Identification of a Microtubule-binding Domain in a Cytoplasmic Dynein Heavy Chain*

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As a molecular motor, dynein must coordinate ATP hydrolysis with conformational changes that lead to processive interactions with a microtubule and generate force. To understand how these processes occur, we have begun to map functional domains of a dynein heavy chain from Dictyostelium. The carboxy-terminal 10-kilobase region of the heavy chain encodes a 380-kDa polypeptide that approximates the globular head domain. Attempts to further truncate this region fail to produce polypeptides that either bind microtubules or UV-vanadate cleave, indicating that the entire 10-kilobase fragment is necessary to produce a properly folded functional dynein head. We have further identified a region just downstream from the fourth P-loop that appears to constitute at least part of the microtubule-binding domain (amino acids 3182–3818). When deleted, the resulting head domain polypeptide no longer binds microtubules; when the excised region is expressed in vitro, it cosediments with added tubulin polymer. This microtubule-binding domain falls within an area of the molecule predicted to form extended α-helices. At least four discrete sites appear to coordinate activities required to bind the tubulin polymer, indicating that the interaction of dynein with microtubules is complex.

In eukaryotic cells, dynein is a ubiquitous high molecular mass ATPase that moves organelles and other cellular cargo toward the minus ends of microtubules (1, 2). The globular head domain, largely encoded by the dynein heavy chain (DHC) gene, couples ATP hydrolysis and microtubule binding to generate conformational changes that provide force for this movement (3). Within the DHC, four P-loop motifs partially identify sites for nucleotide binding. The first of these motifs is highly conserved among cytoplasmic and axonemal dyneins and represents the major ATP catalytic site for force production. To understand how these processes occur, we have begun to map functional domains of a dynein heavy chain from Dictyostelium. The carboxy-terminal 10-kilobase region of the heavy chain encodes a 380-kDa polypeptide that approximates the globular head domain. Attempts to further truncate this region fail to produce polypeptides that either bind microtubules or UV-vanadate cleave, indicating that the entire 10-kilobase fragment is necessary to produce a properly folded functional dynein head. We have further identified a region just downstream from the fourth P-loop that appears to constitute at least part of the microtubule-binding domain (amino acids 3182–3818). When deleted, the resulting head domain polypeptide no longer binds microtubules; when the excised region is expressed in vitro, it cosediments with added tubulin polymer. This microtubule-binding domain falls within an area of the molecule predicted to form extended α-helices. At least four discrete sites appear to coordinate activities required to bind the tubulin polymer, indicating that the interaction of dynein with microtubules is complex.

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1 The abbreviations used are: DHC, dynein heavy chain; PIPES, 1,4-piperazinediethanesulfonic acid; HSS, high speed supernatant(s).

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For both kinesin and myosin motors, the regions believed to interact with their respective filaments are located fairly close to the catalytic P-loop (17–20). It is possible that dynein follows a similar design with a single microtubule binding domain near the primary P-loop. We previously characterized the in situ expression of a 107-kDa fragment of the DHC that contained the first two P-loop motifs (21). Although a small amount of polypeptide appeared to cosediment with microtubules when analyzed by immunoblots, this was substantially less than the native heavy chain and clearly does not represent a native binding activity. This argues that the nucleotide-sensitive interaction of dynein with microtubules is not self-contained within a simple region flanking the first P-loop. Alternatively, there may be weak affinities associated with each of the four P-loop motifs, and microtubule binding is a cooperative effort involving multiple regions. Finally, the microtubule-binding domain may be located elsewhere in the globular head. An important prerequisite for its placement is that binding must be mechanically coupled to ATP catalysis to allow for cyclical on/off interactions.

