Interaction Mapping of a Dynein Heavy Chain

IDENTIFICATION OF DIMERIZATION AND INTERMEDIATE-CHAIN BINDING DOMAINS*

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Cytoplasmic dynein is a multisubunit microtubule-based motor protein that is involved in several eukaryotic cell motilities. Two dynein heavy chains each form a motor domain that connects to a common cargo-binding tail. Although this tail domain is composed of multiple polypeptides, subunit organization within this region is poorly understood. Here we present an in vitro dissection of the tail-forming region of the dynein heavy chain from Dictyostelium. Our work identifies a sequence important for dimerization and for binding the dynein intermediate chain. The core of this motif localizes within an ~150-amino acid region that is strongly conserved among other cytoplasmic dyneins. This level of conservation does not extend to the axonemal dynein heavy chains, suggesting functional differences between the two. Dimerization appears to occur through a different mechanism than the heavy chain-intermediate chain interaction. We corroborate the in vitro interactions with in vivo expression of heavy chain fragments in Dictyostelium. Fragments lacking the interaction domain express well, without an obvious phenotype. On the other hand, the region crucial for both interactions appears to be lethal when overexpressed.

Dynein is a microtubule-associated motor protein that is responsible for several distinct motility processes in eukaryotic cells (reviewed in Refs. 1–3). The dynein superfamily is divided into two major classes based primarily on their cellular function. Cytoplasmic dyneins act in positioning and transport of membrane-bound organelles and help organize spindle assembly during cell division. The more heterogeneous axonemal dyneins are largely responsible for the beating motions of eukaryotic cilia and flagella. All dyneins characterized to date are composed of multiple subunits. A complete dynein molecule typically contains two or three heavy chains (DHC) with molecular masses of ~400 kDa; intermediate chains (DIC) of 70–80 kDa, some of which have been localized to the dynein tail (4, 5); and several light chains of different molecular masses (1, 3, 6–8). Many cytoplasmic forms also contain at least two light-intermediate chains (50–60 kDa) (9, 10).

Comparisons between axonemal and cytoplasmic dyneins suggest a functional division of the DHC into two regions. The carboxy-terminal two-thirds is conserved and comprises the motor domain (11–14). The more divergent amino-terminal third forms at least part of the stalk and likely forms a scaffold upon which most of the other subunits assemble to form the cargo-binding tail (1, 11, 15, 16). The molecular interactions within the dynein tail are not well understood. They are important, however, because variation in the subunit composition is thought to specify and regulate cargo binding either directly or by controlling the interaction of dynein with other proteins such as dynactin (16–20).

Our work in Dictyostelium has shown that overexpression of the motor domain in this organism dramatically alters the interphase microtubule array, but the cells are viable (13). In contrast, a fragment containing the amino-terminal region can only be expressed at low levels; its overexpression appears to be lethal (21). Because this fragment can associate with the native DHC in vivo (21), its overexpression may cause the formation of one-headed or headless dyneins that can bind cargo but cannot function as motors (see also Ref. 22).

To further investigate the toxic effect of this dynein fragment, and characterize its domain structure, we have initiated a functional dissection of the DHC amino terminus from Dictyostelium. We identify here a highly conserved region that is important for DHC homodimerization and for intermediate chain binding. Both these interactive properties are confined to the same region of the DHC; however, they have different sequence requirements and are biochemically distinct in vitro. We further correlate these results with expression of similar regions of the DHC in vivo. This is the first identification of a subunit-interaction domain in a cytoplasmic dynein heavy chain, and it provides a target for investigations into the structure and assembly dynamics of the dynein tail.

EXPERIMENTAL PROCEDURES

Construction of Vectors—A fragment containing the amino-terminal 4007 base pairs of the Dictyostelium DHC cDNA was subcloned into the pET5 expression vector (Novagen) just downstream of the T7 transcription initiation site. Smaller amino-terminal fragments were generated by endo- and exonuclease digestion and cloned into pET5 or pRSET (Invitrogen) expression vectors (see Fig. 5 for summary).

A construct encoding the full-length Dictyostelium DIC cDNA (652 aa, GenBank® accession number U25116 (23)), subcloned into pET 14 (Novagen), was used for the initial stages of this work. However, we found that a large EcoRI fragment of the DIC cDNA (aa 65–613) shows the same binding activity as the full-length DIC but is produced in greater amounts by BL21(DE3) cells. It is used almost exclusively in the data presented here. Plasmids were purified according to the alkaline lysis/polyethylene glycol method (24).

