Outer Dynein Arm Light Chain 1 Is Essential for Controlling the Ciliary Response to Cyclic AMP in *Paramecium tetraurelia*

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The individual role of the outer dynein arm light chains in the molecular mechanisms of ciliary movements in response to second messengers, such as Ca^{2+} and cyclic nucleotides, is unclear. We examined the role of the gene termed the outer dynein arm light chain 1 (LC1) gene of *Paramecium tetraurelia* (ODAL1), a homologue of the outer dynein arm LC1 gene of *Chlamydomonas reinhardtii*, in ciliary movements by RNA interference (RNAi) using a feeding method. The ODAL1-silenced (ODAL1-RNAi) cells swam slowly, and their swimming velocity did not increase in response to membrane-hyperpolarizing stimuli. Ciliary movements on the cortical sheets of ODAL1-RNAi cells revealed that the ciliary beat frequency was significantly lower than that of control cells in the presence of ≥1 mM Mg^{2+}-ATP. In addition, the ciliary orientation of ODAL1-RNAi cells did not change in response to cyclic AMP (cAMP). A 29-kDa protein phosphorylated in a cAMP-dependent manner in the control cells disappeared in the axoneme of ODAL1-RNAi cells. These results indicate that ODAL1 is essential for controlling the ciliary response by cAMP-dependent phosphorylation.

Eukaryotic cilia and flagella are cell organelles for motility and sensing and have various important roles in biological processes. The locomotor behavior of *Paramecium* depends on ciliary movements. The ciliary movements are controlled by changes in the membrane potential that regulate the intraciliary concentrations of Ca^{2+} and cyclic nucleotides. For example, membrane depolarization in response to a mechanical or chemical stimulus applied to the anterior membrane causes an increase in the intraciliary Ca^{2+} concentration (13), which results in a change in the ciliary orientation toward the anterior direction of the cell (ciliary reversal) and a change in the swimming direction (24). Membrane hyperpolarization in response to a mechanical or chemical stimulus applied to the posterior membrane causes an increase in the intraciliary cyclic AMP (cAMP) concentration (38). This induces an increase in the ciliary beat frequency and changes the ciliary orientation to a more posterior orientation, which causes faster forward swimming (11, 25–30). In addition, cAMP suppresses Ca^{2+}-induced ciliary reversal (11, 25–30). However, the molecular bases of the control mechanism of ciliary movements are unclear.

The outer and inner dynein arms, which are multisubunit complexes attached to the outer surface of the peripheral microtubule doublets, generate forces that cause ciliary and flagellar movements. These multisubunit complexes are composed of one or more catalytic heavy chains (HCs) associated with several intermediate chains (ICs) and light chains (LCs). It has been postulated that certain outer dynein arm LCs are responsible for the regulation of ciliary and flagellar movements. For example, the outer dynein arm of *Chlamydomonas reinhardtii* comprises 3 HCs, 2 ICs, and 11 LCs (19). Among the LCs, LC1 associates directly with the catalytic motor domain of γHC (8, 33, 45). The expression of dominant negative LC1 mutant proteins in wild-type C. reinhardtii cells showed significant alterations in the flagellar waveform (33). A *Trypanosoma brucei* outer dynein arm LC1 knockout mutant created by RNA interference (RNAi) exhibited slow backward propulsion and a reversed flagellar beat (6). In addition, the loss of LC1 induced the destabilization of the outer dynein arms. In the planarian *Schmidtea mediterranea*, a reduction in levels of LC1 by RNAi caused a significant drop in the ciliary beat frequency and abolished the ability of beating cilia to form metachronal waves (36). However, the precise role of LC1 of dynein complexes in the molecular mechanisms of ciliary and flagellar movements is unclear. The control of ciliary and flagellar movements depends on second messengers, such as Ca^{2+} and cyclic nucleotides. Therefore, determining how defects of LC1 affect the regulation of the ciliary and flagellar responses to second messengers is essential to an understanding of the role of the dynein subunits in the molecular mechanisms.

We have shown previously that a cortical sheet, an experimental system that we developed, is a useful tool to analyze the ciliary movements of *Paramecium* (26–29, 31). In addition, the *Paramecium tetraurelia* genome database, a ciliary proteome database, and protocols for genetic engineering by RNAi are available (1–4, 14). Therefore, ciliated *Paramecium* could be a useful model organism to study the role of axonemal proteins in the molecular mechanisms of ciliary movements.

