The 78,000-M_r Intermediate Chain of *Chlamydomonas* Outer Arm Dynein Is a Microtubule-binding Protein

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Abstract. A previous study (King et al., 1991. *J. Biol. Chem.* 266:8401–8407) showed that the 78,000-M_r intermediate chain (IC78) from the *Chlamydomonas* outer arm dynein is in direct contact with α-tubulin in situ, suggesting that this protein may be involved in binding the dynein to the doublet microtubules. Molecular genetic analysis of this chain recently demonstrated that it is a WD repeat protein essential for outer arm assembly (Wilkerson et al., 1995. *J. Cell Biol.* 129:169–178). We have now transcribed and translated IC78 in vitro, and demonstrate that this molecule binds axonemes and microtubules, whereas a homologous protein (the 69,000-M_r intermediate chain [IC69] of *Chlamydomonas* outer arm dynein) does not. Thus, IC78 is a bona fide microtubule-binding protein. Taken together with the previous results, these findings indicate that IC78 is likely to provide at least some of the adhesive force that holds the dynein to the doublet microtubule, and support the general hypothesis that the dynein intermediate chains are involved in targeting different dyneins to the specific cell organelles with which they associate. Analysis of the binding activities of various IC78 deletion constructs translated in vitro identified discrete regions of IC78 that affected the binding to microtubules; two of these regions are specifically missing in IC69. Previous studies also showed that IC78 is in direct contact with IC69; the current work indicates that the region of IC78 that mediates this interaction is coincident with two of IC78's WD repeats. This supports the hypothesis that these repeats are involved in protein–protein interactions within the dynein complex.

DYNEINS are molecular motors responsible for ciliary and flagellar movement, the directed movement of membrane-bound organelles, positioning of the Golgi apparatus, nuclear movements, spindle formation and orientation, and possibly some types of chromosomal movement. Dyneins occur as several distinct isoforms, each of which consists of two or more heavy chains (DHCs)[1] associated with a variety of accessory proteins (for reviews see Holzbauer et al., 1994 and Witman et al., 1994). Recent molecular studies have indicated that at least some of the accessory proteins, namely, the intermediate chains (ICs), found in cytoplasmic and flagellar outer arm dynein derive from a common ancestor (Mitchell and Kang, 1991; Paschal et al., 1992; Wilkerson et al., 1995). This observation has raised the possibility that the ICs of flagellar outer arm dynein and cytoplasmic dynein perform related functions. It has been suggested that one function of the ICs is to bind the dynein to its cargo (microtubules in the case of flagellar dynein; membranous vesicles and other cell organelles in the case of cytoplasmic dynein) (King and Witman, 1990; King et al., 1991; Paschal et al., 1992). However, to date, there has been little experimental evidence (see below) to support this proposal.

We have been using the outer arm dynein from *Chlamydomonas* as a model system to learn more about the functions of specific dynein subunits. This dynein (Fig. 1 *A*) consists of 3 DHCs (α, β, and γ; each >500 kD [Mitchell and Brown, 1994; Wilkerson et al., 1994]), 2 ICs (IC78 and IC69 [Also referred to as IC70; Mitchell and Kang, 1991]; 76.5 and 63.4 kD, respectively [Mitchell and Kang, 1991; Wilkerson et al., 1995]), and 10 light chains (LCs; 8–22 kD [King and Patel-King, 1995; Pfister et al., 1982; Piperno and Luck, 1979]). The purified dynein retains both ATP-sensitive (motor) and -insensitive (structural) microtubule-binding domains (Haimo et al., 1979; Haimo and Fenton, 1988). Structurally, each DHC consists of a globular head and a flexible stem. The head, formed from the COOH-terminal ~350 kD, contains the ATPase and motor domains, and is presumed to bind transiently to the B tubule of the opposing doublet microtubule during force generation. The stem, formed from the NH_2-terminal part of the

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1. *Abbreviations used in this paper*: DHC, dynein heavy chain; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; IC, dynein intermediate chain; IC/LC, intermediate chain/light chain complex; LC, dynein light chain; MARP, microtubule-associated repetitive protein.

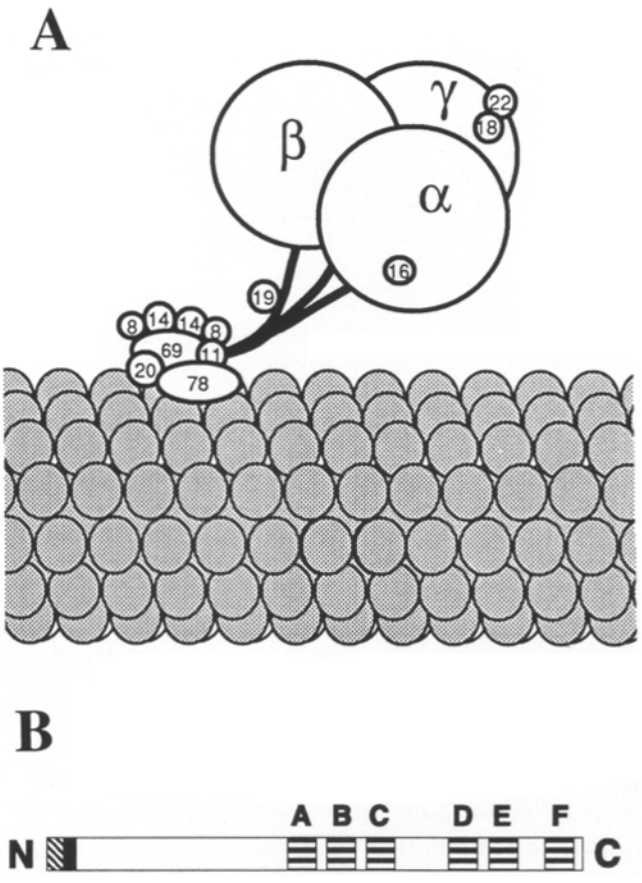


Figure 1. (*A*) Model for the outer arm dynein of *Chlamydomonas* and its association with the outer doublet microtubule (*shaded*). Intermediate chains IC78 and IC69 are closely associated with each other and with the 8-, 11-, 14-, and 20-kD LCs in an IC/LC complex (Mitchell and Rosenbaum, 1986; King et al., 1991) located at the base of the stems of the α, β, and γ DHCs (King and Witman, 1990). The 19-kD LC is associated with the NH_2-terminal one-third of the β DHC (which presumably corresponds to that chain's stem) (Sakakibara et al., 1993), but has not been shown to be a part of the IC/LC complex. The 16-kD LC and 18- and 22-kD LCs are associated with the α and γ DHCs, respectively (Pfister and Witman, 1984), although their location in the heads of these chains is speculative. (*B*) Predicted domain structure of IC78 showing the basic NH_2-terminus (*diagonally hatched box*), the glutamine–proline-rich region that is expected to form a polyproline II helix (*filled box*), and WD repeats A–F (*horizontally hatched boxes*) (Wilkerson et al., 1995).

chain, extends to the base of the dynein and appears to interact with the stems of the other DHCs to hold the dynein together (Johnson and Wall, 1983; Sakakibara et al., 1993; Witman et al., 1983; Goodenough and Heuser, 1984; Sale et al., 1985; for review see Witman et al., 1994). The ICs interact with each other and with several of the LCs to form an IC/LC complex (Mitchell and Rosenbaum, 1986; King et al., 1991) that is located at the base of the soluble dynein particle (King and Witman, 1990). Studies using the zero-length cross-linker l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) revealed that IC78 is in direct contact with α-tubulin in situ, suggesting a role for this component in the ATP-insensitive (or structural) binding of the outer arm to the flagellar doublet microtubule (King et al., 1991). To date, this latter observation provides the sole experimental basis for the hypothesis that ICs are involved in attachment of the dynein motor to its cargo.

