The Inner Dynein Arms I2 Interact with a "Dynein Regulatory Complex" in *Chlamydomonas* Flagella

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Abstract. We provide indirect evidence that six axonemal proteins here referred to as "dynein regulatory complex" (*drc*) are located in close proximity with the inner dynein arms I2 and I3. Subsets of *drc* subunits are missing from five second-site suppressors, *pf*2, *pf*3, sup$_{pf}$3, sup$_{pf}$4, and sup$_{pf}$5, that restore flagellar motility but not radial spoke structure of radial spoke mutants. The absence of *drc* components is correlated with a deficiency of all four heavy chains of inner arms I2 and I3 from axonemes of suppressors *pf*2, *pf*3, sup$_{pf}$3, and sup$_{pf}$5. Similarly, inner arm subunits actin, p28, and caltractin/centrin, or subsets of them, are deficient in *pf*2, *pf*3, and sup$_{pf}$5. Recombinant strains carrying one of the mutations *pf*2, *pf*3, or sup$_{pf}$5 and the inner arm mutation *ida*4 are more defective for I2 inner arm heavy chains than the parent strains. This evidence indicates that at least one subunit of the *drc* affects the assembly of and interacts with the inner arms I2.

THE movement of *Chlamydomonas* flagella is generated by at least six types of dynein including the outer dynein arm, the inner dynein arm I1, and two types of inner dynein I2 and I3 (25). Outer dynein arm and inner dynein arm I1 are formed by three and two distinct heavy chains, respectively, and consist of the same subunits along the axoneme (14, 27). In contrast, the inner dynein I2 and I3 are each formed by two identical heavy chains and consist of different heavy chains depending on their location along the axoneme (25). Each dynein also comprises a distinct set of intermediate and light chains characterized by molecular weight ranging from 140,000 to 8,000 (27, 35).

Two light chains, which are associated with inner arms I2 and I3, were referred to as actin and caltractin/centrin, respectively, on the basis of physical and chemical properties of the two proteins (13, 22, 29). The role of actin and caltractin/centrin in other cytoskeletal structures is to transmit tensile stress and provide Ca^{++}-dependent regulatory mechanisms, respectively (28, 30). In contrast, the function of the same proteins within the inner arm structure has not been identified.

To investigate a possible function of actin and caltractin/centrin in the regulation of inner arm movement, we intended to determine whether the ATPase activity or stability of inner arms I2 and I3 is Ca^{++} sensitive in vitro. Moreover, we aimed to identify other axonemal proteins that interact with actin and caltractin/centrin in vivo. To reach these goals we analyzed complexes formed by actin, caltractin/centrin, and I2 and I3 inner arm heavy chains that were isolated from the outer dynein mutant *pf*28 (18). We also quantitatively analyzed I2 and I3 inner arm deficiency in inner arm defective mutants (9, 16) and suppressors of flagellar paralysis that generate bending of flagella similar to those of inner arm mutants (3, 11). These second-site suppressors are missing different subsets of six axonemal polypeptides and suppress flagellar paralysis of radial spoke mutants without restoring the radial spoke structure (11).

We found that the suppressors are defective for I2 and I3 inner arm subunits to different extents. We also found that the polypeptides missing from the suppressors interact at least with inner arms I2 in wild-type strains. Therefore, we referred to these polypeptides as "dynein regulatory complex" (*drc*).[1] The absence of *drc* components combined with the deficiency of inner arms I2 generate an extensive deficiency of I2 inner arms in recombinant strains carrying both suppressor and inner arm mutations. This and other evidence described here suggests that actin and caltractin/centrin are associated with I2 and I3 inner arms, probably forming a linkage between the *drc* and each of I2 and I3 inner arms.

Materials and Methods

Strains and Culture of Chlamydomonas Cells

Chlamydomonas strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC) and the laboratory of David Luck (Rockefeller University, New York). Nomenclature and phenotype of the strains used for analysis of axonemal components are listed in Table I.

The mutant sup$_{pf}$5 was characterized at the beginning of this study and was isolated as a motility mutant following a mutagenesis of the wild-type strain 137c with nitrosoguanidine (8). It has slower motion than a wild-type strain and lacks four of the six axonemal polypeptides that compose the *drc* (Fig. 1). These four polypeptides are identical to those missing in the mutant *pf*3 (Table II). The motility of the mutant *pf*3, however, is slower than that

1. *Abbreviation used in this paper*: *drc*, dynein regulatory complex.

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Table I. Chlamydomonas Strains Used for Analysis of Axonemal Components

Strain	Motility of flagella	Flagellar length*	Inner arm heavy chain deficiency	Linkage group of defective gene	Defective system in the axoneme
		μm			
137 (wild-type)		12.0 (1.5)			
*pf*28 (oda 2)	Slow motion	9.6 (1.1)		XI	outer arms
*pf*22	None	4.0 (1.2)	2, 3′	I	inner arm I2, I3
*ida*4	Slow motion	10.5 (1.5)	2′, 2	XII	inner arm I2
*pf*23	None	4.0 (1.0)	1α, 1β, 2′, 2, 3′	XI	inner arm I2, I3
*pf*2	Slow motion	8.9 (1.3)		XI	*drc*
*pf*3	Slow motion	9.1 (1.4)		VIII	*drc*
$sup_{pf}3$	Wild-type like motion	12.6 (1.6)			*drc*
$sup_{pf}4$	Wild-type like motion	12.2 (1.5)			*drc*
$sup_{pf}5$	Slow motion	11.0 (1.3)			*drc*
$ida4_{pf}2$	Slow motion	7.6 (0.6)	2′, 2, 3′		*drc* inner arm I2, I3
$ida4_{pf}3$	Slow motion	7.5 (0.8)	2′, 2, 3′		*drc* inner arm I2, I3
*ida*4$sup_{pf}3$	Slow motion	10.0 (1.3)	2′, 2, 3′		*drc* inner arm I2, I3
*ida*4$sup_{pf}4$	Slow motion	11.1 (1.3)	2′, 2		*drc* inner arm I2
*ida*4$sup_{pf}5$	Slow motion	9.1 (1.1)	2′, 2		*drc* inner arm I2

* Flagellar length is expressed as an average of 15 determinations. Standard deviation is reported in parenthesis.

of the mutant $sup_{pf}5$. The analysis of tetrads from a cross between the mutant *pf*3 and $sup_{pf}5$ reveals that they are not alleles; ratios of parental ditype/nonparental ditype/tetratype are 15:3:18.

