Phototactic activity in *Chlamydomonas* 'non-phototactic' mutants deficient in Ca^{2+}-dependent control of flagellar dominance or in inner-arm dynein

Noriko Okita[1], Nahoko Isogai[1], Masafumi Hirono[1], Ritsu Kamiya[1] and Kenjiro Yoshimura[2,*]

[1]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0063, Japan
[2]Doctoral Course of Structural Biosciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan
*Author for correspondence (e-mail: kenjiro@biol.tsukuba.ac.jp)

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Summary

In the mechanism underlying the phototactic behavior of *Chlamydomonas*, Ca^{2+} has been thought to control the dominance between the two flagella so as to steer the cell to correct directions. A newly isolated mutant, *lsp1*, that displays weak phototaxis was found to be defective in this Ca^{2+}-dependent shift in flagellar dominance; in demembranated and reactivated cell models, the trans flagellum (the flagellum farthest from the eyespot) beat more strongly than the other (the cis flagellum) in about half of the cells regardless of the Ca^{2+} concentration between $<10^{-9}$ M and 10^{-6} M, a range over which wild-type cell models display switching of flagellar dominance. This is unexpected because *ptx1*, another mutant that is also deficient in flagellar dominance control, has been reported to lack phototactic ability. We therefore re-examined *ptx1* and another reportedly non-phototactic mutant, *ida1*, which lacks inner arm dynein subspecies *f* (also called I1). Both were found to retain reduced phototactic abilities. These results indicate that both Ca^{2+}-dependent flagellar dominance control and inner-arm dynein subspecies *f* are important for phototaxis, but are not absolutely necessary. Analysis of the flagellar beat frequency in *lsp1* cell models showed that both of the flagella beat at the frequency of the cis flagellum in wild type. In addition, *lsp1* and *ptx1* were found to be deficient in determining the sign of phototactic migration. Hence, the Ca^{2+}-dependent flagellar dominance control detected in demembranated cells might be involved in the determination of the sign of phototaxis. The gene responsible for the *lsp1* mutation was identified by phenotype rescue experiments and found to have sequences for phosphorylation.

Key words: *Chlamydomonas*, Phototaxis, Flagella, Calcium ion, Cell model

Introduction

Almost all kinds of motile protist can sense ambient conditions and move toward environments suitable for their survival. How they display such purposeful behavior is of general interest for the control of motility and behavior in eukaryotic cells. The biflagellate green alga *Chlamydomonas* detects surrounding light conditions and swims to where the light intensity is best suited for photosynthesis. Depending on the cell's condition and the light intensity, cells swim either towards the light source (positive phototaxis) or away from the light source (negative phototaxis). Both kinds of phototaxis are triggered by the light reception by a rhodopsin-related molecule in the eyespot, a discrete membranous structure located on the equator of the cell body (Foster et al., 1984; Sineshchekov et al., 2002; Nagel et al., 2002). The eyespot is most sensitive to the light coming perpendicular to the eyespot; this is because the layers of carotenoid granules lining the eyespot reflect the light coming from the front and the chloroplast in the cell body shields the light coming from behind. This directional sensitivity of the receptor, in combination with cellular rotation around the longitudinal axis, enables the cell to detect the direction of light by scanning the surrounding light conditions (Foster and Smith, 1980; Schallar and Uhl, 1997; Isogai et al., 2000). Upon light reception, the photoreceptor induces an inward current at the eyespot [photoreceptor current (PRC)] and depolarizes the membrane (Litvin et al., 1978; Harz and Hegemann, 1991). Depolarization then produces a Ca^{2+} influx through voltage-dependent Ca^{2+} channels on the flagellar membrane, resulting in an increase in the intraflagellar Ca^{2+} concentration (Beck and Uhl, 1994; Pazour et al., 1995; Yoshimura et al., 1997).

The change in the intraflagellar Ca^{2+} concentration is believed to change the balance of the forces produced by the two flagella, to control the cell's swimming direction. Evidence for such a Ca^{2+}-dependent flagellar dominance control came from a study using demembranated and reactivated cells (Kamiya and Witman, 1984). The flagellum closest to the eyespot (the cis flagellum) was found to beat more strongly than the one farthest from the eyespot (the trans flagellum) at $<10^{-8}$ M Ca^{2+}, whereas the trans flagellum beats more strongly than the cis flagellum at 10^{-7} M to 10^{-6} M Ca^{2+}. Isolation of the mutant *ptx1* supported the importance of the Ca^{2+}-dependent flagellar dominance regulation in the phototaxis

mechanism because, in this non-phototactic mutant, the cis flagellum was more active than the trans flagellum in about half of the total cell models, irrespective of the Ca^{2+} concentration between 10^{-9} M and 10^{-7} M (Horst and Witman, 1993). A later study using mutants lacking flagellar inner-arm dynein subspecies *f* (also called dynein I1) (*ida1*, *ida2*, *ida3* and *ida7*) suggested that the change in the flagellar dominance is brought about through a mechanism involving phosphorylation of a subunit of this dynein. These inner-arm dynein mutants and the mutants *mia1* and *mia2*, which display aberrant phosphorylation forms of the *f* subspecies, were found to exhibit no phototaxis (King and Dutcher, 1997). However, whether the phototaxis loss in these mutants is in fact due to an abnormality in the Ca^{2+}-dependent control of the flagellar balance remains to be studied.

In this study, we aimed to obtain clues to the mechanism of phototaxis by isolating and characterizing mutants deficient in phototaxis, following previous studies that showed the effectiveness of such an approach (Horst and Witman, 1993; Pazour et al., 1995). Of about ten mutants obtained, one (*lsp1*) showed a novel feature: it is weakly phototactic even though its cell models do not show a Ca^{2+}-dependent change in flagellar dominance. This finding prompted us to re-examine the relationship between the flagellar dominance control and phototactic response in other mutants that have been reported to be non-phototactic. Our results indicate that these mutants retain residual phototactic activities, suggesting that there are multiple pathways for generation of phototactic behavior.

