Two Anti-radial Spoke Monoclonal Antibodies Inhibit 
Chlamydomonas Axonemal Motility by Different Mechanisms*

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In the 9 + 2 axoneme, radial spokes are structural components attached to the A-tubules of the nine outer doublet microtubules. They protrude toward the central pair microtubule complex with which they have transient but regular interactions for the normal flagellar motility to occur. Flagella of Chlamydomonas mutants deficient in entire radial spokes or spoke heads are paralyzed. In this study the importance of two radial spoke proteins in the flagellar movement is exemplified by the potent inhibitory action of two monoclonal antibodies on the axonemal motility of demembranated-reactivated Chlamydomonas models. We show that one of these proteins is localized on the stalk of the radial spokes, whereas the other is a component of the head of the same structure and most likely correspond to radial spoke protein 2 and 1, respectively. Fine motility analysis by videomicrography further indicates that these two anti-radial spoke protein antibodies at low concentration affect motility of demembranated-reactivated Chlamydomonas by changing the flagellar waveform without modifying axonemal beat frequency. They also modify wave amplitude differently during motility inhibition. This brings more direct evidence for the involvement of both radial spoke stalk and head in the fine tuning of the waveform during flagellar motility.

Eukaryotic flagella and cilia share a common structure called the axoneme that is responsible for their motility. The main difference between flagellar and ciliary motility resides in that the former is predominantly symmetric (such as that of spermatozoa), and the latter is asymmetric (such as that of tracheal or Tetrahymena cilia). However, depending on environmental conditions, the same axonemal structure is amenable to both types of motility as is the case for the biflagellated green algae Chlamydomonas reinhardtii. This unicellular microorganism has adopted a ciliary type of motility typical of wild-type Chlamydomonas models. We show that one of these proteins is localized on the stalk of the radial spokes, whereas the other is a component of the head of the same structure and most likely correspond to radial spoke protein 2 and 1, respectively. Fine motility analysis by videomicrography further indicates that these two anti-radial spoke protein antibodies at low concentration affect motility of demembranated-reactivated Chlamydomonas by changing the flagellar waveform without modifying axonemal beat frequency. They also modify wave amplitude differently during motility inhibition. This brings more direct evidence for the involvement of both radial spoke stalk and head in the fine tuning of the waveform during flagellar motility.

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The radial spoke of *Chlamydomonas* flagella comprises 23 polypeptides (designated radial spoke protein 1–23 (RSP1–23))^{5,15}, 18 for the stem and 5 for the head (25, 26). Only a few of these have been characterized in detail (10, 25–29), and their specific interactions with each other or with other axonemal proteins and their role in motility remain poorly understood.

Although studies on immotile mutants lacking radial spokes and especially on the reversal of flagellar paralysis by extragenic suppressor mutations provided good insights in the functional role for these axonemal structures, they have serious limitations. First, these mutations are pleiotropic (30, 31). Second, some suppressor mutants in a wild-type background also have aberrant motility, in particular, a low flagellar beat frequency (15). Because of these drawbacks inherent to mutation studies, we chose in the present work to use monoclonal antibodies (mAb) selected from a panel of mAb generated against *Chlamydomonas* axonemal proteins on the basis of their strong motility inhibition of demembranated-reactivated *Chlamydomonas* wild-type cells. This approach with mAb has previously proved to be useful in the study of the role of specific proteins in sea urchin sperm motility (32–36). For example, D316 mAb specifically reacting toward a radial spoke head protein of sea urchin axoneme, changes flagellar beating from a two-dimensional to a three-dimensional type of movement (36).

Two of the mAb generated, L2H12 and L3G4, were used in the present study to evaluate functional properties of radial spoke components in *Chlamydomonas* motility. We first ascertained that antigens recognized by L2H12 and L3G4 are localized on radial spokes and then determined toward which specific RSP they are directed. These mAb were then used to further analyze their effects on various motility parameters by videomicroscopy to assess the mechanism by which these two RSP are involved in the regulation of flagellar motility.