Recently, we described the cloning and sequencing of a full-length cDNA encoding IC78 (Wilkerson et al., 1995). The predicted sequence indicates that the NH_2-terminal 22 residues constitute a highly charged, lysine-rich region (Fig. 1 *B*). This basic region is separated from the rest of the protein by a short glutamine–proline-rich region predicted to form a polyproline II helix. Polyproline II helices generally form flexible linkers between domains of a protein (Adzhubei and Sternberg, 1994), suggesting that the highly charged NH_2-terminal region is a discrete structural domain and probably a discrete functional domain as well. The COOH-terminal half of the protein contains a series of six repeated elements known as WD repeats; such repeats are believed to be involved in subunit–subunit interactions within multisubunit complexes (Neer et al., 1994). Although not previously reported, the WD repeats also are present in *Chlamydomonas* IC69 and in the 74,000-M_r IC (IC74) of cytoplasmic dynein, which are homologous to IC78 (Wilkerson et al., 1995). The fact that the WD repeats are conserved suggests that they may perform similar functions in the different chains. Using the IC78 cDNA as a probe, we identified seven independently isolated strains, generated by insertional mutagenesis and selected on the basis of a slow swimming phenotype, in which the IC78 gene was completely removed from the genome, and one strain in which there was a large insertion within this gene (Wilkerson et al., 1995). Electron microscopic analysis of the disruption mutant and of several of the deletion mutants revealed that in every case the outer arm was completely and specifically lost, indicating that IC78 is essential for outer arm assembly.

To clarify the role of IC78 in outer arm assembly, we have now investigated the ability of in vitro–translated IC78 to bind to microtubules and axonemes. The results demonstrate that IC78 is a bona fide microtubule-binding protein, suggesting that IC78 is essential for outer arm assembly because it provides at least some of the adhesive force for binding the arm to the flagellar microtubules. This finding provides additional support for the general hypothesis that dynein ICs are involved in binding dyneins to the organelles with which they associate. We also have investigated the relationship between IC78 functional domains and the predicted structural domains of the polypeptide. We have identified two, and possibly three distinct regions within IC78 that affect microtubule-binding activity; one of these is contained within the highly charged NH_2-terminal region, the other is located in the center of the molecule and includes another highly basic sequence. The homologous protein IC69 does not bind microtubules in vitro; although IC69 also contains basic regions, the specific sequences that would have been homologous to the regions important for microtubule-binding in IC78 are not present in IC69. Finally, we have identified the region of IC78 that is involved in binding to IC69. This activity is localized to two of the WD repeats in IC78, supporting the hypothesis that these repeats may be responsible for subunit–subunit interactions within the dynein complex. It is likely that at least some of the WD repeats in IC69 of outer arm dynein and IC74 of cytoplasmic dynein have a

similar function, as such subunit–subunit interactions are common to all dynein ICs.

Materials and Methods

Plasmids and Constructions

Two plasmids were used in this study: pc78k3, which consists of a 2.65-kb insert containing the entire coding sequence for IC78 cloned between the EcoRI and XhoI sites of pBluescript II SK− (Wilkerson et al., 1995), and pBc70-16, which contains the cDNA for IC69 cloned into pBluescript II KS+ (Mitchell and Kang, 1991). pBc70-16 was the generous gift of Dr. D. Mitchell (SUNY Health Science Center, Syracuse, NY). NH_2-terminal deletions were constructed in one of two ways. In the first, pc78k3 was digested with SmaI, PstI, and BamHI (each enzyme cuts once within the coding sequence and once within the 5′-polylinker) and religated. The resulting constructs (78ΔSmaI, 78ΔPstI, and 78ΔBamHI) rely on internal codons for translation initiation and are expected to produce fragments corresponding to M_{324}–H_{683}, M_{453}–H_{683}, and M_{480}–H_{683}, respectively. To construct the NH_2-terminal deletion series 78Δ213N-78Δ819N[2], a linker containing part of the 5′-untranslated region of IC78, a translation initiation signal abutted to an EcoRI site, and an in-frame stop codon flanked by an upstream ApaI site and a downstream SalI site was cloned between the XbaI and EcoRI sites of pc78k3. This plasmid then was digested with ApaI to protect the vector and with SalI to provide a substrate for the exonuclease ExoIII. The digestion was stopped at various times, the plasmid cut with EcoRI, blunt ended with S1 nuclease, and the ends repaired with T7 DNA polymerase. The 5′ ends of individual clones then were sequenced to determine the extent of the deletion; only those that retained the correct reading frame were used.

For COOH-terminal deletions of IC78 (the series 78Δ679C–78Δ1951C) and 78ΔSmaI (78ΔSmaIΔC), a linker containing KpnI and NruI sites was inserted between the XhoI and KpnI sites (the latter was not preserved upon religation) in the 3′-polylinker of both plasmids. Plasmids then were cut with XhoI and KpnI to provide appropriate substrates for exonuclease digestion. Individual clones were sequenced to determine the precise point of truncation. Insertion of the linker, which provides a unique site with which to linearize the plasmid for mRNA runoff, was made necessary by our observation that vector protected from digestion by the 3′ overhang of the ApaI site (located between XhoI and KpnI in the original polylinker) apparently could not be digested with S1 nuclease and therefore could not be religated.

Oligonucleotide-directed deletions within 78Δ1309C were made essentially as described by Kunkel et al. (1987). For selection purposes, each oligonucleotide incorporated a restriction site determined by the terminal three bases 5′ to the desired deletion. Therefore, for each construct a single residue encoded by the other half of the restriction site is inserted within the boundaries of the deletion. The restriction sites used and the residue inserted are tabulated in Fig. 8.

In Vitro Transcription and Translation

Plasmid constructs were linearized and transcribed in vitro using T3 RNA polymerase; pBc70-16 was transcribed with T7 RNA polymerase. Individual RNAs were translated using the rabbit reticulocyte lysate system (Promega Corp., Madison, WI) containing [^{35}S]methionine (New England Nuclear, Boston, MA). Before use for axoneme/microtubule-binding experiments, the lysates were spun at ~180,000 *g* for 5 min in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) to pellet any nascent polypeptide chains still attached to polysomes.

The specific activity of the [^{35}S]methionine pool in the lysate was determined as described by Butner and Kirschner (1991). From this value and the number of methionines in IC78, the specific activity of the in vitro translated polypeptide was calculated. The amount of IC78 translated in vitro was then determined by cutting IC78 bands (identified by autoradiography) out of gels and counting the amount of radioactivity in the bands.

Preparation of Axonemes and Microtubules

Chlamydomonas flagella were isolated from the wild-type strain 1132D- and from the outer armless mutant *oda1* by standard methods (Witman, 1986). Flagella were demembranated with HMEK buffer (1% NP-40, washed with 10 mM Hepes, pH 7.5, 5 mM $MgSO_4$, 0.5 mM EDTA, 25 mM KCl), and either used immediately or stored in 50% glycerol at −20°C. Stored axonemes were washed twice with HMEK before use.

DEAE-purified bovine brain tubulin was prepared by the procedure of Vallee (1986) and was kindly provided by Dr. Anthony Moss (Auburn University, Auburn, AL). Polymerized microtubules were stabilized with taxol before use in the sedimentation assays.