Materials and Methods

Strains and culture conditions

A *Chlamydomonas reinhardtii* mutant lacking nitrate reductase, *nit1*, was used as the parental cell for insertional mutagenesis. An arginine autotrophic mutant, *arg7*, was used to make double mutants. *ptx1*, *ptx5*, *ptx6* and *ptx7* are deficient in phototaxis and Ca^{2+}-dependent changes in flagellar dominance (Horst and Witman, 1993; Pazour et al., 1995). *ida1* is a mutant that lacks the subspecies *f* of inner-arm dynein (Kamiya et al., 1991) and has been reported to exhibit no phototaxis (King and Dutcher, 1997). For physiological experiments, cells were cultured in TAP medium (Gorman and Levine, 1965) on a 12 hour light, 12 hour dark cycle for 2-3 days.

Mutagenesis and screening

The ammonium auxotrophic mutant *nit1* was transformed with a linearized plasmid DNA, pMN24 containing the *NIT1* gene using a glass-bead method (Kindle, 1990). The transformed cells were cultured on SGII NO_3 agar plates (Sager and Granick, 1953), which contained no ammonium salts. Each colony of the NIT^+ transformants was transferred to liquid SGII NO_3 medium in 96-well plates. For screening mutants, cells grown in the wells were examined for their ability to gather around a brighter portion of the well. For this purpose, 96-well plates were observed after they had been covered with a piece of black cloth so that light would enter only from one side. Clones were selected that did not exhibit clear photoaccumulation. Those isolates were then examined by Southern-blot analysis using part of the inserted DNA as probe. Co-segregation of the mutant phenotype and insertion of the plasmid was examined by tetrad analysis of the daughter cells formed between each isolate and *nit1*. Genetic techniques used for mating and tetrad analysis were as described (Levine and Ebersold, 1960; Harris, 1989). For physiological experiments, mutants were backcrossed with a wild-type strain (137c) and compared with strain 137c.

A more objective determination of the phototactic ability in a given sample of cells was carried out as follows. Cells grown to a density of 1×10^6 cells ml^{-1} were placed in 10×10×10 mm polystyrene chambers and irradiated for about 5 minutes by a 30-W fluorescent lamp placed ~20 cm behind the chamber. Under these conditions, whereas wild-type cells swam to one side of the chamber and formed a tight band on the bottom of chamber, phototaxis-deficient cells swam in random directions. The difference in the distribution of cell density was clearly identified by the naked eye.

Motility assays

The phototactic abilities of various mutants were quantified by recording and analysing their behavior under continuous unidirectional illumination of various intensities. For this experiment, cells were first washed with an experimental solution {1 mM KCl, 0.3 mM $CaCl_2$, 0.2 mM EGTA, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)} and kept under red light for more than 20 minutes before phototaxis assays. Cells were then put in a 10×10×10 mm polystyrene trough placed on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan). After further adaptation under red light (λ>600 nm) for a few minutes, the cells were illuminated by blue-green light through a filter (λ=500 nm). To avoid adaptation, successive light stimulation was applied after an interval of 1.5 minutes. The behavior of the cells was video recorded with a CCD camera. The angle (θ) between the light direction and the swimming direction of each cell was measured for 1.3 seconds beginning 5 seconds after the actinic light was turned on. As a measure of the phototactic activity in mutant and wild-type cells, the average of $|\cos\theta|$ was calculated. The average is normalized such that it is 0 when all cells are moving randomly (average=$2/\pi$) and it is 1 when all cells are moving towards the light source.

For the assay of photophobic responses, a flash (λ=500 nm) was applied to the cells while their swimming tracks were being video recorded under red light. The proportion of cells that transiently displayed backwards swimming upon the flash application was quantified at various light intensities.

Demembranation and reactivation of *Chlamydomonas* cells

For examination of flagellar dominance at various Ca^{2+} concentrations, cells were demembranated and reactivated as described previously (Kamiya and Witman, 1984). Briefly, cells were washed three times in HES (10 mM HEPES pH 7.3, 0.5 mM EGTA, 4% sucrose) and demembranated by the addition of NP40 (to a final concentration of 0.2%) on ice. The cells were reactivated with ATP at a final concentration of 1 mM in the presence of various concentrations of Ca^{2+} and observed by dark-field microscopy. Many reactivated cell models rotated within a small area because of the inactivation of one of the two flagella. Flagellar dominance was measured by counting the number of cell models rotating with their eyespots on the outer or inner side of the cell body. Only the cell models that clearly showed the eyespot and the two flagella were used for the assay.

Measurement of flagellar beat frequency

Flagellar beat frequencies of live or reactivated cells were measured by Fourier transformation of the light-intensity fluctuation in the microscopic image of the cells (Kamiya, 2000). The beat frequencies of the cis and trans flagella of the reactivated cells were measured in single cell models rotating by beating either flagellum (Kamiya and Hasegawa, 1987).

Electrophysiology

Flash-induced photoreceptor currents were measured by the population

method (Sineshchekov et al., 1992; Sineshchekov et al., 1994). 1 ml cell suspension (1.0×10^7 cells ml^{-1}) in measuring solution [0.1 mM $CaCl_2$, 0.5 mM HEPES (pH 7.3)] was placed in a rectangular polystyrene cuvette (10×10×15 mm) and stimulated by a flash of light. Current was measured with a pair of platinum electrodes set in parallel. The signals were amplified (EPC-7, List, Germany) and stored in a personal computer. This method is based on the fact that cells with their eyespot facing to the light source generate larger photoreceptor currents than cells with their eyespot facing in the opposite direction. Therefore, in a cell suspension illuminated from one side, a net current appears in the direction of the light path. Two electrodes, one placed closer to the light source than the other, can measure this current. This current represents an average membrane current elicited from a population of cells.