**MATERIALS AND METHODS**

**Production of Monoclonal Antibodies**—The hybridoma lines secreting L2H12 and L3G4 mAbs were obtained following procedures already described (32, 37) using purified wild-type *Chlamydomonas* axonemes as immunogens. The two criteria for mAb selection were single band detection on immunoblots made with purified wild-type *Chlamydomonas* axonemal proteins and inhibition of the motility of demembranated-reactivated *Chlamydomonas* cells. To get crude preparations of the mAbs, large pools of supernatants from hybridoma cells cultured in a serum-free protein-free medium (Sigma, catalog number S-2772) were collected, concentrated by ultrafiltration with an Amicon YM100 filter (100-kDa exclusion limit, Millipore Corp., Bedford, MA), and dialyzed against Tris-buffered saline (TBS) (20 mM Tris (pH 7.8), 150 mM NaCl) containing 0.001% sodium azide. Further purification of the mAbs was achieved by Protein A affinity chromatography using the Econo-Pac kit from Bio-Rad (catalog number 732–2020) according to the manufacturer's instructions.

**Chlamydomonas Cell Culture**—The following C. reinhardti strains, all obtained from the *Chlamydomonas* Genetics Center (Department of Botany, Duke University, Durham, NC), were used in this study: wild-type cc1010, unflagellar mutant uni cc1296, radial spoke mutants pf2 cc1024, pf14 cc1032, pf17 cc1035, pf24 cc1384, pf25 cc1385, pf26 cc1386, and pf27 cc1387, outer dynein arm mutants oda1 cc2229 and oda9 cc2244, inner dynein arm f mutant ida2 cc2666, inner dynein arm a, c, and d mutant ida4 cc2670, inner dynein arm e and dynein regulatory complex mutant pf3 cc1026, and the mutant pf15 cc1033 lacking central pair microtubules.

**Chlamydomonas cells** were cultured on a modified medium 1 of Sager and Granic (38); the total phosphate concentration was increased from 1.31 to 16.7 mM, and 14.7 mM of sodium acetate was added. The final pH of that medium was 6.8. Cells were grown with gentle air bubbling at 25 °C on a 14/10-h light/dark cycle. In these conditions *Chlamydomonas* cells were well synchronized in their division with a doubling rate around 12 h.

1. The abbreviations used are: RSP, radial spoke protein; mAb, monoclonal antibody (Ab); pl, isoelectric point; HDE, Hepes, dithiothreitol, and EDTA; TBS, Tris-buffered saline; TBS-T, TBS with Triton X-100.

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**Preparation and Fractionation of Axonemes**—Five to ten liters of *Chlamydomonas* cultures grown to 5 × 10^6 cells/ml were used to prepare axonemes for fractionation. Cells were gently concentrated down to 100 ml using a tangential flow filtration device equipped with a 0.6-μm Durapore membrane cassette (Millipore Corp.). Then axonemes were obtained following a procedure previously described (39) and finally resuspended at a protein concentration of 5–10 mg/ml for immediate use or storage at −70 °C in the presence of 20% glycerol and protease inhibitors (leupeptin 10 μg/ml, aprotinin 10 μg/ml, and 10 mM phenylmethylsulfonyl fluoride).

Radial spokes were extracted using the procedure published by Yang et al. (25). Dynein arms are first removed from the isolated axonemes by two consecutive treatments with 0.6 M NaCl, and the radial spokes then extracted by a 0.5 M potassium iodide (KI) treatment. The other sequential fractionation of axonemes was achieved as follows. Axonemes, adjusted to 5 mg of protein/ml, were first extracted at 4 °C by a 10-min mild detergent treatment (0.6 M NaCl, 5 mM Hepes, 10 mM mercaptoethanol, 0.5 mM EDTA (HDE), and 1 mM phenylmethylsulfonyl fluoride) to solubilize the dynein arms, then subjected to a low salt treatment in HDE buffer, partially extraction the radial spoke heads (31) with a 16-h dialysis against the same buffer, and last, extracted with 0.2% Sarkosyl plus 2 μl urea in HDE for 10 min to remove the remaining radial spokes. In both cases the remaining undissolved axonemal material was resuspended in electrofusible buffer for analysis as well.

