

Centrin Plays an Essential Role in Microtubule Severing during Flagellar Excision in *Chlamydomonas reinhardtii*

M. A. Sanders and J. L. Salisbury

Laboratory for Cell Biology, Department of Biochemistry and Molecular Biology, Mayo Clinic Foundation, Rochester, Minnesota 55905

Abstract. Previously, we reported that flagellar excision in *Chlamydomonas reinhardtii* is mediated by an active process whereby microtubules are severed at select sites within the flagellar-basal body transition zone (Sanders, M. A., and J. L. Salisbury, 1989. *J. Cell Biol.* 108:1751-1760). At the time of flagellar excision, stellate fibers of the transition zone contract and displace the microtubule doublets of the axoneme inward. The resulting shear force and torsional load generated during inward displacement leads to microtubule severing immediately distal to the central cylinder of the transition zone. In this study, we have used a detergent-extracted cell model of *Chlamydomonas* that allows direct experimental access to the molecular machinery responsible for microtubule severing without the impediment of the plasma membrane. We present four independent lines of experimental evidence for the essential involvement of centrin-based stellate fibers of the transition zone in the process of flagellar excision: (a) Detergent-extracted cell models excise their flagella in response

to elevated, yet physiological, levels of free calcium. (b) Extraction of cell models with buffers containing the divalent cation chelator EDTA leads to the disassembly of centrin-based fibers and to the disruption of transition zone stellate fiber structure. This treatment results in a complete loss of flagellar excision competence. (c) Three separate anti-centrin monoclonal antibody preparations, which localize to the stellate fibers of the transition zone, specifically inhibit contraction of the stellate fibers and block calcium-induced flagellar excision, while control antibodies have no inhibitory effect. Finally, (d) cells of the centrin mutant *vtl-2* (Taillon, B., S. Adler, J. Suhan, and J. Jarvik, 1992. *J. Cell Biol.* 119:1613-1624) fail to actively excise their flagella following pH shock in living cells or calcium treatment of detergent-extracted cell models. Taken together, these observations demonstrate that centrin-based fiber contraction plays a fundamental role in microtubule severing at the time of flagellar excision in *Chlamydomonas*.

THE transition zone occurs between the flagellar axoneme and basal body and marks the proximal site where flagella are lost during the process of flagellar excision (Blum, 1971; Lewin and Lee, 1985). In the green alga *Chlamydomonas reinhardtii*, transition zone structure is complex and consists of a pair of central cylinders that have an H shape when viewed in longitudinal section and a stellate array of fibers that interconnect alternate microtubule doublets of the axoneme (Lang, 1963; Ringo, 1967; Sanders and Salisbury, 1989). Transition zone stellate fibers are composed, at least in part, of centrin (Sanders and Salisbury, 1989; Taillon et al., 1992), a 20,000-mol wt protein of the EF hand superfamily of calcium-binding proteins (Errabolu et al., 1994; Huang et al., 1988; Moncrief et al., 1990). At

the time of flagellar excision, the stellate fibers contract and subject the axonemal doublets to mechanical shear force and torsional load which causes the local severing of microtubules just distal to the flagellar transition zone (Sanders and Salisbury, 1989). Here, we present direct evidence that calcium-induced centrin-based stellate fiber contraction generates the forces responsible for microtubule severing in *Chlamydomonas*. These results have important implications for understanding centrin function in other cell types. It is possible that centrin-based microtubule severing may effect the dynamic properties of individual microtubules nucleated at the centrosome and spindle poles. Microtubule severing may predispose individual microtubules to disassemble by creating new free ends which would be susceptible to catastrophic tubulin subunit loss through dynamic instability or, alternatively, to detach microtubules from their nucleation sites and to allow for their movement to distal regions within the cytoplasm.

Address all correspondence to J. Salisbury, Laboratory for Cell Biology, Department of Biochemistry and Molecular Biology, Mayo Clinic Foundation, Rochester, MN 55905.

Materials and Methods

Strains and Culture Conditions

Cultures of *C. reinhardtii*, wild type strain 137c, were obtained from Dr. E. Harris (Chlamydomonas Genetics Center, Botany Department, Duke University, Durham, NC). The variable flagellar number mutant *ylf-2* (isolate 416) was a gift from Dr. J. Jarvik (Carnegie Mellon University, Pittsburgh, PA). Cells were grown in Sager and Granick medium I (Sager et al., 1953) with Hutner's trace element solution (Harris, 1989; Hutner et al., 1950), in 10 ml tubes under a 14:10-h light/dark cycle at 22°C.

Preparation of Detergent-extracted Cell Models

Cells were collected by centrifugation and washed in TEGM buffer (10 mM Tris/HCl, pH 7.0, 5 mM EGTA, 0.5 mM MgSO₄). Cell pellets were gently resuspended in 0.5 ml TEGM buffer and then detergent extracted by adding 10 vol of TEGM extraction buffer containing 0.01% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO). Detergent-extracted cell models prepared in the absence of Mg²⁺ (EDTA models), were made in an identical manner except 5 mM EDTA was added to TEGM buffer.

Free Ca²⁺ Calculations

Calculations of free Ca²⁺ levels were made as described by Potter and Gergely (1975) with computer program modifications of Alan Mandveno (University of Miami, Miami, FL). All constants involving protons were corrected for ionic strength so that pH instead of [H⁺] could be used in the calculations. Free calcium levels were also determined using the fluorescent calcium reporter Fura-2 and the methods of Grynkiewicz and co-workers (Grynkiewicz et al., 1985). Corrected emission spectra were obtained on a spectrofluorometer (model 1680; SPEX Industries, Edison, NJ). Both methods routinely gave free Ca²⁺ concentration determinations within 5% of one another.

Flagellar Excision Assay

Flagellar excision was routinely induced in detergent-extracted cell models (10⁶/ml in a 1.5 ml microfuge tube) by adjusting the free Ca²⁺ concentration to 1 μM above that buffered by EGTA in the TEGM system. Higher free calcium levels (up to 10 mM) were also used in parallel experiments and in all cases gave the same results as the 1 μM treatments. Except where noted, samples were mixed by gentle inversion, and fixed by adding an equal volume of 2% glutaraldehyde in TEGM. Cells were examined for the presence of flagella using a 100X objective; 30 to 130 cells were examined for each replicate treatment. In some experiments, where noted, samples were vigorously vortexed at full speed for 2 min in a fluted tube. We also established a system wherein minimal mechanical shear force would be experienced by the cells during a Ca²⁺-induced flagellar excision assay. Cell models were placed on a flow-through microscope slide where the coverslip was suspended by broken pieces of a second coverslip. Under these conditions a few cells in each field became attached to the slide. 1 μM Ca²⁺ was introduced by further perfusion with Ca²⁺-containing buffers. This low shear assay system was also used for pH and dibucaine treatment of living cells in a modified culture medium (MCM)¹ containing 10 mM Tris-HCl, 1 mM NaH₂PO₄, and 2 mM sodium acetate, pH 7.0. Excision was induced by: (a) pH shock by perfusing living cells for 1 min with MCM adjusted to pH 4.3 with 1 N acetic acid stock, followed by perfusion with MCM adjusted to pH 7.0; or (b) dibucaine treatment by perfusing MCM containing 15 mM dibucaine through the slide chamber. All experimental manipulations were monitored by video microscopy and recorded on video tape.