In this study, we focused on a gene termed the outer dynein arm LC1...
results indicate that the ciliary movements by RNAi using a feeding method (14). Our results indicate that the ODAL1 gene is essential for controlling cAMP-dependent ciliary movement.

**MATERIALS AND METHODS**

**Cell culture.** *P. tetraurelia* (stock 7.2B) cells were cultured in a hay infusion bacterized with *Enterobacter aerogenes* and supplemented with 0.8 μg/ml β-sitosterol according to standard procedures (40). The cells were grown to the late logarithmic phase at 25°C.

**Gene silencing by RNAi using the feeding method.** The open reading frame region of ODAL1 (GenBank accession no. XM_001446309) was amplified by PCR and cloned into the Litmus28i vector (New England Biolabs) between two T7 promoters. The amplification primers used were f1 (ATGGCAAGAACAAGTTGTG) and r1 (TCATTGTGACAGTGG TTGTAG). The resulting constructs were used for the transformation of HT115, an RNAse III-deficient strain of *Escherichia coli* with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase (42). RNAi gene silencing was performed according to a feeding method described previously by Galvani and Sperling (14), with modifications. Wild-type paramecia were incubated in a culture medium containing 0.8 μg/ml β-sitosterol, 100 μg/ml ampicillin, and 0.4 mM IPTG. Gene silencing was initiated by the addition of double-stranded RNA-expressing bacteria to the culture medium (14). One day or two days after the induction of gene silencing, the cells were used for the experiments in this study. The phenotypes of ODAL1-silenced (DAL1-RNAi) cells were the same after 1 day and 2 days of gene silencing. Nonsilenced *P. tetraurelia* cilia were used as the control cells. As a negative control, we used N7 silencing, which affects trichocyst exocytosis without altering the ODAL1 gene or any other cellular function (16, 37). Furthermore, we analyzed the off-target effect of ODAL1 silencing using the ParameciumDB *P. tetraurelia* genome database (http://paramecium.cgm.cnrs-gif.fr) (1).

**Competitor PCR.** One microgram of each poly(A)+ RNA was reverse transcribed by using PrimerScript reverse transcriptase (TaKaRa Bio Inc., Japan). A 2-μl aliquot of cDNA was added to each PCR mixture, which also contained 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl₂, 0.2 μM deoxynucleoside triphosphates (dNTPs), and 0.4 μM each primer described above. The amplification protocol consisted of one cycle at 94°C for 1 min, 54°C for 30 s, and 72°C for 2 min; 25 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. To quantitatively compare the PCR products, we determined the exponential phase of amplification by performing amplification for 15, 20, 25, and 30 cycles, using the ODAL1 primers 5′-CTTTTTTCATCAATGCAATAG-3′ (sense) and 5′-CATTATAGGGTGTACCTTTC-3′ (antisense). In addition, as an internal control for cDNA quantity and quality, we amplified the gene for beta-actin by using the primers 5′-TTGATTATGAAGGAAAATG-3′ (sense) and 5′-TTCGTGGACAAATGGTTGG-3′ (antisense). Amplified PCR products were electrophoresed in 2.0% agarose gels and visualized by ethidium bromide staining. To quantify the relative amounts of each PCR product, the ethidium-stained gels were reversed and analyzed by using ImageJ (National Institutes of Health). The ratios of ODAL1 mRNA to beta-actin mRNA were calculated based on the densities of the PCR products (7, 15, 32, 39).

**Assays of swimming behavior.** Approximately 50 μl of a cell culture containing 40 to 50 live cells was placed by a micropipette onto a depression slide. Membrane-hyperpolarizing stimulation was performed by the addition of CaCl₂ to attain a Ca²⁺ concentration of 2 mM in the cell culture (22). After the addition of CaCl₂, the behaviors of individual cells were observed under a VCT-VBITvB digital microscope (Shimadzu, Japan) and recorded for 1 s. A sequential image of the swimming path was prepared from the recorded frames by using Motic Images Plus 2.1S (Shimadzu, Japan). The forward swimming velocity was determined by measuring the length of the swimming path by using ImageJ. Membrane-depolarizing stimulation was performed by the addition of KCl to attain a K⁺ concentration of 50 mM in the cell culture. After the addition of KCl, backward swimming was observed by using a VCT-VBITvB digital microscope, and the duration of the backward swimming was determined.