**Immunofluorescence on Isolated Axonemes**—Smears of purified axonemes at 10–20 μg of protein/ml in 30 mM Hepes (pH 7.4), were prepared on positively charged slides (Fisherband Super frost/Plus, catalog number 12-550-15, Fisher) that were immersed in 100% methanol at −20 °C for 10 min and then rehydrated in 20 mM Tris (pH 7.8)-buffered saline containing 0.1% Triton X-100 (TBS-T). This was followed by a blocking step performed at 21 °C (pH 7.4) with 5% goat serum in TBS-T. Smears were then incubated overnight at 4 °C with primary antibodies (Ab) diluted from crude preparations (3–5 mg protein/ml) as follows: DMIA 1/1000, L2H12 1/20, and L3G4 1/50 in TBS-T plus 1% goat serum. The next morning slides were washed 3 times for 5 min each by soaking them into TBS-T. The secondary Ab (biotinylated goat anti-mouse IgG, 3/1000 in TBS-T plus 1% goat serum) (Cedarlane Laboratories Ltd., Ontario, Canada) was added, followed by a 1 h incubation at 21 °C. Slides were then washed as above before the addition of fluorescein isothiocyanate conjugate (1/500 w/v) (Molecular Probes, Eugene, OR) for a 45-min additional incubation at 21 °C in the dark. After washing in TBS-T, slides were finally mounted with Prolong Antifade (Molecular Probes) and observed under a Carl Zeiss (Oberkochen, Germany) Axiopt microscope (exciter filter BP 450–490 nm, LP 510 nm; magnification). To assess the background fluorescence, negative controls were performed using the Alexa Fluor 555 streptavidin conjugate alone or the secondary Ab plus the Alexa Fluor 555 conjugate. Fluorescence images were captured with a monochrome Regiga 1300 cooled-CCD camera (QImaging, Canada) and digitized on a computer using the Northern Eclipse Version 6.0 program.

**Axonemal Immunoblot and Motility Analysis**—Fifty to one hundred ml of actively growing *Chlamydomonas* cultures at 1–2 × 10^6 cells/ml were gently spun at 200 × g for 4 min at 20 °C, washed twice with 30 mM Hepes (pH 7.4), and finally, resuspended into 200 μl of the same buffer. This concentrated stock suspension of highly motile cells was then diluted 5-fold into a demembranation solution (30 mM Hepes (pH 7.4), 5 mM MgSO_4, 1 mM dithiothreitol, 1 mM EDTA, 25 mM potassium acetate (HMDEK), supplemented with 0.5% polyethylene glycol 20,000, 1% Ficoll and 0.1% Nonidet P-40). Motility was re-initiated by diluting 20-fold into the reactivation buffer (HMDEK plus 2% polyethylene glycol and 1 mM ATP). This motility assay was performed in a 96-well microtitrator plate at a final volume of 100 μl using the wild-type cells. Percentages of motility were estimated by eye with an inverted microscope at 200× magnification. Controls were done in the absence of mAb or in the presence of mAb L1F1, a mAb reacting against a single epitope on *Chlamydomonas* axonemal immunoblot and of a similar isotype (IgG), as L2H12 and L3G4 but with no significant effect on the motility of demembranated-reactivated *Chlamydomonas* cells at similar concentration.

To facilitate detailed flagellar movement analysis, unflagellated mutants were used for videomicroscopy recording (Olympus inverted microscope, Panasonic WWF camera and recorder). Upon demembranation reactivation into a microtubete, a 20-μl drop of the uni cell mixture was immediately transferred onto a clean slide and covered with a coverslip that was sealed with vacuum grease onto the slide for observation and recording for 30 to 45 min. Successive images were taken to cover a complete cycle of movement of the flagellum by use of dark field microscopy with a 40× lens and oil immersion condenser (Olympus Co. for...
both) combined with hand translation of the microscope stage illuminated at high strobe frequency (Chadwick-Helmut, La Jolla, CA). Detailed images were captured using a 100× oil immersion phase contrast lens (Carl Zeiss, Oberkochen, Germany). Digitization of images was achieved using a digitizer (Formac Studio, GmbH) connected to a Macintosh computer (iMac, Apple Corp., Cupertino, CA). The rotation of the cell body that occurs during motility of uniflagellar mutants was corrected for by rotating the images so that the images could be superimposed on each other, with the anchoring point of the flagellum into the cell body serving as the reference point. The overlapping flagellar images allowed the clear visualization of a beating envelope, which is defined as the plane area covered by the flagellum during a complete beat cycle. iMovie Software (Apple Corp., Cupertino, CA) was used to grab the individual image frames and the Photoshop 7 program (Adobe Systems Inc., San Jose, CA) to trace the flagellum at successive positions and delineate flagellar beating envelopes. The surfaces of those envelopes were calculated through a modified version of the NIH Image program (40). The width of this beating envelope, or wave amplitude, was also measured at three different positions, proximal, median, and distal, located at ⅓, ⅔, and ⅔, respectively, of the distance from the flagellar origin. Beat frequencies were determined on demembranated-reactivated uniflagellar mutant cells as well using a Chadwick-Helmut strobe scope model 236 coupled to a Zeiss microscope, whereas they were observed under an oil immersion 100× objective under phase contrast.