Immunofluorescence Microscopy

Cells were fixed in freshly prepared 4% formaldehyde in 10 mM Hepes buffer, pH 7.0, and allowed to settle for 10 min onto 8-welled microslides (Carlson Scientific, Peotone, IL) coated with 0.1% polyethylenimine (Sigma Chemical Co.). After three washes in 10 mM Hepes buffer, the cells were permeabilized using -20°C MeOH for 10 min and were allowed to air dry. Specimens were blocked for 30 min at 37°C by treating the slides with blocking buffer: PBS containing 5% goat serum (GIBCO BRL, Gaithers-

burg, MD), 5% glycerol, 0.1% BSA, and 0.4% sodium azide. The specimens were incubated for 1 to 3 h in primary anticestrin monoclonal antibody, 20H5, diluted (1:1,000) in blocking buffer, washed three times in PBS, 5 min each, and incubated in FITC-conjugated goat anti-mouse antibody (Cappel, IgG; Oreganon Teknika Corp., West Chester, PA) at 1:400 dilution, for 1 h. Slides were then washed in PBS three times, and mounted in Gelvatol mountant (Rodriguez and Deinhardt, 1960) containing 2% *n*-propyl-gallate, pH 8.5 (Sigma Chemical Co.). Coverslips were sealed with nail polish to prevent desiccation. Preparations were viewed using a Nikon Microphot-FXA photomicroscope equipped with a 100 W mercury lamp and epifluorescence illumination, standard FITC and UV filter sets, and a Fluor 60X, 1.40 NA oil immersion objective. Photographs were recorded on Hypertech film (Microfluor Ltd., Stony Brook, NY) at exposure times of between 30- and 45-s duration and the film was developed in Kodak D-19 developer for 6 min at 20°C. Controls used secondary antibody alone.

Electron Microscopy and Immunogold Labeling

Fixation was carried out according to the procedure of McDonald (1984). Cells were fixed in 3% glutaraldehyde buffered with 10 mM Hepes, pH 7.0, for 2 h at 4°C. Following buffer wash, the samples were post-fixed in 1% osmium tetroxide and 0.8% K₃Fe(CN)₆ in 4 mM phosphate buffer, pH 7.2, for 30 min at 4°C. Samples were washed with deionized water, mordanted with 0.15% aqueous tannic acid for 1 min at room temperature, washed in deionized water, and stained en bloc with 2% aqueous uranyl acetate for 2 h in the dark. After washing with deionized water, the samples were dehydrated through an ascending ethanol series, and embedded in Spurr's resin. Blocks were polymerized at 60°C for 48 h. Silver sections were collected on 0.4% formvar-coated slot grids and post-stained at room temperature with aqueous 2% uranyl acetate for 15 min, followed by Reynold's lead citrate for 15 min. Sections were observed and photographed on a JEOL 1200 EXII electron microscope. For post-embedded immunogold labeling, sections were stained according to methods described previously (Sanders and Salisbury, 1989).

Antibody Neutralization of Flagellar Excision

Monoclonal antibodies used in these studies include the following: anti-centrin 17E10, raised against centrin purified from *Tetraselmis striata* (Salisbury et al., 1988); anti-centrin 20H5 and 11B2, raised against bacterially expressed *Chlamydomonas* centrin (Baron, A., and J. Salisbury, unpublished data); a cocktail of three separate anti-calmodulin preparations, 6G4, 9E3, and 1F11, each raised against *Dictyostelium discoideum* calmodulin and recognize calmodulin from all sources tested, including vertebrate calmodulin (Hulen et al., 1991) and algal calmodulin (Coling and Salisbury, 1992); and anti-α-tubulin or anti-β-tubulin (T9026 and T4026; Sigma Chemical Co.). Cell models were incubated in ascites fluid or protein A/G purified IgG monoclonal antibodies at known concentrations for 15 min. A predetermined amount of 0.1 M CaCl₂ was added to bring the free Ca²⁺ to 1 μM; samples were fixed by adding an equal volume of 2% glutaraldehyde and examined for the presence of flagella as described above.

Results

Contraction of Transition Zone Stellate Fibers Results in Flagellar Excision in Detergent-extracted Cell Models

DIC images of detergent-extracted cell models of two *C. reinhardtii* cells before (a) and after (b) calcium-induced flagellar excision are shown in Fig. 1. Note the loss of flagella in (Fig. 1 B). In these and all subsequent experiments, except where noted, flagellar excision is induced by the simple addition of CaCl₂ to a final concentration of 1 μM free calcium. After calcium addition, the cell suspension is mixed by gently inverting the tube twice in order to minimize shear forces. The ability of these cell models to excise their flagella approached 100% under these conditions. When free calcium levels are maintained below 0.1 μM with EGTA buffers, flagella fail to excise unless the cells are subject to shear forces such as vigorous vortexing, in which case the

1. Abbreviations used in this paper: MCM, modified culture medium; NBBC, nucleus-basal body connector.

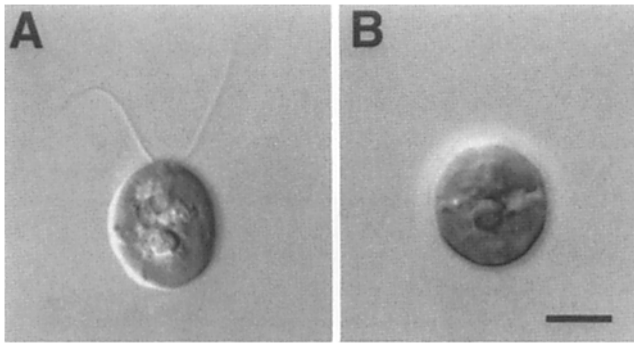


Figure 1. *Chlamydomonas* detergent-extracted cell models, before (A) and after (B) calcium-induced flagellar excision. Note the loss of flagella in B. DIC microscopy. Bar, 5 μm .

flagella may break at inappropriate points along their length (see below).

Fig. 2 illustrates electron micrographs of the flagellar transition zone region of cell models before (Fig. 2, A–C) and after (Fig. 2, D–F) calcium-induced flagellar excision. Detergent treatment effectively removes the membrane from the axoneme and cell body. However, a short cylinder of membrane is resistant to detergent extraction and remains around the region of the transition zone itself; this membrane cylinder is open at both ends. Thus, detergent treatment allows ready access for molecules and ions to the region of the transition zone. Structural features of the transition zone, axoneme, and basal bodies of detergent-extracted cell models remain intact as compared to living cells (Sanders and Salisbury, 1989). Electron-dense material (Fig. 2 B, arrow)

appears associated with each axonemal doublet just above the region of the transition zone and in the region of the stellate fibers; this material is not obvious in unextracted cells. For the purposes of this study, the detailed structure of the transition zone in detergent-extracted models is briefly described below, although most features remain similar to those of unextracted cells (see Ringo, 1967; Sanders and Salisbury, 1989). The transition zone connects the axoneme to the basal body and is characterized by microtubule doublets and a pair of central cylinders that have an H shape when viewed in longitudinal section (Fig. 2 A). The distal cylinder is 80 nm in diameter, 100–120-nm long, and is closed at its proximal end. The proximal cylinder is 80-nm long, and is open at both ends. The H piece cylinder is connected to the A tubules of the microtubule doublets by fibers (~5-nm diam) which form a distinctive stellate pattern when viewed in cross section (Fig. 2 C). The organization of these fibers is complex in that they connect every other A tubule and are arranged in a spiral for six to seven turns along the length of the H piece (Lang, 1963; Manton, 1964). Immunofluorescence, immunoelectron microscopy, and genetic studies have demonstrated that stellate fibers are, in part, centrin based (Sanders and Salisbury, 1989; Taillon et al., 1992). The transition zone undergoes dramatic structural alterations after treatment of detergent-extracted cell models with 1 μM Ca^{2+} (Fig. 2, D–F). The fibers of the stellate structure contract around the outer wall of the central cylinder in the distal portion of the H piece (Fig. 2, D and F). Contraction of the stellate fibers draws the doublet microtubules inward and results in a change in pitch or doublet angle relative to the radius of the transition zone (Sanders and Salisbury, 1989). Microtubules of the axoneme sever just distal to the transition zone H piece, the flagellar axoneme excises, and

