IDENTIFICATION OF A NOVEL REGION OF THE CYTOPLASMIC DYNEIN INTERMEDIATE CHAIN IMPORTANT FOR DIMERIZATION IN THE ABSENCE OF THE LIGHT CHAINS*

Kevin W.-H. Lo¶, Ho.-Man Kan‡, and K. Kevin Pfister¶§

From the ¶Department of Cell Biology, University of Virginia, Charlottesville, VA 22908 and ‡Department of Biology, University of Virginia, Charlottesville, VA 22904

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Address correspondence to:  K. Kevin Pfister, Cell Biology Department, University of Virginia, School of Medicine, PO Box 800732, Charlottesville, VA 22908-0732; Tel.: 434-924-1912; Fax: 434-982-3912; E-mail: kkp9w@virginia.edu

Cytoplasmic dynein is the multi-subunit protein complex responsible for many microtubule-based intracellular movements. Its cargo binding domain consists of dimers of five subunits; the intermediate chains, the light intermediate chains, and the Tctex1, Roadblock, and LC8 light chains. The intermediate chains have a key role in the dynein complex. They bind the three light chains and the heavy chains, which contain the motor domains, but little is known about how the two intermediate chains interact. There are six intermediate chain isoforms and it has been hypothesized that different isoforms may regulate specific dynein functions. However, there is little data on the potential combinations of the intermediate chain isoforms in the dynein complexes. We used co-immunoprecipitation analyses to demonstrate that all combinations of homo- and heterodimers of the six intermediate chains are possible. Therefore the formation of dynein complexes with different combinations of isoforms is not limited by interaction between the various intermediate chains. We further sought to identify the domain necessary for the dimerization of the intermediate chains. Analysis of a series of truncation and deletion mutants showed that a 61 amino acid region is necessary for dimerization in vitro in the absence of the light chains.

Cytoplasmic dynein 11 is a motor complex responsible for the transport of membranous vesicles and different cargo proteins toward the minus ends of microtubules including mitosis, nuclear migration, Golgi and centrosome localization, organelle and viral transport, and axonal transport (1-4). It is a large multisubunit assembly (~1.5 MDa) containing two copies each of DYNC1H1 (the heavy chain), DYNC1I (the intermediate chains) DYNC1LI (the light intermediate chains), and two copies of each of three distinct light chain families (1,5,6). The globular heads of the cytoplasmic dynein heavy chains generate the force for microtubule-based motility (7-9). The heavy chains dimerize via their N-terminal stalks, and the stalks contain both the light intermediate chain and intermediate chain-binding regions (10,11). The intermediate chains, light intermediate chains, and three pairs of light chains are at the base of the cytoplasmic dynein 1 complex and form its cargo binding domain (2,12-15). The two intermediate chains are essential components of the cytoplasmic dynein complex, serving as platforms that interact with the heavy chains, the three light chain dimers, as well as the p150 subunit of dynactin, a cargo adaptor complex, and other protein cargoes (12,13,16,17). The C-terminal half of the protein contains seven WD repeats (18,19) which are presumed to form a beta propeller structure responsible for interacting with the heavy chain (4,20). The N-terminus of the intermediate chain is predicted to form a coiled-coil domain with six heptad repeats that

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may be involved in intra- or inter-chain protein interactions (21), including the interaction with the p150 subunit of dynactin (12). The distinct interaction domains for the three light chain families; DYNLL (LC8), DYNLT (Tctex1), and DYNLRB (Roadblock) are also in the N-terminal half of the intermediate chain (22-24).

Biochemical analyses showed that cytoplasmic dynein 1 can be fractionated into two complexes, one containing the heavy chains and the light intermediate chains and the second composed of the intermediate chains and three light chain families (25-27), but it is not known how the intermediate chains might interact directly, or if their interaction requires one or more of the light chain families. A role for the LC8 light chain as a molecular glue has been suggested by its presence in complexes unrelated to dynein (3,28). Supporting this hypothesis are the observations that an N-terminal fragment of the intermediate chain becomes more ordered upon binding of the LC8 or Tctex1 light chains (29,30). In addition, there are two regions of alternative splicing in the N-terminus of the two intermediate chain genes which generate at least six unique isoforms (1,12,21,31,32). While the functional significance of these intermediate chain isoforms is unknown, there is data supporting the hypothesis that the isoforms of the light intermediate chains and isoforms of the light chain families contribute to the specificity of dynein cargo binding, reviewed in (1,2,33). It is known that the two light intermediate chain family members form only homooligomers producing two distinct cytoplasmic dynein complexes in cells (11). It has been suggested that the two Tctex1 family members also only form homodimers (34). However, homo- and hetero-dimerization has been observed between the two members of the Roadblock light chain family (35,36). Thus it is of great interest to determine whether different intermediate chain isoforms are able to associate with each other.

In this report, we investigate the functional basis for the interaction of the intermediate chains. We find that the six intermediate chain isoforms can interact in all combinations of homo- and hetero-dimers when expressed in cultured cells. In addition, the region of the intermediate chain necessary for dimerization is shown to be a previously uncharacterized region of 61 amino acids. This region is immediately N-terminal to the Roadblock binding region, and it does not overlap with the previously identified intermediate chain subdomains; the N-terminal coiled-coil domain, the C-terminal WD repeat domain, and the three distinct light chain binding domains. Furthermore, in vitro analysis of purified intermediate chain fragments confirms that none of the light chains are necessary for intermediate chain dimerization.