Sedimentation Assays

To assess the axoneme/microtubule-binding activity of in vitro translation products, 10 μl of the reticulocyte lysate was added to a microfuge tube containing 10 μl HMEK buffer and 10 μl axonemes (6 mg/ml) or microtubules (18 mg/ml). The same amounts were used in all experiments. Control tubes contained reticulocyte lysate and 20 μl HMEK, but no axonemes or microtubules. Samples were incubated at room temperature for 30 min and then sedimented for 1 min in a microfuge or, when microtubules were used, in an airfuge. Supernatants were removed and the pellets washed in 500 μl HMEK before preparation for gel electrophoresis (King et al., 1986). It is important to note that all binding experiments took place in the presence of >50 mg/ml cellular protein derived from the reticulocyte lysate. Quantitation of autoradiographs was performed using the program Quantity One (pdi, Huntington Station, NY) running on a Sparc workstation (Sun Microsystems Inc., Mountain View, CA). The amount of binding observed for any one construct was quite reproducible, e.g., in four different experiments performed on different days, values of 67, 70, 72, and 77% were obtained for the amount of 78Δ1309C bound to wild-type axonemes.

Cross-linking experiments were performed as described in King et al. (1991). Briefly, axonemes with bound in vitro translation products were resuspended in HMEK buffer and treated with 0 or 1 mM EDC (Pierce Chemical Co., Rockford, IL) for 60 min at room temperature. The reaction was terminated by the addition of a fivefold molar excess of β-mercaptoethanol before preparation for electrophoresis. Samples were separated in 8 or 10% acrylamide minigels and then subjected to autoradiography. ^{14}C-labeled molecular weight standards were obtained from Amersham Corp. (Arlington Heights, IL).

Immunoprecipitation

mAbs 1878A and 1869A (King et al., 1985; 1986) were purified from ascites fluid and bound to protein A–Affigel beads (Bio-Rad Laboratories, Hercules, CA). The antibody–protein A interaction was stabilized by cross-linking with dimethylpimelimidate (Pierce Chemical Co.) by the procedure of Schneider et al. (1982). For immunoprecipitation experiments, the beads were incubated for 30–60 min with reticulocyte lysates containing in vitro translation products and washed several times with 0.05% Tween 20 in TBS, before resuspension in electrophoresis sample buffer.

Computational Methods

The program PHD was used to predict the secondary structure of IC78 (Rost and Sander, 1993). Hydrophobicity and surface probability were determined using the GCG suite of programs (Devereux et al., 1984).

Results

IC78 Is a Bona Fide Microtubule-binding Protein

Previously, we used the zero-length cross-linking reagent EDC to demonstrate that, within the axoneme, IC78 inter-

2. Nomenclature of the IC78 deletion constructs is based on the numbering of the cDNA clone 78k3 (Wilkerson et al., 1995). For NH_2- and COOH-terminal deletions, the number shown indicates the first or last base in the construct, respectively. For example, the designation 78Δ679C indicates a COOH-terminal deletion that has removed all but the first 679 bases of the clone 78k3. The three constructions made using internal restriction sites to remove NH_2-terminal regions are indicated with the name of the endonuclease used, e.g., 78ΔBamHI. The residues of IC78 encoded by each construct are tabulated in Fig. 3.

acts directly with α-tubulin (King et al., 1991). However, those experiments could not determine whether IC78 merely is in contact with tubulin or is a microtubule-binding protein per se. To address this important question, we investigated the ability of in vitro–translated IC78 to bind to microtubules. The plasmid pc78k3 (Wilkerson et al., 1995), which contains the full-length cDNA encoding IC78, was transcribed in vitro, and the resulting mRNA translated in a rabbit reticulocyte lysate system. Quantitation of radioactivity incorporated into IC78 revealed that ~1 fmol (~80 pg) protein was synthesized per microliter reticulocyte lysate. Subsequently, the ability of the translation products to bind axonemes and taxol-stabilized bovine brain microtubules was assessed in a simple sedimentation assay. In the absence of axonemes, in vitro–translated IC78 remained in the supernatant. In the presence of axonemes (derived either from wild-type or from the outer armless strain *oda1*), significant amounts (up to 60%) of the IC78 translation product were found in the pellet (Fig. 2 *A*, *top panel*). Similar results were obtained when microtubules were substituted for axonemes in the assay (Fig. 2 *B*), indicating that the binding was due to direct interaction between IC78 and tubulin. Binding of IC78 to axonemes was sensitive to the addition of 0.6 M NaCl (not shown), which is the salt concentration required to release >90% of outer arm dynein from the axoneme. Approximately 86% of bound IC78 was released by the salt treatment, indicating that binding is mediated largely by ionic interactions similar to those which attach the arm to the doublet microtubule in vivo. Further evidence that this interaction is similar to that which occurs in vivo was obtained from protein cross-linking experiments described below.

Assuming that tubulin accounts for ~50% of total axonemal protein, each assay contained ~1 nmol tubulin; when brain microtubules were used, the concentration of tubulin was even higher. Inasmuch as the assay mixture contained only ~10 fmol of IC78, these experiments were performed under conditions where a large excess (~10^5-fold in molar terms) of potential IC78 binding sites were present. Because only extremely small amounts of in vitro translated product were available, it was not possible to saturate the available binding sites in the sedimentation assays. However, the fact that much of the IC78 bound to axonemes or microtubules, even though IC78 was present at a concentration of only 80 ng/ml in a background of >50 mg/ml cellular protein from the lysate, strongly suggested that the IC78–microtubule interaction was specific.

As an alternative test of whether the interaction of IC78 with the microtubules was specific, we examined the binding properties of IC69. IC69 is homologous to IC78, has a similar isoelectric point (predicted 5.4 vs. 6.6 for IC78), has similar WD repeats, and also has a basic NH_2-terminus (see Fig. 10) (Wilkerson et al., 1995), but it is not cross-linked to tubulin by treatment of the intact axoneme with EDC (King et al., 1991). IC69 was produced by in vitro transcription and translation of the plasmid pBc70-16 (Mitchell and Kang, 1991). Under the same assay conditions as were used for IC78, IC69 remained exclusively in the supernatant and showed no detectable axoneme-binding activity (Fig. 2 *A*, *bottom panel*). These results indicate that the interaction of IC78 with axonemes and microtubules is specific. We conclude that IC78 is a bona fide microtubule-binding protein. The results also indicate that, in contrast to IC78, IC69 is not a microtubule-binding protein.

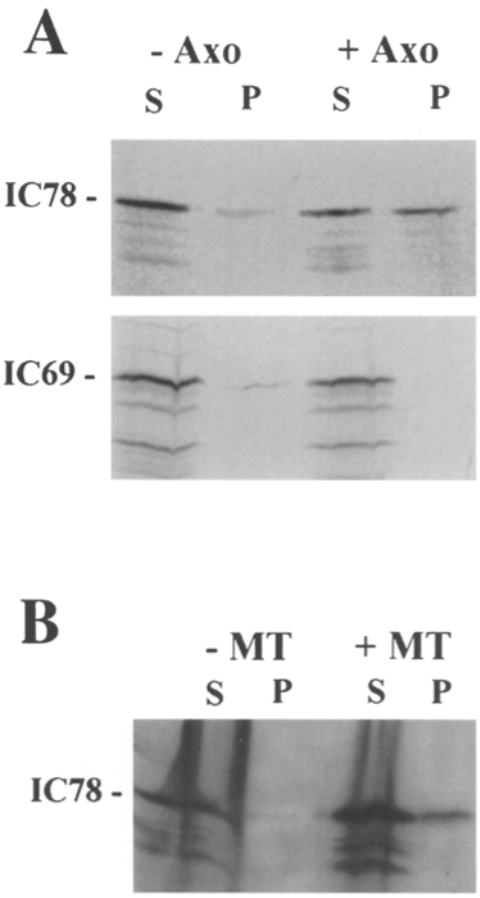


Figure 2. IC78 binds axonemes in vitro. (*A*) IC78 and IC69 were transcribed and translated in vitro. Translation mixes (10 μl) were added to HMEK buffer (20 μl) alone or to buffer containing 3 mg/ml wild-type axonemes. The samples were incubated at room temperature for 30 min, sedimented in a microfuge, and the pellets washed with 500 μl HMEK. Samples were separated in an 8% acrylamide minigel and autoradiographed. In the absence of axonemes (−*Axo*), both IC78 (*top panel*) and IC69 (*bottom panel*) were found in the supernatant (*S*). After axoneme addition (+*Axo*), ~40% of IC78 was found associated with the pellet (*P*), whereas IC69 remained in the supernatant. The minor short fragments visible in these and subsequent autoradiographs derive from translation initiation within the IC78 and IC69 coding regions, presumably at internal methionines. (*B*) In vitro translated IC78 was incubated in the presence or absence of taxol-stabilized brain microtubules. Samples were pelleted in the airfuge and treated as described in *A*.