Results

Isolation of a novel phototaxis-deficient mutant *lsp1*

We generated a panel of mutants using insertional mutagenesis and screened them for deficiency in phototaxis. When a suspension of wild-type cells was illuminated from one side, cells moved towards the light source within minutes (Fig. 1, left). We selected cells that did not show such phototactic accumulation, and obtained about ten strains that stably show defects in phototactic behavior. One typical clone, which was apparently generated by insertion of a single copy of plasmid, was studied further (Fig. 1, right). Because this mutant appeared to have a low sensitivity in phototaxis compared with the wild type (see below), we named it *lsp1*. Although its phenotype as described below was similar to that of *ptx1* (Horst and Witman, 1993), *ptx5*, *ptx6* and *ptx7* (Pazour et al., 1995), genetic crosses indicated that they are independent (Table 1). Hence, it is likely that *lsp1* is a novel mutant.

To examine behavioral defects of the *lsp1* mutant in more detail, we first recorded its swimming tracks in the phototactic light. In the wild type, almost all cells swam towards the light source even at a moderate intensity (8.8×10^{16} photons m^{-2} s^{-1})

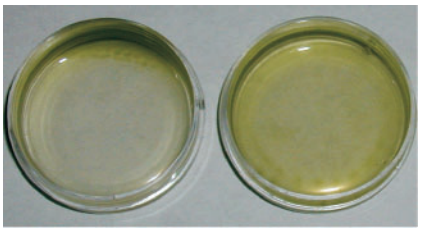

Fig. 1. Photoaccumulation in the wild type (left) and the *lsp1* mutant (right). Cell suspensions are shown after being exposed to light incident from the top of the figure for 5 minutes. Wild-type cells accumulated toward the light, whereas *lsp1* cells remained distributed uniformly.

Table 1. Genetic analysis of phototactic mutants

	ptx1	*ptx5*	*ptx6*	*ptx7*
lsp1	2:0:6	1:1:3	2:3:1	6:7:2

The table indicates the ratios of parental ditypes to nonparental ditypes to tetratypes. The mutant *lsp1* was mated to each *ptx* mutant and the daughter cells in each tetrad were scored for phototactic ability. Occurrence of nonparental ditypes and tetratypes indicates that the *lsp1* mutation differs from these four kinds of *ptx* mutation.

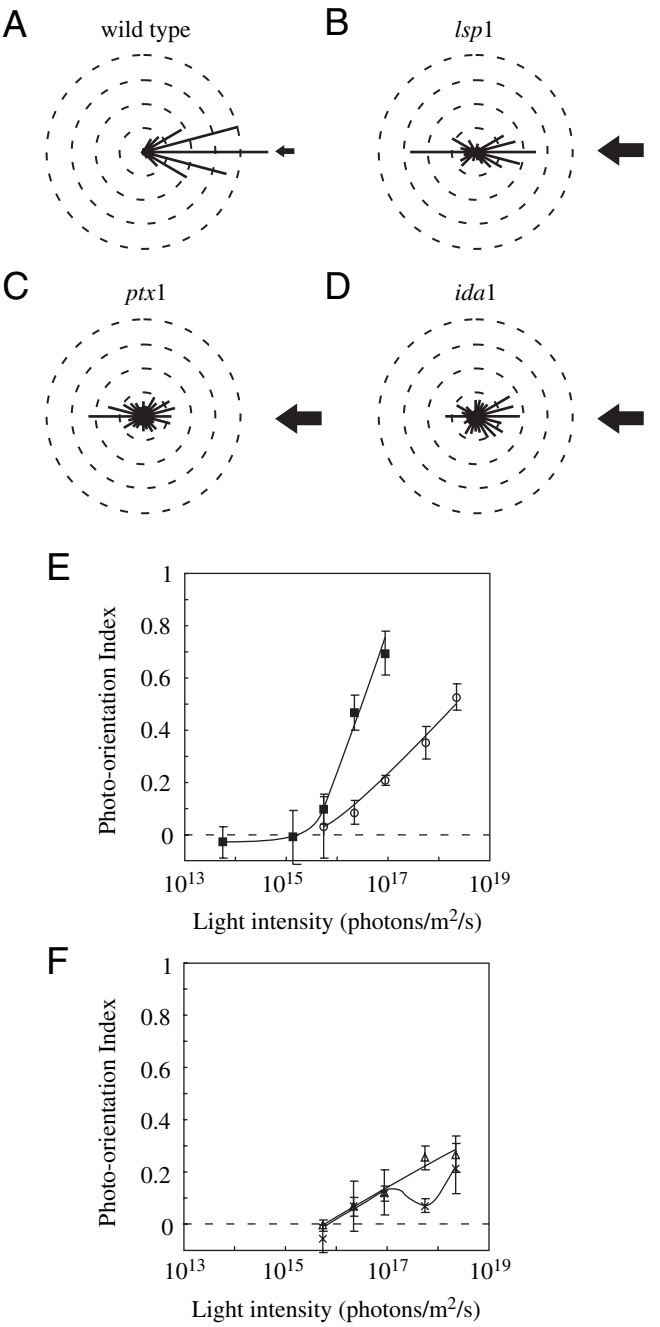

Fig. 2. Swimming directions of the wild-type cells and various phototaxis mutant cells 5 seconds after the phototactic light was turned on. (A-D) Polar histograms showing the swimming directions when the light (wild type, 8.8×10^{16} photons m^{-2} s^{-1}; *lsp1*, *ptx1* and *ida1*, 2.2×10^{18} photons m^{-2} s^{-1}) was applied in the direction indicated by arrows. Bars show the proportions of cells swimming in the direction binned at 15°. The interval between dotted circles represents 5% of the cells. Data on a total of 420 cells were collected from three experiments. (E,F) Stimulus-response curves for the phototaxis in the wild type (squares) and three mutant cells (*lsp1*, circles; *ptx1*, triangles; *ida1*, crosses). The magnitudes of phototaxis are shown by photo-orientation index calculated from the direction of swimming relative to the direction of light. Dotted line shows the value when the cells exhibit no phototaxis. Each point represents the average and standard deviation from three experiments (140 cells in each experiment).

