed, but how the acetyltransferase accesses Lys40 in the lumen of the tubule remains unclear (57, 60–62). The data also suggest that rootlet acetylation is concurrent with initial flagellar outgrowth, likely at or near the completion of cytokinesis, when intraflagellar transport proteins required for flagellar assembly accumulate at the base of the flagella (53). In mammalian cells, the tubulin acetyltransferase, α-TAT1, is associated with the BBSome, a complex of proteins involved in ciliogenesis (63, 64). Perhaps a potential *Chlamydomonas* ortholog of α-TAT1 (Cre07.g345150) is responsible for acetylation of both flagellar and rootlet microtubules (65, 66), and the activity of this enzyme increases following cell division.

Small patches of ChR1 and ChR2 photoreceptors and of EYE2 were observed at the end of the acetylated D4 rootlet in the vast majority of daughter cells with newly emerged flagella (Fig. 3). As the flagella extend, the pigment granule layers assemble, a process that is dependent upon the presence of the EYE2 protein (41, 42) and perhaps is not initiated until a critical level of EYE2 has accumulated at the nascent eyespot. Though eyespot assembly and flagellar assembly are independent processes (eyespot mutants assemble flagella and flagellar assembly mutants contain eyespots [36, 51, 67]), concurrent assembly of these functionally related structures may be triggered by a common postmitotic signal. What then accounts for the specificity of eyespot component localization to the D4 rootlet as opposed to the M4 rootlet microtubules, which are arranged also in a 3-over-1 pattern? Perhaps the D4 is the default choice because M4 is associated with the cleavage furrow (44, 45). In *Spermatozopsis similis* cells, eyespot assembly precedes cell division and occurs in association with a parental rootlet before the rootlet microtubules are observed between the segregating pairs of basal bodies (32). A second possibility, potentially related to the first, is that eyespot assembly is dependent on the accessibility of proteins that are specifically associated with the lengthening microtubules. We have proposed that trafficking along the D4 rootlet moves the photoreceptors away from the anterior end of the cell toward the eyespot, similar to the microtubule-directed movement of the cellulose synthase complex in *Arabidopsis* cells (68) or the cAMP receptor involved in chemotaxis in *Dictyostelium* (69). Perhaps microtubule binding proteins that mediate and/or promote photoreceptor trafficking are specific for microtubules that are lengthening or undergoing acetylation.

Somewhat surprisingly, ChR1, ChR2, and EYE2 localizations to the nascent eyespot appear to be independent of one another. ChR1 localizes to equatorial patches associated with the D4 rootlet in mature *eye2* or *eye3* mutant cells, and EYE2 is correctly localized in *eye3* mutant cells (41). Here, we show that the timing of ChR1, ChR2, and EYE2 localization in eyeless mutant cells is similar to that in wild-type cells. Likewise, ChR2 and EYE2 are correctly localized in ChR1-deficient *cop3Δ* mutant cells, which have eyespots (see Fig. S1A in the supplemental material) (24) and display positive phototaxis in response to white light. In the absence of ChR1, localization of EYE2 and assembly of the pigment granule layers may depend on ChR2, which is closely related to ChR1 (40) and may have a functionally redundant role in eyespot assembly. However, in *mlt1* mutant daughter cells with newly emerged flagella, detectable levels of ChR1 and ChR2 are not observed at the nascent eyespot, yet an EYE2 patch and pigment granule layers are correctly positioned at the end of the acetylated D4 rootlet. Correctly localized patches of EYE2 are also present in *mlt1 eye3* double mutant daughters, which lack eyespot pigment granule layers. These data are not consistent with the hypothesis that eyespot assembly occurs in an obligatory cascade but instead suggest that
the EYE2 protein responds to the localization cue and promotes organization of the pigment granule layers independently of the photoreceptors.

Recently divided mlt1 mutant daughter cells with short flagella have a single “primary” eyespot associated with the D4 rootlet. This first eyespot comprises EYE2 and pigment granule layers but not ChR1 or ChR2, which accumulate at the anterior end of the cell. As the daughter cells mature and the flagella extend beyond 3 μm in length, ChR1 continues to accumulate anteriorly and in secondary eyespots that are <2.0 μm from the basal bodies (Fig. 8D). Eventually, ChR1 moves to the primary eyespot, which in interphase mlt1 cells is at a more anterior position than the wild-type eyespot (36, 70). These data suggest that the MLT1 protein promotes the D4-directed movement of the photoreceptors away from the anterior end of the cell. Previously, we hypothesized that MLT1 primarily affects microtubule rootlet asymmetry, which results in a shorter D4 rootlet and more anterior eyespots in mlt1 cells (36). The hypothesis that D4 rootlet length determines the position of the eyespot along the anterior-posterior axis would also explain the more posterior eyespots in pey1 and cmu1 mutant cells, which have correspondingly long D4 rootlets (70, 71). If, however, the primary effect of the MLT1 protein is on photoreceptor localization, then the shorter D4 rootlet in mlt1 cells may be the result, rather than the cause, of the more anterior position of the primary eyespot. This model is consistent with the recent observation that strains with reduced levels of the pigment granule protein SOUL3 have eyespots that are mispositioned along the anterior-posterior axis, with a corresponding change in D4 length (72). Here, we find that shortly after cell division, the rootlets temporarily extend to the posterior end of the cell. Perhaps, following a net polymerization and lengthening phase, the rootlet microtubules undergo a period of net depolymerization and shortening. During this period, shortening of the D4 rootlet may be blocked at the eyespot by interactions between eyespot-localized proteins and the microtubules. This model could explain both the correlation between eyespot position and rootlet length in mlt1, cmu1, and pey1 mutant cells, and SOUL3-knockdown cells, and the observation that D4 is often the only acetylated rootlet that extends beyond the equator of the cell in mature wild-type cells (36).

How might the absence of the photoreceptors at the nascent primary eyespot in mlt1 cells affect the anterior-posterior positioning of the chloroplast components? As a new daughter cell grows, continued posterior-directed movement of the eyespot complex, tracking along the D4 rootlet, may be required to maintain the eyespot’s equatorial position. Eventually, the eyespot and plus ends of the recently shortened rootlet microtubules might meet at the equator, triggering the formation of a relatively stable complex. Though an EYE2 pigment granule layer “subassembly” is formed in the absence of the photoreceptors, posterior movement of this incomplete complex may be retarded, leaving the eyespot in a more anterior position at the time of rootlet shortening and stabilization. This hypothesis predicts that in the absence of both ChR1 and ChR2, eyespots will be more anterior than in wild-type cells. The cmu1 mutation, which leads to abnormally long cytoplasmic microtubules (71), may inhibit shortening of the rootlet microtubules. In this case, the microtubule-directed movement of the eyespot toward the posterior end of the cell is not halted at the equator. Identification of the MLT1 protein and of eyespot components that interact with the photoreceptors, EYE2, and/or D4 rootlet microtubules will further our understanding of eyespot positioning and of how the asymmetric cytoskeleton of flagellated algae determines the organization of the cell.

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