Mutations in the "Dynein Regulatory Complex"
Alter the ATP-insensitive Binding Sites for Inner Arm Dyneins in *Chlamydomonas* Axonemes

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**Abstract.** To understand mechanisms of regulation of dynein activity along and around the axoneme we further characterized the "dynein regulatory complex" (*drc*). The lack of some axonemal proteins, which together are referred to as *drc*, causes the suppression of flagellar paralysis of radial spoke and central pair mutants. The *drc* is also an adapter involved in the ATP-insensitive binding of I2 and I3 inner dynein arms to doublet microtubules. Evidence supporting these conclusions was obtained through analyses of five *drc* mutants: *pf2*, *pf3*, *supp3*, *supp4*, and *supp5*. Axonemes from *drc* mutants lack part of I2 and I3 inner dynein arms as well as subsets of seven *drc* components (apparent molecular weight from 29,000 to 192,000). In the absence of ATP-Mg, dynein-depleted axonemes from the same mutants bind I2 and I3 inner arms at both ATP-sensitive and -insensitive sites. At ATP-insensitive sites, they bind I2 and I3 inner arms to an extent that depends on the *drc* defect. This evidence suggested to us that the *drc* forms one binding site for the I2 and I3 inner arms on the A part of doublet microtubules.

Various types of dynein arms (13) together cause axonemal microtubules to slide relative to one another and ultimately generate the oscillatory movements of the axonemes. Therefore, axoneme bending results from the coordinated regulation of dynein activities along and around the axoneme structure. Understanding that regulation and, in particular, understanding mechanisms of local activation and/or inactivation of dynein arms would be major achievements in the study of axonemal motility. Towards these goals, we intend to further characterize the "dynein regulatory complex" (*drc*1) (12) a complex that may modify (in wild-type strains) or inhibit (in radial spokes and central complex mutants) (3, 7) the dynein-mediated sliding of doublet microtubules.

The *drc* is composed of six axonemal proteins that in subsets are lacking from the axoneme of nine mutants, representing five loci of the *Chlamydomonas* genome (7, 12). Some mutations of the *drc* were isolated as motility mutants, others as second-site suppressors that release the paralysis of flagella of radial spoke mutants without repairing the original radial spoke defects (7, 12). The same mutations in a wild-type background cause inefficient axonemal beating

1. **Abbreviations used in this paper: drc, dynein regulatory complex; LISB, low ionic strength buffer.**

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The Journal of Cell Biology, Volume 125, Number 5, June 1994 1109-1117
tightly bind to the microtubule lattice. The drc, then, is the first example of a microtubule-associated structure that mediates the interactions between the ATP-insensitive binding domains of dyneins and the microtubule lattice. Further studies of the drc function can be approached by genetics because at least two drc components are putative defective gene products of drc mutations.

Materials and Methods

Strains and Culture of Chlamydomonas Cells

Cell culture and labeling with [35S]sulfuric acid were performed in solid medium (13).

Strains of drc mutants were crossed once to the wild-type strain 137 and tetrads of daughter cells were obtained by standard methods (4). Each mutant could be distinguished from the wild-type strain because mutant cell bodies move less efficiently. Although detailed analysis of the motion of cells from each strain was not performed, the motility phenotype of each drc mutant qualitatively was distinguishable from that of the wild-type strain by optical microscopy. Mutants derived from the cross to the wild-type strain were characterized further throughout this study. The lack or defect of drc components within the axonemes of each mutant was determined by one- and two-dimensional electrophoresis of axonal proteins. This analysis confirmed the existence of a deficiency of different subsets of drc components in each mutant (Table III) as observed by Huang et al. (7) and Pino et al. (12).

Recombinant strains between each of the drc mutant and the central complex mutant pf5 or pf18 (I) or the radial spoke mutant pf1 or pf14 (6) were obtained from nonparental ditypic tetrads. Phenotypic analysis of recombinants was performed by optical microscopy on cells that were grown for 1 d at 25°C in medium containing sodium acetate as described by Sager and Granick (16). Suppression of flagellar paralysis was scored if paralysis or erratic movement of flagella of radial spoke or central pair mutants was changed to regular beating of flagella of the recombinants.

Recombinant strains between the mutant pf28 (11) and each of the drc mutants were obtained from nonparental ditypic tetrads with the exception of pf28pf28 that was obtained from a tetrateype tetrad. The majority of pf28pf28 cells did not have flagella. The recombinants pf3pf28 and supf3pf28 had short and paralyzed flagella. The cell bodies of supf3pf28 did not move but had a minority of flagella beating. The cell bodies of supf3pf28 were all propelled by the movement of flagella.