All recombinant strains carrying the mutation $sup_{pf}5$ in combination with each of radial spoke mutations *pf*1 and *pf*14 (10) and central pair mutations *pf*15 and *pf*18 (1) have motile flagella. Therefore, the mutation $sup_{pf}5$ suppresses flagellar paralysis of radial spoke and central pair mutants.

The mutations $sup_{pf}3$, $sup_{pf}4$, *pf*2, and *pf*3 suppress flagellar paralysis of the mutants *pf*1 and *pf*14 but not the paralysis of the mutants *pf*15 and *pf*18 (11). If the same criterion, namely observation of flagellar beating, was adopted in the analysis of suppression by the mutants $sup_{pf}3$, $sup_{pf}4$, *pf*2, *pf*3 and $sup_{pf}5$, then the range of suppression of the mutant $sup_{pf}5$ is wider than that of the mutants $sup_{pf}3$, $sup_{pf}4$, *pf*2 and *pf*3.

Each of the recombinant strains was isolated from nonparental ditype tetrads with the exception of *pf*2*ida*4 that was isolated from a tetratype

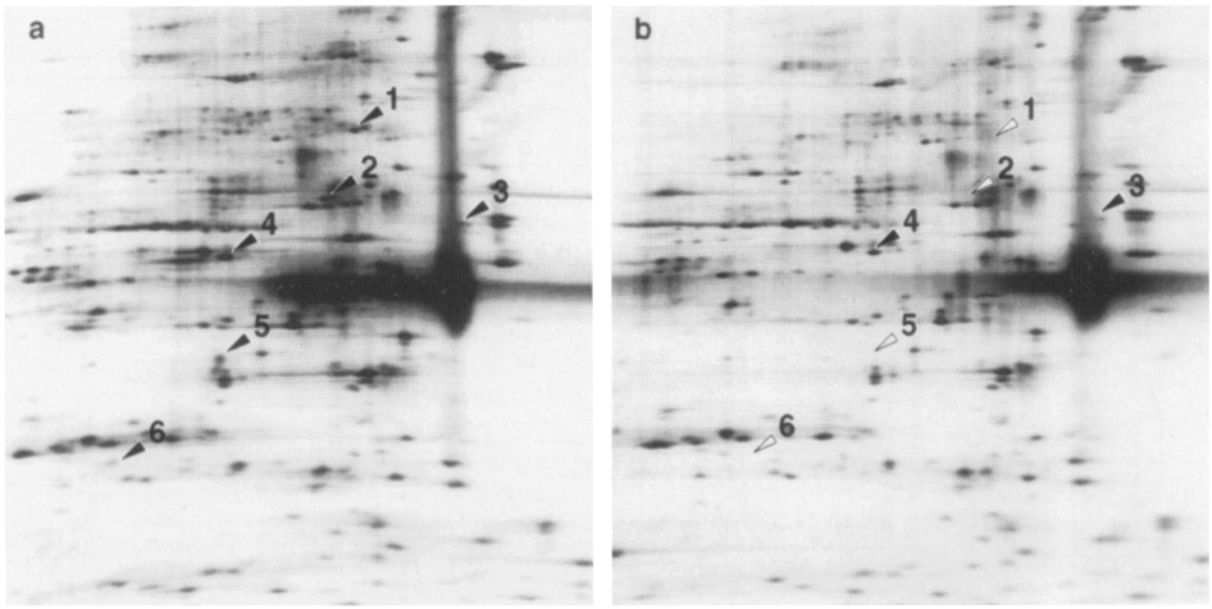

Figure 1. Autoradiograms of ^{35}S-labeled axonemal polypeptides resolved by two-dimensional electrophoresis. Only portions of the original maps resolving polypeptides in the 130,000–15,000 molecular weight range are shown. The gels are oriented with basic polypeptides on the left. (*a*) Wild-type polypeptides. Solid triangles and numbers indicate single or group of polypeptides. Subsets of them are missing in the mutants *pf*2, *pf*3, $sup_{pf}3$ $sup_{pf}4$, and $sup_{pf}5$ (Table II). (*b*) $sup_{pf}5$ polypeptides. Open triangles indicate the positions of wild-type polypeptides missing in $sup_{pf}5$. Other differences between these two maps were not indicated because they were not reproduced in every experiment.

Table II. Deficiency of Axonemal Polypeptides in the Suppressors

$sup_{pf}5$*	Component	Mr × 10^{-3}	*pf*2‡	*pf*3‡	$sup_{pf}3$‡	$sup_{pf}4$‡
−§	1	108	+	−	+	+
−	2	83	+	−	+	+
+	3	65	−	+	+	+
+	4	60	−	+	−	+
−§	5	40	−	−	+	−
−	6	29	−	−	+	−

* This study.
‡ Huang et al. (1982).
§ These components were resolved in two spots differing in apparent molecular weight and isoelectric point.

tetrad. Tetrads were prepared by standard methods (5). Each recombinant was defective for all axonemal polypeptides that are defective in the parent strains.

Cell culture and labeling with [^{35}S]sulfuric acid was performed on solid medium (25).

Electrophoresis in Polyacrylamide Gels

Electrophoresis of dynein heavy chains was performed as described (27). A discontinuous slab gel composed of 3.2% polyacrylamide stacking layer and a 3.6–5% polyacrylamide resolving layer was used. The slab did not contain urea.