Protein Determination and Immunoblotting—Determination of protein concentration was done according to the method of Bradford (41) using bovine serum albumin as standard for the total axonemes and extracts or purified bovine immunoglobulins when measuring concentrations of mAb preparations.

SDS-PAGE and electroblotting were conducted following the procedure of Laemmli (42) and Towbin et al. (43), respectively. All immunodetections were performed with crude preparations of L2H12 and L3G4 mAb at 10 and 1 µg/ml, respectively, using an enhanced chemiluminescence (ECL) substrate (LumiLight, Roche Diagnostics) following the instructions from the manufacturer. Immunoreactivity of L2H12 and L3G4 mAb toward RPS2 was tested in parallel with the control anti-RSP2 rabbit CT220 Ab (5% dilution of a RSP23 blotting preparation) on the same blot using an immunoblotting apparatus (Immunetics, Boston, MA) (Fig. 2B). Both RSP2 fusion protein and CT220 Ab were obtained from Dr. S. King (University of Connecticut Health Center) (26).

RESULTS

L2H12 and L3G4 mAb Inhibit the Motility of Demembranated-reactivated Chlamydomonas Cells—While investigating the effects of a panel of mAb generated against axonemal proteins from Chlamydomonas, we selected two potentially interesting mAb, each recognizing a single protein band on immunoblots and reducing flagellar motility of demembranated-reactivated Chlamydomonas models rapidly and significantly as compared with that of control ones.

In the absence of mAb or in the presence of the absence of Chlamydomonas mAb used as control but not affecting motility (L1F1 polypeptide recognized has a molecular weight (Mr) of 30 on immunoblots (data not shown) the percentage of motile cells remained at a high level (∼70%) within the first 60-min period after the demembranation-reactivation was initiated (Fig. 1). The L1F1 mAb was of the same isotype as L2H12 and L3G4 and did not cause an significant motility inhibition even at concentrations 30–50-fold higher than those of mAb L2H12 and L3G4 (Fig. 1). A similar observation has also been reported in previous studies where mAb generated against sea urchin axonemal proteins but directed to proteins other than RSP1 and RSP2 were tested on sea urchin sperm motility (32–35). When L2H12 or L3G4 mAb was added into the assay mixture, the time needed to inhibit the motility and bring the percentage of motile cells to 10% or below varied according to the concentration and mAb used (Fig. 1). This level of inhibition was reached faster in the presence of L2H12, being almost immediate at 0.1 µg/ml and taking 10–15 min at 0.01 µg/ml, whereas in the presence of L3G4 at similar concentrations, 30 and 60 min, respectively, were needed to attain the same level of inhibition. At 0.001 µg/ml, the percentage of moving cells dropped gradually within 2 h, very similarly for both mAb.

Localization of L2H12 and L3G4 Antigens—To localize the L2H12 and L3G4 antigens on the Chlamydomonas flagellar axoneme, immunoblotting analysis was performed using purified axonemes from wild-type and various flagellar mutants of Chlamydomonas. In the first series of experiments, flagella from mutants missing outer dynein arms (oda1 and oda9), inner dynein arms (ida2), inner dynein arms a, c, and d (ida4), inner dynein arms e and dynein regulatory complex (p73), radial spokes (p14), or central pair microtubules (p15) were tested with the L2H12 or L3G4 mAb. Both antibodies reacted with a single protein band at 105 kDa for L2H12 and at 110 kDa for L3G4 in all mutants except p14 (data not shown), indicating the localization of the antigens recognized by these mAb on radial spokes.