Identification of IC78 Regions that Affect Microtubule Binding

To determine which regions of IC78 are important for binding to microtubules, we generated a series of constructs designed to produce truncated versions of IC78 in which various amounts of the molecule were deleted from the NH_2-terminus, the COOH-terminus, or both (Fig. 3).

These constructs were then transcribed and translated in vitro, and assayed as above to determine if they were capable of binding to axonemes or brain microtubules.

As reported above, the intact IC78 bound to and cosedimented with axonemes or brain microtubules. However, truncation of the NH_2-terminal 20 residues of IC78 (construct 78Δ213N) resulted in loss of binding activity (Fig. 4); the remaining portion of the chain (G_{21}–H_{683}, preceded by a methionine inserted for initiation) was unable to bind to the axonemal or brain microtubules. Fragments in which more extensive portions of the NH_2-terminal region were deleted (up to K_{223}; constructs 78Δ414N, 78Δ522N, 78Δ735N, and 78Δ819N) similarly were unable to bind to microtubules. These results indicate that at least the first 20 residues of IC78 are important for microtubule binding.

Truncated versions of IC78 in which the COOH-terminus was deleted down to L_{386} (constructs 78Δ1951C–78Δ1309C) bound readily to axonemal or brain microtubules. For example, 70% of 78Δ1309C (M_1–L_{386}) bound to and cosedimented with the microtubules (Fig. 5, *top panel*). However, further truncation towards the NH_2-terminus (down to L_{263} or beyond; constructs 78Δ941C and 78Δ679C) completely abolished binding (Fig. 5, *middle and bottom panels*). Therefore, the central region of IC78 (D_{264}–L_{386}) contains a second domain essential for microtubule binding.

Surprisingly, if IC78 was truncated from the NH_2-terminus partially or completely through this second domain (to M_{324} and beyond), then axonemal and brain microtubule-binding activity was restored to the resulting COOH-terminal fragments (constructs 78ΔSmaI, 78ΔPst1, and 78ΔBamHI) (Fig. 6). These results indicate that removal of the entire NH_2-terminal half of IC78 unmasks microtubule-binding activity in the COOH-terminal half of the molecule. COOH-terminal fragments as short as 203 amino acids (residues M_{480}–H_{683}; construct 78ΔBamHI) were capable of binding to axonemal microtubules (Fig. 6, *bottom panel*). A fragment (M_{324}–S_{604}; construct 78ΔSmaIΔC) in which the COOH-terminal 79 residues were deleted also bound to microtubules (Fig. 6, *bottom panel*). Thus, at least some of the COOH-terminal microtubule-binding activity is likely to be contained in the region of overlap between the latter two fragments (residues M_{480}–S_{604}). This does not rule out the possibility that there are additional microtubule-binding sites COOH- or NH_2-terminal to this region. The interaction between 78ΔSmaI and axonemes was partially sensitive to increasing ionic strength; 58% of the bound translation product was released by washing with HMEK buffer containing 0.6 M NaCl, and additional amounts were released by increasing the salt concentration to 1 M (not shown).

NH_2-terminal Half of IC78 Is Cross-linked to α-tubulin by EDC

It was of considerable interest to determine which of the regions identified above were binding to microtubules in a physiologically relevant manner. Inasmuch as IC78 in dynein in situ can be readily cross-linked to axonemal α-tubulin by low concentrations of EDC (King et al., 1991), we investigated whether the intact chain, and fragments representing the NH_2- and the COOH-terminal half of the chain, could be similarly cross-linked to α-tubulin after their binding to *oda1* axonemes in vitro.

IC78 was translated in vitro and bound to *oda1* axonemes. An aliquot of the axonemes was then treated with 1 mM EDC; an identical aliquot was left untreated. After analysis of the aliquots by SDS-PAGE (Fig. 7, *top panels*), a single additional 127-kD band was observed in the EDC-treated sample. This is precisely the size of the IC78/α-tubulin conjugate identified previously after treatment of intact, native axonemes with EDC (King et al., 1991). Because EDC cross-links only proteins in which aspartate or glutamate residues on one protein are in intimate contact with lysine residues on the other protein (Hoare and Koshland, 1966), the IC78/tubulin interaction in vitro is likely to be very similar to that which occurs in vivo. This provides additional evidence that in vitro translated IC78

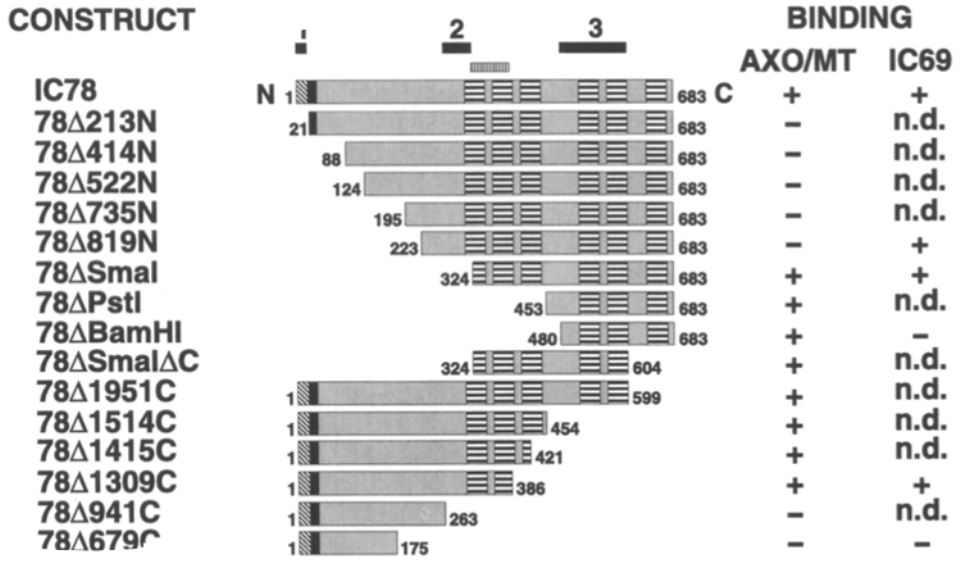


Figure 3. Properties of IC78 and NH_2- and COOH-terminally deleted constructs. This diagram indicates the regions of IC78 covered by the various constructs described in Results, and whether that product was competent to bind axonemes/microtubules and IC69. Predicted structural features of IC78 are as in Fig. 1 *B*. Regions important for microtubule binding (solid bars *1*, *2*, and *3*) and IC69 binding (*vertically hatched bar*) are indicated at the top of the figure; solid bar *2* corresponds to residues D_{264}–D_{323} (see Results). *n.d.*, not determined.