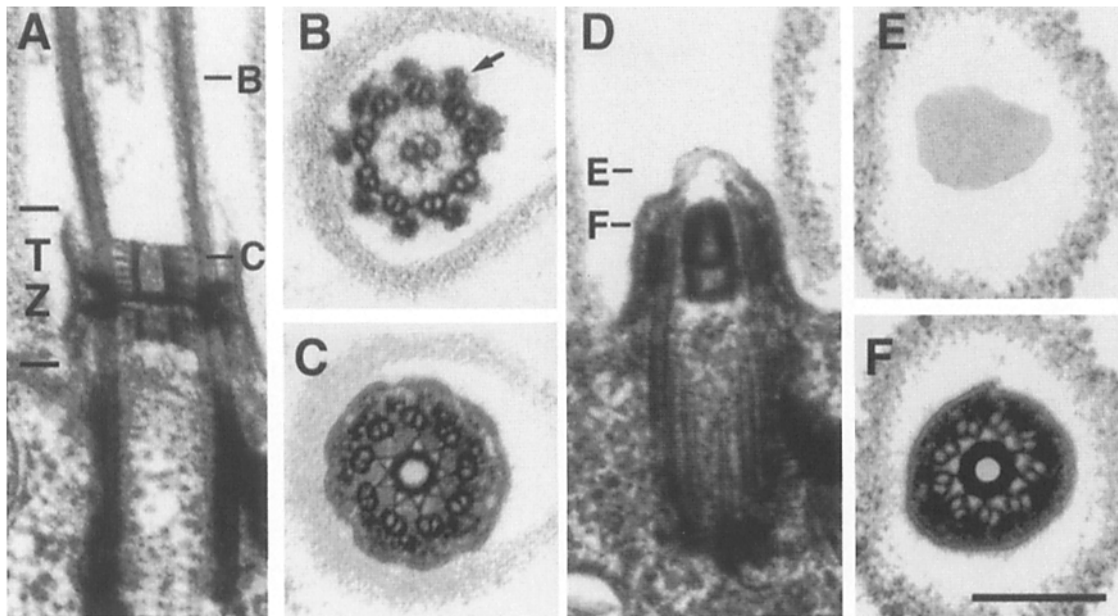


Figure 2. Electron micrographs of flagellar transition zones in detergent-extracted cell models, before (A–C) and after (D–F) calcium-induced flagellar excision. Note that before calcium challenge, the stellate fibers of the transition zone are extended (C) and after calcium challenge they are contracted and the microtubule doublets have been twisted and displaced inward (F). TZ, transition zone; arrow, flocculent material. Bar, 0.2 μm .

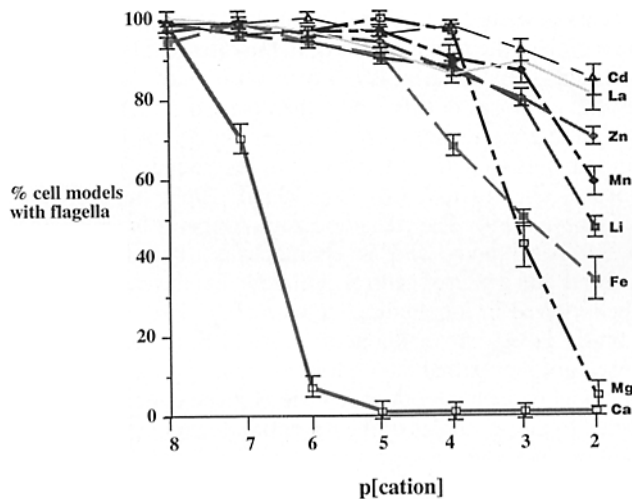


Figure 3. Dose-response curves for various divalent cations in the detergent-extracted cell model flagellar excision assay. Only calcium treatment results in flagellar excision when used at physiological levels, i.e., between 10^{-7} and 10^{-6} M free calcium. All other treatments (Cd, La, Zn, Mn, Li, Fe, and Mg) fail to induce normal flagellar excision. The mean value for three separate experiments with 30 cells scored in each is shown with standard error bars.

membrane remnants appear above the transition zone, even in these detergent-extracted cell models (Fig. 2, D and E).

Calcium Treatment Triggers Flagellar Excision

Calcium treatment alone is sufficient to induce flagellar excision in detergent-extracted cell models. Fig. 3 illustrates the dose-response curves for a variety of cations tested for their ability to induce flagellar excision. Detergent-extracted models rapidly excise their flagella, as described above, when free calcium is raised to micromolar levels. The chloride or sulfate salts of Mg, Mn, Li, Zn, Cd, La, and Fe are ineffective for the induction of flagellar excision, except when used at extremely high (nonphysiological) levels. Although magnesium ions do not induce flagellar excision when used at physiological levels, our observations indicate a loss of flagella in cell models at high Mg^{2+} concentrations. However, flagellar loss does not appear to be due to the excision mechanism described above; i.e., at magnesium levels above 10 mM, the flagella appear kinked and broken at various locations along their length. These flagellar profiles are not consistent with flagellar excision, but rather suggest a fragmentation of the axoneme by some other mechanism.

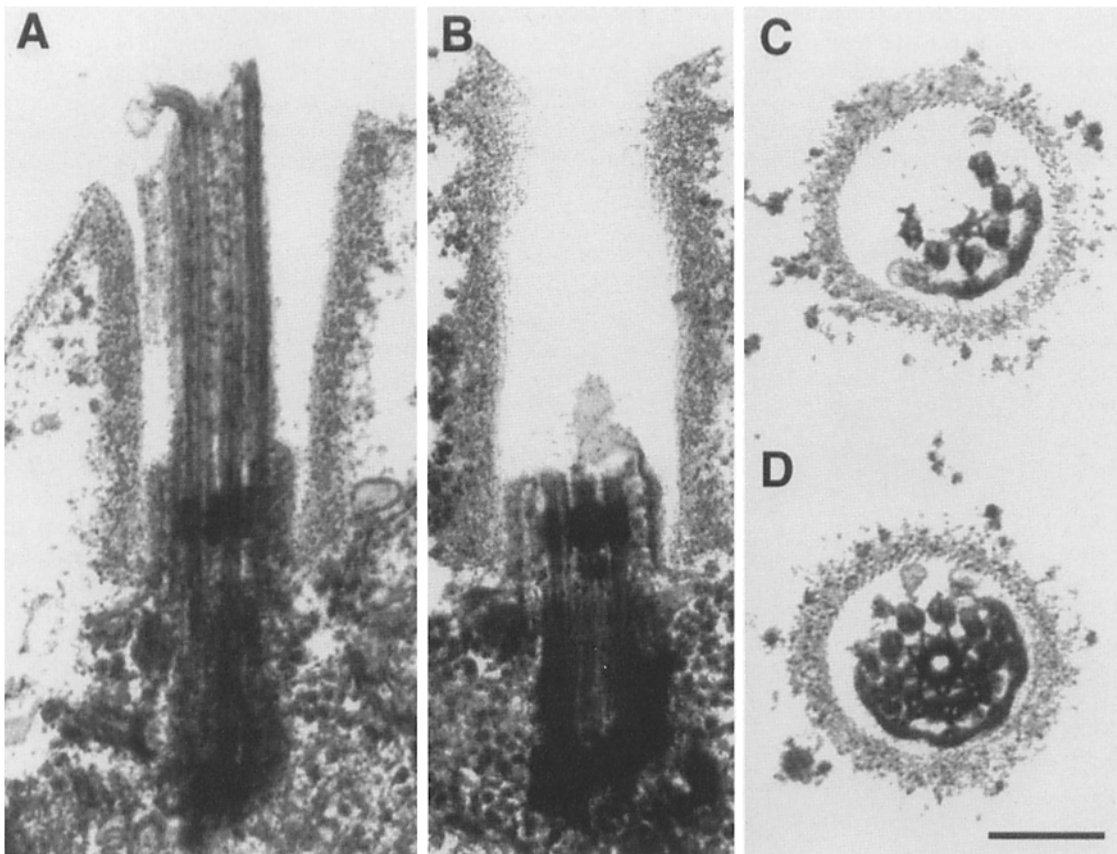


Figure 4. Shear force brakes flagellar axonemes at inappropriate points. (A) Electron micrograph showing the typical site of axoneme disruption following mechanical shearing (vortexing) of a detergent extracted cell model of *Chlamydomonas*. The axonemal doublets rupture unevenly at the top of the flagellar tunnel well above the transition zone. (B-D) In some cases the axoneme ruptures at the normal site of flagellar excision, near the distal-most region of the transition zone, however, the stellate fibers remain extended and the axoneme diameter fails to decrease. Bar, 0.2 μ m.