**Preparation and reactivation of cortical sheets from live cells.** The preparation of cortical sheets from live cells (intact cortical sheets) was performed according to methods described in our previous paper (31), with slight modifications. Concentrated cells were washed by centrifugation with an ice-cold washing medium containing 2 mM EDTA, 50 mM potassium acetate, and 10 mM Tris-maleate buffer (pH 7.0). The loose pellet of cells was resuspended in 1 ml of an ice-cold potassium acetate solution containing 50 mM potassium acetate and 10 mM Tris-maleate buffer (pH 7.0). The cells were pipetted once or twice through a glass pipette with a small inside diameter (approximately 0.15 mm) to tear or nick the cell cortex. This cell suspension was used for the reactivation experiments. A simple perfusion chamber was prepared by placing the sample between a slide and a coverslip. The slide and coverslip were separated by a thin layer of Vaseline applied to two opposite edges of the coverslip. To observe the reactivation of cilia on the sheet of cell cortex, 50 μl of the sample was gently placed onto a glass slide, and a coverslip with Vaseline was placed over the sample. Solutions were then perfused through the narrow opening at one of the edges of the coverslip, while the excess fluid was drained from the opposite end with the aid of small pieces of filter paper. During the first perfusion using a reference potassium acetate solution, some torn cell cortex adhered flat to the glass surface.

Cortical sheets were perfused successively with reactivation solutions. All reactivation solutions contained 50 mM potassium acetate and 10 mM Tris-maleate buffer (pH 7.0) as well as a component(s), such as MgCl₂, ATP, and cyclic nucleotide, as noted in Results and the figure legends. The free Ca²⁺ concentration of 2 × 10⁻⁶ M and lower in the reactivation solutions was controlled by using Ca-EGTA buffer (34), using 1 mM EGTA; a concentration of 2 × 10⁻⁸ M was obtained by the addition of an adequate amount of CaCl₂ to a reactivation solution without EGTA. The reactivation of cilia was carried out at 22°C to 25°C. To determine the ciliary beat frequency, the reactivation of cilia on intact cortical sheets was performed in the presence of 5 μM cyclic GMP (cGMP) throughout, because reactivated cilia without cGMP beat in an abnormal manner to some extent (27).

The ciliary orientation was observed in the presence of 30% glycerol to clearly determine the ciliary orientation (26, 28, 29). Intact cortical sheets were demembranated by perfusion with a Triton solution containing 0.05% Triton X-100, 1 mM EGTA, 1 mM MgCl₂, 50 mM potassium acetate, and 10 mM Tris-maleate (pH 7.0) for 1 min and then washed by perfusion with the same solution without Triton X-100 to remove Triton X-100. The demembranated cortical sheets were incubated in a glycerol solution containing 30% glycerol, 1 mM EGTA, 1 mM MgCl₂, 50 mM potassium acetate, and 10 mM Tris-maleate (pH 7.0) for 5 min. The cilia were reactivated by perfusion with reactivation solutions containing 30% glycerol, 1 mM ATP, 1 mM MgCl₂, 50 mM potassium acetate, and 10 mM Tris-maleate (pH 7.0) as well as Ca²⁺ and cAMP, as noted in Results and the figure legends. In the presence of 30% glycerol, the reactivated cilia on the cortical sheets exhibited only a restricted beat with a small amplitude. However, the pointing directions of the cilia changed in response to Ca²⁺ and cAMP reversibly (26, 28, 29).

**Observation and recording of reactivated cilia.** The reactivated cilia on cortical sheets were observed under a dark-field microscope equipped with a 100-W mercury light source, a heat filter, and a green filter. The ciliary movements were recorded by using an HAS-220 high-speed camera (Ditec, Japan). To determine the ciliary beat frequency, recording was performed at 600 frames per s, and to determine the ciliary direction, recording was performed at 100 frames per s.