(Fig. 2A). By contrast, the *lsp1* cells did not exhibit clear positive phototaxis at this light intensity. When the intensity was increased to 2.2×10^{18} photons m^{-2} s^{-1}, the mutant cells tended to swim parallel to the light direction. In this case, however, about half of the *lsp1* cells swam towards the light source, whereas the rest swam away from it (Fig. 2B). This response is clearly different from that of the wild-type cells, almost all of which swam towards the light source. In other words, although *lsp1* cells did not migrate towards the light source, they did display light-controlled behavior. We measured the angle (θ) between the cells swimming direction and the light direction, and took the absolute value of cosθ as a measure of the photo-orientation. We call this value the photo-orientation index. In wild-type cells, the photo-orientation index was close to zero at light intensities lower than 1.4×10^{15} photons m^{-2} s^{-1}, indicating that the cells are swimming in random directions. As the light intensity was increased up to 8.8×10^{16} photons m^{-2} s^{-1}, the photo-orientation index gradually increased (Fig. 2E). In *lsp1* mutants also, the index took non-zero values at higher light intensities. Although the threshold light intensity in *lsp1* was almost the same as in the wild type, the slope of increase in the photo-orientation index was smaller. The above observation indicates that phototaxis in *lsp1* has two characteristics: bipolar directions and reduced magnitude.

The sign of phototaxis varies with the condition of the cell (Takahashi and Watanabe, 1993), and so it is possible that the bipolar phototaxis in *lsp1* occurs only under a particular condition. However, the presence of 0.1 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which is known to reverse the sign of phototaxis in the wild type (Takahashi and Watanabe, 1993), did not affect the bipolar taxis in *lsp1*. Extended exposure to strong light, which induces negative phototaxis in the wild type, also resulted in bipolar phototaxis in *lsp1*. These observations suggest that bipolar phototaxis in *lsp1* persists under conditions that would induce positive and negative phototaxis in the wild type, although we cannot rule out the possibility that this mutant displays unidirectional phototaxis under some conditions that we have not examined.

Flagellar motility in *lsp1* was almost normal. The flagella of *lsp1* cells beat in a breast-stroke-like pattern, as in wild-type cells (data not shown). The flagellar beat frequency in *lsp1* was 59.0±5.3 Hz, which was not significantly different from the wild type (60.1±4.4 Hz). The swimming velocity of *lsp1* was slightly lower than that of the wild type (wild type, 98.7±7.6 μm s^{-1}; *lsp1*, 70.7±4.5 μm s^{-1}; n=42).

The PRC was also normal in *lsp1*. As shown in Fig. 3A,B, the time course and amplitude of PRC in *lsp1* produced upon application of a flash was almost identical to those of the wild-type PRC. The PRC in *lsp1* increased with light intensity in the same manner as in the wild type (Fig. 3C). These observations indicate that *lsp1* has normal abilities to perceive light and to generate photoreceptor currents.

Another experiment indicated that *lsp1* can display a normal photophobic response, a transient backwards swimming behavior induced by a rapid increase in light intensity. The proportion of the *lsp1* cells exhibiting photophobic response increased with light intensity as in the wild type (Fig. 4). Hence, the defects in *lsp1* did not appear to affect the mechanism underlying the photophobic response.

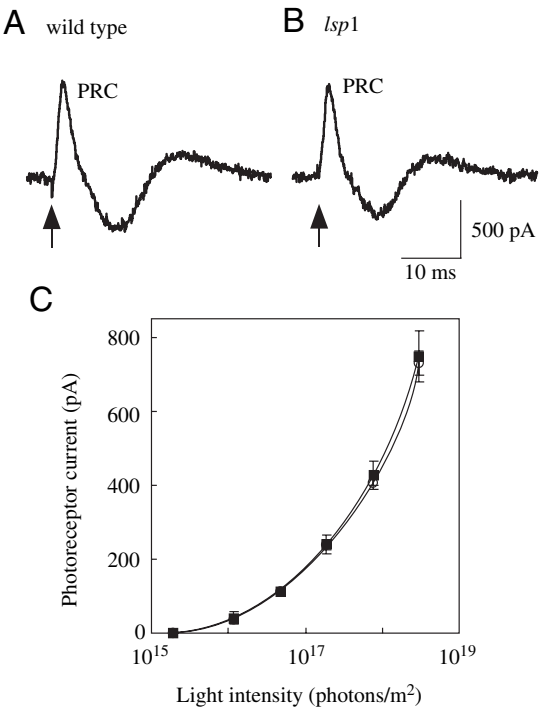

Fig. 3. Photoreceptor current (PRC) in wild-type and *lsp1* mutant cells. (A,B) Typical waveforms observed when a flash (3.0×10^{18} photons m^{-2}) was applied to wild-type (A) and *lsp1* (B) cells at the time indicated by arrows. (C) Light dependence of the PRC in wild type (squares) and *lsp1* mutant (circles). Averages and standard deviations from four experiments are plotted.

