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# The Role of Preassembled Cytoplasmic Complexes in Assembly of Flagellar Dynein Subunits

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Previous work has revealed a cytoplasmic pool of flagellar precursor proteins capable of contributing to the assembly of new flagella, but how and where these components assemble is unknown. We tested *Chlamydomonas* outer-dynein arm subunit stability and assembly in the cytoplasm of wild-type cells and 11 outer dynein arm assembly mutant strains (*oda*1-*oda*11) by Western blotting of cytoplasmic extracts, or immunoprecipitates from these extracts, with five outer-row dynein subunit-specific antibodies. Western blots reveal that at least three *oda* mutants (*oda*6, *oda*7, and *oda*9) alter the level of a subunit that is not the mutant gene product. Immunoprecipitation shows that large preassembled flagellar complexes containing all five tested subunits (three heavy chains and two intermediate chains) exist within wild-type cytoplasm. When the preassembly of these subunits was examined in *oda* strains, we observed three patterns: complete coassembly (*oda* 1, 3, 5, 8, and 10), partial coassembly (*oda*7 and *oda*11), and no coassembly (*oda*2, 6, and 9) of the four tested subunits with HC$\beta$. Our data, together with previous studies, suggest that flagellar outer-dynein arms preassemble into a complete $M_r \simeq 2 \times 10^6$ dynein arm that resides in a cytoplasmic precursor pool before transport into the flagellar compartment.

## INTRODUCTION

*Chlamydomonas* flagella are made up of at least 150 different proteins (Piperno *et al.*, 1977) and show an intricate structural arrangement, with complex components such as inner and outer dynein arms, radial spokes, and a ring of nine doublet microtubules surrounding two central singlet microtubules (Ringo, 1967; reviewed in Dutcher, 1995). At the flagellar base or transition zone (Randall *et al.*, 1967), flagellar components appear to be functionally separated from the cell body (Musgrave *et al.*, 1986; Jarvik and Suhan, 1991). Since neither DNA nor ribosomes have been found within flagella (Ringo, 1967; Johnson and Rosenbaum, 1990, 1993), all flagellar proteins must be transported from the cytoplasm for assembly into the complex flagellar structure. How these proteins are selected and transported through the transition zone is unknown (reviewed by Dentler, 1981).

Studies of many flagellated and ciliated cells have revealed a cytoplasmic pool of precursor protein capable of contributing to the assembly of new flagella or cilia under conditions where synthesis of new proteins is inhibited (Rosenbaum and Child, 1967; Rosenbaum *et al.*, 1969; Child and Apter, 1969; Stephens, 1977). In *Chlamydomonas* this pool is sufficient for assembly of two new half-length flagella (Rosenbaum *et al.*, 1969). In spite of these studies, several questions about flagellar organelle assembly remain little understood, including the location of flagellar precursor proteins in the cytoplasm, how they are transported to the flagellar compartment, whether most proteins are transported as individual subunits or as complexes, and what limits assembly and thus determines flagellar length. In this paper we explore assembly mechanisms of a multisubunit flagellar ATPase, the outer dynein arm.

Recent observations suggest that dynein arms may be preassembled in the cytoplasm and transported to the flagellum as a complex. In *Paramecium*, a dynein complex has been isolated from the cytoplasm that has the same sedimentation rate (22S) as extracted axonemal outer dynein arms and contains a heavy chain

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(HC)[1] with a similar vanadate cleavage pattern and antibody cross- reactivity to an axonemal HC present in extracted 22S axonemal dynein (Fok *et al.*, 1994). In *Chlamydomonas*, an inner-row dynein subunit (p28), extracted from the flagellar cytoplasmic matrix with nonionic detergent, sediments on sucrose gradients as part of a larger complex, suggesting that at least some inner-row dynein subunits assemble before their association with doublet microtubules (Piperno and Mead, 1997).

Indirect evidence suggests that some *Chlamydomonas* dynein mutants with assembly defects accumulate partially assembled complexes in their cytoplasm. When *Chlamydomonas* gametes of opposite mating type are mixed, fusion results in temporary dikaryons with four flagella and a common cytoplasm. Fusion between gametes containing mutations at different loci can lead to cytoplasmic complementation with assembly of functional components into the flagella and restoration of normal or near-normal motility (Luck *et al.*, 1977; Johnson and Rosenbaum, 1993). However, certain combinations of the 14 known outer-dynein arm assembly (*oda*) loci fail to complement in dikaryons (Huang *et al.*, 1979; Kamiya, 1988; Luck and Piperno, 1989; Koutoulis *et al.*, 1997; for a general discussion of nonallelic noncomplementation in *Chlamydomonas*, see Dutcher and Lux, 1989). Kamiya (1988) assayed cytoplasmic complementation by measuring restoration of beat frequency in temporary dikaryons that formed between pairwise combinations of *oda* mutant gametes. Based on his data, *oda* mutants fall into one of three groups defined by their inability to complement either *oda*1 (*oda*1 and *oda*3), *oda*2 (*oda*2, 4, 6, 7, and 9), or *oda*5 (*oda*5, 8, and 10); these data are summarized in Table 1. One explanation for this lack of cytoplasmic complementation is that each gamete preassembles nonmutant subunits into partial dynein complexes that are incapable of dissociation and reassociation into wild-type complexes when gametes of opposite mating type fuse. Alternatively, absence of one protein may cause instability of a binding partner/partners or sequestration of the remaining proteins in a compartment that is unavailable to the assembly mechanism. When outer dynein arms are extracted from *Chlamydomonas* flagellar axonemes, they typically dissociate into three smaller subcomplexes that can be separated by sucrose gradient fractionation into 18S, 12S, and 7S components (Piperno and Luck, 1979; Takada and Kamiya, 1994) as illustrated diagrammatically in Figure 1. Our data show that complexes do exist in the cytoplasm before their attachment onto axonemal microtubules, but these complexes are not identical to the complexes produced by extraction from axonemes.