MATERIALS and METHODS

Plasmids - Mammalian expression constructs of the intermediate chain isoforms (intermediate chain-1A, -1B, -1C, -2A, -2B, and -2C) were constructed using standard polymerase chain reaction, PCR², and DNA ligation methods. Briefly, the full-length intermediate chain 1 isoforms were inserted into the XhoI/NotI sites of both pCMV-HA and pCMV-Myc vectors (Clontech), while the full-length intermediate chains 2 isoforms were inserted into the KpnI/NotI sites of the pCMV-HA and pCMV-Myc vectors. Truncation and deletion constructs of intermediate chain 2C were also cloned into both pCMV-HA and pCMV-Myc vectors using standard recombinant DNA methods. Hexahistidine tagged versions of the intermediate chain 2C truncations 1-150 and 151-250 were generated by cloning the coding region into the pET14b expression vector (QIAGEN Inc.). The coding region for mouse roadblock-1 was obtained from expressed sequence tag using the PCR. A GST-tagged version of roadblock was generated by cloning the coding region into the BamHI/XhoI sites of the pGEX4T-1 expression vector (Amersham Biosciences). All constructs were sequenced to confirm gene sequence and correct reading frame.

Expression and purification of fusion proteins - Bacterial expression of recombinant proteins was carried out using Escherichia coli BL21 (DE3) as the host cells. To express GST-Roadblock-1, or GST, host cells containing the expression plasmid were grown in LB medium at 37 °C to an A₆0₀ of ~0.8. Expression of the fusion proteins were induced by the addition of IPTG, isopropyl-1-thio-β-D-galactopyranoside, to a final concentration of ~0.25 mM. Protein expression continued for ~3 h at 37 °C before harvesting by centrifugation. The fusion proteins were expressed in soluble forms.
and purified using GSH-Sepharose affinity columns (Amersham Biosciences) following the manufacturer's instructions. The purified GST proteins on beads in phosphate-buffered saline (PBS) including various protease inhibitors were directly used for binding assay experiments. Hexahistidine fused intermediate chain 2C truncations were expressed in soluble form and purified using a Ni²⁺-nitrilotriacetic acid affinity column following the procedure described by the manufacturer (Novagen) for proteins under native condition. The 0.5 M imidazole buffer used for elution of the protein from the column was removed by dialysis prior to use.

GST pull-down experiments - GST-Roadblock-1 or GST alone (~10 µg each) prebound to GSH-Sepharose beads were mixed with 293T cell lysates (100 µg of total protein) containing myc-tagged full-length intermediate chain -2C or myc-tagged intermediate chain -2C deletion mutant in binding buffer (50mM Tris-HCl, pH 7.4, 50mM NaCl, 1mM EDTA). The pelleted beads were washed with the binding buffer and subsequently boiled with PAGE sample buffer. The proteins were resolved by SDS-PAGE and subsequently analyzed by immunoblot analysis.

Polyacrylamide gel electrophoresis, and immunoblotting - Protein samples were resolved by SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide-Tris gels and transferred electrophoretically onto PVDF membranes (Biorad). Membranes were blocked 1 h at room temperature in blocking solution containing 10% (w/v) skim milk powder and 0.1% Tween 20 in Tris-buffered saline. Primary antibodies were diluted in blocking solution containing 5% BSA in Tris-buffered saline and incubated with the membrane for 1-2 h at room temperature. Membranes were washed in Tris-buffered saline then incubated with horseradish peroxidase-conjugated secondary antibodies for a further hour at room temperature. Labeled bands were visualized using enhanced chemiluminescence (ECL, Pierce) according to the manufacturer's instructions.

Cell Culture, Transfection, and Co-immunoprecipitations - 293T cells were cultured in Dulbecco's modified essential medium containing 10% calf serum (HyClone) at 37 °C in a humidified, 5% CO₂ incubator. For transient transfection, cells were plated on a 6-cm Petri dish to ~70% confluence and transfected with Fugene 6 (Roche Molecular Biochemicals) following the manufacturer's instructions. Expression of transfected proteins continued for 24–36 h. Transfected 293T cells were lysed into 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM PMSF, 1µg/ml pepstatin A, 1µg/ml Leupeptin, and spun in a microcentrifuge for 10 min at 4 °C. The lysates were then incubated 3 hours at 4 °C with 10 µg anti-HA antibody (12C5) pre-bound to Protein A beads (Zymed). The beads were washed with 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA and boiled with SDS-PAGE sample buffer. The co-immunoprecipitates were analyzed by immunoblotting with anti-myc antibody (9E10). Anti-HA and anti-myc antibodies were obtained from the Lymphocyte Culture Center, University of Virginia.