Two-dimensional electrophoresis of axonemal proteins was performed as described (26). Samples were applied at the anode of a 12 × 14 × 0.075 cm gel containing ampholines. Nonequilibrium pH gradient electrophoresis was run for 17 h at 1.8 mA. Polypeptide maps were obtained through the application of a 0.6 × 14 cm strip of the ampholine-gel on a slab gel containing 4–11% polyacrylamide (20).

Quantitative Analysis of Axonemal Components

Quantitation of axonemal components was performed directly with image data obtained from the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The PhosphorImager is 10 times more sensitive than an X-ray film and gives a linear response in a range of 10:100,000 d.p.m.

Other Procedures

Determination of flagellar length (25), preparation of flagella through the exposure of cells to pH 4 (25), preparation of dynein fractions by exposure of the axoneme to high ionic strength (24), sedimentation of dynein fractions in sucrose gradient (27), isolation of inner arm heavy chains on hydroxyapatite column (23), assay of ATPase activity (24), and immunoblots (21) were performed as described previously.

Nomenclature

The gene encoding *Chlamydomonas* caltractin (12) is identical to that encoding *Chlamydomonas* centrin with the exception of one conservative substitution (J. L. Salisbury, Mayo Clinic Foundation, Rochester, MN, personal communication). Therefore, we referred to one inner dynein arm light chain as caltractin/centrin.

Results

Actin and Caltractin/Centrin Form a Complex with at Least One Inner Dynein Arm Heavy Chain

We intended to confirm that actin and caltractin/centrin are associated with I2 and/or I3 inner dynein arm heavy chains and determine whether the stability or the activity of the complexes is Ca^{++} sensitive.

To confirm that actin, caltractin/centrin, and I2 and I3 inner arm heavy chains are part of the same complexes we analyzed a protein fraction derived primarily from I2 and I3 inner arms. The protein fraction was extracted from axonemes of the outer dynein arm mutant *pf*28 and purified by sedimentation on a sucrose gradient. Inner arm heavy chains I2 and I3 sediment as 11S particles and remain associated with proteins of lower molecular weight (27). An electrophoretogram resolving axonemal polypeptides of molecular weight close to 500,000 shows that the protein fraction containing 11S particles is enriched in I2 and I3 heavy chains 2′, 2, 3, and 3′ and does not contain I1 heavy chains 1α and 1β (Fig. 2). Although the ratio of each heavy chain to the others in the 11S fraction is different than in the axoneme, the 11S fraction is suitable for an analysis of all four heavy chains together.

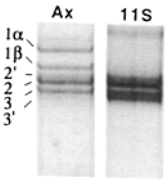

Figure 2. Electrophoretograms of ^{35}S-labeled axonemal polypeptides of the mutant *pf*28. A portion of the original autoradiogram resolving polypeptides in the 500,000–400,000 molecular weight range is shown. Equal amounts of radioactivity were analyzed. (*Left* lane) Unfractionated axonemal polypeptides (*Right* lane) 11S fraction obtained by extraction of axonemal polypeptides, followed by sucrose gradient sedimentation both in the presence of 0.5 M NaCl and 0.1 mM EGTA. Bands referred to as 1α, 1β, 2′, 2, 3, and 3′ are inner dynein arm heavy chains.

We identified actin and caltractin/centrin by immunoblots of all the polypeptides contained in the 11S protein fraction (Fig. 3 *a*). A mAb against chicken gizzard actin (17) (Fig. 3 *b*) and a polyclonal antibody to *Chlamydomonas* caltractin (13) (Fig. 3 *c*) bind actin and caltractin/centrin, respectively. A polyclonal antibody against chicken back muscle bound specifically to actin in a similar experiment (not shown). This evidence confirmed that both actin and caltractin/centrin are bound to at least one of the I2 and I3 inner arm heavy chains. Axonemal actin may not form long filaments because rhodamine-labeled phalloidin (4) does not bind to the axoneme in detectable amounts (result not shown).

Extraction and isolation of the 11S protein fraction used for the immunoblots was performed at high ionic strength, in 0.5 M NaCl, 0.1 mM EGTA, and 10 mM Tris Cl (pH 7.4)

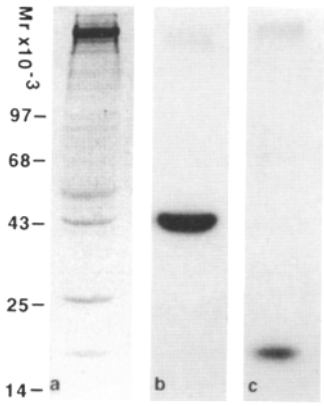

Figure 3. Electrophoretogram of axonemal polypeptides of the mutant *pf*28 and corresponding immunoblots obtained with antibodies specific for actin or caltractin. (Lane *a*) Coomassie blue-stained polypeptides contained in an 11S protein fraction. The position of molecular weight standards is indicated on the left side. Two aliquots of the same 11S fraction were electrophoresed in parallel and then transferred to nitrocellulose. (Lane *b* and *c*) Autoradiograms of the corresponding immunoblots obtained using specific antibodies and ^{125}I- labeled secondary antibodies. (Lane *b*) Immunoblot incubated with anti-actin antibodies. (Lane *c*) Immunoblot incubated with anti-caltractin antibodies.