Deficiency in flagellar dominance control

The above observations indicated that the deficiency in *lsp1* is specific to phototaxis, and so we surmised that the mechanism controlling the balance of the force generation by the two flagella might have been impaired by mutation. Therefore, we examined the dominance between the two flagella in demembranated and reactivated cells at various free Ca^{2+} concentrations, after the method described previously (Kamiya and Witman, 1984). Immediately after reactivation by ATP, the cells swam in straight or helical paths. With time, cell models tended to rotate in a fixed area, because one of the two flagella became less active than the other. A cell with the trans axoneme beating dominantly usually rotates with its eyespot directing towards the center of rotation (Fig. 5A), whereas a cell with the cis axoneme beating dominantly rotates with its eyespot facing outwards (Fig. 5B). We counted the number of rotating cell models with their trans or cis axoneme dominant at various Ca^{2+} concentrations. In the wild type, >70% of the total cell models had their cis axoneme dominant at $<10^{-8}$ M Ca^{2+}, whereas >80% had their trans axoneme dominant at 10^{-6} M Ca^{2+} (Fig. 5C). This result is in good agreement with the previous report (Kamiya and Witman, 1984). By contrast, the flagellar dominance in *lsp1* cell models did not change with Ca^{2+} concentration; about half of the total cell models always had their trans axoneme dominant (Fig. 5C).

Phototactic ability in another phototaxis mutants, *ptx1*

Absence of Ca^{2+}-dependent dominance has also been reported

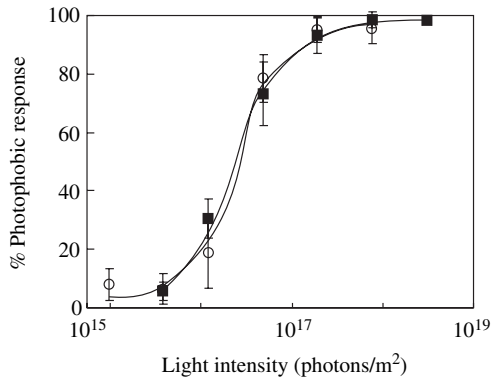

Fig. 4. Photophobic response of wild type (squares) and *lsp1* mutant (circles). The proportions of the cells showing photophobic response was examined at the intensity of flash light indicated on the abscissa. Averages and standard deviations from four measurements each (18-94 cells in each experiment) are shown.

in another phototaxis-deficient mutant, *ptx1*. In this mutant, about half of cell models had their trans flagellum dominant at both 10^{-9} M and 10^{-7} M Ca^{2+} (Horst and Witman, 1993). We re-examined flagellar dominance in this mutant at Ca^{2+} concentrations between $<10^{-9}$ M and 10^{-6} M, and confirmed that the proportion of cell models with the trans axoneme dominant was always about 50% (Fig. 5C).

The mutants *lsp1* and *ptx1* are, therefore, similar in that both showed no Ca^{2+}-dependent change in axonemal dominance. However, *ptx1* has been reported to have a greatly reduced ability to display phototaxis, whereas *lsp1* was found to show weak, bipolar phototaxis as shown above. To understand the reason for this difference, we examined the phototactic ability in *ptx1* at various light intensities over the range wherein *lsp1* cells displayed directional movements. We found that *ptx1* exhibited phototactic migration in both directions although the magnitude is slightly less than in *lsp1* (Fig. 2C,F). The weak positive phototaxis of *ptx1* at high light intensity reported previously (Pazour et al., 1995) might represent the summation of activities in a mixture of cells that display positive and negative phototaxis.

Characteristic flagellar beat frequencies in *lsp1* and *ptx1* cell models

The cis and trans axonemes have been shown to beat at intrinsically different frequencies in demembranated wild-type cell models. The trans axoneme always beats at a higher frequency, irrespective of the Ca^{2+} concentration (Kamiya and Hasegawa, 1987). This difference has been suggested to result from some difference in the outer-arm docking complex (ODA-DC), the site at which outer-arm dynein is attached on the outer doublets (Takada and Kamiya, 1997). To see whether the mutations in *lsp1* and *ptx1* affect beat frequencies, we measured the beat frequencies of the cis and the trans flagella separately in these mutants. In wild-type cell models, as previously reported, the frequency of the trans flagellum was 30-40% higher than that of the cis flagellum and its frequency distribution was broader than that in the cis flagellum (Fig. 6A). In *lsp1*, both flagella displayed a range of beat frequencies similar to those of the cis flagellum of the wild type (Fig. 6B).

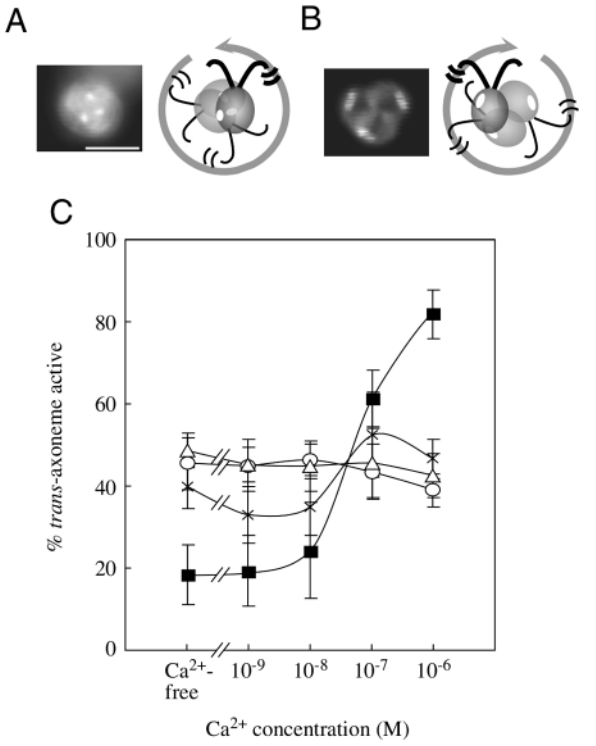

Fig. 5. The effects of Ca^{2+} concentration on the dominance of the activity of cis and trans axonemes. (A,B) Superimposed frames from video records and associated interpretations of rotating wild-type cells. Cells tended to rotate with the eyespot pointing inwards in a solution containing 10^{-6} M Ca^{2+} (A) and outwards in Ca^{2+}-free solution (B). Scale bar, 10 µm. (C) The proportion of reactivated cell models whose trans axoneme beats dominantly at various Ca^{2+} concentrations. Each point shows the average and standard deviation from six experiments on wild-type (squares), *lsp1* (circles), *ptx1* (triangles) and *ida1* (crosses) cells. The number of cells counted in each experiment was 29-159.