In the second set of experiments seven different radial spoke mutants including p14 were used (Fig. 2A). L2H12 antigen was present in all mutants tested except for p14, which totally lacks radial spokes, and p24, which expresses only a small amount of RSP2 (31, 44), suggesting that the latter may be the target for the L2H12 mAb. Moreover, positive reactions with the L2H12 antibody were observed on p11 and p17 (Fig. 2A), both which specifically lack the spoke heads (30), thus strongly indicating the localization of L2H12 antigen on the stalk of the radial spoke. This finding is in agreement with a previous report showing that RSP2 is a component associated with the stalk of the radial spoke (30). From our immunoblotting experiments, we calculated that the apparent Mr of the L2H12 antigen was ~105 kDa (Figs. 2A, 4), a value close to that originally reported for RSP2 (118 kDa) (30). We also showed that the L2H12 mAb was not reacting with RSP23 (Fig. 2B), a novel RSP localized also in the stalk of radial spokes and which has an apparent Mr on SDS-PAGE very similar to that of RSP2 (26).

The L3G4 antigen, although absent in p14 axonemal preparations, was also missing in axonemes from p11 and p17 mutants, strongly suggesting its localization on radial spoke heads. Moreover, it was detected in the other (p24, p25, p26, and p27) radial spoke mutants whose axonemes have been shown by electron microscopy to be decorated with intact, although functionally defective, radial spokes (45). The Mr of the polypeptide reacting with the mAb L3G4 was 110, suggesting that the best candidate for this protein would be RSP1 since the latter has been reported to be a spoke head protein of 123 kDa.

![Image](380x599 to 500x738) FIG. 1. L2H12 and L3G4 mAb dose-dependently inhibit the motility of demembranated-reactivated Chlamydomonas. Demembranation-reactivation assays were performed on wild-type Chlamydomonas cells, as described under "Materials and Methods," in the absence of mAb or in the presence of mAb L1F1 (○, ●) or in the presence of 0.1 (●, ○), 0.01 (□, ○), and 0.001 (●, □) µg/ml of purified L2H12 or in the presence of 0.1 (●, ○), 0.01 (□, ○), and 0.001 (●, □) µg/ml purified L3G4. Values are the averages of data obtained from at least five experiments performed with different Chlamydomonas preparations.
(30). To confirm the identity of the antigens reacting with L2H12 and L3G4 mAb with RSP2 and RSP1, respectively, we also ran a two-dimensional gel electrophoresis of purified *Chlamydomonas* wild-type axonemes immunoblotted with our mAb and found the immunoreacting protein spots at similar isoelectric points (pI) (5.0 and 5.2, respectively) to those reported by Piperno et al. (30) for RSP2 and RSP1 (data not shown).

Immunofluorescence performed with mAb L2H12 and L3G4 on isolated axonemes from *Chlamydomonas* wild-type and pf1, pf14, and pf24 mutant cells was also in agreement with the above results (Fig. 3). Indeed, fluorescence was observed on all axonemes from wild-type *Chlamydomonas*, whereas none was recorded on the spokeless mutant pf14 axonemes in the presence of L2H12 and L3G4 mAb. Fluorescence was detected on pf1 and pf24 axonemes in the presence of L2H12 mAb, whereas with the L3G4 mAb, only pf24 axonemes showed fluorescence, again strongly suggesting the localization of L2H12 and L3G4 antigens on the stalk and head of the radial spokes, respectively. No clear indication of a longitudinal gradient in the fluorescence intensity was noted with these two mAb even though the fluorescent signal was not as strong and perfectly uniform as compared with that observed in the presence of the anti-α-tubulin DM1A, for example (Fig. 3).

**Extraction of L2H12 and L3G4 Antigens**—We further looked for the presence of L2H12 and L3G4 antigens in the radial spokes after an extraction procedure developed recently (25). Both antigens were found in the 0.5 M KI fraction containing the radial spokes. However, although the L2H12 antigen was recovered almost exclusively in the KI extract, the L3G4 antigen was also detected in the other two fractions (0.6 M NaCl and the remaining pellet), although in a lesser amount than in the KI fraction (Fig. 4), raising the possibility that the L3G4 epitope may not be solely found on the radial spokes.