**Analysis of ciliary movements on cortical sheets.** The movements of the reactivated cilia on intact cortical sheets were analyzed by using ImageJ. We analyzed cilia on the left-hand field of the sheet, defining the surface area of the anatomical left-hand side as the left-hand field of the...
cortical sheets (28). The beat frequency of the reactivated cilia was determined by the direct measurement of the ciliary beat cycle by monitoring the recorded video images frame by frame (31). The method for determining the ciliary orientation was essentially the same as that described in our previous papers (26, 28, 29). Three cilia on several independent cortical sheets from at least two independent RNAi preparations were measured for ciliary beat frequency and ciliary orientation.

**Isolation of cilia.** Collected cells were washed twice with a washing solution containing 2 mM KCl, 2 mM CaCl₂, and 10 mM Tris-maleate (pH 7.0). Cells were deciliated by dibucaine treatment according to methods described previously by Mogami and Takahashi (23), with slight modifications (29). Cilia were isolated from cell bodies by centrifugation twice at 600 × g for 5 min. The supernatant was centrifuged at 7,700 × g for 10 min to pellet the cilia. The pellet was resuspended in TMKE solution (10 mM Tris-maleate [pH 7.0], 5 mM MgCl₂, 20 mM potassium acetate, and 1 mM EGTA) containing 0.3 mM phenylmethylsulfonyl fluoride and centrifuged. The pellet was re-washed with TMKE solution. Each step of the isolation procedure was monitored by dark-field microscopy. Isolated cilia were then treated with a demembranation solution containing 0.1% Triton X-100 in TMKE solution for 10 min at 0°C. The suspension was centrifuged to pellet the axonemes. Triton X-100 was removed from the axonemes by washing twice with TMKE solution. The pellet of the axonemes was suspended in a small amount of TMKE.

**Phosphorylation of axonomal proteins.** The *in vitro* phosphorylation of the axonomal proteins was performed according to methods described previously by Hamasaki et al. (18), with slight modifications. The reaction mixture contained 75 μg axonemes and 30% glycerol in 80 μl TMKE solution as well as test substances. Phosphorylation by endogenous protein kinases was started by the addition of 20 μl of [γ-32P]ATP to attain a final concentration of 2 μM ATP. The ATP concentration of [γ-32P]ATP was 10 μM, and the radioactivity was adjusted to 10 μCi with adenosine 5’-[γ-32P]triphosphate (specific activity, 6,000 Ci/mmol; MP Biomedicals Inc., Solon, OH). Immediately after 10 min of incubation at 0°C, the reaction mixture was centrifuged at 10,000 × g for 10 min. The pellet was directly suspended in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.5% bromophenol blue, and 62.5 mM Tris-Cl [pH 6.8]) and incubated at 100°C for 2 min. These SDS-treated samples were then subjected to SDS-PAGE or stored at −20°C for further use. The protein concentration was determined according to methods described previously by Lowry et al. (21), using bovine serum albumin as a standard.

**SDS-PAGE and autoradiography.** SDS-PAGE was performed by a modification of a procedure described previously by Laemmli (20), using 3- to 15% linear gradient acrylamide gels containing 0 to 19% glycerol gradient run on a 20- by 16- by 0.1-cm slab gel. The gels were stained with Coomassie blue R-250 for 15 min or with silver (10) and dried on filter paper. Molecular weight standards were obtained from Bio-Rad (Hercules, CA). To produce the autoradiograms, an imaging plate (IP; Fujifilm Corp., Tokyo, Japan) was placed over the dried gels for 2 days. After exposure, the IP was scanned by using a bioimaging analyzer system (BAS-1800; Fujifilm Corp.).

**Electron microscopy.** For electron microscopy, cells were fixed in 1% (vol/vol) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The cells were washed and postfixed in 1% (wt/vol) OsO₄ for 1 h at room temperature. Postfixation was followed by washing in distilled water and then dehydration in a series of increasing concentrations of ethanol and, finally, 100% propylene oxide. The cells were then embedded in Quetol 812 (Nissin EM Co., Ltd., Japan). Following evaporation and hardening, the cells were cut out of the block and glued onto another polymerized pre-shaped block. These cells were serially sectioned in a longitudinal orientation, and all of the sections were picked up on Formvar-supported grids having one large opening. The grids were placed in chronological order so that the exact position of each section within the entire series could be determined. A diamond knife (Nissin EM Co., Ltd., Tokyo, Japan) and Ultracut E (Reichert, Buffalo, NY) were used for sectioning. Sections were then stained at room temperature with uranyl acetate (43) and lead citrate (35) for 7 min and 3 min, respectively. A transmission electron microscope (H-7000; Hitachi, Tokyo, Japan) operated at 75 kV was used throughout the study.