Dikaryon rescue analyses were performed as described before (9). Gametes were grown for 8 d on solid minimal medium. Rescue of drc components did not require flagellar regeneration from the dikaryons. Mutant cells were labeled by growth on 35S-containing medium. Fusion between 35S-labeled mutant and unlabeled wild-type gametes was carried out in the presence of anisomycin to inhibit protein synthesis in the dikaryon. Putative restoration of function within mutant flagella of the dikaryon was correlated with the assembly of 35S-labeled mutant and unlabeled wild-type polypeptides in mutant axonemes. In the case of the mutant gene product only an unlabeled wild-type polypeptide could be incorporated. Therefore, the component that is lacking from the maps of 35S-labeled axonomal components of mutant wild-type dikaryons was identified as the putative defective gene product of the mutant.

Binding of Inner Arm Heavy Chains to Dynein-depleted Axonemes

We have modified the procedure of Smith and Sale (17) as follows. Axonemes were prepared by the dibucaine method (18) and collected by centrifugation at 10,000 rpm for 15 min in a SS34 rotor (Sorvall, Du Pont Co., Newton, CT). Then, they were resuspended at concentrations close to 2 mg/ml in 50 mM NaCl, 0.5 mM EDTA, 10 mM Hepes, pH 7.4, in the presence of pepstatin A and leupeptin (referred to as low ionic strength buffer (LISB)). In order to extract the dyneins, half of each axoneme suspension was exposed to 0.55 M NaCl, 4 mM MgCl2, 1 mM ATP, 10 mM Hepes, pH 7.4, and sedimented at 9,000 rpm for 5 min at 4°C in an Eppendorf 5402 centrifuge (Brinkmann Instruments Inc., Westbury, NY). Pellets of dynein-depleted axonemes were washed once with LISB and then resuspended in the same solution at a protein concentration close to 2 mg/ml. An aliquot of the dynein-containing salt extract from 35S-labeled pf28 axonemes was recovered (0.3-0.5 ml containing 0.3-0.5 mg/ml of protein, specific radioactivity 50,000 cpm/μg), dialyzed in a membrane tubing (12,000-14,000 mol wt cutoff; Spectra/por, Los Angeles, CA) for 1 h against ice-cooled LISB and centrifuged at 14,000 rpm for 5 min at 4°C in the Eppendorf centrifuge to sediment any aggregate of dynein arms that could be formed during the dialysis. Extracts contained 90% of the II and 80% of the I2 and I3 inner dynein arm heavy chains originally present in the pf28 axonemes. Dynein-containing extracts were then mixed with dynein-depleted or nonextracted axonemes at 0.5:1, 1.5:1, and 2:1 protein ratios. Each sample was processed in double. Incubation of suspensions was performed at room temperature for 15 min. Samples were then centrifuged at 14,000 rpm for 5 min at 4°C in the Eppendorf centrifuge in the presence or absence of 1 mM ATP, 4 mM MgCl2. Pellets were washed once with LISB solution and then solubilized in 1% SDS, 1% -mercaptoethanol.

Protein samples, ~6 μg, were divided in half and subjected to electrophoresis: half on a 3.6-5% and the other half on a 4-11% polyacrylamide gel. Amounts of bound 35S-labeled dynein heavy chains were determined by the Phosphorimager analysis of the 3.6-5% gels, whereas amounts of Coomassie blue-stained tubulin subunits were determined by densitometric analysis of the 4-11% gels. Determinations of tubulin amounts were performed to confirm that each lane of the gel contained equal amounts of protein. Radioactivity and optical density values, as measured by the Phosphorimager or the optical densitometer, were in the linear range of response of the instruments.

Amounts of heavy chains bound to nonextracted axonemes (representing binding to sites other than dynein binding sites, also referred to as non-specific binding) were subtracted from amounts of inner arm heavy chains bound to dynein-depleted axonemes. The second were 3 to 11 times higher than the first, depending on the experiment. The same subtraction was performed for the samples processed in the presence of ATP. In this case both nonextracted and extracted axonemes were processed in the presence of ATP, although amounts of heavy chains bound to nonextracted axonemes were not sensitive to the presence of ATP.

Amounts of II, I2, and I3 inner arm heavy chains bound to axonemes in some cases were normalized for the amounts of tubulin. This correction was introduced if gel lanes did not contain equal amounts of protein, in spite of the fact that we attempted to analyze exactly 3 μg of protein. Amounts of I2 and I3 inner arm heavy chains bound to 3 μg of protein were determined in the absence of ATP. Amounts of I2 and I3 inner arm heavy chains were identical to the amounts of the same heavy chains that were present in 3 μg of 35S-labeled pf28 axonemes.

Other Procedures

Dialysis of dynein-depleted wild-type axonemes was performed at a protein concentration of 1 mg/ml for 2 h at 0°C, against a solution of 0.5 M NaCl, 0.5 mM EDTA, 1.0 mM Hepes, pH 7.4.

One-dimensional resolution of dynein heavy chains or tubulin subunits, two-dimensional resolution of axonomal components (12), determination of flagellar length (13) were performed as we previously described. Resolution of basic proteins by two-dimensional electrophoresis was achieved by nonequilibrium pH gradient electrophoresis run for 14 h at 1.4 mA, other conditions were as in reference 12.

