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Kinesin-II Is Required for Flagellar Sensory Transduction during Fertilization in *Chlamydomonas*

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The assembly and maintenance of eucaryotic flagella and cilia depend on the microtubule motor, kinesin-II. This plus end-directed motor carries intraflagellar transport particles from the base to the tip of the organelle, where structural components of the axoneme are assembled. Here we test the idea that kinesin-II also is essential for signal transduction. When mating-type plus (mt+) and mating-type minus (mt−) gametes of the unicellular green alga *Chlamydomonas* are mixed together, binding interactions between mt+ and mt− flagellar adhesion molecules, the agglutinins, initiate a signaling pathway that leads to increases in intracellular cAMP, gamete activation, and zygote formation. A critical question in *Chlamydomonas* fertilization has been how agglutinin interactions are coupled to increases in intracellular cAMP. Recently, *fla10* gametes with a temperature-sensitive defect in FLA10 kinesin-II were found to not form zygotes at the restrictive temperature (32°C). We found that, although the rates and extents of flagellar adhesion in *fla10* gametes at 32°C are indistinguishable from wild-type gametes, the cells do not undergo gamete activation. On the other hand, *fla10* gametes at 32°C regulated agglutinin location and underwent gamete fusion when the cells were incubated in dibutyryl cAMP, indicating that their capacity to respond to the cAMP signal was intact. We show that the cellular defect in the *fla10* gametes at 32°C is a failure to undergo increases in cAMP during flagella adhesion. Thus, in addition to being essential for assembly and maintenance of the structural components of flagella, kinesin-II/intraflagellar transport plays a role in sensory transduction in these organelles.

INTRODUCTION

Much as animals use cilia as sensory transducers to perceive light, odorants, and chemotactic agents in their environment, gametes of the green alga *Chlamydomonas* use their two flagella as sensory organelles (Solter and Gibor, 1977) to perceive and respond to gametes of the opposite sex in their environment (reviewed by Pan and Snell, 2000b). When *Chlamydomonas* cells are in the vegetative, asexual phase of their life cycle, as they swim through their medium, they undergo frequent, transient collisions with the flagella and cell bodies of other cells in the culture. With vegetative cells, the transient collisions are of no consequence. On the other hand, when vegetatively growing cells are induced to undergo gametogenesis, and the resulting gametes of opposite mating types are mixed together, the random encounters have a different outcome. Collisions between flagella of mating-type plus (mt+) and mating-type minus (mt−) gametes allow interactions between gamete-specific flagellar adhesion molecules, the mt+ and mt− agglutinins (Adair, 1985). Not only do the agglutinin interactions cause the flagella on cells of opposite mating types to adhere to each other but the receptor/ligand-like interactions between the agglutinins induce increases in intracellular cAMP (Goodenough, 1989; Saito *et al.*, 1993) via a protein kinase-dependent pathway (Zhang *et al.*, 1991; Zhang and Snell, 1994). Much as olfactory epithelial cells respond to the odorant-induced increases in intracellular cyclic nucleotides in their cilia (Sklar *et al.*, 1986; Bakalyar and Reed, 1990; Schild and Restrepo, 1998), the interacting *Chlamydomonas* gametes undergo gamete activation in response to the increase in cAMP. The now activated gametes of both mating types undergo cell wall loss, agglutinin synthesis is induced (Snell and Moore, 1980), agglutinins (Goodenough, 1989; Hunnicutt *et al.*, 1990) and an aurora-like protein kinase (Pan and Snell, 2000a) are translocated from the cell body to the flagella, and cell-cell fusion organelles are activated (Goodenough *et al.*, 1982; Wilson *et al.*, 1997). In the final step of fertilization, the activated gametes that had been adhering only via their flagella begin to adhere to each other via the apically localized fusion organelles on their cell bodies. This cell body adhesion is followed rapidly by cell-cell fusion and formation of a quadriflagellated zygote. Although we have learned much about the responses of *Chlamydomonas* gametes to flagellar

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adhesion during fertilization, we still know little about the underlying mechanisms of signal transduction that couple agglutinin interactions to increases in cAMP.

Over the past few years, studies of a newly discovered cellular phenomenon termed intraflagellar transport (IFT) have begun to offer new insights into ciliary and flagellar assembly and maintenance and have provided an inroad to learning more about flagellar signal transduction during fertilization. IFT is a motility process, first discovered in *Chlamydomonas* (Kozminski *et al.*, 1993), in which nonmembrane-bound particles (IFT particles) are ferried along ciliary/flagellar microtubules, from the base to the tip of the organelle, and then back (reviewed by Cole, 1999; Rosenbaum *et al.*, 1999; Marszalek and Goldstein, 2000). The plus-end–directed microtubule motor protein kinesin-II has been shown to be essential for movement of particles toward the tip (anterograde transport; Walther *et al.*, 1994; Kozminski *et al.*, 1995; Piperno *et al.*, 1996; Cole *et al.*, 1998), and the cycle is completed through the action of a cytoplasmic dynein that carries IFT particles back to the cell body (retrograde transport; Pazour *et al.*, 1998, 1999; Porter *et al.*, 1999; Iomini *et al.*, 2001). Studies of *Chlamydomonas* as well as several other organisms have shown that IFT delivers structural components of the microtubular axoneme, including inner dynein arms (Piperno *et al.*, 1996), to the tips of the flagella, where they are involved in flagellar assembly and maintenance.