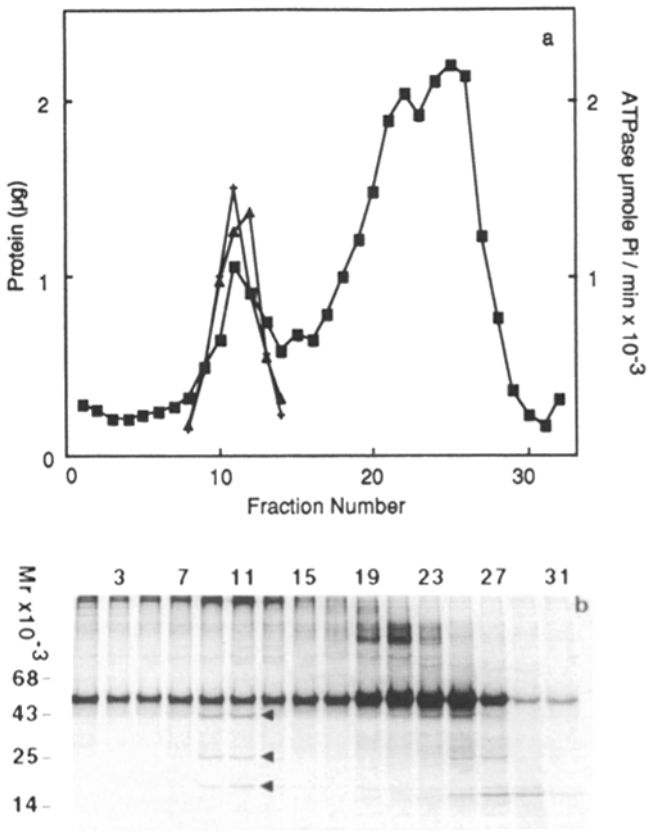

Figure 4. Sedimentation profile and electrophoretograms of ^{35}S-labeled polypeptides from *pf*28 axonemes. (*a*) Sedimentation in a 5–20% sucrose gradient of a protein fraction previously chromatographed on a hydroxyapatite column and then dialyzed against a solution of low ionic strength in the presence of 0.1 mM EGTA. The direction of sedimentation was from right to left. (■) Proteins; (▲) Mg-activated ATPase activity. (+) Mg-activated ATPase in the presence of 0.2 mM Ca^{++}. (*b*) Electrophoretograms of all polypeptides contained in odd fractions 1–31. Triangles indicate actin, p28, and caltractin/centrin cosedimenting with inner arm heavy chains. The position of molecular weight standards is indicated on the left side.

(27). To determine whether the actin and caltractin/centrin, both Ca^{++} binding proteins, bind to inner arm heavy chains or regulate the ATPase activity of the 11S complexes in a Ca^{++}-dependent manner, we isolated the 11S particles by a different procedure.

The I2 and I3 inner arm heavy chains were isolated by chromatography on hydroxyapatite column and then sedimented under conditions of low ionic strength (20) in the presence of 0.1 mM EGTA or 0.1 mM $CaCl_2$. These conditions led to the isolation of 11S fractions that are suitable for ATPase activity assays and similar in purity to those obtained by sedimentation at high ionic strength. An 11S protein fraction containing Mg^{++}-activated ATPases was isolated in a sucrose gradient containing 0.1 mM EGTA, 10 mM Tris Cl (pH 7.4) (Fig. 4 *a*). Electrophoretograms of polypeptides contained in odd fractions of the sucrose gradient show that the 11S peak is formed by dynein heavy chains, actin, and caltractin/centrin (Fig. 4 *b*). A 28,000 molecular weight polypeptide, referred to as p28, is also a component of the 11S complexes. Tubulin subunits and other minor polypeptides are contaminants because they do not form a sedimentation peak coincident to that of the inner arm heavy and light chains. Inner arm heavy chains 2′, 2, 3, and 3′ were identified as components of the 11S peak through gel electrophoresis resolving the inner arm heavy chains (not shown).

A similar 11S protein fraction was obtained when the sedimentation on a sucrose gradient was performed at low ionic strength in the presence of 0.1 mM $CaCl_2$ (not shown). Therefore, the association between I2 and I3 heavy chains and the set of proteins including actin, p28 and caltractin/centrin is Ca^{++} insensitive and stable at high and low ionic conditions.

Mg^{++}-activated ATPase activities of 11S particles are insensitive to Ca^{++} ions. Mg^{++}-activated ATPase activities of I2 and I3 inner arm heavy chains are similar in the absence or presence of 0.2 mM $CaCl_2$ (Fig. 4 *a*).

Actin, Caltractin/Centrin, and p28 Bind to Inner Arm Heavy Chains and Other Axonemal Subunits

To determine whether actin, p28, and caltractin/centrin associate with all I2 and I3 inner arm heavy chains or a subset of them, we analyzed qualitatively and quantitatively axonemal polypeptides of three inner arm mutants, *pf*22, *ida*4, and *pf*23 that are defective for subsets of heavy chains 2′, 2 and 3′ (16, 25).

The mutants *pf*22, *ida*4, and *pf*23 were compared to a wild type strain. An electrophoretogram resolving dynein heavy chains (Fig. 5) shows that the mutant *pf*22 lacks heavy chains 2 and 3′. The mutant *ida*4 lacks heavy chains 2′ and part of heavy chain 2 and finally the mutant *pf*23 lacks heavy chains 2′, 2 and 3′.

Axonemal polypeptides of molecular weight lower than 200,000 from the mutants *pf*22, *ida*4, and *pf*23 were resolved by two-dimensional gel electrophoresis. The positions of actin, p28, and caltractin/centrin in the two-dimensional maps were identified by comparison with a map resolving the low molecular weight components of an 11S protein fraction from the mutant *pf*28 (Fig. 6 *a*; see Fig. 4 for the characterization of the 11S fraction). Actin, p28, and caltractin/centrin are present in axonemes of the mutants *pf*22 and *pf*23 (Fig. 6, *b* and *d*). In contrast, p28 is virtually absent from axonemes of the mutant *ida*4 (Fig. 6 *c*).

Ratios between the radioactivity values of actin, p28, and caltractin/centrin and the radioactivity values of an insoluble axonemal component (26), referred to as s in the maps (Fig. 6, *b–d*) are reported in Table III. Multiple gels containing

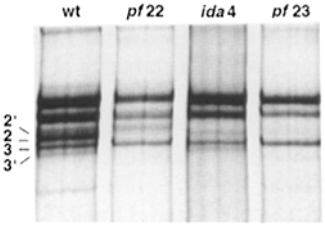

Figure 5. Electrophoretograms of ^{35}S-labeled axonemal polypeptides of a wild-type strain and inner arm mutants *pf*22, *ida*4, and *pf*23. A portion of the original autoradiogram resolving polypeptides in the 500,000–400,000 molecular weight range is shown. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 2′, 2, 3 and 3′ are I2 and I3 inner arm heavy chains.