Construction of Affinity Resins—Expression vectors encoding the DHC amino-terminal fragment and the full-length and partial DIC
cDNAs were expressed in BL21(DE3) cells. Polypeptides were purified from inclusion bodies following the methods of Lin and Cheng (25). Inclusion bodies were solubilized in renaturing buffer (50 mM Tris, 500 mM NaCl, 0.05% Tween 80, 20% glycerol, pH 7.6) containing 8 M urea. Soluble protein was clarified at 25,000 x g for 20 min and adjusted to a concentration of 2 mg/ml. This solution was dialyzed stepwise at 2-h intervals against 25 volumes of buffer with decreasing concentrations of urea (4, 2, 0 M), then against CNBr coupling buffer (100 mM NaHCO3, 500 mM NaCl, pH 8.3) overnight, and clarified at 25,000 x g for 20 min. The concentration of soluble protein was determined by Bradford assay, and the protein was then coupled to one-fourth volume activated CNBr resin (Amersham Pharmacia Biotech) overnight. This resulted in a final protein concentration on the resin of ~3 mg/ml. The affinity resin was picked with a pipette and transferred to a 24-well plate.

The amount of [35S]methionine-labeled polypeptide was determined by Amplify (Amersham Pharmacia Biotech), drying, and exposing to film. Reticulocyte-lysate expressed bands were detected by soaking the gel in 3% SDS-PAGE sample buffer, and boiled. Bound polypeptides were resolved by electrophoresis through a 7.5% SDS-polyacrylamide gel. The data from the DHC-DHC and DHC-DIC cross-linking of the DHC amino terminus were examined using a cluster analysis (28).

RESULTS

Cross-linking of the DHC Amino Terminus—Previous work has suggested that the heavy chains of cytoplasmic dynein associate directly in the absence of other dynein subunits (31). Because the two heavy chains are believed to interact within the amino-terminal third of the DHC, we tested whether this region can dimerize in vitro. A reticulocyte lysate expressing the amino-terminal 1327 aa (151 kDa) of the DHC was exposed to either DSP or EDC cross-linkers to covalently link polypeptides in close contact with one another. DSP is approximately 8 Å in length and cross-links the e-amino of lysine residues; EDC is a zero-length cross-linker that forms a covalent bond between aspartate or glutamate and lysine. As indicated in Fig. 1, both cross-linkers produced labeled bands of MW ~300 kDa, a value close to the predicted dimer weight of the amino-terminal fragment.

Purification of DHC Amino Terminus with DHC Affinity Resin—To further investigate DHC dimerization, a construct encoding the amino-terminal 1327 aa of Dictostelium DHC was expressed in Escherichia coli (see Fig. 2A) and coupled to Sepharose, producing a DHC affinity resin. Two types of control resins were constructed to measure nonspecific interactions: non-dynein proteins (actin or BSA) bound to Sepharose and control resins were constructed to measure nonspecific interactions: non-dynein proteins (actin or BSA) bound to Sepharose at the same concentration, and a similarly-treated protein-free resin.

Incubation of the DHC affinity resin with the same, in vitro expressed polypeptide showed an approximately 3-fold higher retention of the soluble fragment than on control resins (Fig. 2, B and C). This suggests that the bound and soluble fragments interact in the assay. The resin-bound DHC fragment does not retain protein indiscriminately; luciferase does not bind to the affinity resin any more strongly than to the control resins. Moreover, the resins do not retain any of the more abundant protein components of the reticulocyte lysate at levels detectable by Coomassie Blue staining (not shown).

Dimerization Capabilities of DHC Subunits—To determine a minimal HC fragment that supports dimerization, we produced a series of smaller fragments (summarized in Fig. 5) and tested their ability to interact with the DHC resin and to cross-link. As expected, the fragments showed different affinities for DHC resin (Fig. 3A). Specific retention was measured as the ratio of band intensity on the affinity resin to its relevant control. These ratios fell into two groups, suggesting a defined binding site which some, but not all, constructs contain. One group comprises DHC fragments that bind more than twice as efficiently to affinity resin than to control resin; these were considered to contain the binding site. In all cases, these polypep-
tides bound to DHC resin at least as well as did the full-length DHC amino terminus. The second group consists of those that exhibit a mean affinity resin:control resin intensity ratio of less than 2; these were considered noninteractors. Such low ratios are similar to the nonspecific behavior exhibited by luciferase in this system (see Fig. 2B). Statistical analyses showed that, relative to the stated null distribution, these results are significant (P value of 0.04). All of the constructs that showed binding activity contain the region between aa 627 and 780. No fragment lacking this region showed binding ability. However, this region alone is not sufficient for dimer interaction in vitro.