Much of our understanding of IFT and its role in cilia and flagella has come from studies of cells with genetic lesions in IFT components. For example, *Chlamydomonas* mutants with defects in the heavy and light chains of cytoplasmic dynein form short flagella that are filled with 10 times the normal amounts of IFT particle proteins, indicating that cytoplasmic dynein is essential for retrograde IFT (Pazour *et al.*, 1999, 2000; Porter *et al.*, 1999). Similar experiments documented the role of *Chlamydomonas* kinesin-II in anterograde transport. *Chlamydomonas fla10*-1 cells, which express a temperature-sensitive defect in the 90-kDa, kinesin-II motor subunit FLA10 because of a single amino acid substitution in the motor domain (Walther *et al.*, 1994), have normal flagella and are fully motile at the permissive temperature but are unable to form flagella at the restrictive temperature (Huang *et al.*, 1977; Lux and Dutcher, 1991). Moreover, when *fla10* cells previously maintained at the permissive temperature are shifted to the restrictive temperature, anterograde IFT particle movement ceases and IFT particle proteins are depleted from the flagella (Kozminski *et al.*, 1995; Piperno and Mead, 1997; Cole *et al.*, 1998; Iomini *et al.*, 2001). Within 1–2 h after the temperature shift, the flagella gradually begin to shorten, and after several hours most of the cells are aflagellate (Kozminski *et al.*, 1995; Piperno *et al.*, 1996). Lesions in kinesin-II and IFT particle proteins in ciliated cells of multicellular organisms also lead to the absence of cilia and are associated with apoptotic photoreceptor cell death (Marszalek *et al.*, 2000), polycystic kidney disease (Moyer *et al.*, 1994; Pazour *et al.*, 2000; Haycraft *et al.*, 2001), and situs inversus (Nonaka *et al.*, 1998; Marszalek *et al.*, 1999; Okada *et al.*, 1999). Related studies of *Caenorhabditis elegans* chemosensation mutants with defects in formation of sensory cilia revealed that many of the lesions are in genes encoding proteins of the IFT system (Perkins *et al.*, 1986; Shakir *et al.*, 1993; Scholey, 1996; Collet *et al.*, 1998; Signor *et al.*, 2000; Wicks *et al.*, 2000; Haycraft *et al.*, 2001; Qin *et al.*, 2001).

A potentially interesting confluence between sensory transduction during fertilization in *Chlamydomonas* and flagellar motor proteins/IFT emerged from studies by Piperno *et al.* (1996) of flagellar assembly using the temperature-sensitive, kinesin-II mutant *fla10*. These workers reported that *fla10* gametes lost the ability to form zygotes soon after being shifted to the restrictive temperature, well before flagella were lost. Because the block to zygote formation was incidental to the primary focus of the manuscript, the authors noted only that the phenotype could not be ascribed to flagellar loss. More recently it was suggested that the requirement for the kinesin-II motor protein in cell fusion could be due to the need to properly localize and transport flagellar agglutinins (Rosenbaum *et al.*, 1999; Iomini *et al.*, 2001).

Because *Chlamydomonas fla10* gametes with a temperature-sensitive defect in kinesin-II fail to form zygotes at the restrictive temperature, we wanted to gain a better understanding of the possible role of kinesin-II in sensory transduction during *Chlamydomonas* fertilization. To do this, we studied the consequences of loss of kinesin-II function on distinct steps in fertilization and tested the hypothesis that, in addition to its role in ferrying structural molecules required for flagellar assembly and for maintaining flagella length, kinesin-II is involved in cellular signaling. Here, we demonstrate that gametes with a conditional defect in FLA10 kinesin-II fail to undergo proper flagellar sensory transduction during fertilization. Although gametes after 40 min at 32°C undergo flagellar adhesion that is indistinguishable from that of wild-type gametes, *Chlamydomonas* kinesin-II mutants do not form zygotes at the restrictive temperature because the cells fail to couple agglutinin interactions to increases in cAMP.

MATERIALS AND METHODS

Cells and Cell Culture

Chlamydomonas reinhardtii strains *21gr* (mt+; CC-1690), *6145C* (mt−; CC-1691), imp1-15 (mt+; CC-462), *fla10*-1 (mt−; CC-1919), available from the *Chlamydomonas* Genetic Center, Duke University (Durham, NC), were cultured with either medium I or medium II (Sager and Granick, 1954) at 23°C on a 13:11 h light:dark cycle as described previously (Pan and Snell, 2000a). Vegetative cells were induced to become gametes by incubation in medium without nitrogen (N-free medium) followed by culturing in continuous light at room temperature (Pan and Snell, 2000a).

Cell Adhesion and Fusion Assays

mt+ and mt− gametes (1×10^7 cells/ml in N-free medium) were mixed together, and at the indicated times cell-cell adhesion was quantified using an electronic particle counter (Coulter, Palo Alto, CA) as previously described (Snell and Roseman, 1979; Snell and Moore, 1980). Zygote formation was assessed by mixing *fla10* gametes with wild-type mt+ gametes for 15 min at the indicated temperatures followed by fixation in 0.5% glutaraldehyde and determination of the number of biflagellated and quadriflagellated cells by examination in a phase contrast microscope. For each determination, 200 cells were counted. The percentage of cells forming zygotes was calculated from the following formula: % zygotes = 100×2 quadriflagellated cells/($2 \times$ quadriflagellated cells + single cells). Flagellar loss was determined by counting the numbers of cells with and without flagella in glutaraldehyde-fixed samples. Two hundred cells were counted for each determination.

Incubation of Cells with Dibutyryl cAMP

For experiments with dibutyryl cAMP, gametes in N-free medium and vegetative cells in medium II were incubated in 15 mM dibutyryl cAMP and 0.15 mM papaverine for 30 min as previously described (Pasquale and Goodenough, 1987; Pan and Snell, 2000a). The papaverine was from a freshly made 15 mM stock solution in dimethyl sulfoxide (Sigma, St. Louis, MO). Cell wall loss, which is a measure of gamete activation, was assessed by determining whether cells became sensitive to disruption by incubation in 0.075% Triton 100-X, 0.5 mM EDTA, 10 mM Tris, pH 8.0, as described earlier (Snell, 1982).

Cell Fractionation

Flagella were isolated essentially as described by Zhang *et al.* (1991). Typically, 3–4 l of cells were concentrated to 30 ml by centrifugation at 3500 × *g* for 5 min at 4°C, and ice-cold 25% sucrose in 10 mM Tris, pH 7.2, was added to yield a final concentration of 7% sucrose. While stirring the suspension, its pH was rapidly decreased to 4.5 by addition of 0.5 M acetic acid; after the flagella were detached (which typically required ~20 s) the pH was raised to 7.2 with 0.5 M KOH. All subsequent steps were carried out at 4°C. The suspension of cell bodies and flagella was underlayed with 25% sucrose in 10 mM Tris, pH 7.2, and centrifuged for 10 min at 2500 × *g*. The upper phase, which contained flagella and a few remaining cell bodies, was underlayed again with 25% sucrose, 10 mM Tris, pH 7.2, and centrifuged as above. The upper phase containing purified flagella was carefully removed and centrifuged at 9000 × *g* for 8 min to harvest the flagella. The sedimented flagella were resuspended in buffer A (20 mM HEPES, pH 7.2, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 25 mM KCl) (Cole *et al.*, 1998) containing a 1:100 dilution of the Sigma protease inhibitor cocktail for plant cells (Sigma catalogue number P9599) and flash frozen in liquid nitrogen.