Results

Mutations in the drc Suppress Flagellar Paralysis of Both Central Complex and Radial Spoke Mutants

Following the observation of Huang et al. (7) that mutants, pf2, pf3, supf3, and supf4 suppress the paralysis of radial spoke mutants and our observation that the mutation supf5 suppresses flagellar paralysis of both central complex and radial spoke mutants (12) we intended to determine whether the other drc mutants, pf2, pf3, supf3, and supf4, are similar to supf5 for their suppressor effect toward the paralysis of central complex mutants. We isolated recombinant strains between each of the drc mutant pf2, pf3, supf3, and supf4 and the central complex mutants pf15 and pf18 (1). We also isolated recombinants between the same drc mutants and the radial spoke mutants pf1 and pf14 (6).

We observed each recombinant by optical microscopy and found that paralysis or erratic movement of flagella in central...
Flagella of drc Mutants Are Deficient in All Types of Inner Dynein Arms

To address the question of whether the II inner dynein arms are deficient in the drc mutants, we eliminated the outer dynein arms from their axonemes by introducing the mutation pf28 (11). Our past analyses of flagellar proteins from drc mutants determined that I2 and I3 inner dynein arm heavy chains were present in reduced amounts in these mutants (12). However, the same analyses could not determine whether II inner arm heavy chains were also defective because these heavy chains have the same electrophoretic mobility as the outer arm heavy chains.

We isolated recombinant strains pf2pf28, pf3pf28, suppf3pf28, and suppf5pf28 and analyzed inner arm heavy chains from pf3pf28, suppf3pf28, and suppf5pf28 by gel electrophoresis of 35S-labeled flagellar proteins. The recombinant pf2pf28 could not be analyzed because the majority of the cells did not have flagella and the rest had very short stubs. We analyzed flagella and not axonemes to avoid losses of dynein arms that could occur during the isolation of the axonemes. Moreover, we adopted the mass of tubulin subunits as an internal standard of each sample in order to normalize the data. Therefore, we performed qualitative and quantitative analyses of inner arms heavy chains by electrophoretic conditions that resolved heavy chains and tubulin subunits in the same slab gel.

The electrophoretogram of 35S-labeled proteins from flagella of a wild-type strain is in Fig. 1. The major flagellar membrane protein, the dynein heavy chains and the tubulin subunits were resolved in spite of their large difference of apparent molecular weight (14).

Portions of electrophoretograms resolving the inner arms heavy chains from pf28 and recombinants pf3pf28, suppf3pf28, and suppf5pf28 are shown in Fig. 2. They were obtained from Experiment I described in Table I. Flagella of the recombinant pf3pf28, suppf3pf28, and suppf5pf28 lack the 3' inner arm heavy chain and are shorter than 6 μm (Table I) similarly to some inner arm mutants (13).

Results of quantitative analyses of inner arm heavy chains from recombinants pf3pf28, suppf3pf28, suppf4pf28, and suppf5pf28 and the mutant pf28 are reported in Table I. Calculations were performed as follows: (a) radioactivity backgrounds were subtracted from the radioactivity of each inner arm heavy chain; and (b) ratios of inner arm heavy chains to tubulin radioactivities were expressed as percentages of the ratio that was obtained for the mutant pf28. Quantities of II, as well as those of I2 and I3 inner arm heavy chains, are lower in recombinants than in pf28. Recombinant suppf5pf28 is the most defective, whereas suppf4pf28 is the least defective, in both inner arm content and flagellar length. Therefore, some drc defects may affect the binding of all types of inner arms to the axonemes, if a reduction of flagellar length does not affect the inner arm content of the recombinants.

To determine whether the reduction of all inner arm heavy chains depends on flagellar length we prepared 3-μm-long flagella of pf28 by separation and regeneration of flagella from that mutant. Following this preparation we measured the content of inner arms present in the 3-μm-long flagella. We found that short or long pf28 flagella contain approximately the same amount of inner arm heavy chains relatively to the amount of tubulin (Table I). These results indicated that a reduction of inner arm concentration is correlated with a drc defect and not with a reduction of flagellar length. However, the last experiment could be meaningless, if equilibrium of assembly of inner arms in regenerating flagella of pf28 are different from those in stable flagella of recombinant strains.

Additional experiments performed with flagellar proteins from the drc mutants pf2, pf3, suppf3, suppf4, and suppf5 indicated that outer arm heavy chains are not significantly reduced in these mutants. Moreover, experiments performed
with the radial spoke mutant pf14 indicated that the lack of radial spokes does not affect the assembly of I2 and I3 inner arm heavy chains (not shown).

In summary, the lack of a subset of drc components in each recombinant may cause the reduction in concentration of all types of inner arms within flagella. Assuming that the reduction of inner arm concentration is a direct consequence of a drc defect, then, some drc components may form binding sites for inner arms on doublet microtubules. This hypothesis was tested by experiments described in the following sections.