These fragments also displayed differential cross-linking properties (Fig. 3B). Two fragments, encoding aa 567–1327 and 629–1327, generated high molecular weight bands in the presence of cross-linker. Both novel bands are approximately double the predicted mass of the expressed fragments, suggesting dimerization rather than nonspecific cross-linking to reticulocyte lysate proteins. DHC fragments that only contain the central region produced some faint higher molecular weight smearing in the presence of DSP, but no distinct banding patterns could be detected. The fragments of the DHC amino terminus that do not contain the central region do not form high molecular weight species in the presence of cross-linker.

While the affinity resin and cross-linking assays do not generate identical results (summarized in Fig. 5), this may reflect subtle differences between the interactions being measured; the resin work measures the ability of a fragment to interact with the full 1327-aa amino-terminal polypeptide, whereas the cross-linking evaluates the interaction between two identical polypeptides. Nonetheless, the two assays agree that there is a
crucial dimerization core region between aa 629 and 780, whose interaction is supported by flanking sequence on the carboxyl-terminal side. This additional sequence may play a role in stabilizing the structure of the interaction region.

Differential Interaction of DHC Subunits with DIC—Because DHC amino terminus is also predicted to interact with the DIC, we constructed an affinity resin with bacterially expressed DIC and tested its ability to bind the DHC amino terminus. Reticulocyte-lysate-produced DHC also binds to DIC resin (Fig. 4A), with a 4-fold mean enhancement of binding over control. As in the DHC affinity resin, luciferase does not show an increased affinity for the DIC resin. This indicates that the DHC amino terminus also contains a binding site for the DIC.

To search for a DIC interaction site within the DHC amino terminus, we tested the ability of the DHC fragment series to interact with DIC resin (Fig. 4B). The mapping pattern is similar to the DHC dimerization work but defines a smaller minimal region for this activity. The best statistical grouping of fragments predicted to contain the DIC-DHC interaction site is attained when fragments 1–1327, 567–1327, 629–1327, 1–730, 1–940, and 534–760 contain the site (P value = 0.012) (Fig. 5). This suggests that the DIC interaction site is located within aa 629–730, just upstream of the DHC-DHC site.

Heavy Chain and Intermediate Chain Binding Activities—Despite mapping to the same region of the DHC sequence, the DHC and the DIC binding activities are not identical. We analyzed the resin assay interaction under conditions of varying ionic strength. While the homodimerization of the 1327-aa DHC fragments was salt-sensitive, the DHC-DIC interaction was stable even in the presence of 2 M NaCl (Fig. 6). This suggests that the DHC-DHC and the DHC-DIC interact
The sequence on the carboxyl-terminal side, which likely forms a structural support to bind the DIC, suggesting that the DHC central domain of the DHC amino terminus is especially effective in this role.

Conservation of Sequence Among Cytoplasmic Dyneins—The results described above suggest that at least two activities important for dynein subunit assembly are localized to a relatively small region of the DHC amino terminus. Sequences from nine species were compared with the amino-terminal region of the DHC of Dictyostelium (Fig. 8A). Homologous regions were aligned and the degree of identity to the Dictyostelium sequence was determined for intervals of 100 aa. While the amino-terminal region is generally not as conserved as the rest of the polypeptide (1, 11, 27), there are three striking areas of homology evident among the ten cytoplasmic sequences. The most conserved lies between aa 600 and 800 in the Dictyostelium sequence and includes the sequence we experimentally identify as essential for heavy-chain dimerization and heavy chain-intermediate chain binding. This further suggests that the assembly activities localized within aa 629–780 in Dictyostelium DHC are conserved among the other cytoplasmic dynein heavy chains.

Sequences for the axonemal Chlamydomonas, sea urchin, and Paramecium β DHCs, and for Chlamydomonas γ DHC, were also analyzed (Fig. 8B). The amino terminus of the Chlamydomonas β DHC interacts with several other dynein subunits (22), and the homologous regions of the sea urchin and Paramecium β DHCs show regions of good conservation with the Chlamydomonas β sequence. However, the overall pattern of conservation within the β amino-terminal fragments is distinct from the cytoplasmic dyneins.