Shear Forces Break Flagella at Inappropriate Points along Their Length

Detergent-extracted models can be induced to shed their flagella in the absence of added free calcium (EGTA models) by vigorous vortexing; however, the axonemes often break at inappropriate points along their length. Typically, mechanical shearing results in the rupture of axonemal doublets well above the transition zone. Frequently microtubule rupture occurs in the region where the flagellar tunnel ends and the axoneme emerges from the cell wall (Fig. 4 A). It is likely that the axoneme experiences substantial shear force in this region during the period of vortexing. Excision in this region is never seen in cell models or in living cells in the presence of Ca^{2+} (see above and Sanders and Salisbury, 1989). In some instances vortexing in the absence of free calcium (EGTA models) can result in flagellar loss at the normal site of excision, i.e., just distal to the transition zone (Fig. 4, B-D). However, when this is the case, there is no contraction of the stellate zone fibers and there is no obvious inward displacement of the axonemal doublets toward the H piece. For example, Fig. 4, C and D show consecutive serial sections through the distal region of a transition zone where flagellar loss was brought about by vigorous vortexing of EGTA models. Note that the stellate fibers have not contracted, the microtubule doublet pitch or angle relative to the radius of the transition zone remains normal, and the diameter of the transition zone has not decreased. Two conclusions can be drawn from these observations: (a) a weak point in the flagellar axoneme exists just above the transition zone (Blum, 1971) where the axoneme may be predisposed to rupture; and (b) in the absence of calcium, there is no stored tension in the stellate fibers as has been suggested by Jarvik and Suman (1991).

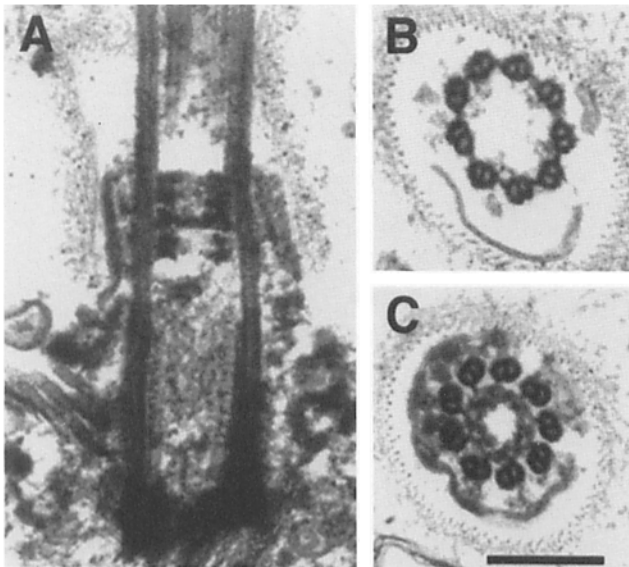


Figure 5. EDTA extraction of cell models disrupts transition zone structure. (A and C) Flocculent material marks the site of the central cylinder. (A) Longitudinal section through a flagellar base. (B) Cross-section of the axoneme. (C) Cross-section of the transition zone. Note that the stellate fibers are no longer present. Bar, 0.2 μm .

EDTA Treatment Disrupts Transition Zone Structure and Renders Cell Models Flagellar Excision Incompetent

Fig. 5 demonstrates that the structure of the transition zone is disrupted when detergent-extracted models are treated with buffers containing EDTA in order to chelate Mg^{2+} from the system (EDTA models). The stellate fibers and central cylinder of EDTA models have undergone a partial disassembly resulting in the appearance of a cylinder of flocculent material in the region where there once was a well organized transition zone (compare Figs. 2, A and C and 5, A and C). As indicated above, EDTA-extracted models fail to excise their flagella even following an increase in free calcium to levels exceeding 2 mM (Table I). When 5 mM MgCl_2 is added back immediately after EDTA extraction, flagellar excision competence is regained by the detergent-extracted models. However, when EDTA extraction is carried out for more than 30 min, flagellar excision competence is completely lost. Thus, disruption of transition zone stellate fibers correlates with an inability to excise flagella using the normal excision mechanism. EDTA treatment also disrupts the distribution of the centrin-based fiber systems in *Chlamydomonas* cells. Fig. 6 illustrates indirect immunofluorescence images of centrin localization in a control detergent-extracted cell model (A) and a model that was subsequently treated with EDTA (B). Note the typical centrin-based fiber distribution in the detergent-extracted cell model (A). In this cell centrin is localized in two descending fibers which extend into the cell and branch at the surface of the nucleus into a system of fimbria that embrace the nuclear envelope; this aspect of the centrin-based fiber system of *Chlamydomonas* has been called the nucleus-basal body connector (NBBC) (Salisbury et al., 1988; Wright et al., 1985). Centrin is also localized to the distal fiber that links the two adjacent basal bodies to one another, and at distal "horns" which are located in the region of the basal body transition zone (Salisbury et al., 1988). However, in the EDTA treated model (Fig. 6 B), most of the centrin-based structures have disassembled. Centrin has been extracted from the cell, leaving only two small spots which correspond to the inner lateral basal body wall where the distal fiber normally attaches adjacent basal bodies. EDTA treatment of cell models, thus, results in disruption of transition zone structure and centrin localization, and this treatment also renders the transition zone incompetent to mediate flagellar excision. These observations suggested that EDTA-sensitive elements of transition zone structure are required for calcium-induced flagellar excision likely to be involved in stellate fiber contraction leading to microtubule severing.

Anti-Centrin Monoclonal Antibodies Neutralize Flagellar Excision Competence

Detergent-extracted cell models allow experimental access of ions and larger molecules to the excision machinery, thereby, enabling a direct test for the active involvement of centrin-mediated fiber contraction. This was accomplished by treating cell models with purified anti-centrin monoclonal antibodies in order to test for potential neutralizing activity of the antibodies in the Ca^{2+} -induced excision assay. Fig. 7 illustrates the assay in a panel of DIC images of *Chlamydo-*

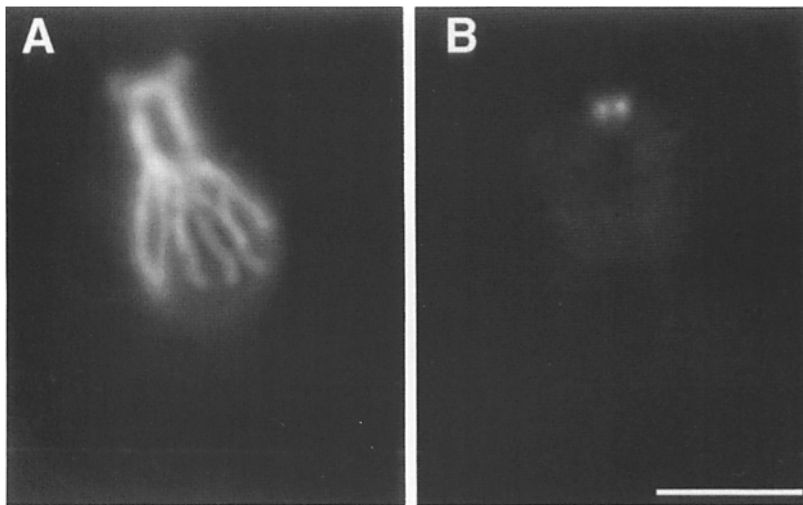


Figure 6. EDTA extraction of cell models disrupts centrin fiber distribution. Indirect immunofluorescence using anti-centrin monoclonal antibody 20H5. (A) Control detergent-extracted model shows the normal distribution of centrin fibers in *Chlamydomonas*. Centrin is localized in the region of the transition zones, distal fiber, descending fibers and fimbria. (B) Most of the centrin has disassembled in the EDTA-extracted model, leaving only two spots at sites where the distal fiber normally links the two adjacent basal bodies. Bar, 3.0 μm .

monas cells before (Fig. 7, A and C) and after (Fig. 7, B and D) treatment with 1 μM free Ca^{2+} . Control cells, not treated with antibody, excise their flagella normally after 1 μM Ca^{2+} treatment (see Fig. 1). However, flagellar excision was inhibited in samples that were pretreated with either of three centrin-specific monoclonal antibodies, 20H5, 17E10, and 11B2 (Fig. 7 A and B, and C and D, respectively, and Table I). Note, that after subsequent Ca^{2+} challenge, the flagella show a characteristic bend at a point about midway

along their length in the antibody treated samples (Fig. 7, B and D, arrows). This flagellar bend may be related to the calcium-induced curvature of detergent-extracted rat sperm described by Lindemann and co-workers (Lindemann et al., 1991; Lindemann and Goltz, 1988). Table I shows quantitative data on the effects of treatment of cell models with three different anti-centrin monoclonal antibody preparations (20H5, 11B2, and 17E10), and four control preparations (no antibody treatment, anti- α -tubulin treatment, anti- β -tubulin treatment, and anti-calmodulin treatment), before and after Ca^{2+} challenge to induce flagellar excision. Each experimental treatment using different anti-centrin monoclonal antibodies effectively blocked Ca^{2+} -induced flagellar exci-