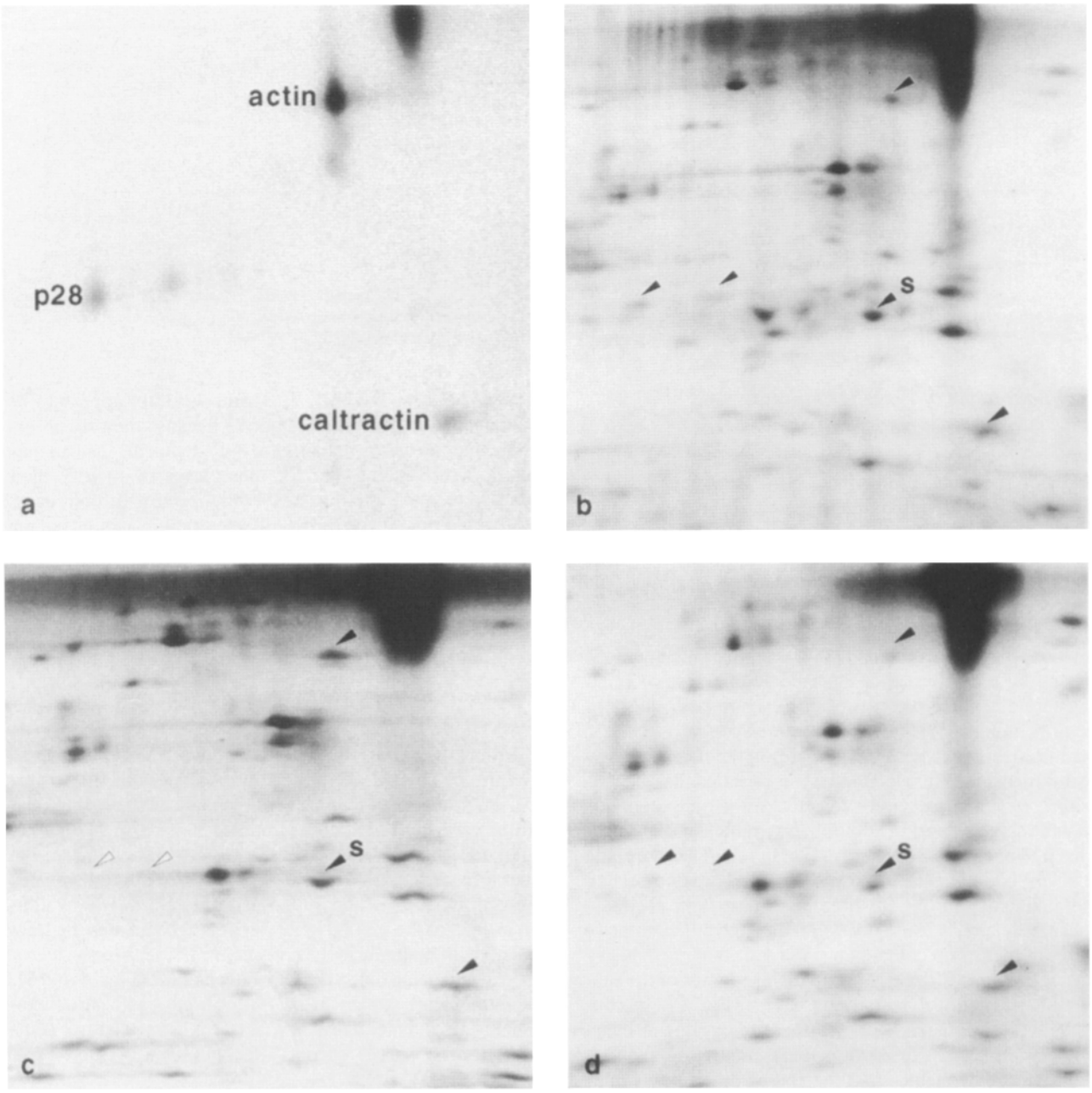

Figure 6. Autoradiograms of ^{35}S-labeled axonemal polypeptides resolved by two-dimensional electrophoresis. Only portions of the original maps resolving polypeptides in the 55,000–15,000 molecular weight range are shown. The gels are oriented with basic polypeptides on the left. (*a*) Map resolving actin, p28, and caltractin/centrin that are present in an 11S protein fraction (see Fig. 4, *a* and *b* for the characterization of the 11S fraction). The protein p28 is resolved into two components that differ in their isoelectric point. (*b*) Polypeptides from the mutant *pf*22. (*c*) Polypeptides from the mutants *ida*4. (*d*) Polypeptides from the mutant *pf*23. Solid triangles indicate the position of actin, p28, and caltractin/centrin. Open triangles indicate the positions of wild-type p28 components missing in the mutant *ida*4. The polypeptide labeled s was adopted as internal standard in each sample.

axonemal polypeptides from wild-type or inner arm mutants *pf*22, *ida*4, and *pf*23 were analyzed.

Both actin and p28 are deficient in all three mutants. Caltractin/centrin is deficient in the mutant *ida*4. No evidence was found suggesting that actin or p28 or caltractin/centrin are associated only to a subset of heavy chains.

In summary, the mutant *pf*23 lacks heavy chains 2′, 2, and 3′ and is deficient in actin and p28, whereas the mutant *ida*4 lacks heavy chain 2′, part of 2 and is more deficient for actin, p28, and caltractin/centrin than the mutant *pf*23. Therefore, a greater loss of heavy chains as in the mutant *pf*23 does not correlate with a greater deficiency of light chains. Conversely, the lesser loss of heavy chains in the mutant *ida*4 does not correlate with a less pronounced deficiency of light chains. This evidence and the evidence obtained by the isolation of 11S particles indicate that all three light chains, or a subset of them, bind to the axoneme through the heavy chains as well as other molecules.