SDS-PAGE and Immunoblot Analysis

Samples for SDS-PAGE were mixed with one-third volume of 4× SDS sample buffer (0.25 M Tris, pH 6.8, 40% glycerol, 16% SDS, 0.4 mM dithiothreitol, 0.1% bromophenol blue) and boiled for 5 min (Pan and Snell, 2000a). In some experiments sample buffer was used at a final concentration of 2×. The samples were subjected to electrophoresis in 9% acrylamide minislab gels at 30 mA in buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and then transferred for immunoblot analysis (see below). Typically 15–30 μg of protein was loaded in each lane. The protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (albumin standard, Pierce, Rockford, IL) as a standard. The immunoblot analysis was essentially as described by Pan and Snell (2000a). After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA) in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol at 100 V for 1 h or at 35 V overnight at 4°C. The membrane was blocked with 5% Carnation dry milk (Nestles, Solon, Ohio) in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20 (TBST) for 1 h and then incubated with primary antibody in 3% Carnation dry milk in TBST for 1 h. The membrane was washed three times for 5 min each with TBST, followed by incubation for 1 h with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) diluted 1:10,000 in TBST containing 3% Carnation dry milk. The membrane was washed as before and incubated in ECL immunoblotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min as described by the manufacturer, exposed to Hyperfilm ECL (Amersham Pharmacia Biotech), and developed in an automatic film processor. Doug Cole (University of Idaho, Moscow, ID) kindly provided polyclonal anti-FLA10 antibody and monoclonal anti-IFT particle protein antibodies.

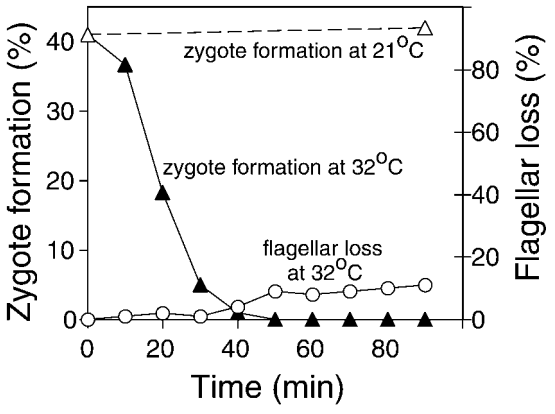

Figure 1. Loss of zygote-forming ability of *fla10* gametes at the restrictive temperature. *fla10* gametes maintained at 21°C were shifted to 32°C at T_0 and at the indicated times were analyzed for their ability to form zygotes after being mixed with mt+ gametes (▲). △, zygote formation by *fla10* gametes maintained at the permissive temperature (21°C). At the same times, the number of cells that had lost flagella also was determined by examination in the phase contrast microscope (○).

Radioimmunoassay of cAMP

To measure cellular levels of cAMP formed during adhesion, mt+ and mt− gametes (1×10^7 cells/ml in N-free medium) were mixed together and at the indicated times aliquots were mixed with 1 volume of 1 N perchloric acid at room temperature. The acidified extracts were analyzed for cAMP by a radioimmunoassay (Domino *et al.*, 1991) with duplicate samples, which typically differed by 5% or less. The results shown are typical of at least two independent experiments.

RESULTS

To establish the time that was required for *fla10* mt− gametes to lose their ability to form zygotes after being changed to the restrictive temperature, we shifted gametes from the permissive temperature (21°C) to the restrictive temperature (32°C) and at various times assessed their ability to fuse with wild-type mt+ gametes to form quadriflagellated zygotes at 32°C. Although the mt+ gametes used as tester cells in our experiments to assess zygote formation of *fla10* mt− gametes were wild type and not the *ida4fla10* mt+ gametes used by Piperno *et al.* (1996), we obtained results similar to those originally reported by those investigators. At the permissive temperature the *fla10* gametes formed zygotes when mixed with tester cells (Figures 1 and 2B, left). After being transferred to 32°C, however, the *fla10* gametes gradually lost the ability to form zygotes, and by 40 min after transfer, the ability to form zygotes had been completely abrogated (Figure 1). As expected, *fla10* gametes kept at 21°C showed no loss in their ability to form zygotes during the 90-min course of the experiment (Figure 1), and control experiments showed that wild-type mt− gametes preincubated for 40 min at 32°C underwent zygote formation with wild-type mt+ gametes similarly to wild-type mt− gametes at 21°C. Importantly, at 40 min after transfer, when zygote formation was blocked, <3% of the *fla10* gametes had lost their flagella (Figure 1). Even at 90 min after