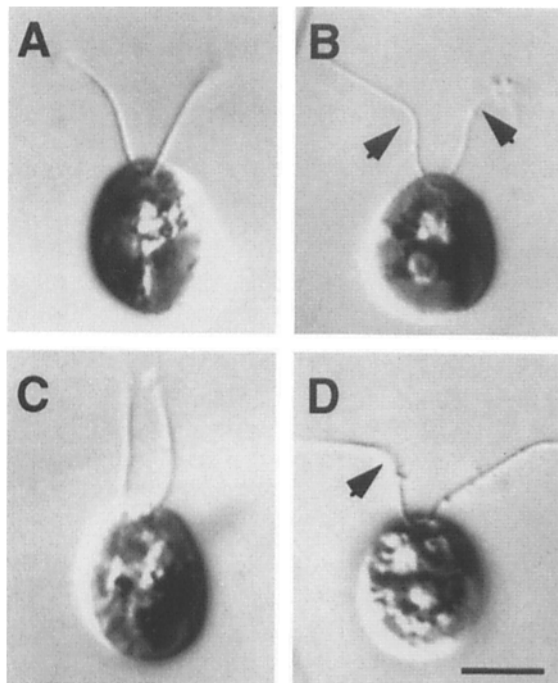


Figure 7. Anti-centrin monoclonal antibodies neutralize flagellar excision competence in detergent-extracted models. Cell models treated with anti-centrin monoclonal antibodies before (A and C) and after (B and D) calcium treatment. Note that the cells in B and D fail to excise their flagella in response to calcium treatment, yet, both display a calcium-induced bend in their flagella (arrows). (A and B) Anti-centrin monoclonal antibody 20H5, (C and D) Anti-centrin monoclonal antibody 17E10.

Table I. Flagellar Excision in *Chlamydomonas* Cell Models and Living Cells: The Effects of Neutralizing Antibodies, EDTA Extraction, and Centrin Mutants

Treatment	Cells with flagella/total cells		Flagellar excision competence
	minus Ca^{2+}	plus Ca^{2+} (1 μM final)	% excision after Ca^{2+} treatment
<i>Detergent-treated EGTA models</i>			
Control	83/90	5/90	94
EDTA Extraction	214/250	207/250 (2 mM Ca^{2+})	03
<i>Monoclonal antibody</i>			
anti-centrin 17E10	103/110	88/110	15
anti-centrin 20H5	120/130	109/130	09
anti-centrin 11B2	90/90	81/90	10
anti- α -tubulin T9026	102/110	9/110	91
anti- β -tubulin T4026	28/30	3/30	89
anti-CaM cocktail	29/30	2/30	93
<i>vfl-2 mutant strain 416</i>	68/110	57/110	12
			% excision after treatment
<i>Living cells no external shear force</i>	<i>pre-shock</i>	<i>pH shock</i>	
Control (wild type)	123/129	27/130	78
<i>vfl-2 mutant</i>	113/127	114/134	04
	<i>pre-shock</i>	<i>dibucaine</i>	
<i>vfl-2 mutant</i>	113/127	106/134	11

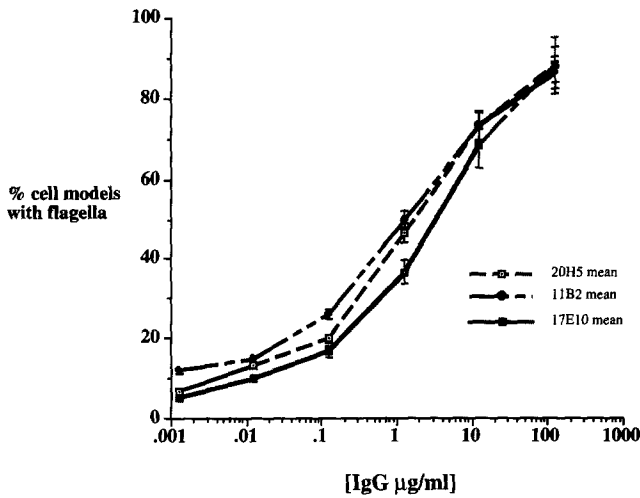


Figure 8. Dose-response curves for anti-centrin monoclonal antibody inhibition of flagellar excision in *Chlamydomonas* detergent-extracted cell models. Three separate IgG fractions of anti-centrin monoclonal antibodies (20H5, 11B2, and 17E10) were used at the concentrations indicated. The mean value for 30 determinations in each of three separate experiments is shown with standard error bars.

sion, whereas, neither anti-tubulin nor anti-calmodulin monoclonal antibodies had any inhibitory effect. A dose-response curve using each of the anti-centrin monoclonal antibodies, to inhibit Ca^{2+} -induced flagellar excision in detergent-extracted cell models, is shown in Fig. 8. These data show inhibition of flagellar excision and demonstrate a constant antigen titration curve, which is typical for neutralizing

antibodies in general (Richmond, 1977). In these experiments the half-maximum inhibition of flagellar excision was achieved at approximately $1 \mu\text{g/ml}$ of antibody for each treatment.

Short-term treatment of cell models with anti-centrin monoclonal antibodies inactivates the contractile properties of transition zone stellate fibers. Fig. 9 *A* illustrates a detergent-extracted cell model that was treated for 15 min with an anti-centrin monoclonal antibody, 20H5, and then processed for indirect immunofluorescence microscopy with FITC-conjugated secondary antibodies. This cell shows bright staining of the flagellar transition zone region, as two bright spots at the base of the flagella, and partial staining of the distal fiber and descending fibers. Similarly treated cells fail to excise their flagella when subjected to millimolar levels of free calcium (see above). An example of a transition zone treated with anti-centrin antibody, 20H5, that was subsequently treated with Ca^{2+} prior to fixation and preparation for electron microscopy, is illustrated in Fig. 9 *B*. Note that the stellate fibers have not contracted as they would in control samples that were not incubated in anti-centrin antibody (compare to Fig. 2 *F*). A sample prepared in parallel for postembedding labeling to the one shown in Fig. 9 *B* and subsequently incubated in colloidal gold-conjugated secondary antibody, is shown in Fig. 9 (*C* and *D*); the stellate fibers (Fig. 9, *C* and *D*) and distal fiber (Fig. 9 *C*) label densely with the gold probe (Fig. 9, *C* and *D*). Longer antibody incubation results in labeling of the entire NBBC complex and prevents their calcium-induced contraction and consequent nuclear movement (Salisbury et al., 1987; and data not shown) further demonstrating the neutralizing capacity of these anti-centrin antibodies. These results, to-