**Inner Arm Heavy Chains Bind to Dynein-depleted Axonemes at ATP-sensitive and -insensitive Sites**

Our previous analyses of drc mutants indicated that at least I2 inner arms interact with the drc (12). However, we did not test the following possibilities: (a) the drc forms a binding site for the inner arms; (b) the drc modifies the inner arm subunits to make them competent to bind other structures; and (c) flagellar matrix proteins are necessary for the inner arm binding to doublet microtubules. To establish that the drc components are responsible for the formation of inner arm binding sites, we performed an assay that allowed us to measure the amount of inner arm heavy chains bound in vitro to axonemes depleted of dynein and matrix proteins.

The binding assay is based on the evidence, which will be described later, showing that the drc is not extracted from the axoneme under conditions that extract inner arm heavy chains. The assay is described in detail in Materials and Methods and was performed as follows. First, all types of outer and inner arm heavy chains were extracted from unlabeled axonemes of wild-type and drc mutants. Second, an 35S-labeled extract containing all inner arm but not outer arm heavy chains was mixed with a suspension of extracted axonemes from wild-type or drc mutants. Following an incubation the axonemes were pelleted, washed, and solubilized by SDS. Finally, 35S-labeled axonemal components were analyzed qualitatively and quantitatively by gel electrophoresis.

Nonextracted axonemes from wild-type and drc mutants were processed in parallel to extracted axonemes from the same strains to measure the level of nonspecific binding of inner arm heavy chains. Samples of extracted and nonextracted axonemes from wild-type or drc mutants were exposed to the same 35S-labeled extract containing inner arm heavy chains. Both samples of extracted and nonextracted axonemes were processed in the same conditions.

Following an incubation with inner arm heavy chains at a protein ratio 2:1, nonextracted and extracted wild-type axonemes generated silver-stained electrophoretic patterns of dynein heavy chains that respectively are similar to the electrophoretic patterns of heavy chains from wild-type and outer arm-less axonemes (Fig. 3 a). Outer arm heavy chains are undetectable in the sample of extracted wild-type axonemes. The corresponding autoradiograms obtained by the PhosphorImager revealed that the 35S-labeled inner arm heavy chains bound much more to extracted axonemes than to nonextracted axonemes (Fig. 3 b). Moreover, nearly the totality (93%) of 35S-labeled inner arm heavy chains bound to extracted axonemes were re-extracted in the presence of 0.55 M NaCl, 1 mM ATP, 4 mM MgCl₂ (not shown). Therefore, the evidence indicates that axonemes exposed to high concentration of NaCl carry a higher number of inner arm binding sites than nonextracted axonemes and bind inner arms in a reversible fashion.

**Table I. Quantitative Analyses of the Content of Inner Arm Heavy Chains in Flagella**

| Flagellar length (μm) | Experiment 1 | | | | | |
|-----------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| I1 inner arms         | 100         | 102         | ND§         | 55          | 71          | 87          |
| I2&I3 inner arms      | 100         | 93          | ND          | 49          | 48          | 69          |
| Flagellar length (μm) | 13.0(0.9)   | 3.0(0.6)    | <1          | 4.5(0.9)    | 6.4(2.2)    | 12.7(1.3)   | 2.4(0.7)    |

| Flagellar length (μm) | Experiment 2 | | | | | |
|-----------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| I1 inner arm          | 100         | 102         | ND          | 71          | 57          | 77          |
| I2&I3 inner arms      | 100         | 92          | ND          | 65          | 43          | 84          |
| Flagellar length (μm) | 10.3(1.0)   | 3.6(0.5)    | 3.4(0.6)    | 3.2(0.6)    | 8.5(1.8)    | 2.7(0.8)    |

* Standard deviations of radioactivity values obtained by the PhosphorImager were lower than 8%. Numbers are percentages of values that were obtained for full-length flagella (a) of the mutant pf28. Numbers in parentheses are standard deviations of length determinations.

† a, full-length flagella; b, regenerating flagella.

§ ND, not determined. The majority of pf2pf28 cells does not have flagella.

**Figure 3.** Electrophoreograms of dynein heavy chains as obtained by electrophoresis in a 3.6–5% polyacrylamide gel. Portions of electrophoreograms resolving polypeptides in the 500,000–400,000 apparent molecular weight range are shown. (a) Electrophoreograms of polypeptides from unlabeled wild-type axonemes that were exposed to a solution containing 35S-labeled inner dynein arms from the mutant pf28, before, first lane, and after, second lane, extraction of dynein heavy chains. Gel was stained by silver. (b) Autoradiogram of the gel portion shown in a. Couples of bands (referred to as 1α and 1β, 2α and 2β, and 3 and 3') are heavy chains of the inner arms II, 12, and 13, respectively. Autoradiogram was obtained by the PhosphorImager.
PhosphorImager indicated that I2 and I3 inner arm heavy chains together bound to extracted axonemes 7.7 times more than to nonextracted axonemes and were retained in stoichiometric amounts. However, the I1 inner arm heavy chains behaved differently than I2 and I3 heavy chains because they bound to extracted axonemes only 4.0 times more than nonextracted axoneme. Furthermore, the Iα heavy chain was retained in an amount that is 20% of the amount of the Iβ heavy chain (the ratio of Iα and Iβ heavy chains is 1:1 in vivo and in the 35S-labeled dynein-containing extract [not shown]).