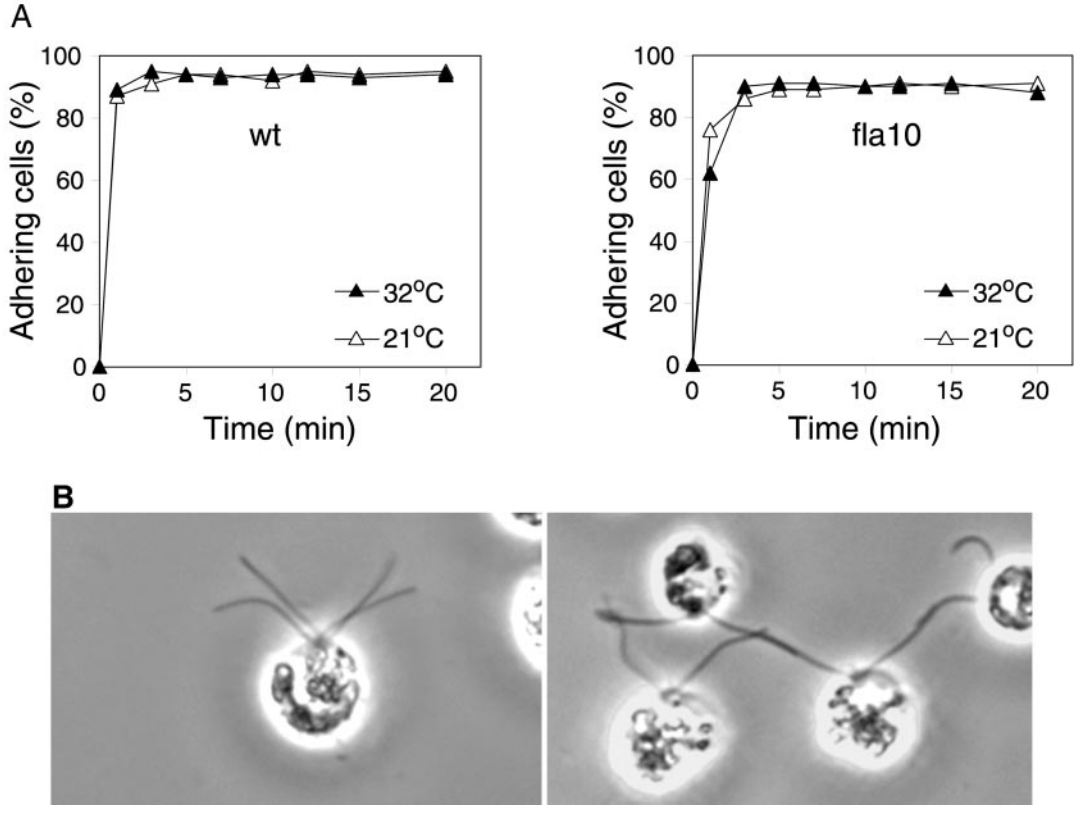

Figure 2. Adhesion of wild-type (wt) and *fla10* gametes at the permissive and restrictive temperatures. (A) Wild-type (left) and *fla10* mt− gametes (right) maintained at 21°C (△) or preincubated at 32°C for 40 min (▲) were mixed with *imp1* mt+ gametes at 21 or 32°C, respectively, and cell adhesion was determined by use of an electronic particle counter. (B) *fla10* gametes maintained at 21°C (left) or preincubated at 32°C (right) were mixed with wild-type mt+ gametes at 21 or 32°C, respectively, fixed with osmium fumes (Hunnicutt and Snell, 1991), and examined by phase contrast microscopy.

the temperature shift, only 10% of the cells had lost their flagella, results consistent with previous studies of these mutants (Kozminski *et al.*, 1995; Piperno *et al.*, 1996). Thus, as reported by Piperno *et al.* (1996), even in cells that are fully flagellated, FLA10 is essential for zygote formation.

Having confirmed that the *fla10* mutant cells were unable to undergo fertilization after being shifted to the restrictive temperature, we began to characterize the fertilization-related phenotype of the cells. Examination by light microscopy indicated that the motility of the cells was indistinguishable from that of the *fla10* cells at 21°C or wild-type cells at either temperature. Thus, the failure to fuse could not be attributed to the inability to move or to undergo collisions with cells of the opposite mating type. It was also possible that kinesin-II was required for maintenance of the differentiated gametic phenotype and that loss of kinesin-II function caused the gametes to dedifferentiate into vegetative cells, which are nonadhesive. Also, a functional kinesin-II might have been required for the presence of agglutinins on the flagella, and after 40 min at the restrictive temperature, agglutinins might have been lost from the flagella.

To test whether the *fla10* gametes shifted to 32°C still retained their gametic properties, we pretreated *fla10* gametes for 40 min at 32°C, mixed them with mt+ gametes at 32°C, and then assessed flagellar adhesion. Because zygotes rapidly become nonadhesive and, therefore, zygote formation interferes with quantitative evaluation of adhesion (Snell and Roseman, 1979), we used an impotent mutant strain, *imp1*, as the mt+ gametes for these experiments. *imp1* gametes undergo normal flagellar adhesion and gamete activation with mt− gametes (Snell and Moore, 1980; Goodenough *et al.*, 1982) but are unable to fuse because of a lesion in the *fus1* gene, which is required for adhesion and fusion of the tips of mating structures during the final steps in fertilization (Ferris *et al.*, 1996). In the experiments described below, qualitatively similar results were obtained with wild-type and *imp1* mt+ gametes. Examination by phase contrast microscopy indicated that the *fla10* gametes at 32°C underwent vigorous adhesion when mixed with the mt+ gametes (Figure 2B, right). This qualitative assessment was confirmed by a quantitative electronic particle counter assay that determines the number of cells adhering by measuring the loss of single cells from the suspension (Snell and Roseman, 1979; Snell and Moore, 1980). As shown in Figure 2A, the initial rates and extents of adhesion of *fla10* gametes with *imp1* mt+ gametes were the same for the 21 and 32°C cells (Figure 2A, right) and were essentially indistinguishable from the results for wild-type mt− gametes at the two temperatures (Figure 2A, left). In all cases adhesion was rapid and ~90% of the cells adhered. These results docu-