Table III. Quantitative Analysis of Inner Arm Light Chains

Strain	Actin*	p28*	Caltractin/centrin*	2D-gels	Axoneme preparations
Wild-type	3.99 (0.76)	1.49 (0.38)	1.02 (0.36)	7	4
*pf*22	0.88 (0.43)	0.45 (0.27)	0.98 (0.34)	3	2
*ida*4	0.74 (0.32)	0.08 (0.02)	0.59 (0.23)	4	3
*pf*23	1.04 (0.56)	0.35 (0.07)	1.55 (0.46)	5	3
*pf*2	2.24 (0.77)	0.94 (0.54)	0.67 (0.30)	4	3
*pf*3	1.72 (0.31)	0.61 (0.24)	0.54 (0.16)	5	2
$sup_{pf}3$	3.30 (0.42)	1.00 (0.35)	0.92 (0.27)	4	3
$sup_{pf}4$	6.15 (2.31)	1.46 (0.36)	1.10 (0.31)	6	3
$sup_{pf}5$	1.84 (0.68)	0.81 (0.48)	0.76 (0.45)	10	5

* Numbers are averages of determinations performed on several two-dimensional gels and ratios between radioactivity values of actin, p28, and caltractin/centrin and radioactivity values of an insoluble axonemal component referred to as s. Standard deviations are indicated in parentheses.

Some Suppressors of Flagellar Paralysis Are Inner Arm Mutants

Second binding sites for actin or p28 or caltractin/centrin could be on the tubulin subunits or other axonemal proteins that are in close proximity with the inner arm heavy chains. To test the latter hypothesis, we turned to the analysis of a group of mutants each lacking a distinct subset of axonemal proteins referred to as *drc* (see Introduction and Materials and Methods).

The position of the *drc* within the axoneme is unknown but an interaction between *drc* and inner dynein arm subunits is suggested by the observation that the suppressors *pf*2 and *pf*3 generate bending patterns of flagella similar to those of inner arm mutants (3).

Quantitative analysis of axonemal polypeptides resolved by two-dimensional gel electrophoresis revealed that the mutants *pf*2, *pf*3, and $sup_{pf}5$ are defective for actin to approximately the same extent. In addition, the mutant *pf*3 is defective for p28 and caltractin/centrin (Table III). Therefore, the lack of *drc* components 1 and 2 or 3 and 4 was correlated with a deficiency of one or three inner arm light chains.

These deficiencies could not be predicted from qualitative analyses of inner arm components. All four inner arm heavy chains 2′, 2, 3, and 3′ are present in the axonemes of the mutants *pf*2, *pf*3, $sup_{pf}3$, $sup_{pf}4$ and $sup_{pf}5$ (Fig. 7). In contrast, quantitative analyses of electrophoretograms revealed that heavy chains 2′, 2, 3, and 3′ are present in reduced amounts in the axonemes and flagella of the mutants *pf*2, *pf*3, $sup_{pf}3$, and $sup_{pf}5$. The ratios between radioactivity values of combined heavy chains 2′, 2, 3, and 3′ and radioactivity values of combined γ and 1β chains of outer arm and inner arm I1 (27) are reported in Table IV.

There is a parallel between these results and the results obtained with the quantitative analysis of actin, p28, and caltractin/centrin. The mutants *pf*3 and $sup_{pf}5$ are the most defective for inner arm heavy chains and actin, whereas the mutant $sup_{pf}4$ is similar to wild-type. For each strain the deficiency of inner arm heavy chains in samples of axonemes is similar to the deficiency of the same components in samples of flagella. Therefore, inner arm heavy chains were not extracted preferentially when flagellar membrane and matrix proteins were separated from the axoneme.

Although we did not identify the axonemal subunits binding actin or p28 or caltractin/centrin, we found that the loss of components 1 and 2 or 3 and 4 of the *drc* is correlated with the defect of assembly of all four heavy chains of inner arms I2 and I3. Components 1, 2, 3, and 4 do not appear to be components of inner arms I2 and I3 because they are absent when inner arm heavy chains are only deficient. Instead they may form a binding site for these arms or modify their subunits to make them assembly-competent. In both cases they should interact directly with one or more subunits of the inner arms I2 and I3. To test this hypothesis, we determined whether the loss of a subset of *drc* components 1, 2, 3, and 4 affects the assembly of a specific type of inner arm in the presence of a preexisting inner arm defect.

Molecular Interactions between drc and Inner Arm Subunits

Assuming that *drc* subunits modify or are contiguous to subunits of inner arms I2 and I3 we expect to find that a deficiency of a specific inner arm is enhanced in recombinant strains carrying both suppressor and inner arm mutations.

For our analysis we isolated five recombinants between the inner arm mutant *ida*4 and each of the five suppressors *pf*2, *pf*3, $sup_{pf}3$, $sup_{pf}4$, and $sup_{pf}5$. The mutant *ida*4 was chosen instead of the mutant *pf*22 or *pf*23 because it is the least defective in inner arm heavy chains, lacking only heavy chain 2′ and being defective for heavy chain 2 (Figs. 5 and 8).

Recombinant strains *pf*2*ida*4, *pf*3*ida*4, and $sup_{pf}5$*ida*4 all show an enhanced loss of inner arm heavy chain 2 compared to the parent strains. Recombinants *pf*2*ida*4 and

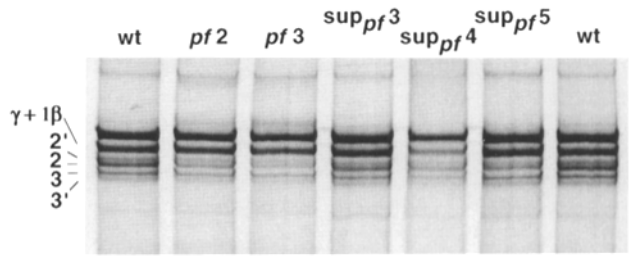

Figure 7. Electrophoretograms of ^{35}S-labeled axonemal polypeptides of wild-type strains and mutants *pf*2, *pf*3, $sup_{pf}3$, $sup_{pf}4$, and $sup_{pf}5$. A portion of the original autoradiogram resolving polypeptides in the 500,000–400,000 molecular weight range is shown. Equal amounts of radioactivity were analyzed in each lane. A lower intensity of $sup_{pf}4$ dynein heavy chains was caused by the presence of cellular contaminants in the axoneme preparation. Bands referred to as γ+1β, 2′, 2, 3, and 3′ are dynein arm heavy chains.