Outer dynein arms in *Chlamydomonas* contain 3 HCs of ~500 kDa each (HC$\alpha$, HC$\beta$, and HC$\gamma$), 2 intermediate chains (IC78, and IC70), about 10 light chains (LCs) ranging from 22 to 8 kDa, and a 7S factor of three proteins that form an outer dynein arm attachment site or docking complex (DC105, DC62.5, and DC25) (Piperno and Luck, 1979; Pfister *et al.*, 1982; King and Witman, 1990; Takada and Kamiya, 1994; Koutoulis *et al.*, 1997). For this report we tested outer-dynein arm subunit stability in the cytoplasm of wild-type and 11 assembly mutant strains by probing Western blots of cytoplasmic extracts with several subunit-specific antibodies. To test the state of dynein assembly in these extracts, we used the same antibodies to probe Western blots of immunoprecipitates prepared with a single subunit-specific antibody. The 11 loci marked by *oda* mutations include those known to encode five of the enzyme subunits and two proteins of the dynein attachment complex (summarized in Table 2 and Figure 1). Gene products of the remaining 6 loci are unknown. Two additional mutations, *pf*13 and *pf*22, also reduce outer-row dynein assembly, but because cells carrying these mutations have short paralyzed flagella (Huang *et al.*, 1979), they represent a different class of flagellar assembly defect than the *oda* mutants and were not included in this study. Our results show that dynein subunits preassemble in the cytoplasm and that both preassembly of dynein complexes and protein instability contribute to dikaryon cytoplasmic noncomplementation. These results provide new information on subunit interactions, dynein mutant phenotypes, and the process of flagellar assembly.

## MATERIALS AND METHODS

### *Mutant Strains*

All of the mutant strains of *Chlamydomonas reinhardtii* used in this study have been described previously (see Table 2). The *oda*2 allele used was *pf*28 (Mitchell and Rosenbaum, 1985). Cells were maintained on minimal medium using standard procedures (Harris, 1989).

### *Cell Cytoplasmic Extract*

*Chlamydomonas* cells were grown in 500 ml of liquid M medium (Sager and Granick, 1953) with aeration in continuous light to a density of $10^6$ cells/ml, harvested by centrifugation (550 $\times$ *g* for 6 min at 22°C), and resuspended in ice-cold HMDEK (10 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 0.1 mM EDTA, 25 mM potassium chloride, pH 7.4) to a total of 500 $\mu$l. The suspension was transferred to a 1.5-ml microfuge tube that contained an equal volume of acid-washed glass beads (1 mm) and vortexed at setting 6.5 on a Genie II vortexer for 1 min. Cell suspensions were then centrifuged

[1] Abbreviations: DC, docking complex; HC, heavy chain; IC, intermediate chain; IP, immunoprecipitation; LC, light chain.

[2] The two thioredoxin-related outer-arm LCs were initially identified as 14-kDa IC-associated and 16-kDa HC$\alpha$-associated proteins (Patel-King *et al.*, 1996), and a Tctex2 homologous LC was identified as a 19-kDa HC$\beta$-associated protein (Patel-King *et al.*, 1997). More recently, this assignment has been modified to assign the two thioredoxin homologues as the 16-kDa (HC$\alpha$) and 19-kDa (HC$\beta$) LCs, and the Tctex2 homologue as the 20-kDa IC-associated LC (S. King, personal communication).

using a Beckman L8 centrifuge at 48,000 × *g*, at 4°C for 2 h. Supernatants were either used for immunoprecipitation as described below or mixed with 0.25 volume of 4× sample buffer (8% SDS, 40% glycerol, 125 mM Tris-HCl, pH 6.8, with Pyronin Y added as tracking dye) and β-mercaptoethanol, to a final concentration of 0.7 M, and stored at −20°C for SDS-PAGE. Pellets were resuspended in 500 μl HMDEK, mixed with 0.25 volume 4× sample buffer, and stored at −20°C for SDS-PAGE. Protein concentration was determined by the Bradford dye binding method using BSA as a standard (Bradford, 1976).

### *Axonemal Preparation*

Axonemes were isolated by the method of Witman *et al.* (1978). Cells were grown in 500 ml of liquid M medium (Sager and Granick, 1953) with aeration in continuous light to a density of $10^6$ cells/ml, harvested by centrifugation at 550 × *g* for 6 min at 22°C, washed with 10 mM HEPES, pH 7.4, centrifuged again, and resuspended in 10 ml HMDS (10 mM HEPES, pH 7.4, 5 mM MgSO4, 1 mM DTT, and 4% sucrose). Resuspended cells were deflagellated with 400 μl 50 mM dibucaine (CIBA Pharmaceutical, CIBA-GEIGY, Summit, NJ) and diluted with 10 ml ice-cold HMDS containing 2 mM EGTA and 2 mM phenylmethylsulfonyl fluoride, and cell bodies were removed by centrifugation at 4°C for 7 min at 1,550 × *g*. The supernatant was collected and recentrifuged as above. Cell-free supernatant was then centrifuged at 31,000 × *g* to pellet axonemes, which were resuspended in HMDEK and an equal volume of 2× sample buffer. β-Mercaptoethanol was added to a final concentration of 0.7 M, and samples were stored at −20°C.

### *SDS-PAGE and Western Blotting*

Samples were prepared and run with Tris-glycine-buffer (Laemmli, 1970) in 5% stacking gels and 5, 7, or 12% separating gels (designated in text) prepared from stocks that contained 30% acrylamide and 0.4% bis-acrylamide. Broad Range protein standards (New England Biolabs, Beverly, MA) of 212, 158, 116, 97.2, 66.4, 55.6, and 42.7 kDa were used, and gels were either stained with Coomassie Blue to show total protein or transferred to immobilon membrane (Millipore, Bedford, MA) for Western blotting following the recommendations of Burnette (1981). Gels were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 10 min and transferred either at 200 mA for 12 h (Figures 2, 3, 4A, and 5) or at 300 mA for 6 h (Figures 4B and 6). Protein standard lanes were separated from sample lanes and stained with amido black. Antibody binding and detection were performed as directed in the POD chemiluminescence kit (Boehringer Mannheim, Indianapolis, IN). Briefly, transferred blots were blocked with 1% POD blocking solution for 1 h at room temperature, incubated with the primary antibody in 0.5% POD blocking solution for 3 h at room temperature, washed with TBST (50 mM Tris base, 150 mM NaCl, pH 7.5, with 0.1% Tween-20 [vol/vol]) 2 × 10 min, washed with 0.5% POD blocking solution 2 × 10 min, incubated with secondary antibody in 0.5% POD blocking solution for 1 h at room temperature, washed with TBST four times for 10 min, and then incubated with developing solution for 1 min and exposed to Biomax film (Eastman Kodak, Rochester, NY). Antigen quantitation was estimated by comparison with a blot of an antigen dilution series (2, 1, 0.8, 0.6, 0.4, 0.2, 0.1 x WT control) processed in parallel.