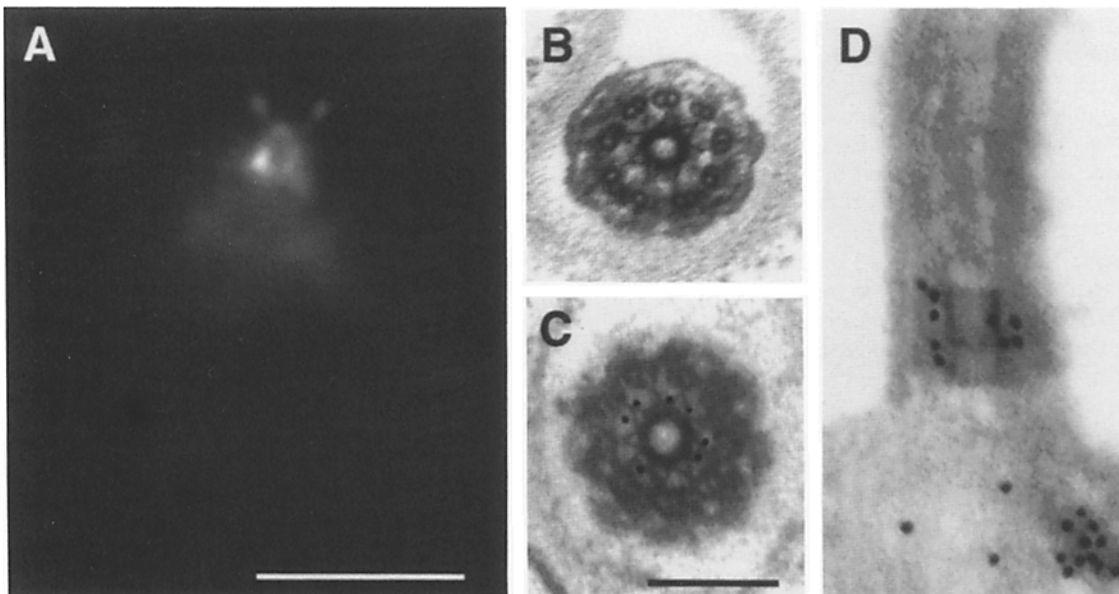


Figure 9. Brief treatment of cell models with anti-centrin monoclonal antibody, 20H5, labels the transition zone and effectively blocks calcium-induced contraction of stellate fibers. (*A*) Indirect immunofluorescence of a cell model after a 15-min treatment with monoclonal antibody 20H5 and a subsequent $1 \mu\text{M}$ calcium challenge. Note that the site of the transition zone is labeled as well as a portion of the distal fiber and descending fibers. (*B*) Electron micrograph of a cross-section of the transition zone of a cell treated as in *A*; note that the stellate fibers have not contracted. (*C* and *D*) Electron micrographs of a cross- and longitudinal section of the transition zone and subsequently processed for post-sectioning immunogold labeling; note that the stellate fibers of the transition zone decorate with anti-centrin monoclonal antibody 20H5 and secondary gold label. Bar, $0.2 \mu\text{m}$.

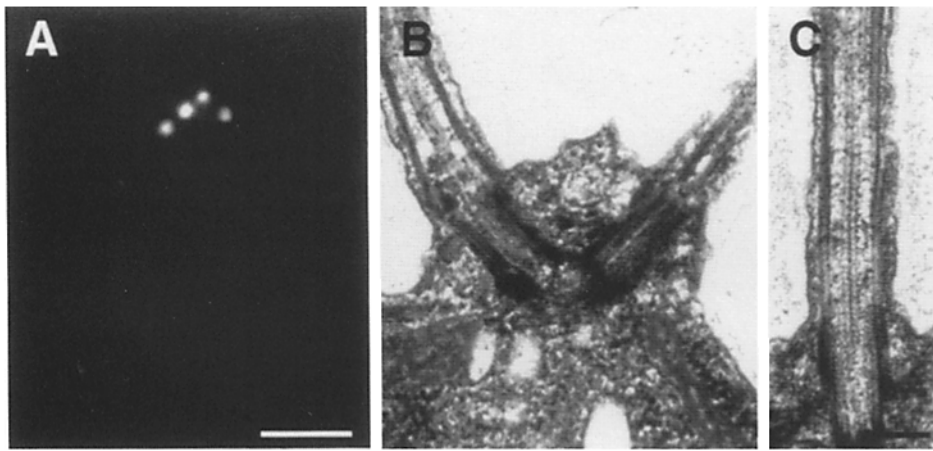


Figure 10. The variable flagellar mutant *vfl-2* has abnormal centrin distribution and defective transition zone structure. (A) Indirect immunofluorescence image of a *vfl-2* cell labeled with anti-centrin monoclonal antibody 20H5. Note that anti-centrin staining is restricted to four spots in this cell that probably mark the outer wall of basal bodies. (B and C) Electron micrographs of the flagellar bases in two *vfl-2* cells. These cells show abnormal transition zone structure as well as defective distal and descending fibers. Bars: (A) 3.0 μm ; (C) 0.2 μm .

gether with the neutralizing anti-centrin antibody treatments presented above, strongly implicate an active role for centrin in the flagellar excision process.

Centrin Mutants Are Defective in the Flagellar Excision Mechanism

Recently, Jarvik and co-workers (Taillon et al., 1992) demonstrated that a single point mutation in the *Chlamydomonas* centrin gene is the underlying defect in the variable flagellar mutant *vfl-2*. This mutation results in a Glu to Lys substitution at amino acid position 101 in the centrin amino acid sequence. *Vfl-2* cells fail to assemble the typical centrin-based fiber system, resulting in an inappropriate separation of basal bodies (precocious separation) during the cell cycle and, thus, giving rise to the variable flagellar number phenotype associated with the *vfl-2* locus (Taillon et al., 1992). The *Chlamydomonas* variable flagellar number mutant *vfl-2* also fails to assemble transition zone stellate fibers (Jarvik et al., 1991; Taillon et al., 1992; and this study). The *vfl-2* mutant, therefore, provides a unique system in which the effect of a specific defect in centrin on the flagellar excision process could be investigated. First, we confirmed that *vfl-2* cells (strain 416) show an abnormal centrin-based fiber distribution by immunofluorescence microscopy (Fig. 10 A). None of the cells in a given *vfl-2* population have NBBC centrin fibers: the descending fibers and fimbria are missing. Also, *vfl-2* cells show no anti-centrin staining of the distal fiber and "horns" located in the region of the transition zone. However, most cells show a "spot" of centrin localized near the base of each flagellum, in a position that we interpret to be at the lateral margin of the basal body wall. Electron microscopy confirms that the centrin-based fibers normally present in wild type cells are lacking in the *vfl-2* mutant (Fig. 10, B and C): the transition zone appears abnormal, typically lacking both the central cylinders and stellate fibers, and the distal fiber which normally connects adjacent basal bodies and the descending fibers. Our studies indicate that flagellar excision is normally mediated by a contraction of the transition zone stellate fibers. However, mechanical shear load from vortexing or stirring of the preparation may result in sufficient shear force to rupture axonemal doublet (see above). Therefore, we established a system wherein minimal mechanical shear force would be experienced by the cells during

a Ca^{2+} -induced flagellar excision assay using detergent-extracted cell models and a microscope slide perfusion system. Cell models were made by perfusing the slide with the detergent-extraction solution and, subsequently, a 1 μM Ca^{2+} challenge was introduced by further perfusion with Ca^{2+} -containing buffers. All experimental manipulations were monitored by video microscopy and recorded on video tape. Under these conditions, wild type cell models effectively excised their flagella within one to two seconds as the Ca^{2+} wave (as monitored by the flow of debris in the field) was seen to pass the cells (Fig. 11, A and B). In contrast, *vfl-2* models failed to excise their flagella, even when the free Ca^{2+} levels were raised to micromolar levels and were allowed to incubate for several minutes (Fig. 11, C and D). Quantitative data on these experiments is shown in Table I. We repeated these experiments using living cells and pH shock or dibucaine treatment to induce flagella excision. We found that wild type cells excise their flagella normally (Fig. 11, E and F), while the *vfl-2* mutants did not (Fig. 11, G and H, and see Table I). Living *vfl-2* cells, did, however, frequently respond to the pH shock by resorbing their flagella within 30 s to 5 min resulting in membrane balloons ("Mickey Mouse ears") at the sites of the flagellar stumps. We conclude, therefore, that *vfl-2* cells are, indeed, defective in the normal flagellar excision mechanism. This is strong genetic and experimental evidence for an essential role for centrin-based fibers of the transition zone in the excision process.