By contrast, both flagella of *ptx1* displayed beat frequencies similar to those in the trans flagellum of the wild type (Fig. 6C).

Phototactic ability in *ida1*, a mutant lacking inner-arm dynein

The above results raise the possibility that mutants previously regarded as non-phototactic might display some light-controlled behavior under our experimental conditions. This is possible particularly because previous studies have paid little attention to the possibility that the same culture has populations of cells that swim towards and away from the light source at the same time. The mutant *ida1*, which lacks subspecies *f* (also called I1) of inner-arm dynein, has been reported to exhibit no phototaxis (King and Dutcher, 1997). This observation is important because, if it was true, it would mean that the sole mechanism that controls appropriate cellular behavior converges on this dynein species. We therefore re-examined the Ca^{2+} control of the flagellar dominance as well as phototactic ability in *ida1*. Like *lsp1* and *ptx1*, *ida1* became oriented to the phototactic light stronger than a threshold intensity (Fig. 2F). However, unlike *lsp1* or *ptx1*, the direction of swimming was

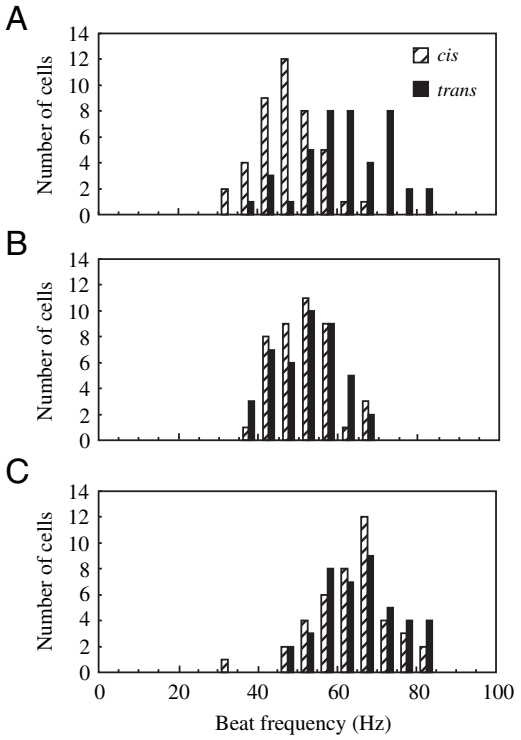

Fig. 6. Flagellar beat frequencies in demembranated and reactivated cell models. The histograms for wild-type (A), *lsp1* (B) and *ptx1* (C) cells are shown.

unipolar (i.e. *ida1* showed weak but positive phototaxis) (Fig. 2D). Furthermore, flagellar dominance in *ida1* cell models depended on the Ca^{2+} concentration, albeit weakly (Fig. 5C). Therefore, *ida1* apparently retains the ability to control flagellar dominance depending on Ca^{2+}, as well as the ability to display positive phototaxis, although both abilities are reduced compared with wild-type cells.

Genomic DNA rescuing the *lsp1* phenotype

Using the inserted plasmid sequence as a probe, we isolated a genomic DNA clone that rescued the *lsp1* phenotype. A 6.5-kb fragment of the clone was found to be the shortest genomic DNA that can rescue the *lsp1* phenotype (Fig. 7A). The program Green Genie predicted that the transcript of the fragment is a single polypeptide of 716 amino acids (Fig. 7B). Despite trials for more than 2 years, we have been unable to confirm the whole open reading frame by isolating its mRNA, except for the three exons at the 5′ end. We speculate that the difficulty is due to its extremely low level of expression and extraordinarily high GC content. The deletion of the first or the last exon in the predicted gene abolished the rescuing ability of the clone (Fig. 7A). The rescuing ability was also abolished by digestion with the restriction enzymes indicated in Fig. 7A. Therefore, it is likely that the *LSP1* gene extends for almost the entire length of the fragment, as predicted by Green Genie.

The predicted polypeptide has several targets for phosphorylation. There are two phosphorylation sites for cyclic-nucleotide-dependent protein kinase, three sites for casein kinase II, and a single site for protein kinase C (Fig. 7B). Hydropathy plots suggest that the polypeptide is not a membrane protein.