### *Antibodies*

Anti-HCβ mAb C11.6 was concentrated by ammonium sulfate precipitation from hybridoma culture supernatants and used at a 1:100 dilution. It was generated from the same fusion as C11.13 (Mitchell and Rosenbaum, 1986). Anti-IC70 mAb 1869A ascites was used at 1:2000 dilution. Anti-IC78 mAb 1878A hybridoma culture supernatant was kindly donated by Dr. G. Witman (King *et al.*, 1985) and was used at a 1:2 dilution. Anti-HCα polyclonal antibody B3B was produced in a rabbit by immunization with a purified bacterial fusion protein. A 1-kilobase (kb) *Pml*I/*Hpa*I fragment of HCα cDNA pBcA6 (Mitchell and Brown, 1997), which encodes amino acids 512–838 of HCα (a region unrelated to other DHC sequence and that contains the HCα EPAA repeat element), was cloned into vector pGEX-4T-2 (Pharmacia Biotech, Piscataway, NJ) at a *Sma*I site and was transformed into DH5αF′ *E. coli*. Fusion protein expression was induced for 3 h with 0.1 mM isopropyl-β-thiogalactopyranoside at 37°C. Fusion protein was solubilized and purified by the method of Frangioni and Neel (1993), run on an SDS-PAGE gel, transferred to nitrocellulose, and visualized with Ponceau S. Nitrocellulose strips containing the protein of interest were dissolved in dimethyl sulfoxide, mixed with adjuvant, and used for immunization. Specific antibodies were affinity purified from whole sera using Western blots of the fusion protein (Frangioni and Neel, 1993) and were used at a dilution of 1:50. Anti-HCγ mAb 25–8, kindly donated by Dr. G. Piperno (King and Witman, 1988), was used at a 1:10 dilution. Peroxidase-labeled goat anti-mouse or goat anti-rabbit secondary antibodies (Bio-Rad Laboratories, Hercules, CA) were used at a 1:6000 dilution.

### *Immunoprecipitation*

Cell extracts (0.5 ml) were mixed in a 15-ml conical tube with an equal volume of ice-cold immunoprecipitation (IP) buffer (HMDEK, 75 mM NaCl, 0.01% thimersol, 0.5 mM PMSF, 3% BSA, 0.1% Triton X-100, pH 7.5) and preabsorbed with 25 μl of 50/50 (vol/vol) protein A agarose in IP buffer for 30 min on ice. mAb anti-HCβ antibody C11.6 was added to preabsorbed extracts and incubated for 3–4 h at 4°C. A 100-μl volume of 50/50 (vol/vol) protein A agarose (Sigma Chemical, St. Louis, MO) in IP buffer was added, and the tubes were mixed gently for 1 h. Agarose beads were washed three times with IP buffer containing 0.05% Triton X-100. Immune complexes were eluted by addition of 2× sample buffer containing 0.7 M β-mercaptoethanol and incubation for 2 min in boiling water. The quantity of HCβ immunoprecipitated with antibody C11.6 from wild-type and *oda* mutant cytoplasmic extracts was determined from preliminary Western blots. Subsequent loads of immunoprecipitate samples were adjusted to include equal amounts of HCβ.

### *Partial Acid Hydrolysis*

Western blots of proteins subjected to partial acid hydrolysis were generated by a modification of the method described by Cleveland *et al.* (1977). Briefly, cytoplasmic extracts or whole axonemes were run on an SDS-PAGE gel along with molecular weight markers. The gel was stained in 0.1% Coomassie blue, 50% methanol, and 10% acetic acid for 30 min and destained in 5% methanol and 10% acetic acid for 45 min. Bands of ~70 kDa molecular mass were cut from the gel and soaked 30 min in 0.125 M Tris/HCl, pH 6.8, 0.1% SDS, 1 mM EDTA. Slices were then lyophilized and either stored frozen at −20°C (controls) or incubated with 70% formic acid for 16 h at 37°C, washed with 50% methanol, and lyophilized. Gel slices were then rehydrated with buffer (1% SDS, 10 mM Tris/HCl, pH 8, 0.1% β-mercaptoethanol, and 10% glycerol) for 6 h, pushed into the bottom of an SDS-PAGE well, and overlayed with 10 μl of Tris/HCl buffer containing 20% glycerol for electrophoresis and transfer to PVDF membranes.

## RESULTS

### *Western Blots of Cytoplasmic Extracts*

Based on previous studies of flagellar regeneration, *Chlamydomonas* cells maintain a cytoplasmic pool of flagellar subunits sufficient to assemble two half-length flagella (reviewed by Johnson and Rosenbaum,

1993). Our first goal was to determine, for several outer-dynein arm subunits, whether similar levels of protein were present in cytoplasmic extracts of wild-type (WT) cells and of outer-dynein arm assembly mutants. Preliminary tests showed that our previously described HC$\alpha$ antibody C2.14 (Mitchell and Rosenbaum, 1986) lacked sufficient sensitivity for these studies. Therefore, a polyclonal rabbit serum (B3B) was produced by immunization with a bacterial GST fusion protein encoding a portion of HC$\alpha$ (see MATERIALS AND METHODS). The specificity of this antibody is demonstrated in Figure 2, in which Western blots of flagellar proteins from WT cells and from cells harboring the *oda*11 mutation (which blocks HC$\alpha$ assembly; see Table 2) were probed with C11.6 (anti-HC$\beta$) and with B3B. Antibody C11.6 detects HC$\beta$ in both WT and *oda*11 flagella (left panel), whereas B3B detects HC$\alpha$ in WT but not *oda*11 flagella (right panel). When cytoplasmic extracts of WT and 11 *oda* mutants were probed with this antibody, it was found that similar signals were present in all but three strains. The amount of HC$\alpha$ was less than half the normal levels in *oda*5 and was completely missing in *oda*7 and *oda*11 (Figure 3). In three independent cytoplasmic extracts, the level of HC$\alpha$ in *oda*5 varied between 40% and 60% of WT, whereas no antigen was detectable in either *oda*7 or *oda*11. Since the HC$\alpha$ gene has been shown to be closely linked to *oda*11, but not *oda*7 (Sakakibara *et al.*, 1991), our data support the assignment of *oda*11 as the HC$\alpha$ locus and suggests that HC$\alpha$ is synthesized normally in *oda*7 but is unstable in this mutant background. Identical results were obtained with extracts from an *oda*7 strain generated by two rounds of backcrosses to wild-type strain 137c, supporting linkage between *oda*7 and this apparent defect in HC$\alpha$ stability.