We describe here efforts to express a series of DHC gene constructs in Dictyostelium. This work roughly defines a minimal functional unit for the dynein motor domain. We further demonstrate that a region centered around a predicted two-part α-helical domain of the heavy chain, just downstream of the fourth P-loop, is able to bind microtubules in vitro. When this domain is deleted, a dynein head fragment expressed in vivo no longer interacts with microtubules and loses its ability to undergo a UV-vanadate cleavage reaction. The sequence encoding this motif is complex and may require substantial secondary structure for its activity. This work identifies an important structural domain of dynein and links this activity to ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Molecular Methods in Dictyostelium—Most of the molecular manipulations and culture conditions have been previously described (21, 22). Briefly, fragments of the dynein heavy chain gene from Dictyostelium were ligated between the DHC promoter and an actin 8 transcriptional terminator on a plasmid containing a G418 resistance marker. Constructs were introduced into AX-2 Dictyostelium cells by CaPO4 precipitation of supercoiled plasmid DNA (23, 24). Colonies were selected for growth in 10 μg/ml G418 and cloned as described previously (21).

Biochemical Methods—Generation of Dictyostelium high speed supernatants, microtubule affinity, UV cleavage, electrophoresis, and immunoblotting were performed as described in Refs. 26 and 27. Purified rabbit skeletal muscle actin was purchased from Cytoskeleton (Denver, CO).

In Vitro Transcription/Translation—For in vitro expression, an initial EcoRI fragment of the DHC gene (encoding amino acids 3182–3679)
In Vivo Expression—The Dictyostelium DHC encodes an open reading frame of 4725 amino acids (6). Previous work showed that a 380-kDa carboxyl-terminal fragment corresponds to a single globular head (21). This fragment binds to microtubules in an ATP-sensitive fashion indistinguishable from native dynein and undergoes a UV-vanadate cleavage, a reaction diagnostic for dynein and for a structurally active ATP extract from cells expressing the 380-kDa head domain fragment. The 380-kDa polypeptide is marked with an arrowhead. Lanes 5 and 6 show an equivalent HSS and ATP extract from cells expressing the 380-kDa head domain fragment. The 380-kDa polypeptide is marked with an arrowhead. Lanes 7 and 8 show an immunoblot of 318 kDa HSS supplemented with 1 mM ATP and 0.1 mM sodium orthovanadate. The HSS was evenly divided. Lanes 7 shows the unirradiated aliquot; lane 8 shows the sample after irradiation with 365-nm UV light for 60 min. The native DHC (arrowhead on the left) nearly disappears following irradiation, indicating cleavage. A faint band representing the native lower molecular mass cleavage product can be detected (arrowhead on the right). The higher molecular mass cleavage product would be obscured by the 318-kDa band. The 318-kDa product is not appreciable in the microtubule pellet or ATP extract. For comparison, lanes 5 and 6 show an equivalent HSS and ATP extract from cells expressing the 380-kDa head domain fragment. The 380-kDa polypeptide is marked with an arrowhead. Lanes 7 and 8 show an immunoblot of 318 kDa HSS supplemented with 1 mM ATP and 0.1 mM sodium orthovanadate. The HSS was evenly divided. Lanes 7 shows the unirradiated aliquot; lane 8 shows the sample after irradiation with 365-nm UV light for 60 min. The native DHC (arrowhead on the left) nearly disappears following irradiation, indicating cleavage. A faint band representing the native lower molecular mass cleavage product can be detected (arrowhead on the right). The higher molecular mass cleavage product would be obscured by the 318-kDa band. The 318-kDa product is not appreciable in the microtubule pellet or ATP extract.