DISCUSSION

The organization of the cytoplasmic dynein tail and the specific interactions between its subunits at the amino acid level have not been well characterized. Electron microscopy has been useful in localizing some of the subunits (4, 5), and considerable progress has been made in investigating interactions of these subunits with other dynein-associated proteins (15, 18–20). Subunit organization of axonemal dyneins has been primarily studied through isolation of dynein gene mutations (reviewed in Ref. 2). These often produce organisms with nonlethal motility defects, which are amenable to biochemical and structural analyses. Similar analyses are becoming informative for cytoplasmic dyneins (21, 23, 32–35), mapping out the heavy chain domain structure and identifying the microtubule-binding motif. However, the physical organization of the cytoplasmic dynein tail has not been dissected in sufficient detail to identify heavy chain domains that mediate interactions with other subunits. Because the tail region links the motor to a cargo, understanding the interactions here is important in determining how dynein is targeted and regulated.

Previous work in vitro suggested that the amino-terminal third of the dynein heavy chain is responsible for interactions between the DHCs and with other dynein subunits (21, 22). We used both chemical cross-linking and in vitro affinity methods here to identify a region that promotes DHC dimerization. This supports an earlier report suggesting recombinant cytoplasmic DHCs directly form multimers, even in the absence of other subunits (31). We have further determined that the sequences crucial for this interaction lie within an approximately 150-aa region of the DHC. While this core region is necessary for dimerization, it seems to function most efficiently in the context of flanking sequences. The additional amino acids may be important in stabilizing the structure of this central domain. The sequence on the carboxyl-terminal side, which likely forms at least part of the stalk connecting the tail to the globular head, is especially effective in this role.

We also show that the central domain of the DHC amino terminus directly interacts with the dynein intermediate chain in vitro. The sequence does not appear to require an additional structural support to bind the DIC, suggesting that the DHC.
and DIC interactions are distinct and that their activities are not dependent on one another. The different biochemical properties of the two interactions lend strength to this hypothesis. The in vitro work is supported by in vivo expression of DHC fragments. The amino- and carboxy-regions of the DHC amino terminus can be expressed at high levels in Dictyostelium; selective immunoprecipitation of these fragments shows no evidence that they bind the DHC or DIC. We cannot rule out the possibility that the fragments are misfolded and their activities do not reflect native behavior. However, previous expression of the amino-terminal region indicated that larger fragments (158 kDa) are able to dimerize with a native heavy chain, suggesting some degree of proper folding (21). Attempts to overexpress the entire amino terminus in Dictyostelium led to the idea that this fragment contains a functional motif that can perturb native dynein activity, resulting in cell death. This lethal activity appears to be preserved within the central 29 kDa of the amino terminus, within the region we indicate contains the dimerization and DIC binding sites. We do not know whether the toxic effect is because of perturbation of DIC binding, dissociation of the two heavy chains, or both.

Because most dyneins form multi-polypeptide assemblies through interactions in their tail, it seems reasonable to expect some degree of conservation within this region. While the amino-terminal tail region of the DHC is less conserved than the carboxyl-terminal head domain (1, 11, 12), it does contain small areas of strong conservation. The highest degree of sequence identity corresponds to the area we identify here to mediate DHC and DIC interactions. Among cytoplasmic heavy chains, this region is generally 40–65% identical to the Dictyostelium sequence. Even the most divergent yeast sequences show some degree of conservation in this region. In contrast, axonemal dynein heavy chains show very little sequence conservation with cytoplasmic dyneins in this region (1, 2, 36–38, and this study). Given their conserved structures seen by electron microscopy, this seems surprising. It is certainly possible that tertiary structure is preserved in this region and that similar interaction mechanisms occur. However, axonemal dyneins more often form heterodimers or heterotrimers of distinct DHC polypeptides, whereas cytoplasmic dyneins dimerize from identical heavy chains. While the amino-terminal portion of the DHC is important for assembly in both cytoplasmic and axonemal dyneins, the mechanisms of interaction may be distinct.

The current work suggests a functional division of the DHC amino terminus into three regions. The central core contains an interaction domain that assembles two heavy chains and binds the intermediate chain. Moreover, expression of this region in vivo appears to generate a tool to disrupt native dynein assembly and activity. Directed mutagenesis and in vivo expression should prove useful here in determining the exact location and mechanism of the DHC dimerization and DIC interaction sites, thereby providing information on the interaction dynamics of the cytoplasmic dynein tail.
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