HC$\beta$, like HC$\alpha$, was present at reduced levels in *oda*5 cytoplasm and was undetectable in *oda*4 cytoplasm (Figure 4A). Absence of HC$\beta$ in *oda*4 is expected based on previous identification of *oda*4 as the HC$\beta$ locus, whereas its reduction in *oda*5 suggests that HCs may have reduced stability in the absence of the *oda*5 gene product. In addition, proteolytic fragments of HC$\beta$ were always more prominent in *oda*1 and *oda*3 than in any of the other strains. These two loci encode gene products that form part of the DC (Table 2), and lack of HC$\beta$ stability therefore indicates that the DC likely interacts with HC$\beta$ in the cytoplasm, altering its susceptibility to proteolysis. Similar analysis of HC$\gamma$ revealed the presence of this HC in the cytoplasm of all *oda* mutants except *oda*2 (Figure 4A), which has previously been identified as a mutation in the HC$\gamma$ gene. No quantitative conclusions could be drawn regarding HC$\gamma$ levels in the remaining strains using this antibody.

When these same blots were probed with anti-IC78 and anti-IC70 (Figure 4A, bottom two panels) two unanticipated results were found. The anti-IC78 antibody, whose specificity for a single flagellar dynein IC has already been documented (King *et al.*, 1985, 1986), cross-reacted with cytoplasmic protein bands of slightly lower apparent molecular weight than IC78 (asterisk in Figure 4). These bands showed variable levels of signal intensity unrelated to the strain used, but their presence in *oda*9 cytoplasm suggests that they are not due to breakdown of the IC78 protein since *oda*9 is a mutation in the IC78 gene. Since flagellar and cytoplasmic dynein ICs are homologous (Wilkerson *et al.*, 1995), these bands might be due to antibody cross-reactivity to a cytoplasmic dynein IC. IC78 was not only absent in *oda*9 cytoplasm, but was also reduced in *oda*5 (40% of WT) and *oda*6 (10% of wt) as well. Similarly, IC70 was not only missing in *oda*6, but was also reduced to 60% in *oda*5 and 30% in *oda*9. Both the level of IC70 in *oda*9 cytoplasm and the level of IC78 in *oda*6 cytoplasm varied among independent sample preparations, from the levels seen in the bottom two panels of Figure 4A to those illustrated in Figure 4B. We were unable to determine the cause of this variation, but the reduction of IC78 in *oda*6 was consistently greater than the reduction of IC70 in *oda*9.

When blots of *oda*6 cytoplasmic extract probed with anti-IC70 were exposed for extended periods, small amounts (2% of WT) of antigenic protein that migrated identically to flagellar outer dynein arm IC70 were detectable (Figure 4C). Sequence analysis has shown that *oda*6–95, the allele used in this study, is a frameshift that encodes a truncated protein 10% the size of full-length IC70, and Western blots with an anti-IC70 mAb confirmed that *oda*6–95 flagella lack detectable IC70 (Mitchell and Kang, 1993). To further test the nature of this antigen, patterns generated by partial acid hydrolysis of the *oda*6–95 cytoplasmic 70-

**Table 1.** Complementation in temporary dikaryons between *oda* strains[a]

| | *oda*1 | *oda*2 | *oda*3 | *oda*4 | *oda*5 | *oda*6 | *oda*7 | *oda*8 | *oda*9 | *oda*10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *oda*1 | − | | | | | | | | | |
| *oda*2 | + | − | | | | | | | | |
| *oda*3 | − | + | − | | | | | | | |
| *oda*4 | + | − | + | − | | | | | | |
| *oda*5 | + | + | + | + | − | | | | | |
| *oda*6 | + | − | + | − | + | − | | | | |
| *oda*7 | + | − | + | − | + | − | − | | | |
| *oda*8 | + | + | + | + | − | + | + | − | | |
| *oda*9 | + | − | + | + | + | − | + | + | − | |
| *oda*10 | + | + | + | + | − | + | + | − | + | − |

[a] + Indicates complementation, defined as dikaryon beat frequency greater than 35 Hz, and is based on the data reported in Table 3 of Kamiya (1988) in which the mean frequency of all dikaryons indicated above as noncomplementing (−) was 26 ± 4 Hz (n = 22), while that of all complementing (+) dikaryons was 42 ± 3 Hz (n = 33).

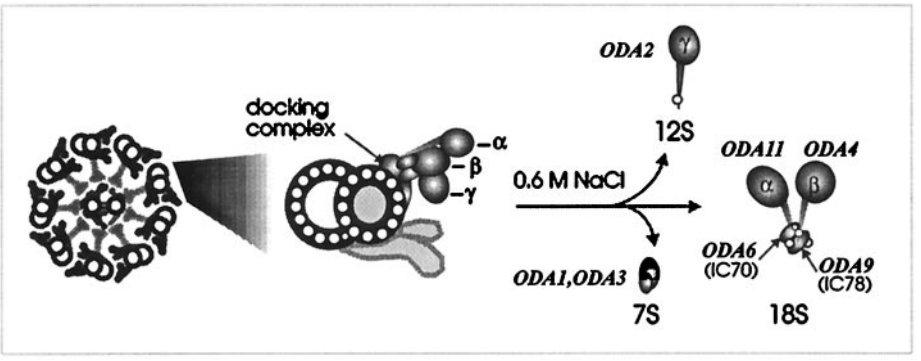


**Figure 1.** Diagram illustrating the relationship between outer-row dynein arms in situ and after dissociation by 0.6 M NaCl into 7S, 12S, and 18S particles. The loci (*ODA1, ODA2,* etc.) that are known to encode subunits of each particle are also indicated.