Covalent Chemical Cross-linking Experiments – Cross-linking of the bacterially expressed intermediate chain 2C truncations were performed in a 20mM Hepes, pH 8.0, 150 mM NaCl, 5 mM EDTA, and incubated with 0.1 mM disuccinimidyl glutarate (DSG, Pierce) for 30 min at room temperature. Reactions were terminated by addition of gel sample buffer, and the samples were resolved by SDS-PAGE. DSG was dissolved in N,N-dimethylformamide, and the final N,N-dimethylformamide concentration in the cross-linking reaction mixture was 5% (v/v). Cross-linking of the mammalian expressed full-length 2C was performed in a similar manner except that the cross-linking buffer was 20mM Hepes, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, with protease inhibitors.

Analytical Gel Filtration Chromatography - The homogeneity of the complexes formed by the truncated forms of intermediate chain 2C was determined by analytical gel filtration chromatography using a Superose 6 column (Amersham Biosciences) which was calibrated with the following standard proteins (Sigma) with diffusion coefficients, D₂₀,ₙ, (X10⁻⁷); sweet potato beta-amylose (5.77), bovine serum albumin (6.19), bovine erythrocyte carbonic anhydrase (9.0), horse heart cytochrome C (11.1) from the Handbook of Biochemistry (37).

Sequence Analysis – To compare the homology of the dimerization regions from various species, Blast searches were used to obtain cytoplasmic
dynein intermediate chain sequences and the accession numbers of the species used are: Human (*Homo sapiens*) IC-1 AAH22540, IC-2 AAC33445; Mouse (*Mus musculus*) IC-1 AAC33445, IC-2 O88487; Rat (*Rattus norvegicus*) IC-1A X66845, IC-2C U39046; Red jungle fowl, (*Gallus gallus*) IC-1 XP_418672; Dog (*Canis familiaris*), IC-2 XP_860043; African clawed frog (*Xenopus laevis*) IC-2 AAK28507; Zebrafish (*Danio rerio*) IC-1 XP_688516; Fruit fly (*Drosophila melanogaster*) AAX33607; Fungi (*Cryptococcus neoformans* var. neoformans JEC21) AAW45478; Nematode worm (*Caenorhabditis elegans*) AAC02580; Filamentous fungi (*Aspergillus nidulans*) AAL67574; Slime mold (*Dictyostelium discoideum*) XP_640973.

**RESULTS**

The six intermediate chain isoforms form homo-oligomers and hetero-oligomers *in vivo*. Initial cloning of the intermediate chains found in rat indicated that there were two alternative splicing sites in gene 2, and that there was one alternative splicing site in gene 1, which aligned with the second alternative splicing site of gene 2 isoforms (12,21,31). That data thus suggested that there were two intermediate chain 1 isoforms and three intermediate chain 2 isoforms. However, in a more recent PCR analysis of cDNAs obtained from rat brain mRNA, a second alternative splicing site was identified in intermediate chain gene 1, at a position that aligns with the first alternative splicing site of gene 2 isoforms (data not shown). Re-examination of the expression patterns of the intermediate chain 1 alternatively spliced mRNAs indicates that the brain intermediate chain isoforms originally identified as 1A and 1B (31,32) actually correspond to the newly defined 1B and 1C isoforms respectfully (data not shown). The protein products of the two intermediate chain genes are closely related, with 79% similarity and 69% identity. The longest intermediate chains (the A isoforms) have no regions removed by splicing.

There are also two genes for the cytoplasmic dynein 1 light intermediate chains in mammals. Co-overexpression and co-immunoprecipitation studies demonstrated that their gene products only form homo-oligomers (33). We therefore used a similar approach to test the ability of the six intermediate chain isoforms to form homo- or hetero-oligomers. Pairs of the intermediate chain isoforms tagged with the HA or myc epitopes were co-expressed in cultured cells. The HA-tagged intermediate chain was then immunoprecipitated with an anti-HA antibody and blots of the co-immunoprecipitates were probed with an anti-myc antibody. The data presented in Figure 1A and 1B demonstrate that both intermediate chain 1 and intermediate chain 2 isoforms can associate in all possible combinations of homo- and hetero-oligomers. In contrast, there was no detectable immuno-complexes when cells were singly transfected with myc-tagged intermediate chains (Figure 1C), demonstrating the specificity of the immunoprecipitating anti-HA antibody. Furthermore, no gross differences in the strength of interaction between homo-oligomers and the hetero-oligomers were observed.

Region of the intermediate chain involved in oligomerization. To investigate the region of the intermediate chain necessary for oligomerization, we made intermediate chain 2C truncations tagged with the HA and myc epitopes. The intermediate chain 2C isoform was chosen as the study protein because intermediate chain 2C is the ubiquitously expressed isoform (38), and to ensure that the analysis would not be complicated by the presence of regions that can be removed by alternative splicing. The co-immunoprecipitation assay was then used to identify the truncations of the intermediate chain that were able to oligomerize. The mapping data are summarized in Figure 2A. A truncation containing only the predicted N-terminal coiled-coil domain, 1-65, did not oligomerize, while the complementary construct, 66-612, did oligomerize. A larger N-terminal truncation construct, 1-150, which includes the minimal p150 binding domain (12), also did not oligomerize. These data indicate that the N-terminal coiled-coil region is not required for oligomerization. An essential role for the seven WD repeat domain in oligomerization was excluded by the observations that truncation constructs 1-250, and 151-250 oligomerize in the co-immunoprecipitation assay.