Results

head domain fragments that do express well show substantial dynein-like activity. Therefore it seems reasonable to predict that the difference between these two sets of constructs (i.e. the central head region) contains an activity important for dynein’s structure or function. An SpkI restriction digest of the DHC gene removes an in-frame 1.6-kilobase fragment of sequence (amino acids 3105–3643) within this region. This excised segment encodes a structurally unique region of the heavy chain, containing two predicted α-helical motifs. Fig. 2 shows that...
AX-2 cells transformed with the 380KΔSph construct abundantly produce the 318-kDa polypeptide. However, unlike either native dynein or the 380-kDa head domain fragment, this 318-kDa polypeptide does not cosediment with microtubules in binding assays and fails to cleave in the presence of UV light. However, because tubulin is an acidic polymer (pI 5.5), it is possible that the in vitro expressed polypeptides nonspecifically interact through charged residues. Actin also forms long polymers and carries a net overall charge similar to that of tubulin (pl 5.4) (30). Fig. 5 compares the sedimentation of the 57- and the 30-kDa polypeptides in the presence of molar excesses of microtubules and actin filaments. Although actin sedimentation results in a slight increase of pelletable 57-kDa product over buffer alone, there is substantially more polypeptide in the tubulin pellet. The 30-kDa polypeptide does not pellet in the presence of microtubules nor in the actin polymer control. These results strengthen the argument that the interactions described here are tubulin-specific.

The results presented in Figs. 3–5 indicate that this predicted helical region of the DHC contains an ability to cosediment with tubulin and thus may at least in part define the microtubule-binding domain of dynein. They further indicate that this region is complex and binding requires either substantial secondary structure or that multiple contact sites with the polymer are involved. In the in vitro pelleting assays, only a small percentage of the total expressed polypeptide appears to cosediment with microtubules. Although this is consistent with what we have found in Dictostelium high speed supernatants, where only a fraction of the total native DHC cosediments with bovine microtubules, it may also indicate a partial microtubule binding ability. Native dynein contains both high and low affinity microtubule binding states, which are likely

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2 M. P. Koonce, unpublished observations.
the product of geometrical changes within the microtubule-contact site. Without detailed information on the tertiary structure of this region in both the native molecule and the fragment expressed here, quantitative assessments on the binding efficiency are probably not meaningful.

**DISCUSSION**

The work presented here describes two related efforts to understand the domain structure of a dynein heavy chain gene. The first describes efforts to express functional units of the globular head domain, and the second identifies a region of the molecule that appears to contain a microtubule binding activity.

**A Minimal Motor Domain for Dynein**—Biochemical and structural data indicate that a 380-kDa carboxyl-terminal fragment of the *Dictyostelium* DHC corresponds to a functionally active, single globular head of dynein (21). The data presented here describe efforts to produce substantially smaller head domain fragments that retain an ATP-sensitive microtubule binding activity. In our hands, several constructs truncated from the carboxyl-terminal end fail to produce detectable product, indicating that the specified polypeptide either is highly unstable or is toxic to the cell. Of the smaller constructs that are produced *in vitro*, none show a substantial ability to bind microtubules and, for the ones containing the first P-loop, fail to undergo the UV-vanadate cleavage normally associated with this motif (29). Truncations in from the amino terminus of the head domain would remove the P-loop associated with ATP hydrolysis and thus would fail to make complete motors.

A deletion from the middle of the head domain produces a polypeptide that appears stable but does not interact with microtubules or UV-vanadate cleaves. Moreover, the region excised contains an ability to bind microtubules in *vitro*. If an ATP-insensitive microtubule binding activity is retained in constructs expressed *in vivo*, then these polypeptides would likely coat the surface of the microtubules, inhibit dynamics and associated organelle motility, and likely lead to cell death. This may at least partially explain our difficulty in expressing some of the head domain constructs.

Although the first P-loop in the DHC sequence plays a prominent role in binding the nucleoside triphosphate, additional residues are necessary for ATP hydrolysis (31). Kinesin and myosin share at least three other motifs in common with some GTPases that fold together forming the ATP catalytic domain (reviewed in Ref. 32). Although identical sequences are not obvious in the *Dictyostelium* DHC, it seems reasonable to predict that similar structural motifs are required for dynein; these could be distributed anywhere along the linear sequence. Thus even though the ΔSph truncation occurs over 1100 amino acids downstream from the first P-loop, the inability of the 318-kDa polypeptide to UV-cleave indicates that the catalytic pocket is not folded properly or is missing a functional element necessary for ATP hydrolysis. Together with the data discussed in the previous paragraphs, these results suggest that the entire 380-kDa carboxyl-terminal fragment is necessary for ATP-sensitive microtubule binding activity and thus roughly defines the minimal functional head domain for dynein.