kDa antigen were compared with patterns generated from WT flagellar IC70 and from 70-kDa antigens present in the cytoplasmic extract of an *oda*2 strain (*pf*28 allele). All three samples consisted of SDS-PAGE–purified 70-kDa proteins, which were then subjected to hydrolysis by formic acid, separation of hydrolysis products by SDS-PAGE, and Western blotting with mAb 1869A. All three samples showed identical fragment patterns (Figure 5). From this we concluded that the 70-kDa antigen in *oda*6 cytoplasm represents a bona fide IC70 protein present at very low levels. In spite of its presence, no IC70 has been seen in *oda*6 flagella (Mitchell and Kang, 1993) or in coprecipitates with HC$\beta$ from *oda*6 cytoplasm (detailed below). We do not believe the IC70 seen in Figure 4C results from the appearance of spontaneous revertants in our cultures since the spontaneous reversion frequency of *oda*6–95 is low (Mitchell and Kang, 1991), and since we have never observed phenotypic revertants in these cultures. However, residual levels of gene expression in frameshift mutations such as *oda*6–95 can result from +1, +2, −1, or −2 shifts during ribosomal translation (Weiss *et al.*, 1987), and a string of four cytosines upstream of the *oda*6–95 mutation site could allow a −1 translational shift that would result in synthesis of a full-length protein with only eight amino acids different from WT. We previously analyzed an intragenic pseudo-revertant of *oda*6–95 (*oda*6-r88), in which a second frameshift mutation had occurred 23 codons upstream of the *oda*6–95 mutation. Since this revertant allele generates an IC70 protein that assembles into flagellar outer dynein arms (Mitchell and Kang, 1993), the hypothesized gene product generated by a translational frameshift of *oda*6–95 mRNA should also sup-

**Table 2.** Characteristics of outer-dynein arm assembly mutants

| Mutation | Gene product | Beat frequency | Assembly defect | Reference |
|---|---|---|---|---|
| *oda*1 | DC63 | 25 Hz[a] | Docking complex + outer arm | [bc] |
| *oda*2 (*pf*28) | HC$\gamma$ | 25 Hz[a] | Outer arm | [bd] |
| *oda*3 | DC105 | 25 Hz[a] | Docking complex + outer arm | [be] |
| *oda*4 | HC$\beta$ | 25 Hz[a] | Outer arm | [bf] |
| *oda*5 | ? | 25 Hz[a] | Outer arm | [b] |
| *oda*6 | IC70 | 25 Hz[a] | Outer arm | [bg] |
| *oda*7 | ? | 25 Hz[a] | Outer arm | [b] |
| *oda*8 | ? | 25 Hz[a] | Outer arm | [b] |
| *oda*9 | IC78 | 25 Hz[a] | Outer arm | [bh] |
| *oda*10 | ? | 25 Hz[a] | Outer arm | [b] |
| *oda*11 | HC$\alpha$ | 55 Hz | HC$\alpha$ + 16 kD LC | [bi] |

[a] Wild-type beat frequency = 60 Hz.
[b] Kamiya, 1988.
[c] Takada and Kamiya, 1994; Takada *et al.*, 1996.
[d] Mitchell and Rosenbaum, 1985; Wilkerson *et al.*, 1994.
[e] Koutoulis *et al.*, 1997.
[f] Huang *et al.*, 1982; Mitchell and Brown, 1994; Porter *et al.*, 1994.
[g] Mitchell and Kang, 1991.
[h] Wilkerson *et al.*, 1995.
[i] Sakakibara *et al.*, 1991.

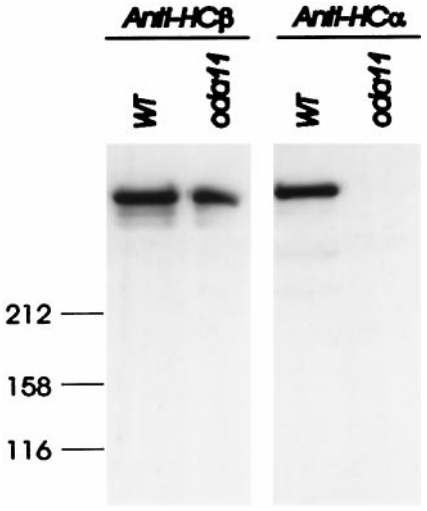


**Figure 2.** Specificity of antibody B3B. WT and *oda*11 flagella, run on 5% gels and blotted to PVDF membrane, were probed with C11.6 (left panel), which detected equal levels of HC$\beta$ in both samples. A parallel blot probed with antibody B3B (right panel) detected a single band in WT flagella that is missing in flagella of HC$\alpha$ assembly mutant *oda*11. Antibodies were detected with a peroxidase-linked secondary antibody and chemiluminescent detection.

port dynein assembly, but may be synthesized at a rate too low to allow coassembly with other subunits.

### *Westerns of Immunoprecipitates from Cytoplasmic Extracts*

Our second goal was to determine whether immunoprecipitation of one dynein subunit from a native cytoplasmic extract would result in coprecipitation of a preassembled complex. For these experiments we chose an anti-HC$\beta$ mAb, since it has already been demonstrated that an outer-dynein arm complex containing two HCs, two ICs, and several LCs can be immunoprecipitated from crude flagellar extracts using anti-HC$\beta$ mAb C11.13 (Mitchell and Rosenbaum, 1986). mAb C11.6, which was prepared from the same hybridoma fusion as C11.13 and recognizes the same HC$\beta$ epitope (see MATERIALS AND METHODS), was used because of instability of the C11.13 hybridoma cell line. When C11.6 immunoprecipitates of WT cytoplasmic extracts were transferred and probed with anti-HC$\alpha$, anti-HC$\beta$, anti-HC$\gamma$, anti-IC78, and anti-IC70 antibodies, all five subunits were found to be present (Figure 6, WT lanes).