Discussion

Flagellar Excision Is Mediated by a Contraction of Centrin-based Stellate Fibers of the Transition Zone

We have developed a detergent-extracted cell model of *C. reinhardtii* which retains the ability to excise flagella. These cell models are amenable to experimental manipulation of the cellular machinery involved in the flagellar excision process. Simply raising the free Ca^{2+} levels to above 10^{-6} M is sufficient to induce flagellar excision in these models. Calcium treatment alone is necessary and sufficient to induce flagellar excision, while Mg^{2+} is required to maintain the structural integrity of the excision machinery. No other diva-

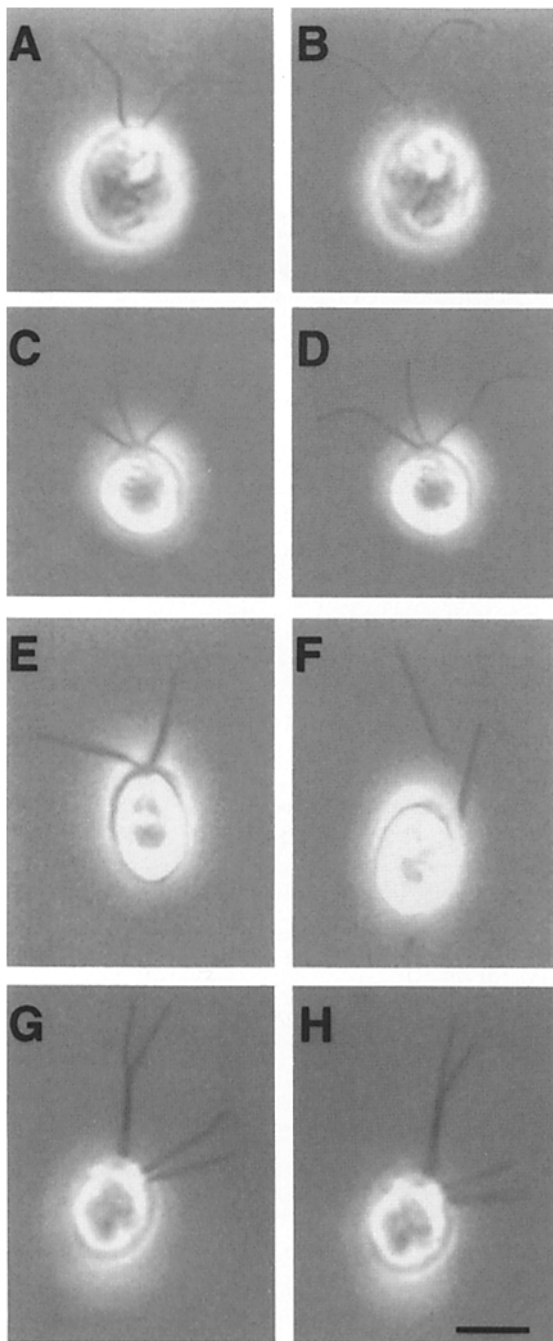


Figure 11. Living wild type cells and cell models excise their flagella, while living *vfl-2* mutant cells and cell models fail to do so. The frames were taken from video tape records of individual cells on a flow through microscope slide. Wild type (*A* and *B*) and *vfl-2* (*C* and *D*) detergent-extracted cell models before (*A* and *C*) and after (*B* and *D*) 1 μ M calcium challenge. The control cell model excised its flagella in response to the calcium challenge (*B*), while the *vfl-2* mutant (*D*) failed to do so. Wild type (*E* and *F*) and *vfl-2* (*G* and *H*) living cells before (*E* and *G*) and after (*F* and *H*) pH shock. The control cell excised its flagella (*F*) in response to pH shock, while the *vfl-2* mutant (*H*) failed to do so. Bar, 5.0 μ m.

lent cations tested (i.e., Cd, La, Li, Mn, Fe, Zn) cause flagellar excision when used at physiological concentrations. Studies at the electron microscopic level demonstrate that cell models excise their flagella in a manner similar to that observed in living cells: contraction of transition zone stellate fibers draws the axonemal microtubules inward and subjects them to shear and torsional load forces which we suggest leads to a physical severing of the microtubules just distal to the transition zone structure.

Earlier, we proposed the hypothesis that flagellar excision in *C. reinhardtii* is mediated by calcium-induced contraction of centrin-based fibers of the transition zone stellate structure (Sanders and Salisbury, 1989). In summary, here we present structural, physiological, immunological, and genetic evidence which supports our original hypothesis. This evidence is as follows: (a) Flagellar excision is rapid and is induced by Ca^{2+} and Ca^{2+} alone, both features are hallmarks of centrin-based contractile fiber systems (Bazin et al., 1990; Melkonian, 1989; Salisbury, 1982; Salisbury, 1983). (b) Centrin is localized at the transition zone stellate fibers which contract at the time of flagellar excision (Sanders and Salisbury, 1989). (c) Extraction of *Chlamydomonas* cell models, using EDTA-containing buffers, disrupts transition zone stellate structure morphology, extracts centrin from centrin-based fiber system, and renders the transition zone unable to excise flagella in response to treatment with elevated free Ca^{2+} levels. (d) Monoclonal anti-centrin antibodies block contraction of the transition zone stellate fibers and effectively neutralize flagellar excision. Finally (e), mutants (*vfl-2*) defective in centrin content and distribution, fail to excise their flagella, in the absence of external shear forces, when living cells are subjected to pH shock, or when cell models experience elevated levels of free calcium. In addition, our studies and those of others (Blum, 1971; Jarvik et al., 1991; Lewin et al., 1985) and observations concerning the rescue of flagellar excision competence in another *Chlamydomonas* excision-defective mutant, *fa-1* (Lewin et al., 1983; Sanders, M. A., and J. L. Salisbury, to be published elsewhere), also suggest that a "weak point" in the flagellar axoneme exists just distal to the transition zone which may predispose microtubules for local severing. We conclude that flagellar excision is mediated by a Ca^{2+} -induced contraction of centrin-based fibers of the transition zone and that centrin is essential to this process.

Two reports from Jarvik and co-workers (Jarvik et al., 1991; Wright et al., 1989) suggested that a *Chlamydomonas* mutant, *vfl-2*, which is defective in centrin-based fiber structures and in centrin itself, was able to excise flagella after treatment with the local anesthetic dibucaine. We assayed the ability of *vfl-2* cells to excise their flagella when external shear forces were first minimized. Our experiments clearly demonstrate that *vfl-2* cells fail to excise their flagella following pH shock of living cells or treatment of detergent-extracted cell models with elevated free Ca^{2+} . In addition, living *vfl-2* cells rarely excise their flagella when treated with dibucaine in the absence of external shear forces: more commonly the cells will cease swimming and rapid resorption of the axonemes occurs. However, when even mild shear is applied (e.g., brief vortexing or stirring), dibucaine-treated cells will shed their flagella. We conclude, therefore, that *vfl-2* mutant cells are, indeed, defective in the normal exci-

sion mechanism and that external shear may substitute for the normal cellular mechanism.

Microtubule Severing in Other Cell Types

At the present time, the functional role of centrin in higher eukaryotic centrosomes and mitotic spindle poles remains unknown; however, the high degree of conservation of centrin's molecular structure across diverse species (Errabolu et al., 1994) suggests that functional properties may be conserved as well. Consequently, centrin-based severing of microtubules may provide a mechanism for releasing a subset of microtubules anchored at the centrosome in higher eukaryotes. Microtubules have been demonstrated to detach from centrosomes in cultured epithelial cells and neurons and to subsequently depolymerize (McBeath and Fujiwara, 1990) or to be transported to distal regions within axons (Baas and Joshi, 1992; Yu et al., 1993). In such cells, newly generated microtubule fragments may be predisposed to unique behavior, including rapid disassembly through the dynamic instability pathway first described by Mitchison and Kirschner (1984). Release of microtubules from the centrosome would also allow for new polymerization from nucleation sites, and/or allow for the transport of free microtubules away from their original site of nucleation. Microtubule severing may, likewise, contribute to microtubule dynamics seen at the onset of mitosis where microtubule turnover increases and microtubule half-life decreases dramatically (Salmon et al., 1988; Saxton et al., 1984; Snyder and McIntosh, 1975). Significant progress has been made recently toward the identification of microtubule-severing factors from the cytoplasm of higher eukaryotic cells. Vale and co-workers reported a microtubule severing factor from mitotically activated *Xenopus* oocytes (Hollenbeck et al., 1991; Vale, 1991; McNally and Vale, 1993), and Shiina and co-workers have identified a novel homo-oligomeric protein of 56 kD from oocytes which has MPF-dependent microtubule severing activity (Shiina et al., 1992). The possible relationship between these microtubule severing factors and centrin-mediated microtubule severing is currently not clear. It is possible, however, that redundant mechanisms exist for microtubule severing, or that regulatory or accessory properties may link centrin's involvement to the activities described by Vale and Shiina and co-workers (1992). In either case, the observations that we have presented strongly implicate an essential role for centrin in the process of microtubule severing during flagellar excision in *Chlamydomonas*. These observations may ultimately prove relevant to microtubule stability in higher eukaryotic cells.