The capacity of binding of extracted axonemes is saturated at a protein ratio of 1.5:1 between 35S-labeled extract and extracted axonemes for all types of inner arm heavy chains (Fig. 4).

To determine whether the inner arm heavy chains bind through their ATP-insensitive portion (as they do in vivo on the A part of doublet microtubules) and/or through their ATP-sensitive portion (as they do in vivo on the B part of doublet microtubules) we measured the amounts of bound inner arm heavy chains that remain on extracted axonemes after an exposure to 1 mM ATP and 4 mM MgCl₂. The amount of each inner arm heavy chain was reduced to ~60% of the amount bound in the absence of ATP (not shown). Saturation of binding was obtained at a protein ratio of 1.5:1 between 35S-labeled extract and extracted axonemes also in the presence of ATP.

In summary, the binding of stoichiometric amounts of I2 and I3 inner arm heavy chains is irreversible, saturable, and mostly occurs at ATP-insensitive sites. Moreover, this binding does not require the presence of matrix proteins or ATP because it occurs on extracted axonemes and in the absence of ATP. This evidence indicates that I2 and I3 inner arm heavy chains can be used to determine whether specific axonemal components form inner arm binding sites. In contrast, the II inner arm heavy chains are not suitable for the same purpose because a large portion of II inner arms is dissociated irreversibly during the experiment.

**Binding of I2 and I3 Inner Arms to ATP-insensitive Sites Is Lower in drc Mutants Than in Wild-type Axonemes**

To determine whether some drc components form a binding site for the inner arms we measured the amount of 35S-labeled inner arm heavy chains that remain bound to unextracted axonemes of each of the drc mutants at the protein ratio 2:1 between inner arm and extracted axoneme. Inner arm heavy chains first were bound to unextracted axonemes in the absence of ATP-Mg, and then were exposed to the presence of ATP-Mg. We limited our analysis to the I2 and I3 inner arm heavy chains bound to ATP-insensitive sites because the drc is located on the A part of the doublet microtubules, the site where the inner arm base is connected in an ATP-insensitive fashion.

The amounts of I2 and I3 inner arm heavy chains bound to extracted axonemes of a wild-type strain were higher than those bound to extracted axonemes of pf2, pf3, and sups5 and similar to those bound to extracted axonemes of sups4 and sups3 (Table II).

This evidence indicates that the number and/or the affinity of I2 and I3 inner arm binding sites in axonemes of a subset of drc mutants is lower than in wild-type axonemes. The decrease of inner arm binding to extracted axonemes of drc mutants is correlated to the extent of the drc defect, which is more severe in pf2, pf3, and sups5 (Table III). Therefore, the absence of some drc components in pf2, pf3, and sups5 decreases the number of inner arm binding sites. Alternatively, the absence of these drc components decreases the affinity of a subset of the inner arm binding sites. In both cases, a subset of drc components, missing in pf2, pf3, and sups5, is involved in the formation of I2 and I3 inner arm binding sites in the A part of doublet microtubules.

**The drc Is As Insoluble As Radial Spoke Stalks and As Large As a Dynein Heavy Chain**

A necessary condition to evaluate the drc's competence to bind inner arm heavy chains is that the drc itself remains bound to doublet microtubules during extraction and re-binding of the inner arms. To determine whether the drc remains insoluble after exposure to 0.6 M or 0.5 mM NaCl (ionic conditions that are higher and lower, respectively, than those adopted for extraction and re-binding of inner arms) we analyzed the molecular composition of wild-type axonemes that were exposed to these conditions. Polypeptides components of extracted axonemes were resolved by two-dimensional gel electrophoresis and compared to untreated axonemes, Fig. 5.

**Table II. Quantitative Analyses of the I2 and I3 Inner Arm Heavy Chains Bound in Vitro to Extracted Axonemes**

| Experiment | Wild-type | pf2 | pf3 | sups3 | sups4 | sups5 |
|------------|-----------|-----|-----|-------|-------|-------|
| 1          | 100       | 46  | 36  | 86    | 100   | 29    |
| 2          | 100       | 69  | 58  | 93    | 97    | 34    |

* Standard deviations of radioactivity values obtained by the PhosphorImager were lower than 8%. The amount of inner arms heavy chains bound to drc mutants is expressed as a percentage of the inner arm heavy chains bound to wild-type axonemes.