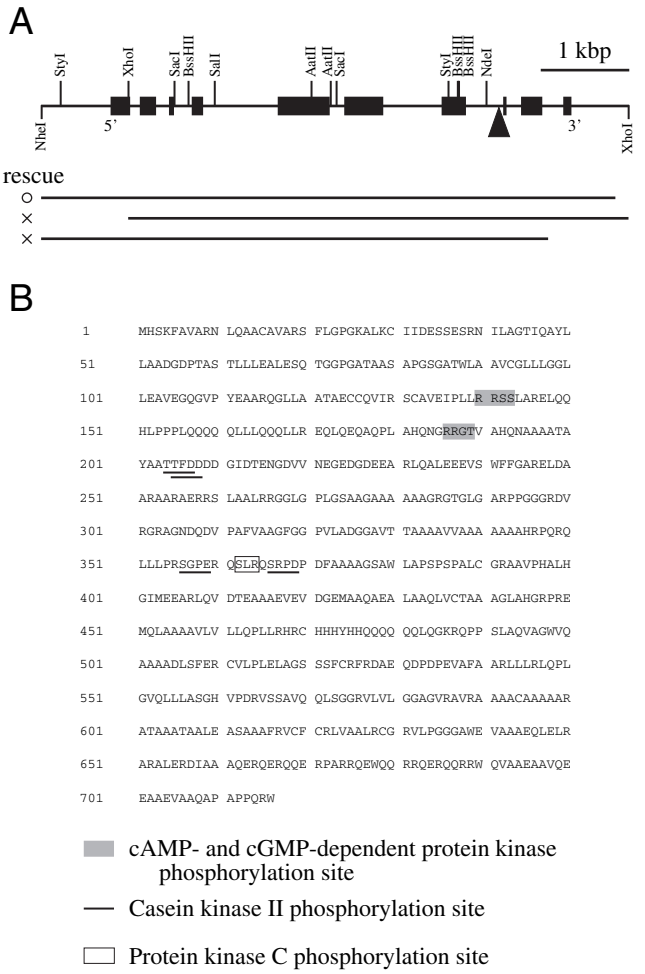

Fig. 7. (A) A genomic DNA segment that rescues the *lsp1* phenotype. Boxes represent the open reading frames predicted by the exon-prediction software Green Genie. The sites of restriction enzyme digestion by which rescue was abolished are indicated on the top. The triangle shows the site of the plasmid insertion. The *lsp1* phenotype was not rescued when the first or the last exon of the predicted gene was deleted (bottom). (B) Predicted primary structure of Lsp1. Phosphorylation sites are indicated. The sequences have been deposited in DDBJ/EMBL/GenBank with accession number AD194902.

Discussion

In this study, we showed that *Chlamydomonas* mutants *lsp1* and *ptx1* displayed weak phototactic behavior even though their demembranated 'cell models' did not show Ca^{2+}-dependent switching of flagellar dominance. In addition, *ida1*, a mutant lacking subspecies *f* (I1) of inner-arm dynein, also displayed weak but positive phototaxis. These observations disagree with previous reports that those mutants are non-phototactic, and thus require a reconsideration of the mechanism that produces phototactic behavior in *Chlamydomonas*.

An important step in the mechanism of behavioral control in *Chlamydomonas* is the control of the balance of beating between the two flagella, which must produce unequal propulsive forces when a cell makes a turn. A previous finding that the flagellar dominance in demembranated and reactivated wild-type cell models varies with submicromolar Ca^{2+} concentrations led to the idea that the balance between the two flagella in a swimming cell might be controlled by submicromolar concentrations of intraflagellar Ca^{2+} (Kamiya and Witman, 1984). This idea gained strong support from the finding that a non-phototactic mutant, *ptx1*, fails to display Ca^{2+}-dependent changes in flagellar dominance in the cell model (Horst and Witman, 1993). However, the present finding that *ptx1* and *lsp1* display a reduced degree of phototaxis, although confirming a close correlation between the flagellar dominance control and the cell's phototactic ability, requires that we elaborate the mechanism further. The new finding seems to suggest either of the following: (1) the balance between the two flagella in swimming cells is controlled by a Ca^{2+}-independent mechanism as well as by a Ca^{2+}-dependent mechanism; (2) the balance of the two flagella is solely controlled by a Ca^{2+}-dependent mechanism, but this Ca^{2+}-dependent process in live swimming cells is not correctly assessed by the dominance analysis of demembranated cell models. At present, we cannot distinguish between these two possibilities. As a Ca^{2+}-independent mechanism, we might posit the involvement of other ions; for example, we might consider H^+, which has been shown to be translocated upon activation of the *Chlamydomonas* photoreceptor (Ehlenbeck et al., 2002). Alternatively, we might think of signal transduction pathways that involve cAMP or GTP (Hasegawa et al., 1987; Habermacher and Sale, 1995; Patel-King et al., 2002). In this respect, it is interesting that the predicted Lsp1 polypeptide has multiple cAMP- and cGMP-dependent phosphorylation sites. We must also seriously consider the relevance of the flagellar dominance assessment using cell models, because the behavior of demembranated cell models differs greatly from that of live cells; in most cell models, one flagellum tends to be inactivated completely, a situation not frequently observed in vivo. Possibly, the Ca^{2+}-dependent behavioral change observed in cell models show only partial aspects of Ca^{2+}-control operating in vivo. Simultaneous observations of the Ca^{2+} concentration and flagellar beating in live cells should be important for clarifying these points.

The mutant *lsp1* was found to be similar to *ptx1* except for the axonemal beat frequency in cell models. That is, both axonemes of *ptx1* beat at the same frequency as the trans axoneme of the wild type, whereas both axonemes of *lsp1* beat at the frequency of the cis axoneme. These observations are interesting when considering the nature of the *lsp1* and *ptx1* mutations in the light of the findings by Rüffer and Nultsch (Rüffer and Nultsch, 1997), who found that the cis and trans flagella in live wild-type cells undergo distinct changes upon photostimulation of the cell, and that both flagella in *ptx1* cells respond in a manner similar to that of the trans flagellum in the wild type. They therefore suggested that the *ptx1* mutant has two equal flagella with the properties of the trans flagellum. Our data from the frequency measurement are consistent with this idea, and further suggest that the *lsp1* mutation is a mutation that causes the cell to have two cis flagella (i.e. a mutation opposite to *ptx1*). Presumably, the two flagella of *lsp1* cells are cis for the mechanism that controls Ca^{2+}-dependent flagellar dominance as well as for the beat frequency, but not for the Ca^{2+}-independent mechanism revealed here for the first time, because they retain some ability to steer.