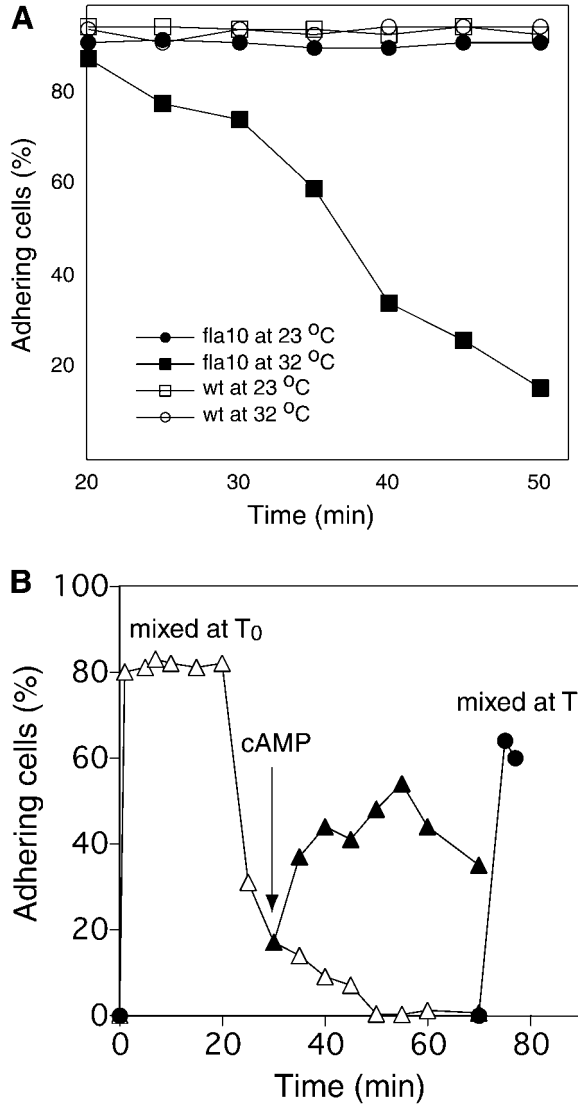

Figure 3. Loss and restoration of flagellar agglutinins in *fla10* gametes at the restrictive temperature. (A) Cell adhesion from the samples in Figure 2A was followed for an additional 30 min. Only the *fla10* samples at 32°C lost their adhesiveness. wt, wild type. (B) *fla10* gametes preincubated at 32°C for 40 min were mixed with *imp1* mt+ gametes at 32°C at T_0, and cell adhesion was determined using an electronic particle counter (△). After 30 min, when the majority of the cells had deadhered, a portion of the sample was incubated with dibutyryl cAMP (▲). ●, cell adhesion results from a control sample in which *fla10* gametes that had been preincubated at 32°C for a total of 70 min were mixed with *imp1* gametes.

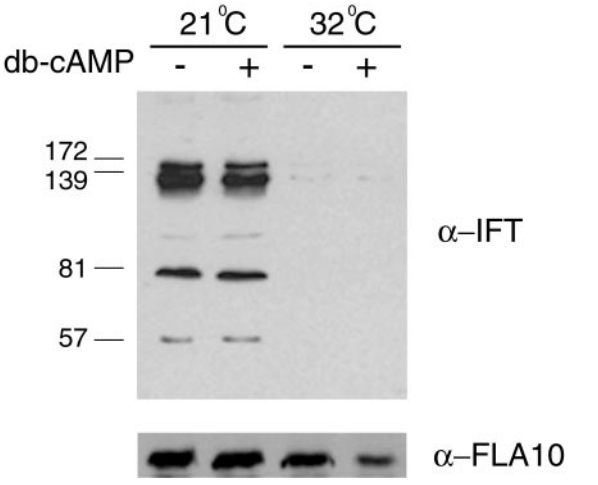

Figure 4. Incubation in cAMP does not induce IFT particle movement into flagella *fla10* gametes incubated either at 21 or 32°C for 40 min were incubated with dibutyryl (db) cAMP for 30 min, flagella were isolated from non-cAMP–treated and -treated gametes, and the presence of IFT particle proteins and FLA10 protein was examined by SDS-PAGE and immunoblotting. Monoclonal antibodies against 172-, 139-, 81-, and 57-kDa IFT particle proteins were mixed together for the blot shown in the top panel and the blot was stripped and reprobed with a polyclonal antibody against the 90-kDa FLA10 protein for the blot shown in the bottom panel.

mented that, even though the *fla10* gametes were unable to fuse after 40 min at 32°C, they retained the ability to undergo flagellar adhesion. Thus, the cells indeed were gametes and their flagella contained functional agglutinins.

In the course of these experiments we noticed that, while *fla10* gametes at the restrictive temperature showed wild-type levels of flagellar adhesion for 20 min after being mixed with gametes of the opposite mating type (Figure 2), when we examined the *fla10*, 32°C samples after they had been mixed together for longer than 20 min, they began to lose their adhesiveness (Figure 3A, ■). Loss of adhesiveness occurred only in the *fla10* samples and only at the restrictive temperature; *fla10* samples at 21°C (Figure 3A, ●) and wild-type mixtures at both 21°C (□) and 32°C (○) retained their adhesiveness for at least 50 min, observations consistent with previous studies (Snell and Moore, 1980; Pasquale and Goodenough, 1987). By 50 min after mixing, all adhesiveness had been lost in the *fla10*, 32°C sample.

Because previous studies of *Chlamydomonas* gametes in which flagellar adhesiveness was experimentally impaired have shown that flagellar adhesion can be restored by incubating cells in dibutyryl cAMP (Goodenough, 1989; Hunnicutt *et al.*, 1990), it became possible to take advantage of this new observation to determine whether *fla10* cells at 32°C retained their ability to respond to cAMP. To test for responsiveness to cAMP, we preincubated *fla10* gametes at 32°C for 40 min, mixed them with mt+ *imp1* gametes until adhesiveness was lost, added dibutyryl cAMP, and measured flagellar adhesiveness using the electronic particle counter assay. As above, the cells underwent rapid flagellar adhesion, remained adhesive for 20 min after mixing, and then began to deadhere (Figure 3B, △). By 50 min flagellar adhesion had been lost completely. That the loss of adhesiveness was a consequence of adhesion and not just due to prolonged incubation at 32°C was demonstrated by the experiment shown in Figure 3B (●). In this experiment instead of mixing the 40 min, 32°C pretreated *fla10* gametes with *imp1* gametes immediately after the 40-min pretreatment (T_0), we kept the cells at the restrictive temperature for an additional 70 minutes before mixing them with the tester cells (T_{70}). As expected, the cells adhered to essentially the same extent as the

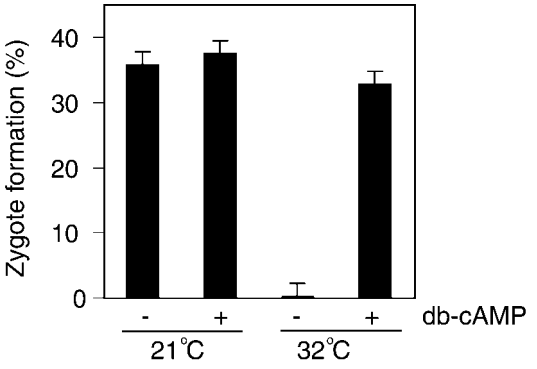

Figure 5. Rescue of *fla10* gamete fusion at 32°C by incubation in dibutyryl (db) cAMP. *fla10* gametes maintained at 21°C or 40 min after being shifted to 32°C were incubated with or without dibutyryl cAMP for 30 min, and zygote formation was measured.

samples mixed at T_0 (Figure 3B, ●). Finally, we tested whether the T_0 samples that had deadhered were able to respond to cAMP by incubating them with dibutyryl cAMP. As shown in Figure 3B (▲), flagellar adhesiveness was restored.