We then repeated this procedure with *oda* mutant cytoplasmic extracts. Since HC$\beta$ is present in all *oda* mutants (except *oda*4), this experiment should reveal proteins associated with HC$\beta$ in each mutant. Immunoprecipitates from cytoplasmic extracts of *oda*1 contained all five tested subunits (HC$\alpha$, HC$\beta$, HC$\gamma$, IC78, and IC70) as did immunoprecipitates of *oda*3, *oda*5,

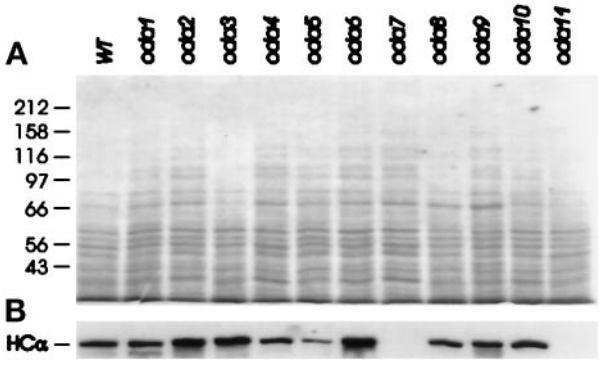


**Figure 3.** Western blots of HC$\alpha$ in WT and *oda* cytoplasmic extracts. Samples of cytoplasmic extracts from wild-type and *oda*1-*oda*11 cells were separated on 7% gels and blotted to PVDF membrane. Each lane was loaded with 20 $\mu$g of total protein. (A) Gel stained with Coomassie blue. (B) Western blot probed with B3B (anti-HC$\alpha$). No HC$\alpha$ was detectable in either *oda*7 or *oda*11; levels in *oda*5 are 50% of WT.

*oda*8, and *oda*10 extracts, demonstrating that none of these five mutations prevent association of HCs and ICs. The immunoprecipitate from *oda*4 cytoplasm showed no antigenic protein in any blot, which was expected since the *oda*4 mutation disrupts the antigen target used for these immunoprecipitations. Immunoprecipitates from *oda*11 contain all the major outer dynein arm proteins except HC$\alpha$, but the *oda*2, *oda*6, *oda*7, and *oda*9 mutations had more drastic effects. Those from extracts of *oda*2, *oda*6, and *oda*9 showed the presence of only HC$\beta$ (Figure 6). Absence of HC$\gamma$ (*oda*2) or the IC dimer (*oda*6 and *oda*9) thus prevent preassembly of HC$\beta$ with the remaining tested subunits, even though those subunits are present in the cytoplasm at approximately WT levels. Although faint HC$\alpha$ bands are detectable in *oda*2 and *oda*9 immunoprecipitates (Figure 6, top panel), with longer exposures such bands became visible in all lanes except those already shown to completely lack this antigen (see Figure 3) and are due to nonspecific binding to the protein A agarose used in the immunoprecipitation. Immunoprecipitates of *oda*7 revealed a complex between HC$\beta$, HC$\gamma$, and faint traces of IC78 and IC70, but did not contain HC$\alpha$. Both IC70 and IC78 are present at normal levels in *oda*7 cytoplasm (*oda*7 lanes in Figure 4); therefore, their reduction in *oda*7 immunoprecipitates indicates an effect on IC–HC interactions. The IC dimer appears essential for joining HCs together since neither HC$\alpha$ nor HC$\gamma$ coprecipitate with HC$\beta$ when the IC dimer is missing (*oda*6 and *oda*9 lanes in Figure 6). The *oda*7 defect thus appears to allow an association between the ICs and HCs that is strong enough to support assembly between HC$\beta$ and HC$\gamma$, but not strong enough to withstand immunoprecipitation.

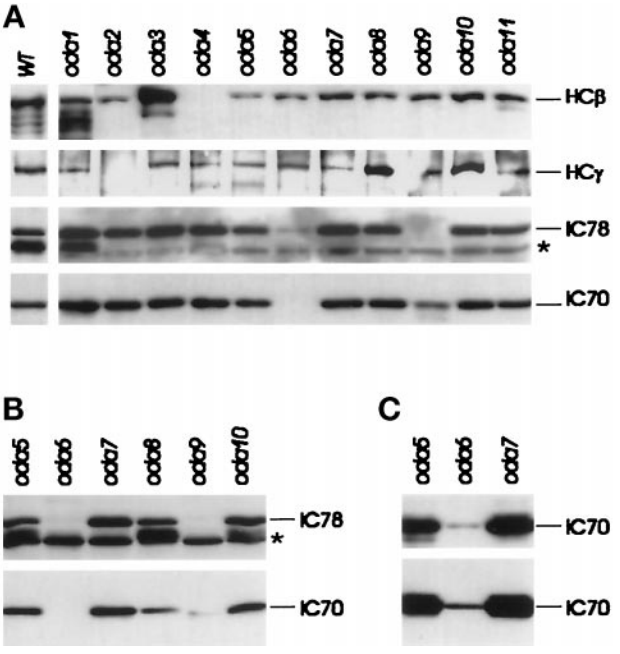


**Figure 4.** Western blots of WT and *oda* cytoplasmic extracts test subunit stability. Cytoplasmic extracts of WT and *oda*1-*oda*11 prepared as in Figure 2 were probed with mAbs C11.6 (anti-HC$\beta$), 25–8 (anti-HC$\gamma$), 1878A (anti-IC78), and 1869A (anti-IC70). (A) Outer dynein arm proteins (indicated along the right margin) present in cytoplasmic extracts of WT and *oda*1–11. Most *oda* mutant samples display antigen levels similar to WT, but there are several exceptions as discussed in text. Multiple bands below HC$\beta$ in WT, *oda*1, and *oda*3 are due to the breakdown of HC$\beta$. No detectable HC$\beta$ was found in *oda*4, no HC$\gamma$ was found in *oda*2, no IC78 was found in *oda*9, and no IC70 was found in *oda*6 (at this exposure; see text and Figure 3C). 1878A (anti-IC70) cross-reacted with cytoplasm proteins migrating faster than 78 kDa (asterisk) that are apparently unrelated to flagellar dynein. (B) Western blots of a second set of independently prepared cytoplasmic extracts from *oda*5-*oda*10 probed with 1878A (anti-IC78) and 1869A (anti-IC70) that illustrate the variability in the reductions of IC70 in *oda*9 and IC78 in *oda*6 (compare with bottom two panels in panel A). (C) Upper and lower panels show the results of a 10-fold increase in exposure time for lanes *oda*5–*oda*7 from the bottom panels of Figure 3A and Figure 3B, respectively, and reveal an IC70 band in *oda*6 cytoplasmic extracts.