As a possible explanation of the regulation of the cis-trans flagellar dominance control and the occurrence of those mutants, we present here a model of the two flagella (Fig. 8A). Because the *ptx1* mutant shows a motility change in response to light stimulus (Rüffer and Nultsch, 1997), its axoneme should retain the mechanism for changing its activity. Hence, we speculate that its failure to change flagellar imbalance depending on the Ca^{2+} concentration (Fig. 5) is caused by the two flagella undergoing the same response. Likewise, the two flagella in *lsp1* are assumed to have normal properties as cis flagella. Now, we assume that, in the wild-type cell, the signals from the light reception are transmitted to the cis and trans flagella through transducers specific to each kind of flagellum (Fig. 8A), and that the mutants *ptx1* and *lsp1* lacks either one of the transducers. In *ptx1* cells, for example, the cis transducer is absent and both flagella contain the trans transducer. By contrast, *lsp1* has two cis flagella and no trans flagellum. The cis and trans transducers change the flagellar activity depending on the Ca^{2+} concentration. We assume that the intraflagellar Ca^{2+} concentration changes depending on the light reception by the eyespot, as well as on the cell's internal conditions. It must oscillate when the direction of the photoreceptors rotates with respect to the light ray, because the cell body rotates while swimming. We suggest that a correct phototactic turn occurs because the Ca^{2+} change causes opposite effects to the cis and trans flagella because the two transducers have opposite Ca^{2+} dependencies.

A striking finding with *lsp1* and *ptx1* is that these mutant cells tended to swim either towards or away from the light

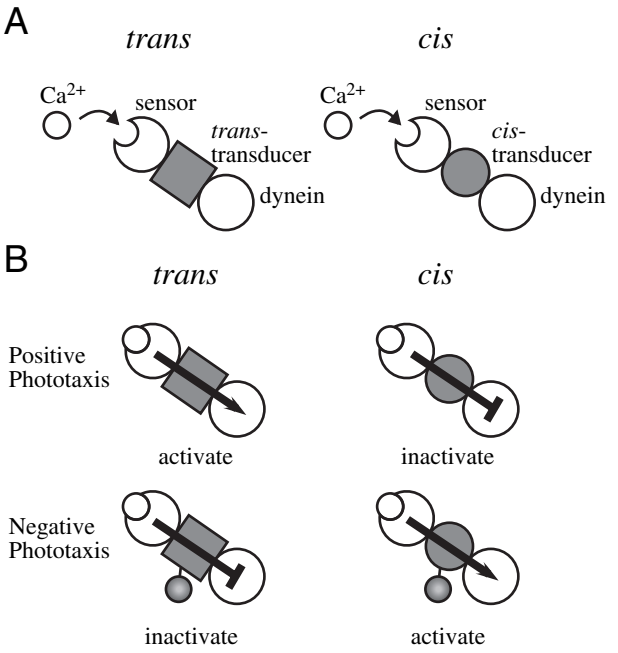

Fig. 8. (A) Hypothetical trans and cis transducers that transmit information from the sensor to dynein. (B) Reciprocal activation of the cis and trans complex in positive (top) and negative (bottom) phototaxis.

source; in other words, cells from the same culture form two populations that display either positive or negative phototaxis. The sign of phototaxis in *Chlamydomonas* and other motile algae has been shown to be variable, depending on circadian rhythm, activity of photosynthesis or other factors (Diehn, 1973; Sakaguchi and Tawada, 1977; Takahashi and Watanabe, 1993). However, how the sign is determined has not been understood. In theory, a reversal of the direction of photomigration could be brought about by a reversed response at any step in the signal transduction pathway. Because *lsp1* displays normal photoreceptor currents (Fig. 3), its inability to decide between positive and negative phototaxis probably results from some defects in the later stage of signal transduction. The simultaneous loss of the ability to determine the phototactic direction and the ability to change the cis-trans flagellar dominance in *ptx1* and *lsp1* indicates that those abilities are controlled by a common factor or process. Therefore, we speculate that positively and negatively phototactic cells might occur because their cis-trans transducers have opposite properties, possibly caused by some reversible modification such as phosphorylation (Fig. 8B). The importance of protein phosphorylation for flagellar activity control has been fully documented (Habermacher and Sale, 1995; Habermacher and Sale, 1996; Habermacher and Sale, 1997; King and Dutcher, 1997). The presence of phosphorylation sites found in the predicted Lsp1 polypeptide (Fig. 7B) raises the interesting possibility that Lsp1 is involved in the phosphorylation pathway for the determination of the sign of taxis and/or the Ca^{2+}-independent control of flagellar dominance.

The mutant *ida1* lacks the *f* subspecies of inner-arm dynein and was found to display weak phototaxis. Although this observation is consistent with a previous report (King and Dutcher, 1997), in that it indicates the distinctive importance of dynein *f* for the phototactic mechanism, it at the same time suggests that dynein *f* is not the sole dynein responsible for phototaxis. Changes in the swimming direction during phototaxis appear to be brought about by alterations in both flagellar waveform and beat frequency (Rüffer and Nultsch, 1987; Rüffer and Nultsch, 1991). Because the loss of the *f* subspecies results in a change in the flagellar waveform without much change in the beat frequency (Brokaw and Kamiya, 1987), the *f* subspecies might be responsible for modifying the flagellar waveform during phototaxis. Thus, other dyneins might function in the regulation of beat frequency. A good candidate is outer-arm dynein, the loss of which has been shown to result in a decrease in the beat frequency (Kamiya and Okamoto, 1985; Mitchell and Rosenbaum, 1985).

Overall, our results indicate that multiple pathways of signal transduction are involved in the phototactic behavior of *Chlamydomonas*. The pathway present in *ptx1* or *lsp1* apparently functions to orient a cell parallel to the light direction without deciding between 0° and 180°. Such a pathway might enhance the performance of the main pathway, which determines the direction of phototaxis. Some pathways might be activated only in certain ranges of light intensities; the irregular dose-response curve in *ida1* (Fig. 2F) might be a reflection of the functioning of multiple pathways with different light-intensity dependencies. Such multiple pathways for motility control might be important not only for producing phototactic behavior but for integrating various signals from external conditions, including chemicals (Hirschberg and Rogers, 1978; Ermilova et al., 1996), gravity (Bean, 1977; Yoshimura et al., 2003) and light.

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