We next investigated the requirements for the light chain binding domains in intermediate
chain dimerization. Oligomerization of the 151-282 and 151-250 truncations demonstrated that the Tctex1 and LC8 light chain binding regions are not required for oligomerization. Supporting this conclusion is the observation that the 1-150 and 66-150 truncations, both of which contain these two light chain binding domains, fail to oligomerize. Co-immunoprecipitation of the 1-211 truncation suggested that the Roadblock binding region was also not required for oligomerization. In earlier work, we defined a region of the intermediate chain 1A that binds to Roadblock light chains (24). This region partially overlapped with the first WD repeat. However, it was proposed that a 39-residue fragment, corresponding to amino acid residues 211-250 of intermediate chain 2C, that excluded the portion of the first WD repeat was the actual roadblock-binding domain (24). We therefore sought to confirm this hypothesis, so as to more precisely define the Roadblock light chain binding region. To accomplish this, we synthesized an intermediate chain 2C construct which contains a deletion of the 39-residue fragment (amino acids 211-250) fused to the myc epitope (ΔRobl).

Collectively, the data obtained with these intermediate chain truncation and deletion constructs indicated that amino acids 151-211 contained the region of the intermediate chain 2C that was necessary for oligomerization. To test this conclusion, the 151-211 truncation construct was synthesized and expressed in cultured cells, but it did not oligomerize. The smallest continuous intermediate chain region that was expressed in cultured cells, and which was competent to oligomerize, was the 151-250 polypeptide. One possible explanation for the failure of 151-211 truncation polypeptide to oligomerize was that it was unable to fold into the proper conformation for oligomerization. Therefore to further investigate the role of this region in dimerization, several constructs with a deletion of this region were synthesized. While we were unable to express a full length construct with a deletion of that region, an N-terminal construct composed of amino acids 1-282, with a deletion of the 151-211 region (Δ151-211), was expressed. The Δ151-211 construct was unable to oligomerize in the co-immunoprecipitation assay. As a positive control for this result, it was observed that the 1-282 truncation construct was competent to oligomerize. These data confirm the essential role of the domain defined by amino acids 151-211 in intermediate chain oligomerization. We also note that the Δ151-211 construct which is unable to oligomerize contains all three light chain binding domains.

In vitro analysis of the intermediate chain dimerization domain. Two in vitro approaches were utilized to further analyze the interactions of the truncation polypeptides. The 151-250 polypeptide, which is the smallest polypeptide competent to oligomerize in vivo and the 1-150 polypeptide which does not oligomerize in vivo were tagged with hexahistidine, expressed in bacteria, and purified. The hydrodynamic behavior of these proteins was then characterized by analytical gel filtration chromatography (Figure 3). The dimerization competent 151-250 truncation construct eluted as a single symmetrical peak and no minor peaks were detected. This indicates that this polypeptide exists in a single molecular state in solution. In contrast, the 1-150 truncation protein eluted as multiple small peaks, suggesting the existence of several molecular states. The formation of these multiple molecular
states may be due to the non-specific association between monomeric units.

To further characterize the purified 151-250 protein sample, it was treated with an amine selective cross-linking reagent, DSG, with a linking spacer length of 7.72 Å (Figure 4A), to crosslink lysine residues that possessed the appropriate accessibility and spatial orientation in solution. The high pH of the reaction buffer and low protein concentration ensured the amine selectivity of the cross-linking reaction. The native and crosslinked 151-250 polypeptides were then analyzed by SDS-PAGE and Coomassie blue staining (Figure 4B). A higher mass reaction product was found in the lane with the crosslinked sample. This result indicates that the purified protein also self-associates, consistent with the results obtained in the co-immunoprecipitation assay. The elution pattern of the crosslinked 151-250 protein sample was then analyzed by gel filtration chromatography (Figure 3). The elution profile of the crosslinked protein was similar to that of the uncrosslinked sample, indicating that the crosslinking reagent stabilized an existing conformation, and that it did not generate higher order artifacts. Since the 151-250 protein oligimerizes in vivo, the elution of the uncrosslinked protein in a single discrete peak suggests a high affinity between the monomers. The slight shift in elution pattern that was observed in the crosslinked protein sample may be the effect of the crosslinking reagent. These results with purified protein further confirm that the light chains are not required for association of the intermediate chains.

It was observed that the uncrosslinked 151-250 truncation polypeptide, which has a calculated mass of ~12.2 kDa, migrates slower than the 20 kDa mass marker protein on SDS-PAGE (Figure 4B). Similar differences between mass of N-terminal intermediate chain fragments as estimated by SDS-PAGE and the calculated mass have previously been observed by Barbar and co-workers (30). They suggested that the anomalous migration was the result of reduced binding of SDS to the negatively charged N-terminal fragments. We also considered the possibility that the increased mass observed in the crosslinked sample represented not the interaction of two 151-250 polypeptides, but rather the increased mass added to the monomer by the addition of the crosslinking reagent to the eight lysines present in the polypeptide. As a control, samples of the 1-150 truncation, which does not oligomerize, with and without added crosslinking reagent, were analyzed by SDS-PAGE. While this polypeptide has nineteen lysines, no higher mass bands were found in the crosslinked sample. This indicates that addition of the crosslinking reagent mass does not significantly change the migration pattern of the polypeptides. These observations are also in agreement with observation that this polypeptide does not oligomerize in the in vivo co-immunoprecipitation assay, and they are consistent with the chromatography elution profile. It is of particular note that the N-terminal 1-150 truncation polypeptide which migrates close to the 30 kDa mass standard protein also runs slower on SDS-PAGE than expected for its calculated mass of 20.7 kDa. Further these data show that the N-terminal hexahistidines do not induce dimerization or oligomerization of the fusion proteins. When the various proteins are resolved on comparable gels were transferred to a solid support and analyzed by immuno-blotting, the major bands in both the truncations 1-150 and the 151-250 sets of lanes, identified with arrow or star were shown to react with antibodies to hexahistidine (data not shown).