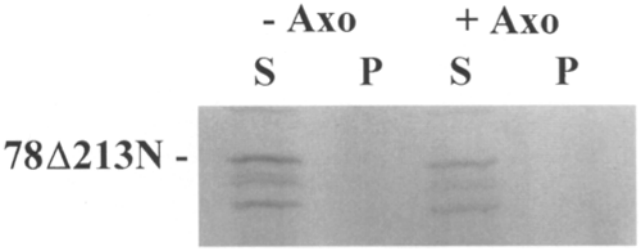


Figure 4. The NH_2-terminus of IC78 is essential for microtubule binding. The fragment 78Δ213N, which lacks only the first 20 amino acids of IC78, was expressed in vitro and assayed for microtubule binding activity as in Fig. 2. All of the product remained in the supernatant in both the presence and absence of axonemes.

binds to axonemal microtubules in a specific and physiologically relevant manner.

A similar experiment was carried out with the fragment 78Δ1309C (M_1–L_{386}), which contains both the NH_2-terminal and the central regions that were observed to be important for microtubule binding in vitro. Reaction of axoneme-bound 78Δ1309C with EDC generated a single additional band with a relative molecular mass of 92,000 (Fig. 7, *bottom panels*). The mass of this conjugate is consistent with the cross-linking of the 43-kD translation product to tubulin. In contrast, when the same experiment was carried out with bound 78ΔSmaI (M_{324}–H_{683}), which contains the COOH-terminal microtubule-binding activity, no cross-linked products were observed, even at higher concentrations of EDC (Fig. 7, *middle panels*). Therefore, it appears that the sites responsible for cross-linking of IC78 to α-tubulin reside exclusively in the NH_2-terminal half of the molecule.

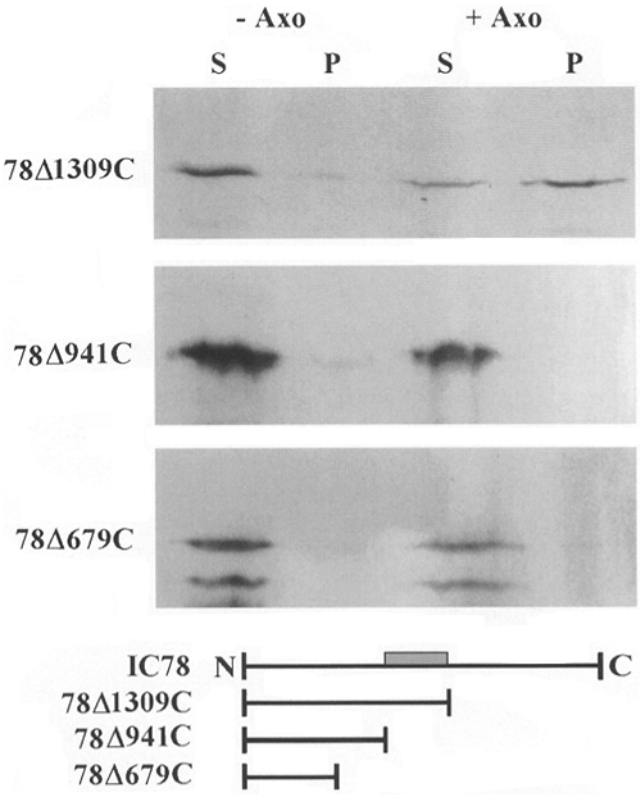


Figure 5. The central region of IC78 is important for microtubule binding. The axoneme-binding behavior of three COOH-terminally truncated versions of IC78 are shown. Constructs 78Δ1309C, 78Δ941C, and 78Δ679C represent IC78 residues M_1–L_{386}, M_1–L_{263}, and M_1–Q_{175}, respectively. After the sedimentation assay, samples were separated in a 10% minigel and autoradiographed. In the absence of axonemes, all three products remained in the supernatant. However, in the presence of *oda1* axonemes, >70% of 78Δ1309C was found in the pellet. Neither 78Δ941C nor 78Δ679C showed detectable axoneme-binding activity. The lower panel diagramatically illustrates the regions of IC78 covered by these constructs; the shaded box indicates the region important for microtubule binding.

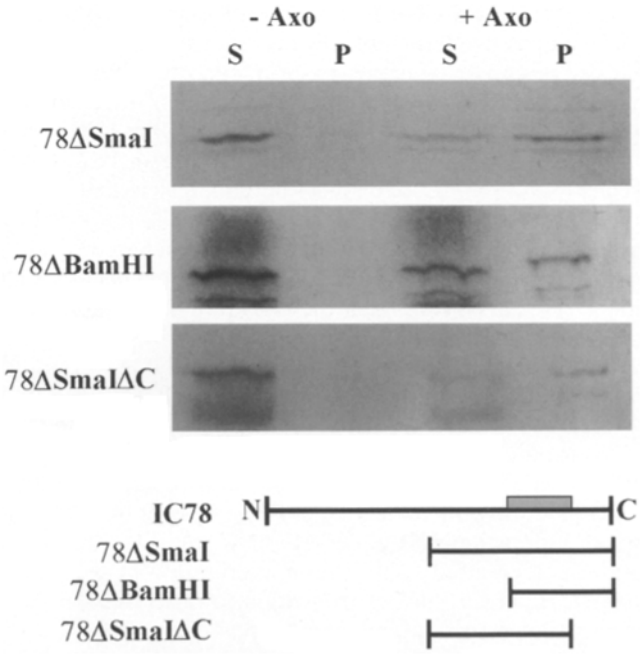


Figure 6. Microtubule-binding activity in the COOH-terminal region of IC78. Three NH_2-terminal deletion constructs (78ΔSmaI, 78ΔBamHI, and 78ΔSmaIΔC), encoding overlapping sections of the COOH-terminal region of IC78, were transcribed and translated in vitro. The ability of each translated product to bind *oda1* axonemes then was assessed in the sedimentation assay. Samples were separated in an 8% minigel and autoradiographed. All three products bound avidly, indicating that IC78 contains a microtubule-binding domain within the region of overlap (*shaded box in diagram*). Note that for 78ΔBamHI, the region containing the centrally located site that is important for microtubule binding (see Fig. 5) has been completely removed. The multiple bands are due to translation initiation at several internal residues.

Further Definition of Regions in the NH_2-terminal Half of IC78 Important for Microtubule Binding

Because microtubule binding was observed only for those large NH_2-terminal fragments which contained both the extreme NH_2-terminal domain (M_1–T_{20}) and the region from D_{264}–L_{386}, it was not possible to use simple truncations to assess the importance to microtubule binding of parts of the molecule located between these two regions. To investigate the contribution of these other parts to this activity, we produced a series of constructs in which various small internal regions were deleted from the fragment