**RESULTS**

**Characteristics of ODAL1.** The *ODAL1* gene of *P. tetraurelia* (GenBank accession no. XM_001446309) encodes a protein whose predicted sequence is 38% identical to outer dynein arm LC1 of *C. reinhardtii* (accession no. AF112476), and such a protein was detected in the *P. tetraurelia* genome database and the ciliary proteome database (1–4). The *ODAL1* gene includes the conservation of most residues that directly bind the outer dynein arm γHC catalytic motor domain and tubulin (8, 33, 45) (Fig. 1). It is presumed that the *ODAL1* product has a molecular mass of 22 kDa. The CAMP-dependent phosphorylation site was predicted by using NetPhosK 2.0 (9) (Fig. 1).

**Confirmation of ODAL1 silencing by RNAi.** We confirmed the effect of *ODAL1* silencing by RNAi using competitive PCR. *ODAL1* mRNA was expressed over 2-fold compared with beta-actin mRNA in nonsilenced cells (control) (Fig. 2). When the paramecia were fed *E. coli* including a knockdown plasmid, however, the amount of *ODAL1* mRNA was strikingly decreased, even within 1 day after feeding (Fig. 2). Compared to control cells, the level of *ODAL1* mRNA was 6-fold lower in *ODAL1*-silenced paramecia.

Furthermore, the off-target effect of *ODAL1* silencing was analyzed. For *ODAL1* silencing, the RNAi off-target has four genes: 3
genes are itself and its ohnologues, and the other has a completely different function, and its inactivation by this RNAi is very unlikely, since only a single 23-nucleotide (nt) siRNA can target it (data not shown). Therefore, it seems that there are no off-targets and that the RNAi insert is specific.

**Phenotypes of ODAL1-silenced cells.** We examined the phenotypes of ODAL1-silenced (ODAL1-RNAi) cells. Control cells swim at approximately 0.8 mm/s and showed a significant increase in the forward swimming velocity in response to hyperpolarizing stimulation by the addition of CaCl2 to attain a Ca2+ concentration of 2 mM (Fig. 3A and B). In contrast, ODAL1-RNAi cells swam at half the swimming velocity of the control cells. Moreover, ODAL1-RNAi cells did not show a significant increase in the forward swimming velocity in response to hyperpolarizing stimulation (Fig. 3A and B).

Control cells exhibited backward swimming for approximately 10 s in response to depolarizing stimulation by the addition of KCl to attain a K+ concentration of 50 mM. In contrast, ODAL1-RNAi cells exhibited a long period of backward swimming after the depolarizing stimulation (Fig. 3C). They also exhibited a short period of backward swimming (within 1 s), an “avoiding reaction,” frequently seen in culture medium without the depolarizing stimulus (see Movie S1 in the supplemental material). The phenotypes of ND7-silenced (ND7-RNAi) cells were the same as those of the control cells (data not shown).

**Effects of ODAL1 silencing on ciliary beat frequency in response to Mg2+-ATP.** The effects of ODAL1 silencing on the ciliary beat frequency were determined at various concentrations of Mg2+-ATP by using intact cortical sheets. The ciliary beat frequency of the control cells increased with increasing Mg2+-ATP concentrations up to 8 mM (Fig. 4, and see Movie S2A in the supplemental material). The apparent Km and Vmax were 0.80 mM and 52 Hz, respectively. In contrast, the ciliary beat frequency of ODAL1-RNAi cells did not increase in the presence of ≥1 mM Mg2+-ATP (Fig. 4, and see Movie S2B in the supplemental material). The apparent Km and Vmax of ODAL1-RNAi cells (2 days after the induction of gene silencing) were 0.22 mM and 21 Hz, respectively. However, the reactivated cilia of ODAL1-RNAi cells showed a normal beat cycle that consisted of an effective stroke and a recovery stroke (37) (see Movie S2B in the supplemental material). The ciliary beat frequency of ND7-RNAi cells was essentially the same as that of the control (Fig. 4).