While the uncrosslinked truncation polypeptides migrate slower on SDS-PAGE than expected for their calculated molecular weights, the higher mass product found in the crosslinked 151-250 polypeptide sample (Figure 4B, arrow), migrates close to the position predicted for the mass of a 151-250 dimer. In agreement with this observation, we found that when lysates of cells expressing myc-tagged full length intermediate chain 2C were treated with the crosslinking reagent, a higher mass product approximately twice that of the intermediate chain was obtained (Figure 4C).

**Species comparison of the dimerization domain amino acid sequences.** The identified dimerization domain is in the middle of the intermediate chain and C-terminal to the alternative splicing sites. Thus it is present in the three alternative splicing isoforms derived from the intermediate chain gene 2. Since the intermediate chain polypeptides encoded by rat genes *Dync1i1* and *Dync1i2* are not identical, but do heterodimerize in vivo (Figure 1), we compared
the sequences of the 61 residues from the rat intermediate chains 2C and 1A (Figure 5A). As would be expected for a region important for dimerization, there is exceptional conservation between the sequences found in the two proteins. Of the C-terminal most forty four amino acids of the two rat proteins all but three are either identical or contain conserved and semi-conserved substitutions. While the N-terminal portion is not as highly conserved, only ten of the remaining N-terminal amino acids are non-conserved. We also compared the cytoplasmic dynein intermediate chain dimerization domain sequences of other vertebrate species. There was exceptional conservation in the dimerization region, comparable to that observed between the rat gene 1 and gene 2 sequences. We note for example that of the C-terminal thirty five amino acids, nineteen are identical in the ten different sequences, and that there would be eight other identical columns of amino acids in the C-terminus except for amino acid substitutions found in one of the analyzed sequences. When the invertebrate protein sequences are compared, there is significant conservation in the region despite their evolutionary divergence (Figure 5B). While there is less conservation in the N-terminal portion of the domain than was observed in the vertebrates, twenty of the C-terminal most amino acids are either identical or conserved. We particularly note that there is near identity in the C-terminal seven amino acids of the fifteen vertebrate and invertebrate sequences.

**DISCUSSION**

In this study, we demonstrate that all the cytoplasmic dynein intermediate chain isoforms are competent to form hetero- and homo-dimers in vivo. We further identify a 61 amino acid region of the intermediate chain upstream of the Roadblock light chain binding region that is necessary for intermediate chain dimerization in vivo. This portion of the intermediate chain does not contain the Tctex1, or the LC8, or the Roadblock light chain binding domains and it does not include the N-terminal coiled-coil domain. In vitro biochemical analyses with purified recombinant intermediate chain fragments further demonstrate that none of the three light chain families are required for the dimerization of the intermediate chains.

Different intermediate chain isoforms are expressed in different tissues and cell types, for example intermediate chain 2C is the only intermediate chain isoform found in many cultured cells, including astrocytic glia, while most of the isoforms are found in cultured neurons (31,40). The expression levels of the intermediate chain isoforms are regulated during brain and neuronal development (31,32,41). Most interestingly, the fast and slow components of axonal transport have dynein complexes with different intermediate chain and light intermediate chain isoforms (32,42,43). The data reported here indicate that there is no structural impediment to the formation of dynein complexes with different combinations of the intermediate chain isoforms and provide a mechanism for generating diverse cargo binding domains. These diverse domains may create specific cargo attachment sites on the individual dynein motor units. Alternatively, the diverse domains may be permissive for differential regulation of cytoplasmic dynein by kinases and phosphatases (16,41,44-47). Interestingly, under experimental conditions similar to ours, the isoforms of the light intermediate chain subunit formed only homo-oligomers (33).

Prior to this work, little was known about the association of the cytoplasmic dynein intermediate chains in the dynein complex. The WD repeat domain of the intermediate chains was shown to be important for their binding to the heavy chains and it was known that the three light chains bound to separate locations on the intermediate chain (20,24,26). Also, the cytoplasmic dynein complex could be dissociated by urea and other chaotropic agents into two subcomplexes. One complex contained the heavy chains and light intermediate chains, and a second complex was composed of the intermediate chains and the light chains (25,26). The intermediate chain – light chain complex could be further fractionated and yielded intermediate chain monomers, with and without light chains, and intermediate chain dimers also with and without light chains (27).