**A Microtubule-binding Domain for Dynein**—The *in vivo* deletion and the *in vitro* expression work suggest there are microtubule binding activities located within the predicted α-helical region just after the fourth P-loop in the DHC. A preliminary report describing a similar finding for another cytoplasmic dynein has also recently appeared (33). Comparative sequence analysis shows this region to be well conserved among dynein heavy chains. Within this region, the *Dictyostelium* sequence is approximately 50% identical to most cytoplasmic dyneins and close to 30% identical to axonemal dyneins sequenced to date. Our work suggests that at least four discrete units can act in different combinations to mediate the binding of dynein to microtubules. Comparisons do not show an obvious
sequence conservation between these four domains, suggesting that they do not contain simple repeated motifs similar to other microtubule-binding proteins, e.g. MAP-2, MAP-2C, MAP-4, and tau (34).

The predicted α-helical regions just after the fourth P-loop represent a unique structural domain in the dynein molecule. The borders of these regions are arbitrarily defined here by proline residues. Although the true structure of this domain has yet to be determined, the position and spacing of the proline residues are well conserved among axonemal and cytoplasmic dyneins. Previous discussions of functional properties for this domain include: 1) the helices could form a projection off the head domain and provide the ATP-sensitive B-link to the adjacent microtubule found in several axonemes (4, 35); 2) they may combine with another, shorter predicted helical domain preceding the first P-loop and participate in forming the tertiary structure of the head domain or the dynein molecule (1, 36); 3) they may form coiled-coil interactions with other polypeptides (4, 36); and 4) they may play a role in the conformational changes associated with force production (37). Our data strengthen the arguments presented for 1) and 4). If the region does form two or more helical domains, they could serve to project a microtubule contact site or could serve as mechanical levers to raise/lower a binding domain in conjunction with structural changes produced by ATP catalysis.

**Dynein-Microtubule Interactions**—A microtubule-binding sequence motif has been proposed in common with dyneins and kinesins (38, 39). P-X<sub>6</sub>-L represents the core consensus sequence of this motif and is surrounded by several conserved hydrophobic, polar, and charged regions (39). This sequence was described just after the first P-loop, about the same distance downstream in dynein, kinesin, and kinesin-like proteins. Although it falls within a region of kinesin shown experimentally to bind microtubules (17), a similar relationship to functional activity within dynein has not been reported. Interestingly, this core motif is also found within the domains we suggest have microtubule binding activity. Prolines 3198, 3366, 3648, and 3712 are followed by a glutamic acid 6 residues downstream and a leucine (or in one case, isoleucine) 3–5 residues further. The latter three of these “motifs” are well conserved among cytoplasmic dyneins.

Here we present data suggesting that the entire carboxyl-terminal two-thirds of the dynein heavy chain from *Dictyostelium* is essential to comprise a functional head domain. We also suggest that there are at least four domains, each containing approximately 125 amino acids, clustered around a predicted α-helical region downstream from the fourth P-loop that collectively participate in binding the motor to the microtubule polymer. Data also exist to suggest that dynein binds to both α- and β-tubulin (40), and structure work indicates that the dynein head is large enough to interact with multiple dimers. If a single dynein can bind to both α- and β-tubulin, the contact regions would not necessarily be identical. However, they might contain a similar core structure that coordinates affinity with ATP hydrolysis. Taking this together, we propose that cytoplasmic dynein makes more than one functional contact with a microtubule. This could be important in binding (does it dock to or does it grip the tubulin surface) or during its mechanochemical stroke (instead of rocking a lever arm, does the head roll?). At a minimum, this work associates a relatively defined region of the DHC with a functional activity and will permit a more focused analysis of how dynein binds microtubules.

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