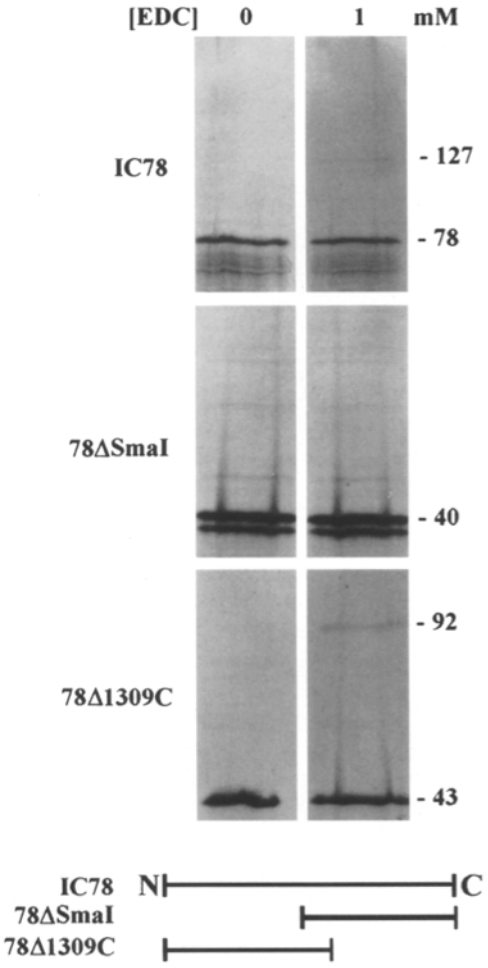


Figure 7. EDC cross-linking of in vitro–translated IC78 and fragments. In vitro–translated IC78, 78ΔSmaI, and 78Δ1309C were bound to axonemes, washed with HMEK buffer, and then treated with 1 mM EDC. The samples were separated in an 8% minigel and autoradiographed. For IC78, reaction with EDC generated a cross-linked product with a relative molecular mass of 127,000 that appeared to be identical to the product previously shown to derive from the cross-linking of IC78 to α-tubulin (King et al., 1991). No detectable difference between EDC-treated and untreated samples was observed for 78ΔSmaI. However, after treatment with EDC, an extra product with a relative molecular mass of 92,000 was obtained with 78Δ1309C. The apparent mass of this product is consistent with the cross-linking of 78Δ1309C (43 kD) to tubulin. The diagram indicates the regions of IC78 represented by the constructs.

78Δ1309C (Fig. 8). These fragments were then assayed for the ability to bind to and cosediment with axonemes. The deletion of the region from S_5–D_{29} reduced by 58% the amount of fragment cosedimenting with axonemes, confirming the importance of this region for microtubule binding. The deletion P_{298}–D_{323} reduced binding by 35%. In contrast, deletion of the adjacent regions T_{272}–E_{293} or A_{326}–S_{339} had little or no effect on binding. This confirms that most if not all of the microtubule-binding activity in this central part of the molecule is contained within the region extending from D_{264}–D_{323} (see above), and probably within the smaller region from P_{298}–D_{323}. None of the other small deletions (ΔS_{116}–D_{140}, ΔK_{181}–E_{204}, ΔA_{236}–S_{253}) had a noticeable effect on the binding activity of the fragment. Therefore, within the NH_2-terminal half of IC78, the highly charged NH_2-terminal domain and the region from D_{264}–D_{323} appear to be uniquely important for microtubule binding.

IC78 Region that Binds IC69 Is Coincident with Two WD Repeats

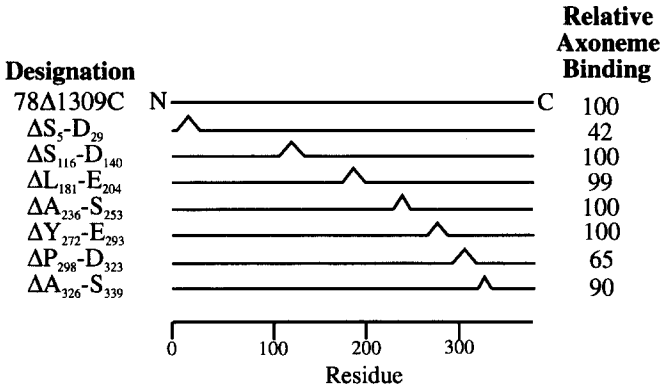
IC78 interacts with IC69 in situ (Mitchell and Rosenbaum, 1986; King et al., 1991). To determine the region of IC78 responsible for this interaction, IC69 was translated in vitro and allowed to interact for 30 min with various IC78 constructs that likewise had been translated in vitro. The samples were then immunoprecipitated with either antibody 1878A or 1869A (both antibodies are highly specific for their respective IC; see King et al., 1985; 1991). The immunoprecipitates were then examined by SDS-PAGE to determine if the nonimmunoreactive intermediate chain or fragment was coprecipitated.

When intact IC78, 78ΔSmaI, and 78Δ1309C were immunoprecipitated with 1878A, significant amounts of IC69 also were found in the pellets (Fig. 9). These data suggest that the region of IC78 involved in binding IC69 is located in residues M_{324}–L_{386} (i.e., the overlap region between 78ΔSmaI and 78Δ1309C). The 1878A epitope is correlated with the sequence extending from A_{326} to F_{334} (King, S. M., and G. B. Witman, unpublished results); hence, fragments truncated further towards the NH_2- or COOH-terminus (e.g., 78Δ679C or 78ΔBamHI) lack the 1878A epitope and could not be used here.

In the converse experiment, the antibody 1869A immunoprecipitated IC69 together with minor amounts of IC78, 78ΔSmaI, and 78Δ1309C (Fig. 9), but did not coprecipitate 78Δ679C or 78ΔBamHI along with IC69 (not shown). We previously observed that binding of the 1869A antibody to native outer arm dynein causes significant disruption of the complex (see King and Witman, 1990) and this likely accounts for the small amounts of IC78 and constructs coprecipitated with IC69 by this antibody. In any case, the data obtained with antibody 1869A support the assignment of residues M_{324}–L_{386} of IC78 as containing the site of interaction with IC69. This section of IC78 coincides closely with WD repeats A and B (S_{307}–S_{339} and H_{358}–V_{390}, respectively) (see Fig. 1 *B* and Wilkerson et al., 1995).

Discussion

In the studies reported here, we found that the 78,000-M_r intermediate chain (IC78) of *Chlamydomonas* outer arm dynein, produced by translation in a rabbit reticulocyte lysate, is able to bind to axonemes and bovine brain microtubules in vitro. The interaction appears to be specific and similar to that which occurs in vivo based on the following observations: (*a*) Binding occurs when IC78 is present at a concentration of only ~80 ng/ml in a background of >50 mg/ml cellular protein in the reticulocyte lysate. (*b*) The bound protein is released by 0.6 M NaCl, which is the concentration that releases >90% of outer arm dynein from the axoneme. (*c*) Binding occurs under conditions where IC69, a homologous protein with similar WD repeats, a similar basic NH_2-terminus, and a similar size and isoelec-



Restriction Site	Residue Inserted
-----	-
XhoI	E
HindIII	L
SacII	R
PvuI	S
NsiI	H
PstI	Q
BstBI	E

Figure 8. Location of oligonucleotide-directed deletions made within 78Δ1309C. For each product, the percent binding to axonemes relative to 78Δ1309C is reported. Also indicated is the restriction site used to select for each deletion and the single amino acid residue inserted between the boundaries of the deletion.

tric point (Mitchell and Kang, 1991; Wilkerson et al., 1995; Ogawa et al., 1995), does not bind. (*d*) EDC treatment of IC78 bound to axonemes in vitro results in the formation of a 127-kD complex which appears to be identical to the IC78/α-tubulin conjugate formed by EDC treatment of intact axonemes (King et al., 1991). Based on these results, we conclude that IC78 is a bona fide microtubule-binding protein. This is the first evidence for a specific function for any dynein intermediate chain. That at least one polypeptide of *Chlamydomonas* outer arm dynein is a microtubule-binding protein is consistent with the earlier finding that this dynein is capable of binding directly, in an ATP-independent manner, to microtubules assembled from purified brain tubulin (Haimo et al., 1979; Haimo and Fenton, 1988).