Using a variant method of extractions to solubilize the L2H12 and L3G4 antigens from wild-type purified axonemes (Fig. 4), we found that the high salt treatment (0.6 M NaCl), where most of the dynein arms are released, contained no L2H12 antigen but some of the L3G4 as observed in the previous experiment. The second extraction step, which consisted of a 16-h dialysis against a low salt buffer, released a very small amount of the L2H12 antigen but a fairly large quantity of the L3G4 antigen. Finally, treatment of the residual axonemes in harsher conditions with 0.2% Sarkosyl/2M urea solubilized the entire L2H12 antigen, and most of the remaining epitopes associated with L3G4, with a small amount, in this case, still detectable in the corresponding pellet.

**Flagellar Movement Analysis in the Presence of L2H12 and L3G4 mAb**—To better understand how L2H12 and L3G4 mAb affect the flagellar movement, a more detailed analysis of the flagellar beat cycle was performed from video recordings of
FIG. 5. Video sequences of flagellar movement in the presence of L2H12 and L3G4 mAb. Video images of the demembranated-reactivated uniflagellar mutant uni1 cells were recorded under dark-field microscopy as described under “Materials and Methods.” For the sake of clarity, black and white colors were inverted. The time frame of each sequence of six cell positions was 1/50 of a s, covering about one beat cycle; therefore, successive positions are separated by 1/300th of a second intervals. Movement of the flagellum is shown in the absence (control) or the presence of L2H12 (0.01 μg/ml, purified) and L3G4 mAb (0.5 μg/ml, crude) after 2–3 min and 15–20 min of the onset of the demembranation-reactivation assay. Although no significant change was observed in the beat cycle pattern of control cells over time, axonemes of cells reactivated in the presence of L2H12 or L3G4 showed even after 2–3 min of incubation an incomplete recovery phase that was further reduced after 15–20 min of the beginning of the demembranation-reactivation assay. Bar, 10 μm.

demembranated-reactivated *Chlamydomonas* uniflagellar models in the presence and absence of mAb. In this case unflagellar mutant cells were used instead of the normal biflagellated cells because they swim in circles in one plane, thus facilitating the recording in proper focus of the beating flagellum (14).

In the absence of active mAb, the axonemes of *Chlamydomonas* showed a typical ciliary pattern of movement in which a power stroke is followed by a full recovery phase (Figs. 5 and 6). This beat cycle pattern in control cells was maintained over the usual 30–45-min observation period on the slide. In the presence of L2H12 (0.01 μg/ml of purified mAb) or L3G4 (2 μg/ml of a crude mAb preparation), a reduction in the extent of the power stroke was first observed due to improper recovery of axonemes to their initial position. After 15–20 min of exposure to mAb, complete failure of the axonemes to recover was apparent, the latter being arrested in a rigid position (Figs. 5 and 6). At this point obviously cell motility inhibition was in most instances complete, as observed in Fig. 1.

To further characterize the inhibitory process triggered by L2H12 and L3G4 mAb, we measured at different time points of the demembranation-reactivation assay done on unflagellar *Chlamydomonas* cells the flagellar beat frequencies, the surface of axonemal beating envelope, and the maximal wave amplitudes at three different positions on the flagellum (Fig. 7).

In the presence of either L2H12 or L3G4 mAb, a decrease of the beating envelopes from 30 to 5 μm² was observed as soon as 10 min after the beginning of the demembranation-reactivation assay. On the other hand, the flagellar beat frequencies of L2H12- or L3G4-treated models did not change significantly over time until the flagellum became completely paralyzed after prolonged exposure to the mAb.

Further refinement for the analysis of the motility interference by the two mAb was gained by measuring the wave amplitudes at three equally spaced positions along the flagellum. Thus, we found that after 10 min of exposure to L2H12 mAb, the flagellar wave amplitudes had dropped by more than 60%. By 30–45 min amplitude values measured at the proximal and median sites along the axoneme did not differ significantly from those observed at 10 min, whereas the wave amplitude at the distal portion further decreased by another 60% (Fig. 7). In the case of L3G4 mAb, decreases of amplitudes were observed in the first 10 min of incubation at median and distal positions on the axoneme, with a further 50% decrease after a longer period of incubation (30–45 min). In the proximal region though, amplitude values did not change significantly throughout the 45-min period of observation.