*Table IV. Quantitative Analysis of Inner Arm Heavy Chains**

	Wild-type	*pf*2	*pf*3	sup$_{pf}$3	sup$_{pf}$4	sup$_{pf}$5
Axonemes	0.95	0.81	0.50	0.77	1.11	0.55
Flagella‡	0.95	0.78	0.54	0.78	0.93	0.60

* Numbers are ratios between radioactivity values of combined heavy chains 2′, 2, 3, and 3′ and radioactivity values of combined heavy chains $\gamma + 1\beta$. Five determinations of radioactivity were performed on the same electrophoretogram. Standard deviations were lower than 7% in each case.
‡ Flagella or axonemes were prepared from different cultures. Flagella were analyzed to establish that losses of dynein arms did not occur after the solubilization of flagellar membrane.

*pf*3*ida*4 lack heavy chains 2 and part of 3′, whereas sup$_{pf}$5*ida*4 lacks heavy chain 2. In contrast, sup$_{pf}$3*ida*4 is only slightly more deficient for heavy chain 2 than *ida*4 and sup$_{pf}$4*ida*4 is indistinguishable from *ida*4 (Fig. 8). The loss of inner arm heavy chain 2 was not observed by qualitative analysis of the recombinant *pf*2sup$_{pf}$5 (result not shown).

In summary, *ida*4, an inner arm mutation that causes a partial defect in inner arms I2, combined independently with each of the suppressor mutations *pf*2, *pf*3, and sup$_{pf}$5, resulted in a total loss of inner arms I2. The suppressors *pf*2, *pf*3, and sup$_{pf}$5, differ from the suppressor sup$_{pf}$3 and sup$_{pf}$4 because they are deficient for actin and *drc* components 1, 2, or 3. Therefore, the loss of heavy chain 2 in *ida*4 recombinants is caused by a synergistic effect of defects affecting different parts of the inner arm structure. This evidence supports a model where part of the *drc* interacts with inner arms I2.

Discussion

The Presence of Caltractin/Centrin and Actin within the Axoneme

The transition between ciliary and flagellar types of motion of *Chlamydomonas* axonemes can be observed in vivo (31), with demembranated cell models (15) and with isolated axonemes (2). In each case the transition depends on the Ca^{++} concentration of the medium where the axoneme moves. At Ca^{++} concentrations above 10^{-6} M axonemes assume a flagellar type of waveform instead of the usual ciliary type, even in the absence of outer dynein arms (3). This indicates that the formation of flagellar bending waves is triggered by at least one axonemal component that responds to concentrations of Ca^{++} greater than 10^{-6} M and regulates the function of inner dynein arms.

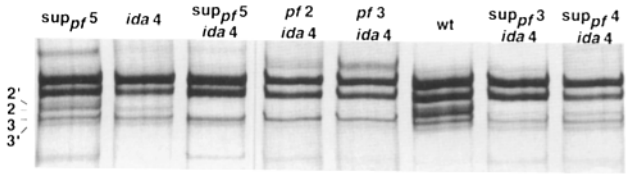

Figure 8. Electrophoretograms of ^{35}S-labeled axonemal polypeptides of a wild-type strain, mutants sup$_{pf}$5 and *ida*4 and recombinant strains *pf*2*ida*4, *pf*3*ida*4, sup$_{pf}$3*ida*4, sup$_{pf}$4*ida*4 and sup$_{pf}$5*ida*4. Portions of the original autoradiograms resolving polypeptides in the 500,000–400,000 molecular weight range are shown. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 2′, 2, 3, and 3′ are I2 and I3 inner arm heavy chains.

The characterization of a calcium binding protein, caltractin/centrin from *Chlamydomonas* basal bodies (13), provided suggestive evidence that caltractin/centrin was also an inner arm subunit. Caltractin/centrin has the same molecular weight and isoelectric point as a protein subunit associated with inner dynein arm heavy chains and axonemal actin (23). On the basis of these observations we were interested in confirming the identification of caltractin/centrin as an inner arm component. In addition we wanted to determine whether Ca^{++} ions regulate the structure or function of inner dynein arms in vitro.

We have confirmed that caltractin/centrin is associated in vitro and in vivo with the heavy chains of inner dynein arms I2 and I3, actin, and a third small protein referred to as p28. However, a change of Ca^{++} concentration does not affect the Mg^{++}-activated ATPase activity or the molecular composition of caltractin/centrin-containing complexes in vitro.

Evidence of an association among I2 and I3 inner arm heavy chains, actin, p28, and caltractin/centrin was obtained by qualitative analyses of polypeptide complexes or axonemes that were isolated from motility mutants. The analysis of polypeptides did not determine whether all three light chains bind to each of the heavy chains. As a consequence, we do not know if any light chain is located only in the distal or proximal part of the inner arm row, as the inner arm heavy chains 2′ and 3′ are (25). Instead, quantitative analyses suggested that actin, p28, and caltractin/centrin bind to the axoneme through I2 or I3 inner arm heavy chains as well as other molecules.

Axonemal molecules binding actin, p28, and caltractin/centrin may be found among components 1, 2, 3, and 4 of the *drc*, which interacts at least with I2 inner arm heavy chains. Although the lack of *drc* components 5 and 6 from the mutant sup$_{pf}$4 does not cause an inner arm defect, the absence of components 1, 2, 5, and 6 from the mutant *pf*3 is paralleled by the highest deficiency of actin, caltractin/centrin, and I2 and I3 inner arm heavy chains. Therefore, *drc* components 1 and 2 may affect the binding of inner arms I2 and I3 to the axoneme directly or through an interaction with actin and caltractin/centrin.