The *in vivo* domain mapping experiments presented here clearly demonstrate that an evolutionarily conserved region, defined by residues 151 to 211 of intermediate chain 2C, is
necessary for intermediate chain dimerization (Figure 6). An intermediate chain construct lacking this region, Δ151-211 is unable to dimerize in an in vivo co-immunoprecipitation assay, while a construct that is identical except that it contains this region, is competent to dimerize (Figure 2A). However, when expressed as a single truncation, this 61 amino acid region alone is not competent to dimerize. The simplest explanation of this result is that some neighboring amino acid sequence is required for the 61 residues to obtain the proper conformation. This analysis is supported by the finding that constructs with the addition of either the neighboring C-terminal thirty two amino acids of the roadblock light chain binding region (151-282) or sequence farther C-terminal from the region (ΔLC) are competent to dimerize. This interpretation is also similar to the findings of Mok and co-workers (23). They identified a minimal deletion construct if the intermediate chain that would not bind the Tctex1 light chain. However, they also found that a synthetic peptide corresponding to this region was not sufficient to bind to the light chain.

Since the Δ151-211 construct that does not dimerize contains the binding regions for all three light chains, we conclude that the light chain binding domains are not necessary for intermediate chain dimerization. Supporting this conclusion is the finding that constructs deleted for all three of the light chain binding regions are competent to dimerize. It has been suggested that one or more of the light chains may serve as an intramolecular glue responsible for holding the two intermediate chains together (3,24,28). The light chains exist as dimmers with two intermediate chain binding domains in vivo and there is good evidence that LC8 and Tctex1 light chain binding to an intermediate chain N-terminal peptide imposes order on the fragment (30,39,48-50). Furthermore, LC8 is associated with several dimeric proteins including myosin V (51), and it has been shown that a dimeric coiled-coil of the protein Swallow, a transcription factor linked to asymmetric mRNA distribution, becomes more stable upon LC8 binding (28). Thus, in principle the presence of the light chains would induce an interaction between intermediate chain truncations which contain those light chain binding domains and this should be detected in our co-immunoprecipitation assay. However, it seems likely that in our experiments the high levels of proteins synthesized during over-expression minimizes the contribution of indirect interactions that might be mediated by endogenous proteins such as the light chains. Consistent with this analysis, no Roadblock light chain was found when the co-immunoprecipitates of the 1-282, 151-250, and 151-282 constructs, which contain that light chain binding domain were probed with antibodies to the light chain (data not shown). While our data strongly demonstrate that none of the light chains are necessary for intermediate chain dimerization, we do not exclude the possibilities that binding of the light chains to the intermediate chain will enhance or stabilize intermediate chain dimerization or contribute to the formation of intermediate chain homodimers.
REFERENCES


**FOOTNOTES**

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1. **Cytoplasmic dynein 1 subunit nomenclature.** There are two distinct cytoplasmic dynein complexes. The designations of three of the subunits of cytoplasmic dynein 1, the cytoplasmic dynein analyzed in this paper, start with *DYNC1*. They are then grouped into families of related proteins and an additional letter(s) was added to their names to describe their relative size, *DYNC1H*, the heavy chain; *DYNC1I*, the intermediate chain family; *DYNC1LI*, the light intermediate chain family. There are also three functionally distinct light chain families, *DYNLT*, the Tctex1 family; *DYNLRB*, the Roadblock family; and *DYNLL*, the LC8 family. The light chains are not exclusive to cytoplasmic dynein 1, so do not use the C1 designation. A dynein polypeptide subunit was identified at first mention with its formal name followed by the common name in parentheses, with most subsequent mentions being to the common name, for example *DYNC1LI*, (light intermediate chain). The subunit families are encoded by two or more genes and their products are distinguished by adding numbers to the name; alternative splicing isoforms are referred to with letters; and for clarity hyphens may be used, for example *DYNC1I-2C* is the cytoplasmic dynein 1 intermediate chain 2, alternative splice isoform C. This nomenclature has been endorsed by the Human Genome Organization Nomenclature Committee (HGNC) and the International Committee on Standardized Nomenclature for Mice (5)

2. The abbreviations used are: GSH, glutathione-Sepharose; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinyl fluoride; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; HA, hemagglutinin.
FIGURE LEGENDS

Figure 1. Homomeric and heteromeric association of the cytoplasmic dynein intermediate chain isoforms. 293T cells were co-transfected with the pairs of full length intermediate chain isoforms tagged with the HA- and myc- epitopes indicated by +. The HA-tagged intermediate chain isoforms in the cell lysates were then immunoprecipitated with anti-HA antibody. The cell lysates and immunoprecipitates were analyzed by SDS-PAGE, immunoblotted, and probed with anti-myc antibody to detect co-immunoprecipitating myc-tagged intermediate chain isoforms. The co-immunoprecipitation of the myc-tagged intermediate chain with the HA-tagged intermediate chain is shown in the blot at the bottom of each panel probed with the antibody to myc (Anti HA IP). The expression of the myc-tagged intermediate chains is demonstrated in cell lysates shown in the blot at the top of each panel (Myc inputs). A. Left Panel, all combinations of the gene 1 intermediate chain isoforms co-immunoprecipitate. A. Right Panel, all combinations of the gene 2 intermediate chain isoforms co-immunoprecipitate. B. All combinations of the gene 1 and gene 2 intermediate chain isoforms co-immunoprecipitate. C. In control experiments which were transfected with myc-tagged intermediate chains alone, no myc-tagged intermediate chains were immunoprecipitated with the antibody to HA.