Figure 4. Diagram representing the amounts of inner arm heavy chains bound to dynein-depleted axonemes as function of the ratio between the amounts of 35S-labeled dynein extract from the mutant pf28 and unlabelled dynein-depleted axonemes from a wild-type strain. The amounts of inner arm heavy chains bound to dynein-depleted axonemes are expressed as percentage of the homologous heavy chains bound in vivo in pf 28 axonemes. (●●●●), curve representing the I2 and I3 heavy chains. (■■■■), curve representing the II heavy chains.
### Table III. Characteristics of drc Components

| Component | Mr \( \times 10^{-4} \) | Lacking in* | Putative gene product of |
|-----------|-----------------|-------------|-------------------------|
| drc 1     | 83              | pf3, supp3 5 | pf3                     |
| drc 2     | 70              | pf3, supp3 5 | Unknown                 |
| drc 3     | 62              | pf2, supp1 3 | Unknown                 |
| drc 4     | 55              | pf2, supp3 5 | Unknown                 |
| drc 5     | 40              | pf2, pf3, supp3 3, supp4 5 | supp4                  |
| drc 7     | 29              | pf2, supp3 5 | Unknown                 |
| drc 6     | 192             | pf2         | Unknown                 |

* Estimates of apparent molecular weights of drc components were obtained by comparing molecular weight standards to drc components that were partially purified by FPLC chromatography, then identified by two-dimensional gel electrophoresis and finally isolated by one-dimensional gel electrophoresis (Michel Le Dizet, unpublished results). Previous estimates of molecular weights were calculated from two-dimensional maps of polypeptides (7).

* The drc components 3, 5 and 6 are reduced in amount but not lacking in the mutant supp5.

Each of the drc components (Fig. 5, a and b, arrowheads) is present in extracted axonemes and is as insoluble as a subset of components of radial spoke stalks (Fig. 5 b, empty arrowheads). In contrast, a number of dynein components were extracted (Fig. 5 a, lines).

The resolution of axonemal components by the two-dimensional electrophoresis shown in Fig. 5 is limited to polypeptides of apparent molecular weight approximately <180,000 and isoelectric point comprised between 7.5 and 4. To determine whether the drc is composed of polypeptides not resolved under these conditions we compared axonemal components from drc mutants and wild-type strains by a variety of procedures, including two-dimensional electrophoresis of basic proteins and one-dimensional electrophoresis of large polypeptides (apparent molecular weight above 180,000).

We were able to observe all the deficiencies in axonemal polypeptides reported before for the mutants pf2, pf3, supp3 5, and supp4 4 (7). Moreover, we identified a new drc component. Fig. 6 shows that an axonemal component of apparent molecular weight 192,000 is missing from the axonemes of the mutant pf2 but not from axonemes of other drc mutants. The same polypeptide in wild-type axonemes is as insoluble as other drc components (not shown). Therefore, it can be regarded as a drc component and will be referred to as component 7 (Table III).

The drc component 7 is the heaviest. Together with all other drc components it may form a complex of at least 530,000 apparent molecular weight and therefore a structure that is as large as a dynein heavy chain or more.

### Some of the drc Components Are Putative Defective Gene Products

The identification of a function of each drc component will require additional in vivo studies to be performed by approaches of genetics and molecular genetics. As preliminary approach to that development we intended to determine...
whether any of the drc components is a gene product of a drc mutant. Dikaryon rescue analysis of the mutants sups3 and sups4 was performed before (7). That analysis identified as putative defective gene products drc components 4 and 5, respectively. Our analysis confirmed only the identification of the putative defective gene product of sups4.

We analyzed the drc components by the dikaryon rescue procedure and found that drc components 1 and 5 are putative defective gene products of the mutants pf3 and sups4, respectively (Table III). None of the drc components appeared to be SUPs5 product. Surprisingly, drc component 4 appeared to be gene product of both sups3 and pf2. The study of pf2-wild-type dikaryons was performed by both one- and two-dimensional gel electrophoresis of axonemal proteins to include drc component 7 in the analysis (not shown).

Two-dimensional maps of 35S-labeled axonemal polypeptides from sups5-wild-type and pf3-wild-type dikaryons are shown as examples of results obtained by the dikaryon rescue procedure. The map of axonemal components from sups5-wild-type dikaryon (Fig. 7 a), is indistinguishable from that of a wild-type strain (compare with Fig. 5 a), whereas, the map of axonemal components from pf3-wild-type dikaryons (Fig. 7 b) is lacking drc component 1, the putative defective gene product of pf3.

Discussion

The Mechanism of Suppression of Flagellar Paralysis

Our conclusion that the drc mutants pf2, pf3, sups3, and supps4 suppress the paralysis of flagella of central complex as well as radial spoke mutants is in contrast with the statement that these mutants do not suppress the paralysis of flagella of central complex mutants (7). The origin of this discrepancy is unknown and may derive from conditions for cell growth before the phenotypic analysis and/or criteria to score suppression.