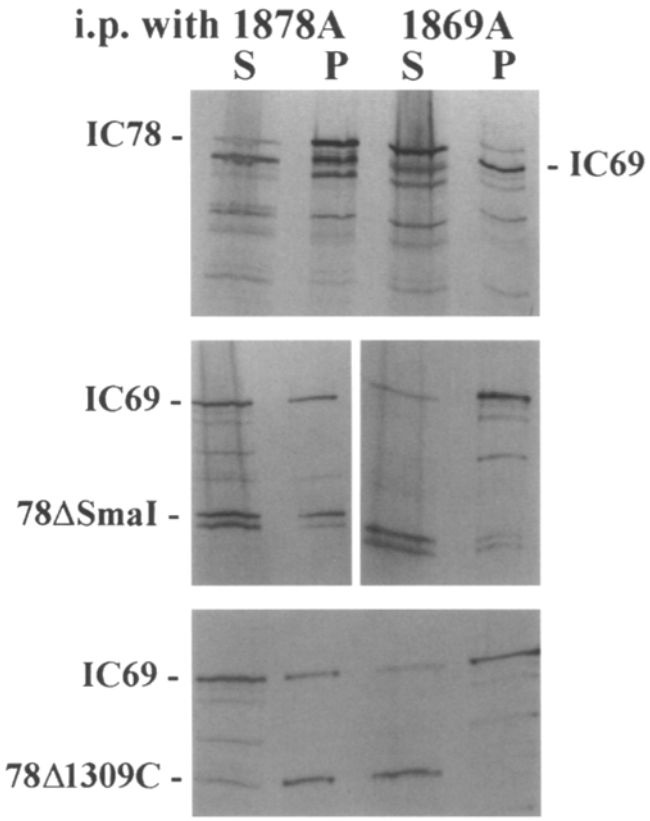


Figure 9. IC78 binds IC69 in vitro. 10 μl of the IC78, 78ΔSmaI, or 78Δ1309C translation mixture was added to an equal volume of lysate containing in vitro–translated IC69. After incubation for 30 min, the samples were divided into two aliquots and immunoprecipitated either with antibody 1878A or 1869A. The supernatants (*S*) were saved and the pellets (*P*) washed several times with 0.05% Tween 20 in TBS before electrophoresis in an 8% acrylamide minigel and autoradiography. Some of the short fragments of IC69 resulting from translation initiation at internal methionines do not contain the epitope recognized by mAb 1869A and hence are not immunoprecipitated by that antibody. Similarly, some of the IC69 fragments apparently lack the region of IC69 that interacts with IC78 and are not coprecipitated by antibody 1878A.

Previous results showed that IC78 is located at the base of the soluble outer arm dynein (King and Witman, 1990), that it is in direct contact with α-tubulin in the axoneme (King et al., 1991), and that it is essential for outer arm assembly (Wilkerson et al., 1995). Taken together with the current finding that IC78 is a microtubule-binding protein, these results indicate that IC78 is almost certainly involved in attaching the outer arm dynein to the doublet microtubule by virtue of its microtubule-binding activity. This raises the possibility that the ICs of other dynein isoforms may have a similar role in attaching those dyneins to the specific cell organelles with which they associate.

By analysis of the microtubule-binding properties of truncated versions of IC78, and of IC78 fragments containing small internal deletions, we were able to identify three regions of IC78 that appear to be important for microtubule binding in vitro.

The first of these regions corresponds to the NH_2-terminal ~20 amino acids; truncation of these residues prevents the remainder of the chain from binding to microtubules. Sequence analysis of IC78 (Wilkerson et al., 1995) revealed that the NH_2-terminal 24 amino acids form a highly charged, lysine-rich region that is separated from the rest of the molecule by a short glutamine–proline-rich segment predicted to form a polyproline II helix. Such helices form flexible linkers between domains in proteins (Adzhubei and Sternberg, 1994), so the highly charged NH_2-terminus is likely to form a discrete structural and functional domain. The current findings imply that one of its functions is to bind microtubules.

The second region important for microtubule binding is located in the center of the polypeptide, within the region D_{264}–D_{323} and probably between P_{298} and D_{323}. Truncation through the wider region from the COOH-terminus eliminated binding of the remaining N-terminal portion of the molecule; deletion of the smaller section within this region reduced the amount of binding of a large NH_2-terminal

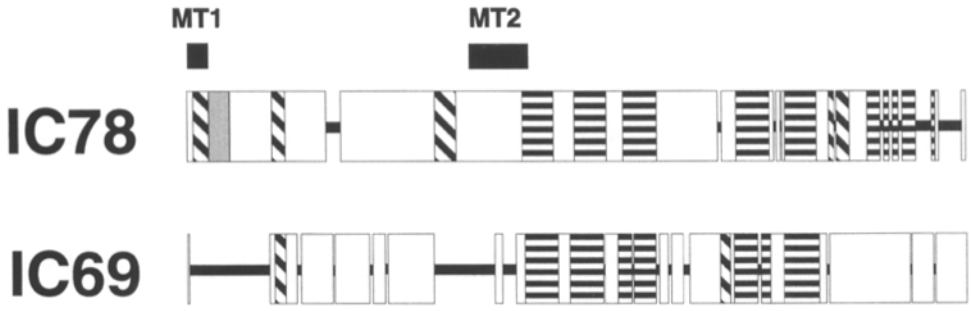


Figure 10. Comparison of IC78 and IC69. An objective, computer-generated alignment of IC78 and IC69 in which gaps have been inserted to optimize the match (Ogawa et al., 1995). Most of the difference in mass between the two chains is due to large gaps in the NH_2-terminal and central regions of IC69; these gaps correspond to the regions (MT1 and MT2) of IC78 that are implicated in microtubule binding. ▨, Basic; ■, GLN-PRO; ▤, WD Repeat; —, Gap.

fragment. The wider region contains two charged sequences of interest. The first ($_{273}$EEVAMDFKYWDDASD$_{287}$) is acidic, shares substantial similarity to part of the repeated sequence in the microtubule-associated repetitive protein (MARP-1) from *Trypanosoma brucei* (Schneider et al., 1988; Hemphill et al., 1992):

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MARP-1    E E V A T D M R H V D E
IC78      E E V A M D F K Y W D D
          * * * *   *   +     * +
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and is predicted to form an α helix (results not shown). The second sequence is very basic ($_{304}$SDKSKRRQ$_{311}$) and has the highest surface probability of any region in the entire molecule. Because of its similarity to the MARP-1 protein, the acidic sequence would seem to be a likely candidate for a microtubule-binding domain. However, deletion of the region Y_{272}–E_{293} had no effect on the ability of the NH_2-terminal fragment 78Δ1309C to bind to microtubules. In contrast, deletion of P_{298}–D_{323} significantly reduced binding, suggesting that the basic residues in this region are likely to be the ones important for microtubule binding.

The third region that has microtubule-binding activity is located in the COOH-terminal half of the molecule, between residues M_{480} and S_{604}. This region contains WD repeats D (S_{518}–L_{550}) and E (D_{567}–L_{594}), which are likely to be involved in interactions between protein subunits (Neer et al., 1994, and see below). However, there are several reasons to question the physiological relevance of this binding. First, the microtubule-binding activity of this region is only revealed when the entire NH_2-terminal half of the molecule is deleted. Second, the interaction between axonemes and fragments containing this region is not completely disrupted by washing with 0.6 M NaCl. Third, bound fragments containing only this region are not cross-linked to tubulin by EDC. Finally, the homologous protein IC69 contains similar WD repeats, but does not bind to microtubules in vitro. These observations do not rule out the possibility that a portion of this region functions as a microtubule-binding domain in vivo, but there is no independent evidence to support such a role. This region may be binding to the axonemes nonspecifically, possibly through residues exposed due to misfolding as a result of deletion of the NH_2-terminal half of the molecule, although the fact that binding is observed in the presence of >50 mg/ml of other protein argues against this. Alternatively, binding of this region to microtubules may be regulated by sequences present in the NH_2-terminal half of the chain; such regulation might prevent microtubule binding until the dynein has docked at the correct location on the axoneme (see below). In this case, the absence of the NH_2-terminal regulating sequence might then leave the COOH-terminal domain free to bind to microtubules.