Taken together, the results from the video images and the data generated from them strongly suggest that the primary effect of these two anti-radial spoke mAb is on the bending formation of the axonemal wave rather than on the beat frequency.

**DISCUSSION**

The data presented here strongly support the implication of the stalk and head of radial spokes in the process regulating the wave amplitude during flagellar movement. The mAb generated (L2H12 and L3G4) against two individual RSP, each one found to be localized on a different region of the radial spoke, showed potent inhibitory action on the motility of demembranated-reactivated *Chlamydomonas* cells (Fig. 1). Although the importance of radial spokes in axonemal motility is well recognized in *Chlamydomonas* because of the availability of paralyzed mutants lacking the entire radial spokes (*pf14* mutant; 23 polypeptides missing) or more specifically the spoke heads (*pf1* and *pf17*; 5 polypeptides missing), our approach using mAb on a demembranated-reactivated model has the advantage of targeting a restricted epitope of an individual component of the radial spoke and could, therefore, facilitate the attribution of a more specific role for that component or the substructure in which that component is localized. Furthermore, applying low concentrations of mAb on the cells permitted time-dependent evaluation of changes in the motility parameters in a more progressive manner (Figs. 5–7).

In calculating the stoichiometric ratio of mAb to antigen from the motility inhibition curve obtained after incubation with 10 ng/ml of L2H12 or L3G4 mAb, we estimated that there were around three mAb for one antigen. We calculated that with the cell concentration at 5 × 10⁶/ml, the length of one flagellum at 10 μm, and the number of radial spokes per 96-nm axonemal cross-section at 18 (3, 46), that there is one antigen per radial spoke. This 3 to 1 mAb:antigen ratio reinforces the idea that L2H12 and L3G4 mAb act on peptides or epitopes that are crucial for the proper function of flagella.

Using *Chlamydomonas* mutants missing specific axonemal structures, we determined both by immunoblotting and immunofluorescence that the proteins recognized by mAb L2H12 and L3G4 are localized on the stalk and the head of the radial spokes, respectively (Figs. 2 and 3). The proteins targeted by L2H12 and L3G4 are most likely RSP2 and RSP1, based on their apparent Mr and pI, which were very similar to those reported by Piperno et al. (31). The fact that the L2H12 antigen is virtually absent in axonemes from the mutant *pf24* (Figs. 2 and 3), which is greatly reduced in RSP2 (12, 30), further supports the conclusion that the protein recognized by L2H12 is RSP2. Neither L2H12 nor L3G4 showed an immunological reaction toward RSP29 (Fig. 2B), a radial spoke stalk protein with characteristics (Mr, pI, and low expression in *pf24* mutant) very similar to those of RSP2 (26), giving additional support to the conclusion that RSP2 is the target of L2H12 mAb.

In agreement with these findings, the L2H12 and L3G4 antigens were found in the radial spoke extract (0.5 M KI) of *Chlamydomonas* axonemes as expected (Fig. 4). It is worth noting that, whereas the L2H12 antigen was solely recovered in the 0.5 M KI fraction, the L3G4 antigen was only partially extracted by the KI solution, a small proportion being solubilized with the high salt (0.6 M NaCl) treatment or left over in the remaining pellet. In a sequential extraction with high salt, low salt, and Sarkosyl/urea, we found a significant portion of the L3G4 antigen in the low salt fraction (Fig. 4), in agreement with results of Piperno et al. (31) who have previously reported partial purification of spoke heads from *Chlamydomonas* ax-
onemes using a low salt dialysis after 0.5 M NaCl treatment. Moreover, the L3G4 antigen was never completely extracted, contrary to the L2H12 antigen, which was restricted to the Sarkosyl/urea extract (Fig. 4), suggesting that the L3G4 antigen may not be solely positioned on the radial spokes. However, the absence of L3G4 antigen specifically from the pf1, pf17, and pf14 mutants (Figs. 2 and 3) did not support this interpretation. Differences in the degree of association of proteins to the axoneme have also been observed previously without any indication that these proteins varied in their molecular nature (35, 36). It is more likely to reflect differences in their association with distinct axonemal structures or proteins. Localization of RSP1 on the spoke head, which has transient interactions with the central microtubule complex (47), could induce variation in the binding affinity of RSP1 to the spoke head according to the state of the radial spoke/central pair microtubule interaction, and therefore, RSP1 extracted more or less easily. In contrast, the position on the stalk of the radial spoke could make the extraction of L2H12 antigen (RSP2) much more difficult and would explain that it was confined exclusively in the KI or Sarkosyl/urea fractions.