**Effects of ODAL1 silencing on ciliary orientation in response to Ca2+ and cAMP.** The effects of ODAL1 silencing on the ciliary orientation in response to Ca2+ without cAMP were determined by using cortical sheets. The orientation of the reactivated cilia of the control cells was toward the 6-o’clock position (posterior direction of the cell) at ≈0.2 μM Ca2+ (Fig. 5A). When the cortical cells were perfused with a reactivation solution containing ≥1 μM Ca2+, the orientation of the cilia was toward the 12-o’clock position (anteroposterior direction of the cell) (26, 28, 29). The changes in the
The ciliary orientation of ND7-RNAi cells on cortical sheets in response to Ca\(^{2+}\) were very similar to those of the control cells (Fig. 5A).

The effects of ODAL1 silencing on the ciliary orientation on cortical sheets in response to cAMP were determined in the presence of 2 μM Ca\(^{2+}\). In the case of the control cells, the ciliary orientation began to change from an anterior to a posterior direction of the cell in the presence of 1 μM cAMP. At ≈10 μM cAMP, the orientation of the reactivated cilia was toward the 5-o’clock position. On the contrary, the ciliary orientation of ODAL1-RNAi cells did not change from the anterior to the posterior direction of the cell, even in the presence of 100 μM cAMP (Fig. 5B).

The ciliary orientation of ND7-RNAi cells in response to Ca\(^{2+}\) and cAMP was essentially the same as that of control cells (Fig. 5A and B).

**Change in composition of axonemal proteins after ODAL1 silencing.** The axonemal proteins of ODAL1-RNAi cells were analyzed by SDS-PAGE using 3-to-15% polyacrylamide gradient gels. The two HC bands were decreased to some extent in the 1-day- and 2-day-silenced ODAL1-RNAi cells (Fig. 6, lanes b and c). The composition of axonemal proteins was not affected by ND7 silencing (data not shown).

**Effects of ODAL1 silencing on cAMP-dependent phosphorylation of axonemal proteins.** We examined the effects of ODAL1 silencing on the cAMP-dependent phosphorylation of axonemal proteins. In control and the ND7-RNAi cells, the 29-kDa and 65-kDa axonemal polypeptides were phosphorylated with 10 μM cAMP (5, 17, 26, 27, 29, 30) (Fig. 7).

In ODAL1-RNAi cells, the 65-kDa polypeptide was phosphorylated with 10 μM cAMP, but the phosphorylation of the 29-kDa polypeptide was not detected after 1 day of silencing (Fig. 7A). After 2 days of silencing, no phosphorylation was detected for the 29-kDa or 65-kDa polypeptide (Fig. 7B).

**Effects of ODAL1 silencing on the presence of outer and inner dynein arms within axonemes.** Cross sections of cilia from the control, ODAL1-RNAi, and ND7-RNAi cells were observed by using a transmission electron microscope. In the ODAL1-RNAi
axonemes, a couple of the outer dynein arms had disappeared randomly (indicated by arrowheads in Fig. 8A and B). The mean number of missing outer dynein arms was 3.22 ± 1.64 (n = 36) (Fig. 8D). However, the inner dynein arms of ODAL1-RNAi were not affected. In the ND7-RNAi cells, both the outer and inner dynein arms were not affected (Fig. 8C).

**DISCUSSION**

Outer dynein arm LC1 has been thought to be responsible for regulating ciliary and flagellar movements. For example, C. reinhardtii LC1 binds the outer arm dynein HC motor domain and a doublet microtubule within the axonemal superstructure and may regulate outer dynein arm activity through a conformational switch for flagellar motility (8, 33, 45). In T. brucei, LC1 is necessary for proper forward flagellar motility and for a stable outer dynein arm assembly (6). LC1 of the planarian *S. mediterranea* acts in a mechanosensory feedback mechanism controlling outer arm activity (36). However, the role of outer dynein arm LC1 in the molecular mechanisms of ciliary and flagellar movements is unclear. In this study, we cloned ODAL1 from *P. tetraurelia* using the *Paramecium* genome database and the ciliary proteome data-base (1–4). To clarify the role of ODAL1 in the ciliary movements of *P. tetraurelia*, we created ODAL1-silenced cells by RNAi using the feeding method (14) and examined the effects of ODAL1 silencing on the ciliary movements and the compositions of axonemal proteins. We confirmed that the ODAL1 gene was properly silenced (Fig. 2) and that ODAL1 silencing could have no off-target effects. Furthermore, as a negative control, we examined the