Although the simplest explanation for the restoration of flagellar adhesiveness was that the cellular response to dibutyryl cAMP was independent of kinesin-II and was due to a direct effect on agglutinin mobilization, it was also possible that the dibutyryl cAMP incubation restored kinesin function and IFT. We tested this possibility by using immunoblotting to assay for IFT particle proteins in flagella isolated from gametes at the permissive and restrictive temperatures that were incubated with or without dibutyryl cAMP. Consistent with previous studies (Kozminski *et al.*, 1995; Piperno and Mead, 1997; Cole *et al.*, 1998), we found that IFT particle proteins were present in flagella isolated from 21°C *fla10* gametes and greatly diminished in flagella isolated from *fla10* gametes that had been shifted to the restrictive temperature (Figure 4A). Moreover, dibutyryl cAMP treatment of *fla10* gametes at either 21 or 32°C failed to increase the amount of IFT particles proteins in the flagella (Figure 4A). Similarly, immunoblotting of these samples with an anti–kinesin-II antibody showed that, as expected, the kinesin-II protein was present in flagella isolated from cells at both the permissive and restrictive temperatures (Kozminski *et al.*, 1995; Cole *et al.*, 1998) and the levels were not increased by incubation with dibutyryl cAMP (Figure 4B). Thus, the restoration of flagellar adhesiveness by dibutyryl cAMP was not associated with restoration of IFT particle transport.

Having documented that the *fla10* gametes at 32°C retained their ability to respond to dibutyryl cAMP similarly to wild-type gametes, we next wanted to determine whether their failure to fuse was due to a direct requirement for kinesin-II in cell-cell fusion itself. To test whether the cell fusion machinery was functional in the *fla10* gametes at 32°C, we incubated *fla10* gametes at 21 and 32°C for 40 min, followed by an additional incubation for 40 min at 32°C with and without dibutyryl cAMP (Pasquale and Goodenough, 1987). Then, we tested the cells for their ability to undergo cell fusion with wild-type mt+ gametes. As before, fusion was completely abrogated in the nondibutyryl cAMP-treated, 32°C *fla10* gametes (Figure 5). On the other hand, treatment of the 32°C *fla10* gametes with dibutyryl cAMP restored their ability to form zygotes to nearly the levels seen with control and dibutyryl cAMP-treated, 21°C cells (Figure 5). Thus, failure of the cells to fuse was not due to a kinesin-II–related defect in the cell-cell fusion machinery.

Taken together the results presented above suggested that gametes required a functional kinesin-II to carry out an early step in gamete activation downstream of flagellar adhesion but upstream of increases in cAMP. To test this idea, we used a radioimmunoassay to measure cAMP levels during adhesion of gametes at the permissive and restrictive temperatures. For consistency, *imp1* cells were used at the mt+ gametes in these assays. Similar to previous reports (Pasquale and Goodenough, 1987), when wild-type mt− gametes were mixed with *imp1* mt+ gametes at 21°C, cAMP levels increased from <0.1 pm/10^7 cells in the unmixed gametes to nearly 1.5 pm/10^7 cells within 1 min after the cells were mixed (Figure 6A). When wild-type mt− gametes were preincubated at 32°C before mixing, the cAMP reached nearly 2.3 pm/10^7 cells within 1 min, which was ~1.7-fold higher than the 1.3 pm/10^7 reached at 21°C (Figure 6A). After 3 min the level began to decrease.

Whereas the results at 21°C with *fla10* gametes were similar to those of wild-type mt− gametes at 21°C, much different results were obtained with *fla10* gametes at the restrictive temperature. In the experiments with *fla10* gametes that had been preincubated at the restrictive temperature for 40 min, rather than being higher than the level of cAMP present in the 21°C samples, the amount of cAMP present at 1 min was dramatically lower (Figure 6B). A small increase in cAMP (to a level slightly more than one-half of that observed at the permissive temperature) occurred immediately after mixing, and at 1 min, the level was ~0.2 pm/10^7 cells (Figure 6B). Figure 6C summarizes results on cAMP present at 1 min after mixing from this experiment and two comparable experiments that showed similar kinetics, one of which was carried out in the presence of a phosphodiesterase inhibitor. Although the absolute amounts of cAMP differed in the three experiments, the 32:21°C ratios of cAMP levels at 1 min were much higher in the experiments with wild-type gametes than for those with *fla10* gametes (Figure 6C). These results, in combination with the observations above that *fla10* gametes at the restrictive temperature are capable of responding to exogenously added dibutyryl cAMP, indicated that gametes require a functional kinesin-II to undergo the adhesion-induced increase in cAMP that normally accompanies flagellar adhesion.

DISCUSSION

A Role for Kinesin-II in Sensory Transduction in Intact Flagella

We have shown that cell-cell fusion fails in kinesin-II, temperature-sensitive mutants of *Chlamydomonas* at the restrictive temperature because the gametes require a functional kinesin-II for coupling flagellar adhesion to increases in cAMP. Results obtained after using several approaches support this idea. First, microscopic examination and quantitative flagellar adhesion assays demonstrated that the rate and extent of flagellar adhesion of *fla10* gametes after 40 min at the restrictive temperature were indistinguishable from