Previous functional studies using flagella from unflagellar Chlamydomonas recombinant strains lacking radial spoke heads lead to the conclusion that the radial spokes are involved in the conversion between symmetrical and asymmetrical waveforms (13–15). The fact that the radial spoke mutants are missing several polypeptides brings limitations to ascertain a precise role for individual RSP. Our own approach in the present study partially circumvents this drawback as we used cells with intact axonemal structures and targeted two restricted epitopes present only in single radial spoke components (RSP1 and RSP2). Motility analysis of demembranated-reactivated unflagellar Chlamydomonas mutant cells treated with the two anti-radial spoke antibodies clearly indicate the involvement of radial spokes in bending formation and perhaps propagation during the beat cycle rather than in the regulation of the beat frequency (Figs. 5–7).

Most interestingly, the wave amplitude data (Fig. 7) indicated that the inhibition did not proceed exactly the same way for both mAb. At the beginning of the inhibition process, the L2H12 mAb primarily affected the proximal region, whereas L3G4 seemed to first affect the distal part of the axoneme, both mAbs leaving some partial bending in the opposite portions. These data may reflect a functional difference between RSP2 and RSP1 and/or between the stalk and the head of radial spokes. This could also suggest that the antigen distribution along the axoneme is not constant but rather follows a gradient, having a lower density toward the tip for L2H12 and conversely for L3G4 toward the basal body. However, it is not possible to conclude from immunolocalization images (Fig. 3) that there is an intensity gradient toward the tip or the base of the axoneme. According to the current axonemal model, ultrastructurally the base of radial spokes are in close proximity to the dynein regulatory complex and the inner arms and more distant from the outer arms (21). In this regard, our results support the view proposed by earlier studies that inner dynein arms play a larger role in determining the symmetry of flagellar waveform, whereas outer dynein arms control beat frequency and add power (for review, see Refs. 3, 48, and 49).

During flagellar motility in Chlamydomonas, the projections

FIG. 6. Digital representations of flagellar movement in the presence of L2H12 and L3G4 mAb. Complete flagellar beating cycles were digitally reconstituted by superimposing the images recorded under phase-contrast microscopy as detailed under “Materials and Methods.” Five to six different positions (every 50th of a second and numbered 1–6) of the flagellum during ciliary beating are depicted in each micrograph. In untreated cells (control), a large beating envelope was observed with important bending in the recovery phase of the beat cycle that did not change significantly during the 20-min observation period. In the presence of L2H12 or L3G4 mAb, a decrease of the wave amplitude and beating envelope was already noticeable after 2–3 min after the beginning of the demembranation-reactivation assay. After 15–20 min of incubation with the mAb, the axonemes showed very little amplitude of their beating wave. Bar, 5 μm.
The fine modulation of this pathway is dependent on multiple phosphorylations, which in turn can be modified by effectors such as Ca$^{2+}$ and cAMP. Interestingly, RSP2 is one of the five stalk components subjected to phosphorylation (31) and to which calmodulin and Ca$^{2+}$ can bind (44).

For the first time through specific interference by mAb on individual radial spoke proteins, our results stress the importance of head and/or stalk radial spoke substructures in modifying the shape and the amplitude of the flagellar bending waves while having little or no effect on the sliding velocity determining the beat frequency. They further suggest a model in which both RSP1 and RSP2 would be crucial participants in the extent of the power stroke in the flagellar beat cycle of Chlamydomonas. During the recovery phase though, the radial spoke stalk via RSP2 would preferentially be involved in the proper wave propagation in the proximal portion of the axoneme, whereas the radial spoke head via RSP1 would primarily affect the fine wave amplitude tuning in the distal axonemal region.

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