We thank Drs. A. Baron, W. Lingle, N. Maihle, W. Lutz, M. Uzawa, and U. Wolfrum for helpful comments on the manuscript and Dr. J. Jarvik for cultures of the *Chlamydomonas vfl-2* mutant. Phyllis J. Fisher's assistance with the free calcium calculations and fluorometric determinations is greatly appreciated.

This work was supported by a grant from the National Institutes of Health (GM 35258) to J. L. Salisbury.

Received for publication 4 October 1993, and in revised form 10 December 1993.

References

Baas, P., and H. Joshi. 1992. γ -tubulin distribution in the neuron: implications for the origins of neuritic microtubules. *J. Cell Biol.* 119:171-178.

- Bazinot, C., A. Baron, and J. Salisbury. 1990. Centrin: A calcium-binding protein associated with centrosomes. In *Stimulus Response Coupling: The Role of Intracellular Calcium-binding Proteins*. V. L. Smith and J. R. Dedman, editors. 39-56.
- Blum, J. J. 1971. Existence of a breaking point in cilia and flagella. *J. Theor. Biol.* 33:257-263.
- Coling, D., and J. Salisbury. 1992. Characterization of the calcium-binding contractile protein centrin from *Tetraselmis striata* (Pleurostrophyceae). *J. Protozool.* 39:385-391.
- Errabolu, R., M. Sanders, and J. Salisbury. 1994. Cloning of a cDNA encoding human Centrin, a calcium-binding protein of centrosomes and mitotic spindle poles. *J. Cell Sci.* In press.
- Grynkiwicz, G., M. Poenic, and R. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
- Harris, E. H. 1989. *The Chlamydomonas source book: a comprehensive guide to biology and laboratory use*. San Diego, California, Academic press. pp. 780.
- Hollenbeck, P., and W. Dentler. 1991. Microtubules get the chop. *Nature (Lond.)* 350:378-379.
- Huang, B., A. Mengersen, and V. D. Lee. 1988. Molecular cloning of cDNA for caltractin, a basal body-associated Ca^{2+} -binding protein: homology in its protein sequence with calmodulin and the yeast *cdc31* gene product. *J. Cell Biol.* 107:133-140.
- Hulen, D., A. Baron, J. Salisbury, and M. Clarke. 1991. Production and specificity of monoclonal antibodies against calmodulin from *Dictyostelium discoideum*. *Cell Motil. Cytoskeleton.* 18:113-122.
- Hutner, S. H., L. Provasoli, A. Schatz, and C. P. Haskins. 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. *Proc. Am. Phil. Soc.* 94:152-170.
- Jarvik, J., and J. Suhan. 1991. The role of the flagellar transition region: inferences from the analysis of a *Chlamydomonas* mutant with defective transition region structures. *J. Cell Sci.* 99:731-740.
- Lang, N. 1963. An additional ultrastructural component of flagella. *J. Cell Biol.* 19:631-634.
- Lewin, R. A., and C. Burrascano. 1983. Another new kind of *Chlamydomonas* mutant with impaired flagellar autonomy. *Experientia.* 39:1397-1398.
- Lewin, R. A., and K. W. Lee. 1985. Autonomy of algal flagella: electron microscope studies of *Chlamydomonas* (Chlorophyceae) and *Tetraselmis* (Prasinophyceae). *Phycologia.* 24:311-316.
- Lindemann, C., T. Gardner, E. Westbrook, and K. Kanous. 1991. The calcium-induced curvature reversal of rat sperm is potentiated by cAMP and inhibited by anti-calmodulin. *Cell Motil. Cytoskeleton.* 20:316-324.
- Lindemann, C., and J. Goltz. 1988. Calcium regulation of flagellar curvature and swimming pattern in Triton X-100-extracted rat sperm. *Cell Motil. Cytoskeleton.* 10:420-431.
- Manton, I. 1964. The possible significance of some details of flagellar bases in plants. *J. R. Microsc. Soc.* 82:279-285.
- McBeath, E., and K. Fujiwara. 1990. Microtubule detachment from the microtubule-organizing center as a key event in the complete turnover of microtubules in cells. *Eur. J. Cell Biol.* 52:1-16.
- McDonald, K. 1984. Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. *J. Ultrastruct. Res.* 86:107-118.
- McNally, F. J., and R. Vale. 1993. Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell.* 75:419-429.
- Melkonian, M. 1989. Centrin-mediated motility: A novel cell motility mechanism in eukaryotic cells. *Botanica Acta.* 102:3-4.
- Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature (Lond.)* 312:237-242.
- Moncrief, N., R. Kretsinger, and M. Goodman. 1990. Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. *J. Mol. Evol.* 30:522-562.
- Potter, J., and J. Gergely. 1975. The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar ATPase. *J. Biol. Chem.* 250:4628-4633.
- Richmond, M. H. 1977. Enzyme/antiserum interactions of β -lactamases. In *Immunochemistry of Enzymes and Their Antibodies*. M. R. J. Salton, editor. John Wiley & Sons, New York. 29-55.
- Ringo, D. L. 1967. Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. *J. Cell Biol.* 33:543-571.
- Rodriguez, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. *Virology.* 12:316-317.
- Sager, R., and S. Granick. 1953. Nutritional studies with *C. reinhardtii*. *Annu. NY Acad. Sci.* 56:831-838.
- Salisbury, J. L. 1982. Calcium-sequestering vesicles and contractile flagellar roots. *J. Cell Sci.* 58:433-443.
- Salisbury, J. L. 1983. Contractile flagellar roots: the role of calcium. *J. Submicrosc. Cytol.* 15:105-110.
- Salisbury, J. L., M. Sanders, and L. Harpst. 1987. Flagellar root contraction and nuclear movement during flagellar regeneration in *C. reinhardtii*. *J. Cell Biol.* 105:1799-1805.
- Salisbury, J., A. Baron, and M. Sanders. 1988. The centrin-based cytoskeleton of *C. reinhardtii*: distribution in interphase and mitotic cells. *J. Cell Biol.* 107:635-641.

- Salmon, E., W. Saxton, R. J. Leslie, M. Karow, and J. McIntosh. 1988. Spindle microtubule dynamics in sea urchin embryos: Analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J. Cell Biol.* 99:2165-2174.
- Sanders, M., and J. Salisbury. 1989. Centrin-mediated microtubule severing during flagellar excision in *C. reinhardtii*. *J. Cell Biol.* 108:1751-1760.
- Saxton, W., D. Stemple, R. Leslie, E. Salmon, M. Zavortink, and J. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175-2186.
- Shiina, N., Y. Gotoh, and E. Nishida. 1992. A novel homo-oligomeric protein responsible for an MPF-dependent microtubule severing activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4723-4731.
- Snyder, J., and J. McIntosh. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* 67:744-760.
- Taillon, B., S. Adler, J. Suhan, and J. Jarvik. 1992. Mutational analysis of centrin: an EF-hand protein associated with three distinct contractile fibers in the basal body apparatus of *Chlamydomonas*. *J. Cell Biol.* 119:1613-1624.
- Vale, R. 1991. Severing of stable microtubules by a mitotically activated protein in *Xenopus* egg extracts. *Cell.* 64:827-839.
- Wright, R., S. Adler, J. Sanier, and J. Jarvik. 1989. The nucleus-basal body connector of *C. reinhardtii*: Evidence for a role in basal body segregation and against essential roles in mitosis or in determining cell polarity. *Cell Motil. Cytoskeleton.* 14:516-526.
- Wright, R., J. Salisbury, and J. Jarvik. 1985. A nucleus-basal body connector in *C. reinhardtii* that may function in basal body localization or segregation. *J. Cell Biol.* 101:1903-1912.
- Yu, W., E. Centoze, F. Ahmad, and P. Bass. 1993. Microtubule nucleation and release from neuronal centrosomes. *J. Cell Biol.* 122:349-359.