Because axoneme-bound fragments containing the NH_2-terminal and central domains appear to be cross-linked to tubulin by EDC, it is likely that this binding is physiologically relevant. One or both of these regions is probably in direct contact with tubulin after binding. As noted earlier, both outer arm dynein and IC78 are released from outer doublet microtubules by 0.6 M NaCl and thus appear to be attached to the microtubule by ionic bonds. Both of the above regions contain highly basic stretches that have the potential to interact with tubulin, which is acidic, via electrostatic bonds. Our finding that truncation of either of these regions completely eliminated binding suggests either that these regions interact to form a single binding site, or that they act cooperatively to increase the affinity of IC78 for microtubules.

An objective alignment of IC78 and IC69 (Ogawa et al., 1995) reveals that most of the difference in mass between these two chains is due to large gaps in the IC69 sequence corresponding to IC78's L_4–D_{82}, K_{229}–D_{287}, and L_{296}–K_{308} (Fig. 10, and see Fig. 7 in Ogawa et al., 1995). The first gap eliminates essentially all of the sequence in IC69 that would have been homologous to the NH_2-terminal region important for microtubule binding in IC78; the other two gaps eliminate most of the IC69 sequence that would have been homologous to the central region important for microtubule binding in IC78. IC69 is not cross-linked to tubulin in the native, intact axoneme, and IC69 produced by in vitro translation does not bind to axonemes. Therefore, the absence of these regions appears to be specifically correlated with the lack of microtubule-binding activity in IC69. Apparently, IC69 either did not retain, or did not evolve, the specific sequences necessary for microtubule binding.

The sequences of IC78 that are important for microtubule binding also are not highly conserved in IC74 of cytoplasmic dynein. This is consistent with the presumed absence of a site within cytoplasmic dynein for structural binding to microtubules. However, the fact that IC78 binds outer arm dynein to flagellar microtubules strength-

ens the argument that the homologous IC74 may be involved similarly in targeting cytoplasmic dynein to the organelles with which it associates. Multiple forms of IC74 generated by phosphorylation (Dillman and Pfister, 1994) and alternative splicing (Paschal et al., 1992) have been described, and these variants may well play a role in the attachment of cytoplasmic dynein to different cellular structures.

Multiple microtuble-binding sites have been identified within other MAPs, including tau (Butner and Kirschner, 1991), MAP-2 (Lewis et al., 1988) and MAP-4 (Chapin and Bulinski, 1991); in these cases at least some of the contact sites are arranged as a series of imperfect repeats ending in the glycine-rich sequence PGGG. In MAP-4, these repeats are adjacent to a basic proline-rich region; in vitro studies indicate that both regions bind to microtubules, although the proline-rich region appears to bind most tightly (Aizawa et al., 1991). This interaction may thus be analogous to that of IC78 and microtubules. However, with the possible exception of MARP-1 (which also exhibits a multiple repeat structure [Schneider et al., 1988; Hemphill et al., 1992]), IC78 shows no overt similarity with other MAPs, indicating that it contains at least one and perhaps two novel microtubule-binding motifs.

After high salt extraction from the axoneme, the *Chlamydomonas* outer arm routinely is obtained as two discrete particles: a γ subunit containing the γ DHC and 2 LCs, and an α/β dimer consisting of the α and β DHCs, IC78 and IC69, and the remaining LCs (Pfister et al., 1982). Efficient rebinding of either particle to dynein-depleted axonemes requires the presence of the other particle (Fay, R. B., and G. B. Witman. 1977. The localization of flagellar ATPases in *Chlamydomonas reinhardtii. J. Cell Biol.* 75:286*a*; Takada and Kamiya, 1994; King, S. M., and G. B. Witman, unpublished results), suggesting that the α/β dimer must associate with the γ subunit before strong binding can occur. Thus, although we demonstrate here that IC78 is a microtubule-binding protein, this component apparently is not sufficient for strong binding of the outer arm to the doublet microtubule. It is possible that other dynein polypeptides also contribute to the ATP-insensitive microtubule-binding of the outer arm to the doublet microtubules; for example, the α DHC from sea urchin sperm flagella has been suggested to participate in this interaction (Bell et al., 1982; Moss et al., 1992). Alternatively, the interaction of the γ subunit with the α/β dimer may cause a conformational change in IC78 that allows binding to occur; this would ensure that outer arm binding sites were not occupied by the α/β dimer alone.

It also is likely that the outer arm interacts with other axonemal proteins in addition to tubulin. Flagellar outer arm dynein decorates the entire surface of microtubules assembled from purified brain tubulin (Haimo et al., 1979; Haimo and Fenton, 1988), whereas outer arm dynein binds to a specific site on outer doublet microtubules both in vitro (Fay, R. B., and G. B. Witman. 1977. The localization of flagellar ATPases in *Chlamydomonas reinhardtii. J. Cell Biol.* 75:286*a*; Takada and Kamiya, 1994) and in vivo. Thus, the outer doublet microtubules probably contain some protein which specifies the outer arm binding site. Recently, Takada and Kamiya (1994) reported that a 7S factor was necessary for efficient assembly of outer arms onto outer doublet microtubules. This factor was correlated with an ~70-kD polypeptide, and with a small pointed structure at the site where the outer arm normally attaches to the outer doublet. Thus, the 70-kD polypeptide and this structure may specify the outer arm binding site. Further experiments will be necessary to determine if this factor interacts with IC78 or with some other dynein polypeptide. In any case, it should be noted that some of the experiments reported here used axonemes from the mutant *oda1*, which lacks the 7S factor; thus, the binding we observed using *oda1* axonemes, as well as that observed with brain microtubules, could not have been mediated by the 7S factor.

Previous studies have used detergent-induced dissociation (Mitchell and Rosenbaum, 1986) and direct chemical cross-linking (King et al., 1991) to determine that IC78 and IC69 interact directly with each other and with several of the dynein LCs. Such IC/LC complexes also have been found in dyneins from sea urchin (Tang et al., 1982; Moss et al., 1992) and trout (King et al., 1990) sperm flagella and probably are a general feature of outer arm design (for review see Witman et al., 1991). In the present study, we found that the site in IC78 that interacts with IC69 apparently is localized to the central region (M_{324}–L_{386}) of IC78. This region correlates very closely with IC78's WD repeats A (S_{307}–S_{339}) and B (H_{358}–V_{390}) (Wilkerson et al., 1995). It has been proposed that WD repeats are involved in subunit–subunit interactions within multisubunit complexes, and that they may act in pairs to form a site for subunit binding (Neer et al., 1994). Our findings are in good agreement with this proposal. It will be of considerable interest to determine if IC78's other WD repeats are similarly involved in binding the dynein LCs and perhaps the β DHC. Inasmuch as subunit–subunit interactions appear to be a feature common to all dynein ICs, it is likely that the WD repeats in IC69 and IC74 have a similar role.

The present study has identified two, and possibly three, regions of IC78 that appear to be important for binding the outer arm to the outer doublet microtubule, as well as one region that appears to be important for binding IC78 to IC69 within the dynein complex. Recently, several insertional mutants of *Chlamydomonas* were identified in which the IC78 gene has been completely deleted (Wilkerson et al., 1995). Thus, it should now be possible to use site-directed mutagenesis to alter those regions of the IC78 cDNA or gene that encode the presumptive functional domains identified in this study, and then transform the altered DNA into the null mutants to test the roles of these domains, and of specific residues within these domains, in vivo.

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