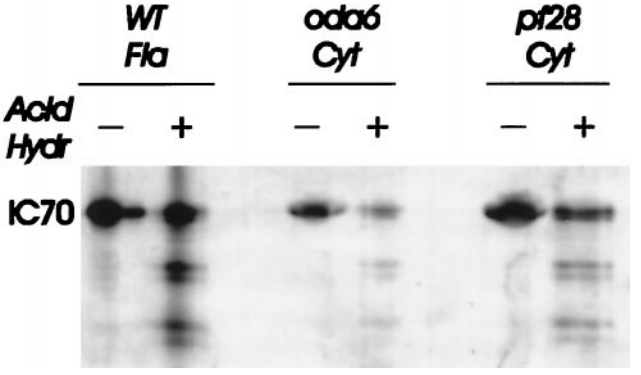


**Figure 5.** Fragmentation of 70-kDa antigens by acid hydrolysis. Proteins of ~70 kDa were purified by SDS-PAGE from WT axonemes, *oda*6 cytoplasmic extract, and pf28 cytoplasmic extract, digested by partial hydrolysis with formic acid, separated on a 12% gel, blotted to PVDF membrane, and probed with 1869A (anti-IC70). All three samples generate similar fragmentation patterns.

## DISCUSSION

In this report we have examined the stability and assembly state of several outer-dynein arm subunits that reside in a cytoplasmic pool of flagellar precursor proteins. Although the existence of a *Chlamydomonas* flagellar precursor pool has been established, the assembly state of proteins within this precursor pool has not been previously examined. We show, by coprecipitation of five flagellar outer-dynein arm subunits with a mAb against one subunit, that large preassembled flagellar complexes exist within this cytoplasmic pool (Figure 6). A preassembled complex of flagellar radial spoke proteins has been observed in *Chlamydomonas* cytoplasmic extracts (Diener *et al.*, 1996), and inner-row dyneins may also preassemble (Piperno and Mead, 1997), which suggests that many flagellar components assemble to varying degrees in a cytoplasmic pool before flagellar assembly.

We then examined 11 outer-dynein arm assembly mutants, to determine whether lack of flagellar assembly reflected loss of subunit stability, a block to cytoplasmic preassembly, or potential defects in transport and binding of preassembled complexes onto flagellar doublet microtubules. Defects in each of these three categories were observed. We confirmed the previous identification of gene products for *oda*2 (HC$\gamma$), *oda*4 (HC$\beta$), *oda*6 (IC70), and *oda*9 (IC78) loci, since each antigen is missing in the corresponding mutant strain (Figures 3 and 4). The absence of detectable HC$\alpha$ antigen in either *oda*11 flagella (Figure 2 and Sakakibara *et al.*, 1991) or cytoplasm (Figure 3) also supports the identification of *oda*11 as the HC$\alpha$ locus (although the *oda*11 and HC$\alpha$ loci are genetically linked, direct evidence of allelism is lacking [Sakakibara *et al.*, 1991]). At least four *oda* mutations affect the abundance of a subunit that is not the mutant gene product, a result that we interpret as an alteration in subunit stability. There was no detectable HC$\alpha$ antigen in *oda*7 cytoplasmic extracts even though all other tested outer-dynein arm proteins were present at approximately WT levels (Figure 3), which may indicate that the WT *oda*7 gene product interacts with and stabilizes HC$\alpha$. The only subunit known to directly interact with HC$\alpha$ is its tightly associated 16-kDa LC (Mitchell and Rosenbaum, 1986), which has recently been cloned and identified as a thioredoxin homologue (Patel-King *et al.*, 1996). The *oda*7 locus probably does not encode the 16-kDa LC, since absence of this LC (along with HC$\alpha$) from outer-dynein arms of *oda*11 flagella does not prevent assembly of the remaining outer-dynein arm subunits (Sakakibara *et al.*, 1991), whereas the

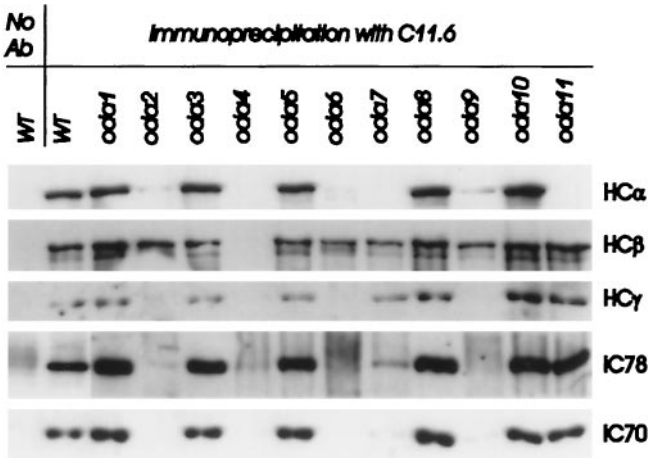


**Figure 6.** Immunoprecipitation of preassembled complexes. Immunoprecipitates with C11.6 (anti-HC$\beta$) or no antibody (control) from WT and *oda*1–*oda*11 cytoplasmic extracts were separated on 7% gels, blotted to PVDF membrane, and probed with B3B (anti-HC$\alpha$), C11.6 (anti-HC$\beta$), 25–8 (anti-HC$\gamma$), 1878A (anti-IC78), or 1869A (anti-IC70). Immunoprecipitates of *oda*1 extracts showed the presence of all five major outer-dynein arm proteins at equivalent levels to WT, as did immunoprecipitates of *oda*3, 5, 8, and 10. The immunoprecipitate of *oda*11 lacked only HC$\alpha$; the immunoprecipitate of *oda*7 lacked HC$\alpha$ and had greatly reduced levels of IC70 and IC78, whereas no proteins coprecipitated with HC$\beta$ in *oda*2, *oda*6, or *oda*9. The *oda*4 lanes serve as a no-antigen control.

*oda*7 mutation clearly does (Kamiya, 1988). In addition to its affect on HC$\alpha$ stability, *oda*7 also prevents preassembly of the remaining subunits into a stable complex; the two remaining HCs remain associated during immunoprecipitation, but the ICs are largely lost. Could *oda*7 be essential for a posttranslational modification that is needed both for stability of HC$\alpha$ and for a strong HC–IC interaction? Two possibilities include regulation of the redox states of the 16-kDa (HC$\alpha$-associated) and 19-kDa (HC$\beta$-associated) thioredoxin homologous LCs (Patel-King *et al.*, 1996), and regulation of the phosphorylation state of HC$\alpha$, which is the only outer-dynein arm subunit known to be phosphorylated in vivo (Piperno and Luck, 1981; King and Witman, 1994). The significance of thioredoxin homology in these LCs has not yet been determined, and the physiological role of HC$\alpha$ phosphorylation is also presently unknown.