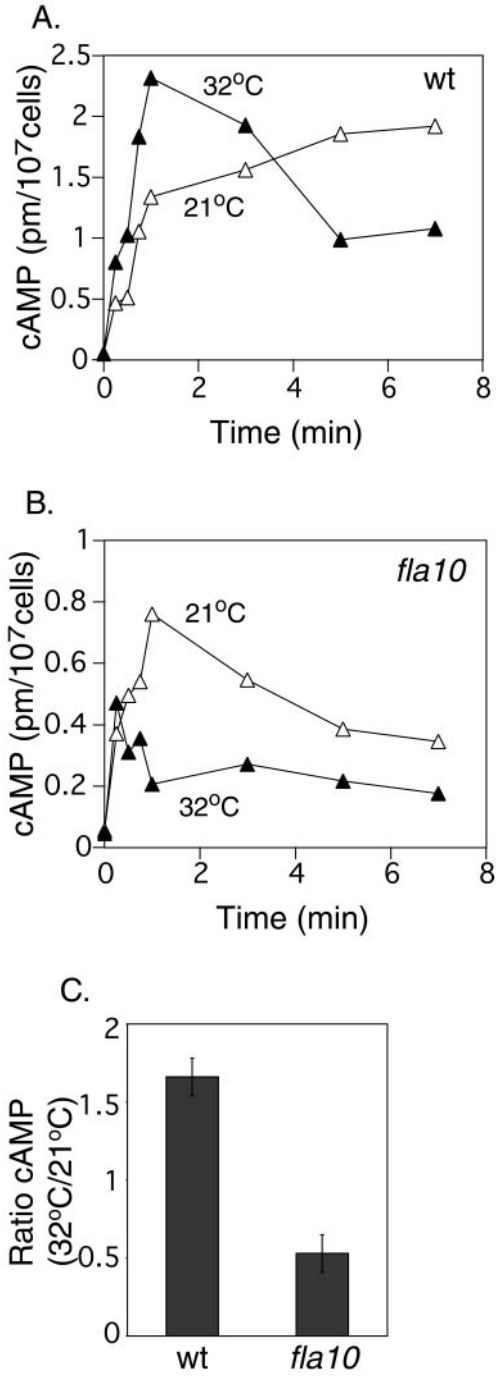

Figure 6. Changes in cAMP levels during adhesion of wild-type (wt) and *fla10* gametes at the permissive and restrictive temperatures. Wild-type (A) and fla10 mt− (B) gametes maintained at 21°C or preincubated at 32°C for 40 min were mixed with *imp1* gametes at the indicated temperatures, and at the indicated times after mixing, samples were analyzed for cAMP. C, ratios of cAMP present at 32/21°C, 1 min after mixing wild-type or *fla10* gametes from the panel A/B experiment and from two separate experiments, in one of which the mixing was carried out in the presence of the phosphodiesterase inhibitor, papaverine (Pasquale and Goodenough, 1987; Hunnicutt *et al.*, 1990).

those of wild-type gametes at both temperatures and of *fla10* gametes at 21°C (Figure 2); yet, the *fla10*, 32°C gametes were incapable of cell-cell fusion (Figure 1). Second, *fla10* 32°C gametes retained the ability to respond to cAMP as assessed by their ability to mobilize flagellar agglutinins (Figure 3) and undergo cell fusion after incubation in dibutyryl cAMP (Figure 5). Finally, direct assays of cAMP showed that the samples containing *fla10* gametes at 32°C did not undergo the substantial flagellar adhesion-dependent increase in cAMP that was observed with wild-type gametes at both temperatures (Figure 6) and with *fla10* gametes at 21°C.

Although the requirements for kinesin-II in assembly of cilia and flagella as well as in maintenance of organelle length are well documented (reviewed by Scholey, 1996; Cole, 1999; Rosenbaum *et al.*, 1999; Marszalek and Goldstein, 2000), the availability of the temperature-sensitive *fla10* mutant made it possible to examine the role of kinesin-II in sensory transduction in a structurally intact cilium/flagellum. The cells used in the experiments reported here, i.e., *fla10* cells at 40 min after shift to the restrictive temperature, have distinctive properties. Although IFT particles are absent from the flagella, no structural defects are detectable in the flagellar axoneme (Kozminski *et al.*, 1995); the cells are fully flagellated (Figure 1; Kozminski *et al.*, 1995; Piperno *et al.*, 1996), and they are motile and contain functional flagellar surface adhesion molecules as evidenced by their wild-type rates and extents of flagellar adhesion (Figure 2). The only two functional defects detected so far in these cells are the absence of IFT particle movement (Kozminski *et al.*, 1995; Piperno *et al.*, 1996; Piperno and Mead, 1997) and the failure of flagellar adhesion to induce signal transduction.

Several IFT mutants in *C. elegans* were first identified by their defects in sensory transduction (Perkins *et al.*, 1986; Shakir *et al.*, 1993; Cole, 1999; Orozco *et al.*, 1999; Signor *et al.*, 1999, 2000; Wicks *et al.*, 2000; Qin *et al.*, 2001; Haycraft *et al.*, 2001). These defects, however, are all due to an indirect consequence of the requirement of IFT for production of a structurally intact cilium. Each of these sensory-defective IFT worm mutants, whether they are kinesin-II (or heteromeric kinesin) mutants, IFT particle protein mutants, or mutants in genes encoding proteins that have been shown to move via IFT, produce abnormal cilia (Perkins *et al.*, 1986). In most cases the cilia are completely absent or shorter than wild-type organelles. For example, *osm-3* worms, with lesions in the gene that encodes the heteromeric kinesin protein OSM-3, fail to assemble the distal segments of sensory cilia (Perkins *et al.*, 1986; Signor *et al.*, 1999). Worms with lesions in the OSM-1 and OSM-6 molecules, which are cargoes for CeKinesin-II, lack both the middle and distal segments of sensory cilia (Cole *et al.*, 1998; Collet *et al.*, 1998). The *che-11* and *daf-10* mutants, with defects in IFT particle proteins (Qin *et al.*, 2001), form nearly full-length cilia, but the organelles are of abnormal structure, being irregular in contour or containing amorphous material in their centers (Albert *et al.*, 1981; Perkins *et al.*, 1986). Thus, the sensory transduction lesions in these *C. elegans* mutants have underlying structural correlates, which is not the case for the *Chlamydomonas fla10* gametes used in our experiments.

Kinesin-II/IFT: Direct Participant in Signaling?