We could not obtain direct evidence that actin or p28 or caltractin/centrin binds to *drc* components after extraction from the axoneme because we could not isolate complexes containing *drc* components and inner arm light chains. The *drc* components are not dissociated from the axoneme under conditions that lead to the solubilization of nearly all other axonemal substructures, with the exception of radial spoke stalks (26 and G. Piperno, unpublished results). In this respect the *drc* differs from outer and inner dynein arms and radial spoke heads that were isolated as complexes from wild-type axonemes. Nearly all the polypeptides that tentatively were identified as components of dynein arms and radial spoke heads by the analysis of motility mutants (9, 10), were identified also as components of the same substructures after isolation (26, 23, 24).

The Location of the "Dynein Regulatory Complex"

The test for the existence of an interaction between *drc* and dynein arms through the analysis of recombinant strains car-

rying both *drc* and inner arm defects has a precedent in the analysis of a recombinant carrying $sup_{pf}1$ and *pf*22 mutations (11). The mutant $sup_{pf}1$ has outer dynein arms but carries an outer arm β heavy chain that is defective. The mutant *pf*22 is deficient for outer dynein arms and lacks inner arms components 2 and 3′ (25). In contrast, the recombinant $sup_{pf}1pf22$, lost completely the outer dynein arms. Therefore, more than one structural defect is needed to cause the loss of outer dynein arms, which likely are bound to the axoneme through more than one site. Similarly a loss of *drc* subunits in combination with a deficiency of inner arms I2 resulted in the loss of inner arms I2 in each recombinant *pf*2*ida*4, *pf*3*ida*4, and $sup_{pf}5ida4$. This evidence supports the conclusion that the *drc* and inner arms I2 interact directly.

The enhancement of inner arm I2 defect in recombinants *pf*2*ida*4, *pf*3*ida*4, and $sup_{pf}5ida4$ is correlated with the deficiency of actin in suppressors *pf*2, *pf*3, and $sup_{pf}5$ (Tables III and IV). Therefore, suppressor defects that lead to loss of I2 inner arms in the recombinants may derive from defective interactions between inner arm light chains and *drc* components. These polypeptides may include the defective gene products of suppressors *pf*2, *pf*3, and $sup_{pf}5$.

The location of the *drc* within the axoneme has never been determined. We propose that the *drc* is located in close proximity with inner arms I2 and I3 since it affects the assembly of inner arms I2 and I3 when it is defective. In a position close to inner arms I2 and I3, the *drc* could be located between the radial spokes S1 and S2 and the inner arms that are a target of the *drc* activity.

A complex double-rowed organization of inner arms was described by Muto et al. (19) as the result of tilt-series thin section EM of *Chlamydomonas* flagella. Within a repeating unit of 96 nm the inner arms appeared to be formed by a row of structures consisting of four electron dense structures and three pairs of densities located centrifugally and centripetally, respectively. Muto et al. (19) postulated that both rows of densities are formed by inner arm heavy chains. In contrast, we propose that one row of densities contains the *drc* and is located in a centripetal position closer to the radial spoke stalks. This hypothesis can be tested through EM of axonemes from mutants lacking *drc* components.

From its putative location between the stem of inner arm I2 and radial spoke stalks the *drc* may regulate the activity of radial spokes. The spokes in bend regions of the axoneme form an angle with central pair and doublet microtubules instead of being perpendicular as they are in straight regions of the axoneme (34). The *drc* also may regulate the motion of inner arms I2 and I3 toward the proximal region of the axoneme (6). Lastly, it may form a specific binding site of inner arms I2 and I3 on the surface of doublet microtubules (33).

The Regulation of Axonemal Motility

The dynein arms at opposite sides of axoneme bends must be in an active and passive state, respectively, as far as concerns their contribution to the formation of the bend. Moreover, axoneme bending occurs between points having different rates of active sliding among doublet microtubules. These conditions indicate that dynein arm activity is highly regulated and may require the function of complexes like the *drc* that interact only with specific types of dynein.

It was proposed that the mechanisms regulating the formation of bending waves may operate primarily through variations of mechanical properties of the axonemal substructures (7). A position of the *drc* close to radial spokes and inner arms would allow the formation of a physical linkage between these substructures. This linkage could generate a conformational and not a chemical change of protein subunits of the three systems in response to axonemal bending.

Within 96-nm sections of each outer doublet actin, p28 and caltractin/centrin may regulate the function of both inner arms I2 and I3 and the *drc*. The possible integration with other axonemal substructures appears to be a characteristic of every intermediate and light chain that was identified as an inner arm component (9). A polypeptide referred to as 1′ (molecular weight 110,000) was found to be missing in the radial spoke mutant *pf*5 in association with the loss of two subunits of the radial spoke stalk (10). Moreover, a second inner arm component referred to as 2′ (molecular weight 83,000) is missing in all *mbo* mutants, mutants that are both defective for proximal beak-like projections and able to generate only flagellar type of bending of the axonemes (32). Therefore, several kinds of regulation may be mediated through inner arm intermediate and light chains and have inner arm heavy chains as a main terminal.

The assembly and/or function of complexes formed by I2 and I3 inner arm heavy chains, p28, and caltractin/centrin may be regulated also through posttranslational modifications because each of these subunits is phosphorylated in vivo (23). In addition, the presence of caltractin/centrin as part of these complexes provides a site for functional regulation through changes of Ca^{++} concentration. Both kinds of regulations have the inner dynein arms as a main terminal since the outer dynein arms do not have a strong influence toward changing the waveforms of flagella (3).

In summary, we have shown that the *drc* interacts with inner arms I2 in vivo. To integrate this observation in a more general model of the mechanism generating specific bending patterns of flagella, we intend to determine whether the radial spokes form a continuous structure with the *drc* and whether Ca^{++} ions above 10^{-6} M level change the signaling occurring between radial spokes and inner arms.

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