Suppression of flagellar paralysis in recombinants carrying one drc mutation and one central complex or radial spoke mutation is caused by either gaps within the inner arm row (as they occur in pf2, pf3, sups3, and sups4 flagella [12]) or defective control of inner arm activity (this may be caused by the sups4 mutation, as discussed below). In both cases the cause of suppression is a defect of a dynein arm. The same occurrence was observed also in the suppressors sups1 (7) and pf9-2 (15), which are defective for the β-chain of the outer arms and II inner arms, respectively. Therefore, an inhibition of flagellar movement may derive from an inhibition of dynein functions that occurs at different points of the dynein structure.

The inhibition can be bypassed if an axonemal component is missing. This axonemal component can be a dynein arm or part of a subunit of a dynein arm that is the site where the inhibition occurs. If this hypothesis is correct we should be able to find that also the mutant sups2 is a dynein arm mutant. A molecular phenotype of the mutant sups2 is unknown, although the mutant sups2 is a suppressor of flagellar paralysis of central complex and radial spoke mutants (7) like nearly all other suppressors of paralysis that are known (with the exception of the mutant pf9-2; reference 15).

Flagella of drc Mutants Lack Some of the Inner Arms

The first observation of a quantitative deficiency of I2 and I3 inner arms within flagella of drc mutants was obtained by the analyses of 35S-labeled dynein heavy chains. The γ outer arm heavy chain and Iβ inner arm heavy chain together were adopted as internal standards. Results from these analyses clearly indicated that flagella of some drc mutants contained low amounts of I2 and I3 inner arm heavy chains. The amounts of I2 and I3 inner arm heavy chains were 98%, in sups4, and 57%, in pf3, of the amounts present in a wild-type strain. The mutants were ordered as follows depending on the I2 and I3 inner arm heavy chains loss: sups4 < sups3 < pf2 < sups5 < pf3 (12). This evidence suggested to us that some of the drc proteins could form a binding site for I2 and I3 inner arms or be involved in a mechanism that makes I2 and I3 inner arms heavy chains competent to bind.

We considered that it was important to test these hypotheses because the outcome could help us to understand the pro-

Figure 7. Autoradiograms of 35S-labeled axonemal polypeptides that were resolved by two-dimensional electrophoresis. Only portions of the original maps resolving polypeptides in the 130,000-150,000 molecular weight range are shown. The gels are oriented with basic polypeptides on the left. Filled arrowheads and numbers indicate the drc components. (a) Axonemal components from sups5 wild-type dikaryons. (b) Axonemal components from pf3 wild-type dikaryons. An empty arrowhead indicates the position of the drc component 1. That component is the putative defective gene product of pf3.
cess of flagellar assembly and function. Before testing the competence of axonemes from drc mutants to bind inner arm heavy chains in vitro we first intended to determine whether the II inner arms are lacking from flagella of drc mutants similarly to I2 and I3 inner arms.

This experiment had priority because our previous measurements of the amounts of I2 and I3 inner arm heavy chains were relative to the amounts of combined outer arms and II inner arm heavy chains. Therefore, they could be affected by a defect of II inner arms. Moreover, the observation that a drc defect affects also the binding of II inner arms to the axonemes could be relevant to explain the phenomenon of suppression of flagellar paralysis of radial spoke and central pair mutants.

Our inability to resolve the II inner arm heavy chains from the outer arm heavy chains prompted us to isolate drc recombinant strains carrying the pf28 mutation and consequently lacking the outer dynein arms. Through the recombinants we were able to measure the quantities of II inner arm heavy chains and found that II inner arms were defective similarly to I2 and I3 inner arms. Although this result was straightforward it lead us to the following consideration.

The presence in the same strain of two mutations affecting the binding of dynein arms to the axonemes resulted in the formation of flagella that were shorter than those of pf28 or each of the drc mutants (with the exception of supAPf28 flagella) (Table I). This evidence indicated that the absence of outer arms and part of inner arms have a negative and synergistic effect toward the assembly of flagella. Moreover, the amounts of I2 and I3 inner arms are lower in the recombinants than in the drc mutants (Table I of this article and Table IV of reference 12). These results suggested to us that together pf28 and drc mutations negatively affect inner arm binding to doublet microtubules. Nevertheless, we concluded that a defect of II inner arms in the recombinants likely is caused by the presence of the drc mutation because the II inner arm defect in pf3pf28 and supAPf28 is as extensive as that of I2 and I3 inner arms.

The molar ratio between the γ outer arm heavy chain and the 1β inner arm heavy chain is 4:1 (13) and the loss of 1β in supAPf28 is at the most 70%. Therefore, the internal standard adopted in our previous measurements of I2 and I3 inner arms in supAPf28 could be 14% lower than in a wild-type strain. As a consequence it is possible that our assessment of I2 and I3 inner arm defect in supAPf28 was underestimated. For the same reason other drc mutants also could be slightly more defective for I2 and I3 inner arms than previously reported (12).