**FIG 7** cAMP-dependent phosphorylation of the axonemal proteins from ODAL1-RNAi cells. Axonemes were labeled *in vitro* with adenosine 5'-[γ-32P]triphosphate. The phosphorylated proteins were run on 3-to-15% linear gradient acrylamide gels. CBB, band pattern stained with Coomassie blue R 250; Autorad., autoradiogram. (A) Effect of ODAL1 silencing (1 day after the induction of gene silencing) on the cAMP-dependent phosphorylation of axonemal proteins. (B) Effects of ODAL1 silencing (2 days after the induction of gene silencing) on the cAMP-dependent phosphorylation of axonemal proteins. (C) Effects of ND7 silencing on the cAMP-dependent phosphorylation of axonemal proteins. 65 k and 29 k indicate the cAMP-dependent phosphorylated 65-kDa and 29-kDa polypeptides in the autoradiogram, respectively. + and − indicate the presence and absence of 10 μM cAMP, respectively. HC, outer dynein arm HCs; T, tubulins.
effects of ND7 silencing, which affects trichocyst exocytosis without altering the ODAL1 gene or any other cellular function (16, 37). We confirmed that ND7 silencing did not affect the ciliary movements and the compositions of axonemal proteins (Fig. 4, 5, 7C, and 8C).

We initially examined the phenotypes of ODAL1-RNAi cells. They swam more slowly than the control cells, and their swimming velocity did not increase in response to hyperpolarizing stimulation (Fig. 3A and B). In addition, the silenced cells showed a long period of backward swimming in response to depolarizing stimulation and, frequently, a spontaneous avoiding reaction in the absence of depolarizing stimulation (Fig. 3C, and see Movie S1 in the supplemental material). These observations suggest that ODAL1 silencing resulted in two types of defects in the ciliary activities. The first defect is the impairment of the ability to increase the ciliary beat frequency. The second defect is apparent hypersensitivity to Ca\(^{2+}\).

The ciliary beat frequency depends on the Mg\(^{2+}\)-ATP concentration (31) (Fig. 4, and see Movie S2A in the supplemental material). We found that the ciliary beat frequency on intact cortical sheets of ODAL1-RNAi cells did not increase with high concentrations of Mg\(^{2+}\)-ATP (≥1 mM) (Fig. 4, and see Movie S2B in the supplemental material). This indicates that ODAL1 silencing impairs the ability to increase the ciliary beat frequency. Therefore, the slow swimming of ODAL1-RNAi cells (Fig. 3A and B) is a consequence of the impairment of the ability to increase the ciliary beat frequency. The reactivated cilia of ODAL1-RNAi cells showed a normal beat cycle that consisted of an effective stroke and a recovery stroke (41) (see Movie S2B in the supplemental material). This suggests that ODAL1 silencing does not impair the ciliary waveform. In C. reinhardtii and Tetrahymena thermophila, an inner dynein arm has been thought to be responsible for the regulation of the ciliary and flagellar waveforms (12, 44). Therefore, the normal ciliary waveform in ODAL1-RNAi cells indicates that ODAL1 silencing does not affect the inner dynein arms of Paramecium (Fig. 8).

Previous analyses of ciliary movements using permeabilized cell models (Triton models) and cortical sheets have shown that ≥1 μM Ca\(^{2+}\) induces a ciliary reversal and backward swimming (11, 24–30). We expected that if ODAL1-RNAi cells showed hypersensitivity to Ca\(^{2+}\), the ciliary orientation on cortical sheets would show a ciliary reversal in the presence of lower Ca\(^{2+}\) concentrations compared with that of the control cells. However, the threshold Ca\(^{2+}\) concentration for the ciliary orientation reversal of the control cells was almost similar to that of ODAL1-RNAi cells (Fig. 5A). This indicates that ODAL1 silencing does not affect sensitivity to Ca\(^{2+}\) in the ciliary motor mechanism.