Figure 2. Mapping of the cytoplasmic dynein intermediate chain oligomerization region in vivo. A. Summary of the interaction of intermediate chain 2C truncations. The domain map of the full length cytoplasmic dynein 2C intermediate chain (FL-2C) is shown at the top of the figure; N-terminal coiled coil region (yellow); C-terminal WD-repeat domain, with 7 WD repeats (A-E’, purple); the binding site for the p150 subunit of dynactin (N-terminal dark blue bar); dynein heavy chain binding region (C-terminal dark blue bar). The binding regions for the three cytoplasmic dynein light chains are also indicated (central brown bars). The lower rows show the self association analysis of full length, truncation, and deletion constructs of rat intermediate chain 2C. On the left are either the amino acid sequences of intermediate chain 2C truncations or the name of the deletion constructs which are tagged with the HA- and myc-epitopes. The ∆Robl construct has a deletion of the Roadblock light chain binding domain, amino acids 211-250 in the full length protein. The ∆LC construct lacks all three light chain binding domains. It spans amino acids 151-447 and contains the 211-250 deletion of the Roadblock light chain binding domain. The ∆151-211 construct contains a deletion of the putative dimerzation domain. It spans amino acids 1-282 and contains a deletion of amino acids 151-211. The central bars are a graphical description of the location of the constructs in the intermediate chain 2C sequence relative to the top panel. The individual truncations tagged with both HA- and myc-epitopes were co-transfected into cells and immunoprecipitated with antibody to HA. Positive co-immunoprecipitation of the myc-tagged truncation with the HA-tagged truncation is indicated in the graphical representation by a green bar; negative co-immunoprecipitation is indicated by a red bar. The panels on the right (Anti-myc) show immuno-blots probed with anti-myc antibody which document the presence or absence of the myc-tagged intermediate chain truncations in the anti-HA immunoprecipitates (IP), and the cell lysates (Lysate). In single expression controls which were transfected with myc-tagged intermediate chains alone, no myc-tagged intermediate chains were immunoprecipitated with the antibody to HA (Control). B. Deletion analysis of the Roadblock light chain binding domain. The Roadblock-1 light chain binds to the full length 2C intermediate chain, but not to the ∆Robl intermediate chain 2C deletion construct in vitro. GSH beads with bound GST-Roadblock-1 or GST alone were incubated with 293T cell lysates expressing either myc-tagged full-length intermediate chain isoform 2C [Myc-FL-2C] or the intermediate chain deletion construct [Myc-∆Robl]. The proteins bound to the beads were resolved by SDS-PAGE and transferred to PVDF and the blots were probed with antibodies to myc and GST as indicated on the right. Inputs lanes show that both constructs were expressed. The anti-GST blot shows the presence of the GST and GST-fusion proteins in the binding assay.

Figure 3. Size-exclusion chromatography of cytoplasmic dynein intermediate chain 2C 1-150 and 151-250 truncations. The column elution profiles of purified recombinant Histagged 151-250...
polypeptide (blue line), crosslinked 151-250 (red line) and 1-150 polypeptide (black line), are shown. Both of the 151-120 truncation samples eluted as a single peak, indicating a uniform and homogeneous complex. Chemical crosslinking of the 151-250 polypeptide did not result in the creation of a larger complex. The 1-150 polypeptide has a heterogeneous elution profile. The arrows mark the peak elution fraction of standards with the indicated diffusion coefficients, $D_{20,w}$, ($X \times 10^7$).

**Figure 4.** SDS-PAGE Analysis of covalent chemical cross-linking of the full length 2C intermediate chain, and truncations 1-150 and 151-250.

A. Molecular structure of disuccinimidyl glutarate (DSG), a primary amine specific cross-linker. The spacer arm length is 7.72Å. B. Crosslinking of the 151-250 truncation but not the 1-150 truncation. Purified recombinant His$_6$-tagged intermediate chain truncation proteins (10 µM) were treated with 0.1 mM DSG in Hepes buffer, pH 7.4 for 30 min at room temperature. The reactions were quenched by the addition of gel sample buffer. The samples were resolved on 15% SDS-PAGE gels and stained with Coomassie blue. When the purified 151-250 truncation was treated with the crosslinking reagent, there is a significant increase in intensity of a new higher molecular weight band (arrow) in the presence of DSG. In addition the relative amount of the monomer (*) is reduced, indicating that this truncation forms dimers or other oligomers in vitro. When the samples of the purified 1-150 truncation were analyzed, no higher mass polypeptides were detected in the sample treated with DSG crosslinking agent, and no differences in the intensity of the monomer band (*) with and without DSG treatment were observed. This suggested that this truncation does not form dimers or other oligomers. C. Cross-linking of cytoplasmic dynein intermediate chain 2C. A lysate of 293T cells expressing intermediate chain 2C with a myc epitope tag was incubated with DSG as described. Equal amounts of the untreated and treated lysates were resolved on SDS-PAGE, blotted to PVDF and probed with the antibody to myc. In the control lane (DSG -) only the expressed intermediate chain was detected. In the crosslinked sample (DSG +) the intermediate chain band and a band approximately twice the mass of the intermediate chain were observed. The molecular weight markers in kDa are indicated with arrow heads.