The two other mutations that alter stability have reciprocal effects, a mutation in IC70 (*oda*6), reducing IC78 abundance, and a mutation in IC78 (*oda*9), affecting IC70 levels (Figure 4). These two proteins can be purified from partially dissociated flagellar extracts as a stable heterodimer (Mitchell and Rosenbaum, 1986), and although full-length in vitro translation products are apparently stable as individual proteins (King *et al.*, 1995), dimerization may be required for stability in vivo. Absence of this dimer, in turn, prevents cytoplasmic preassembly of HC$\alpha$, HC$\beta$, and HC$\gamma$ (Figure 6).

In five strains (*oda*1, 3, 5, 8, 10) all five HC and IC subunits tested were preassembled in the cytoplasm, but this preassembled complex could not be transported from the cytoplasm and bound onto flagellar doublets. The data of Kamiya (1988) suggest that these loci encode subunits of two additional preassembled complexes (Table 1). We hypothesize that loci in each group encode proteins that form preassembled complexes, and that the HC–IC complex described here is the complex disrupted by *oda*2, 4, 6, 7, or 9. This is the first direct demonstration that lack of complementation among *oda* mutants results from the prevention of complex preassembly. The *oda*1 and *oda*3 gene products copurify from flagella as subunits of a 7S DC that can assemble into flagella independently of other outer-dynein arm subunits (Takada and Kamiya, 1994; Koutoulis *et al.*, 1997); therefore, lack of cytoplasmic complementation between *oda*1 and *oda*3 (Kamiya, 1988) suggests that the gene products of these loci also preassemble into a separate complex in the cytoplasm. The gene products of *oda*5, 8, and 10 are not known, but since a mixture of 12S and 18S dynein complexes purified from flagella can rescue either *oda*2 or *oda*5 axonemes in vitro (Takada and Kamiya, 1994), 12S and 18S dynein may contain all of the components of both the preassembled HC–IC complex (disrupted in *oda*2) and the hypothesized *oda*5, 8, 10 complex. The combined 12S and 18S fractions consist of only three HCs (encoded by *oda*2, 4, and 11), two ICs (encoded by *oda*6 and 9), and eight LCs (Piperno and Luck, 1979; Pfister *et al.*, 1982), which suggests that the *oda*5, 8, and 10 complex consists of LCs. A model of outer-arm dynein assembly based on the data and these assumptions is presented in Figure 7. In wild-type cells, all three complexes come together in the cytoplasm to form a complete dynein arm, which then moves into the flagellar compartment for attachment to doublet microtubules (heavy arrows). Mutations block this process at the steps indicated by the numbers, each of which refers to one of the *oda* mutations. Dashed lines in the model show assembly steps that still occur even when other pathways are blocked (e.g., the DC can assemble onto doublets in the absence of other components, and the remainder of the outer arm can then add onto the docking site in the flagellum, as occurs after the fusion of *oda*1 and *oda*2 gametes). Evidence that the entire arm preassembles in wild-type cells includes the observations that *oda*5 affects the level of HC$\alpha$ and HC$\beta$ in cytoplasmic extracts (Figures 2 and 3), and that HC$\beta$ is more susceptible to proteolysis in *oda*1 and *oda*3 extracts (Figure 4), as well as the data of Fok *et al.* (1994) on *Paramecium* extracts. As an alternative to this model, the *oda*5, 8, and 10 complex could be required only in the cytoplasm, where it could act

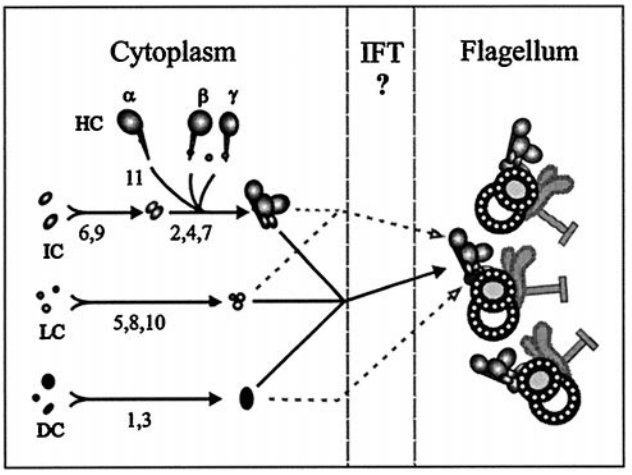


**Figure 7.** Model of the dynein assembly process. Solid arrows indicate steps thought to occur in wild-type cells, where all of the subunits are hypothesized to preassemble in the cytoplasm (left side) before moving into the flagellum (right side). An intermediate compartment (IFT) may be required for transport between the cytoplasm and flagellum. Assembly mutants *oda*1–*oda*11 block this process at the steps indicated by each number in the diagram and affect the preassembly of one of three complexes, an IC–HC complex (top), a LC complex (middle), or a docking complex (bottom). Dashed arrows show assembly pathways occurring during cytoplasmic complementation of assembly mutants.

to modify the HC–IC complex into an assembly-competent form.

In conclusion, our data reveal that outer-dynein arm HC and IC subunits exist as a preassembled complex in a cytoplasmic precursor pool, and that at least three *oda* mutations that disrupt this complex have major effects on the abundance of another subunit in addition to the mutant gene product. It is unknown whether there is a sequential order in the wild-type assembly process, although temporary dikaryon complementation studies suggest that many paths can lead to assembly of a complete complex. Only the identification of gene products of the remaining *oda* loci will reveal whether they all encode enzyme subunits or whether some encode cytoplasmic assembly factors. When flagella and axonemes of several *oda* mutants were tested for the presence of outer-dynein arm subunits by Western blot, trace amounts of some subunits were seen, but in every case these subunits remained tightly associated with axonemes after detergent extraction (Mitchell, unpublished observation). It thus appears that dynein subunits that are not bound to doublet microtubules do not accumulate in the flagellar compartment in these mutants, and that the flagellar pool is very small relative to the total cytoplasmic pool. We do not know whether this flagellar pool size increases during flagellar assembly or dikaryon complementation. Having established that dynein subunits undergo extensive cytoplasmic assembly before their movement into the flagellar compartment, it remains to be determined whether specific transport or targeting mechanisms such as the recently identified intraflagellar transport (IFT) particles (Cole *et al.*, 1998; Pazour *et al.*, 1998) are involved in bringing these complexes to their ultimate destinations.

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