cAMP makes the ciliary orientation more posterior (26, 27, 29). Furthermore, it was shown previously that cAMP and Ca\(^{2+}\) act antagonistically in setting the ciliary orientation and that cAMP suppresses Ca\(^{2+}\)-induced ciliary reversal (11, 25–30). We analyzed the effect of ODAL1 silencing on cAMP-dependent ciliary responses using cortical sheets. As shown in Fig. 5B, the ciliary reversal induced by 2 μM Ca\(^{2+}\) was not suppressed by cAMP in cortical sheets of ODAL1-RNAi cells. This result indicates that ODAL1 silencing impairs the ciliary response to cAMP. Therefore, the phenotypes of ODAL1-RNAi cells, such as the longer period of backward swimming (Fig. 3C) and the spontaneous avoiding reaction in the absence of any stimulation (see Movie S1 in the supplemental material), are probably due to the apparent hypersensitivity to Ca\(^{2+}\) that is a consequence of the defect in the ciliary response to cAMP.

To test whether ODAL1 silencing affects axonemal proteins other than the ODAL1 product, we analyzed the composition of axonemal proteins in ODAL1-RNAi cells using SDS-PAGE. In the axonemes of ODAL1-RNAi cells, two HC bands (>200 kDa) were decreased to some extent (Fig. 6, lane b). The upper band corresponds to the outer dynein arm HC (indicated by open circles in Fig. 6). In addition, after 2 days of silencing, several bands of the axonemal proteins also decreased (Fig. 6, lane c). We observed the cross-sectional images of the ODAL1-RNAi axonemes with missing outer dynein arms at the level where inner dynein arms were present (Fig. 8). While such defects were rare, we did not see such axoneme cross sections in nonsilenced cells and in ND7-RNAi cells. Our observations indicate that as previously shown for T. brucei (6), in P. tetraurelia, the loss of ODAL1 also destabilizes the outer dynein arms.

In Paramecium, a 29-kDa polypeptide (p29), an LC of the outer dynein arm (22S dynein), is phosphorylated in a cAMP-dependent manner (5, 17, 26, 27, 29, 30) (Fig. 7A and B). The sliding velocity between the outer dynein arm containing p29 and the outer doublet microtubules increased in a cAMP-dependent
manner (5, 17). Therefore, p29 is thought to play a key role in ciliary movements in response to cAMP. The deduced amino acid sequence of ODAL1 includes several phosphorylation sites (Fig. 1), and the ciliary orientation on cortical sheets from ODAL1-RNAi cells lost cAMP-dependent control (Fig. 5B). This may be due to the absence of the cAMP-dependent phosphorylation of some axonal proteins induced by ODAL1 silencing. To test this possibility, we examined the effects of ODAL1 silencing on the cAMP-dependent phosphorylation of axonal proteins. As a result, the cAMP-dependent phosphorylation of p29 was not detected in the axonesomes from ODAL1-RNAi cells (Fig. 7A and B). This result indicates that ODAL1 silencing impairs the cAMP-dependent phosphorylation of p29. The SDS-PAGE band pattern and the observation of cross-sectional images of the ODAL1-RNAi axonesomes showed that the outer dynein arms were decreased to some extent (Fig. 6 and 8). These results suggest that the defect in the ciliary response to cAMP caused by ODAL1 silencing may be due to a reduction in the levels of p29 in the outer dynein arm. Our results also suggest that the structural integrity of the outer dynein arm may be essential to produce a high ciliary beat frequency. Further studies, e.g., determining ciliary movements using gene silencing for the other outer dynein arm components, will be required to clarify the mechanisms regulating the ciliary beat frequency.

In conclusion, we demonstrated that the ODAL1 gene is essential for controlling the ciliary response by cAMP-dependent phosphorylation. The ODAL1 product may be the p29-phosphorylatable LC of the Paramecium 22S dynein. The use of gene silencing by RNAi using the feeding method and the analysis of ciliary movements using cortical sheets could provide further information for an understanding of the molecular mechanism of ciliary movements.

ACKNOWLEDGMENT

This work was supported by a grant-in-aid for scientific research (C) from MEXT of Japan (grant no. 21590358 to M.H.).

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