**Figure 5.** Comparison of the dimerization regions of vertebrate and invertebrate intermediate chains. A. Vertebrate species. Forty five of the sixty one amino acids are either identical or conserved in the analysis of ten sequences, both IC-1 and IC-2, from seven species. There is considerable identity in the sequences in the C-terminal region. B. Invertebrate model organisms. There is substantial conservation in the C-terminal of the putative dimerization domain, less in the N-terminal portion. Species names and accession numbers of the compared sequences are in Materials and Methods The dimerization region sequences were aligned and analyzed using ClustalW. The symbols at the bottoms of the columns describe the similarity of the amino acid sequences, "*" the residues in the column are identical in all sequences in the alignment; ":" conserved substitutions are found in the column; "." semi-conserved substitutions are present; no symbol indicates lack of conservation.

**Figure 6.** Intermediate chain domain map with dimerization region. The structural motifs and known polypeptide binding regions of the intermediate chain 1A, which is not alternatively spliced; N-terminal coiled coil region (yellow); C-terminal WD-repeat domain, with 7 WD repeats (A-E', purple). The N-terminal regions removed by alternative splicing in other isoforms, A1 and A2 (red) flank a serine rich region, S (green). The N-terminal coiled-coil region makes up part of the binding site for the p150 subunit of dynactin (N-terminal dark blue bar). The WD repeat region is involved in binding to the cytoplasmic dynein heavy chain (C-terminal dark blue bar). The binding regions for the three cytoplasmic dynein light chains are also indicated (central brown bars). The intermediate chain – intermediate chain dimerization region, Dimer (light blue box), is between the amino acids 183-242 of rat intermediate chain 1A, and amino acids 151 to 211 of rat intermediate chain 2C.
Lo et al., Figure 1.
B. Myc-FL-2C  Myc-ΔRobl  Inputs

GST-Robl-1  GST  GST-Robl-1  GST  Myc-FL2C  Myc-ΔRobl

Anti-myc

Anti-GST

Lo et al., Figure 2B.
Lo et al., Figure 4.
A. Vertebrate Species

Mouse IC-1          DEEMVEPKIG HDSELENQEK KQETK--EAP PRELTEEEKQ QILHSEEFLI FFDRTIRVIE RAL
Human IC-1          DEEMVESKVQ QDSELENQDK KQE VK--EAP PRELTEEEKQ QILHSEEFLI FFDRTIRVIE RAL
Fowl IC-1           DEEMVEPKAQ DDLETENQDQ KQETK--EAP PRELTEEEKQ QVLHSEEFLI FFDRTIRVIE RAL
Zebrafish IC-1      DEELSVPKSE PASQLEEEK TQEVE--EVH PRELTNEEKE QIVHSEDFLF FFDRSIRVVE RVL
Rat IC-1A           DEEMVEPKVG HDSELENQDK KQETK--EAP PRELTEEEKQ QILHSEEFLI FFDRTIRVIE RAL
Rat IC-2C           --DVAAPKPP VEPEEEKILK KDEENDSKAP PHELTEEEKQ QILHSEEFLS FFDHSTRIVE RAL
Mouse IC-2          --DVATPKPP VEPEEEKTLLK KDEENDSKAP PHELTEEEKQ QILHSEEFLS FFDHSTRIVE RAL
Human IC-2          --DVATPKPP VEPEEEKTLLK KDEENDSKAP PHELTEEEKQ QILHSEEFLS FFDHSTRIVE RAL
Dog IC-2            --DVTTPKPP IEPEEEKTLLK KDEENDSKAP PHELTEEEKQ QILHSEEFLS FFDHSTRIVE RAL
Frog IC-2           EEETVAPKAV TVQEEDKPEK K-EE-APTEAP PHELTEEEKQ QILHSEEFVS FFDHSTRILE RAL

B. Invertebrate Species

Drosophila          -THGLPTVKD VAAPITPLEI KKETEVKKEV N-ELSEEQKQ MIILSENFQR FVVRAGRVIE RAL
Dictyostelium       -TDSINGDS STSVANSAN DDDDKDEIEI P-ELDENNEK DILESDFSKS FFGRSSRVIE RAL
Aspergillus         -REEIEEEER ATQESAKNES GADASIRYPL R-QLDDDELK AVTSSEDFLD FVERSAKVIE RAL
Cryptococcus        ILEELDEERK ALEKELR-EL R-KTEKEKI A-ELSDEQRQ AIFAAPDFTS FIEESTRIVQ RAL
Caenorhabditis      ----LGQTKQ GDKGLTRQET EKKTQTVQI VPPLSDEEKY RLTLDFEFLD SFNRSCKVIS RAL
Lo et al., Figure 6
Identification of a novel region of the cytoplasmic dynein intermediate chain important for dimerization in the absence of the light chains
Kevin W.-H. Lo, Ho.-Man Kan and K. Kevin Pfister

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