Given that an obvious defect in axonemal structure fails to explain the inability of the *fla10* gametes to undergo flagellar

adhesion-dependent increases in cAMP, what could be the role of kinesin-II in coupling agglutinin interactions to increases in cAMP? One idea is that kinesin-II participates directly in sensory transduction by moving molecules or molecular complexes within the flagella after the initial interactions between mt+ and mt− agglutinins occur. According to this idea, once mt+ and mt− flagellar agglutinins interact with each other, coupling of the interaction to increases in cAMP would require that the flagellar agglutinins undergo kinesin-II-dependent movement within the flagellar membrane. For example, clustering of the interacting flagellar agglutinins could be required for increases in cAMP, or possibly agglutinins must be moved from the membrane along the shaft of the flagella to the flagellar tips to signal maximally (Mesland *et al.*, 1980; Goodenough, 1993; Piperno *et al.*, 1996). We should note in this regard that Reese and Haimo (2000) demonstrated that cAMP-dependent protein kinase activates kinesin-II binding to microtubules in *Xenopus* melanophores. Interestingly, Saito *et al.* (1993) reported that gametes of the *Chlamydomonas imp3* mutant, which can adhere but not fuse, undergo only small adhesion-dependent increases in cAMP compared with wild-type gametes. The molecular lesion associated with the *imp3* mutation has not been identified. Future studies should indicate whether the *imp3* mutation is related to kinesin-II function or IFT.

Another possibility is that the IFT particle itself participates in signaling, because by 40 min after the shift to 32°C, particle proteins no longer are detectable in the flagella (Figure 4). To date, none of the characterized IFT particle proteins is reported to exhibit properties that make it an obvious candidate for a signaling molecule. On the other hand, only a few of the ~16 IFT particle proteins have been characterized and it could be that one of them is directly involved in signal transduction. One idea that emerges from this speculation is that IFT particles could play a dual role in flagella: one as cargo transporters dependent on kinesin-II and another as supramolecular signaling complexes in close association with the flagellar membrane and the agglutinin molecules (Rosenbaum *et al.*, 1999).

We also cannot not rule out that kinesin-II has roles in the flagella that are independent of IFT particles and possibly even independent of its role as one of the subunits of heterotrimeric kinesin-II (Signor *et al.*, 1999). Recently, several reports have linked cellular signaling events and molecular signaling complexes in nonciliated cells to members of the kinesin superfamily, including kinesin-II (reviewed by Goldstein, 2001; Hollenbeck, 2001; Verhey and Rapoport, 2001). For example, Shimizu *et al.* (1998) showed that SMAP [SMAP (Smg GDS-associated protein; Smg GDS: small G protein GDP dissociation stimulator)], a proposed mammalian homologue of the nonmotor subunit of sea urchin kinesin-II, binds to a regulator of small G proteins called Smg GDS. SMAP has armadillo repeats and is phosphorylated by Src tyrosine kinase (Shimizu *et al.*, 1996). Also, Nagata *et al.* (1998) reported that the MAP kinase kinase kinase MLK2 interacts with mammalian members of the kinesin-II/KIF3 family of kinesin-related proteins and with KAP3A, a nonmotor protein subunit of the kinesin-II/KIF3 motor complex. Additionally, the conventional kinesin-related protein, COS2, encoded by the *costal2* gene, was shown to be an essential component of a multiprotein signaling complex that is regulated by the *hedgehog* gene product in *Drosophila* embryos (Robbins *et al.*, 1997; Sisson *et al.*, 1997). Although COS2 has not been shown to exhibit motor activity, its regulated microtubule binding activity is implicated in determining the cytoplasmic versus nuclear localization of key molecules in the hedgehog-signaling pathway. Finally, JIP scaffolding proteins and associated signaling molecules that are part of the c-jun NH_2-terminal kinase (JNK)-signaling pathway bind to rat conventional kinesin light chain proteins (Bowman *et al.*, 2000; Verhey *et al.*, 2001) and this motor protein is important for concentration of the JIP complex in nerve terminals (Verhey *et al.*, 2001).

Finally, the importance of kinesin-II in IFT particle movement is well documented, and one straightforward explanation for the failure of the *fla10* gametes to undergo increases in cAMP during adhesion is that kinesin-II/IFT plays an indirect role in sensory transduction by maintaining proper levels of signaling components in the flagella. Another possibility is that IFT particles and cytoplasmic dynein might carry adhesion-activated signals from the flagella to the cell body. These ideas can be tested in future experiments by use of newly described IFT mutants available in the collection of Iomini *et al.* (2001).

Kinesin-II Is Not Required for Flagellar Mobilization of Active Agglutinins

One surprising result from our experiments was that *fla10* gametes at the restrictive temperature underwent dibutyryl cAMP-induced mobilization of flagellar agglutinins. In previous experiments we and others have shown that gametes are able to translocate flagellar agglutinins from their cell bodies onto their flagella (Goodenough, 1989; Hunnicutt *et al.*, 1990). In experiments from our laboratory, flagellar agglutinins were inactivated by an anti-agglutinin mAb under conditions in which the inactive agglutinins on the surface of the cell body were unaffected (Hunnicutt *et al.*, 1990). Incubation of the nonadhesive cells with dibutyryl cAMP restored active agglutinins onto the flagella. Similarly, Goodenough (1989) has shown that incubation of gametes with dibutyryl cAMP led to an increase in flagellar agglutinins. Once the existence of kinesin-II-dependent IFT was reported, IFT became the best candidate for the motility process responsible for agglutinin translocation. Thus, our result that flagellar adhesiveness was restored by incubation of the newly nonadhesive *fla10* gametes in dibutyryl cAMP (Figure 3B) was unexpected and argues against the model that kinesin-II/IFT is essential for agglutinin movement from the cell body to the flagella (Piperno *et al.*, 1996; Rosenbaum *et al.*, 1999; Iomini *et al.*, 2001).

Another possible mechanism for moving agglutinins onto the flagella is flagellar surface motility, a poorly understood process visualized in the laboratory as the movement of latex microspheres up and down the surfaces of flagella (Bloodgood, 1995). Additionally, agglutinins might move via the as yet unidentified mechanism responsible for movement of outer dynein arm components into flagella. Piperno *et al.* (1996), showed that the outer dynein arm IC protein IC69 translocated into flagella of *fla10* gametes at the restrictive temperature in a manner indistinguishable from that observed at the permissive temperature. In the context of our experiments, the result that flagellar adhesiveness was restored by dibutyryl cAMP treatment demonstrated that

the cells still were functional gametes whose flagella were capable of responding to and participating in a complex signaling event in the absence of a functional kinesin-II. Nevertheless, it will be interesting to learn more about the mechanisms responsible for this kinesin-II–independent agglutinin mobilization.

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