The drc Components Alter the Binding of I2 and I3 Inner Arms to the Axonemes

We have measured the amounts of inner arms that bind in vitro to ATP-insensitive sites of dynein-depleted axonemes of wild-type and drc mutants. We found that the extraction of dynein arms increased the number of binding sites for inner arms as much as 11 times in a wild-type strain, whereas the absence of some drc components decreased the binding of I2 and I3 inner arms to approximately one third of the wild-type level. The concentration of rebound inner arms was reduced to an extent that depends on the drc defects. From these observations we deduced that the lattice of outer doublet microtubules does not bind I2 and I3 inner arms with high affinity, whereas the drc forms at least one type of inner arm binding site. The alternative that drc components modify and make I2 and I3 inner arm heavy chains competent to bind is unlikely because neither matrix proteins nor ATP are required for the binding.

A dual function of the drc in binding the inner arms and regulating their function is suggested by the behavior of the mutant supA4. The mutant supA4 is a suppressor of paralysis of radial spoke and central complex mutants like the rest of the drc mutants but is similar to a wild-type strain in inner arm content both in vivo and in vitro. As a consequence drc components 5 and 6, which are lacking from supA4 as well as from other drc mutants, may be unnecessary for the inner arm binding but sufficient to cause flagellar paralysis in radial spoke or central pair mutants. Therefore, the drc may be formed by two parts: one regulating the function of I2 and I3 inner arms and the other binding the same arms to the A microtubule.

Among the drc components that form a putative binding site, components 1 and 2, which lack in pf3 and supA5 axonemes, affect I2 and I3 inner arm binding in vivo more than components 3, 4 and 7, which lack in pf2. Components 1 and 2 of the drc may function as a complex that binds the inner arms, whereas components 3, 4 and 7 may increase the affinity of the binding site for the inner arms.

All parts of the drc may be located between the inner arms and the outer dynein arms, as was deduced from electron microscopy images of the mutant pf2 (10). We noticed that the lack of drc components 1, 2, 5, 6, and 7 and a protein mass of at least 378,000 is correlated with the lack of an electron-dense structure from the axoneme of pf2 that is touching both the I2 inner arm and one every four outer arms. From that location the drc may interact differently with each type of inner arm.

The drc also could coordinate the activity of both outer and inner arms within a 96-nm repeat along the doublet microtubules. The existence of such coordination is suggested by results of in vitro experiments. The movement of outer dynein arms in disintegrating axonemes generates a sliding velocity of doublet microtubules that is higher than that of inner dynein arms (8). However, in vivo both types of arms must move at a similar speed in order to work efficiently.

The Function of the drc In Vivo

The following evidence indicated that formation of asymmetric waveforms and therefore efficient swimming of Chlamydomonas require a flow of information passing from the central complex to the inner dynein arms through radial spokes and drc. First, axonemes deprived of central complex form symmetric waveforms after reactivation in vitro instead of the asymmetric waveforms that are formed by intact axonemes (5). Second, radial spoke mutants move the flagella with symmetric waveforms, if they also carry a mutation suppressing their paralysis (3). Third, the row of inner dynein arms must be complete in order to form the asymmetric waveforms of wild-type Chlamydomonas flagella (2). Finally, defects of the drc reduce the number of I2 and I3 inner arms bound to the axoneme (12).

Further evidence that the drc is a major part in the chain of structures linking the central complex to inner arms is described in this report. Defects of the drc affect the binding
of every type of inner arm to the axonemes in vivo and affect the ATP-insensitive binding of I2 and I3 inner arms to the axonemes in vitro. Therefore, the drc mediates the binding of inner arms to the lattice of the microtubule wall and regulates the functions of the same arms along and around the axoneme.

The fact that the same drc mutations reverse the paralyzing effects of either central complex or radial spoke mutations reinforces the evidence that there is a flow of information passing through the central complex, the radial spokes, the drc and, finally, the dynein arms.

Signals passing from the central complex to the dynein arms do not require necessarily a physical contact. For instance, the II inner arm mutant pf9-2 was obtained as a bypass suppressor of the temperature-sensitive central complex mutant pf16BR3. However, it does not suppress the paralyzed flagella phenotype of other central complex or radial spoke mutations (15). Therefore, the interaction between the pf9-2 and pf16BR3 mutations represent a specific interaction between the central complex and the II inner arms and not a more general bypass mechanism such as that shown by all other mutations suppressing flagellar paralysis of central complex and radial spoke mutants.

**Perspectives**

Our study of inner arm binding through quantitative analyses of I2 and I3 inner arm bound in vitro extends previous observations indicating that inner arms must rebind to axonemal doublet microtubules at specific sites (17). We have focused on the ATP-insensitive binding sites of inner arms and identified the drc as the adapter that binds I2 and I3 inner arms. The fact that inner arm reconstitution on the outer doublets also restores the ability of the axoneme to disintegrate (17) will allow us to determine whether the absence of drc components alters the sliding velocity of microtubules.

This work was supported by the grant GM-44467 from the National Institutes of Health.

Received for publication 22 November 1993 and in revised form 7 February 1994.

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