The Third P-loop Domain in Cytoplasmic Dynein Heavy Chain Is Essential for Dynein Motor Function and ATP-sensitive Microtubule Binding

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Sequence comparisons and structural analyses show that the dynein heavy chain motor subunit is related to the AAA family of chaperone-like ATPases. The core structure of the dynein motor unit derives from the assembly of six AAA domains into a hexameric ring. In dynein, the first four AAA domains contain consensus nucleotide triphosphate-binding motifs, or P-loops. The recent structural models of dynein heavy chain have fostered the hypothesis that the energy derived from hydrolysis at P-loop 1 acts through adjacent P-loop domains to effect changes in the attachment state of the microtubule-binding domain. However, to date, the functional significance of the P-loop domains adjacent to the ATP hydrolytic site has not been demonstrated. Our results provide a mutational analysis of P-loop function within the first and third AAA domains of the *Drosophila* cytoplasmic dynein heavy chain. Here we report the first evidence that P-loop-3 function is essential for dynein function. Significantly, our results further show that P-loop-3 function is required for the ATP-induced release of the dynein complex from microtubules. Mutation of P-loop-3 blocks ATP-mediated release of dynein from microtubules, but does not appear to block ATP binding and hydrolysis at P-loop 1. Combined with the recent recognition that dynein belongs to the family of AAA ATPases, the observations support current models in which the multiple AAA domains of the dynein heavy chain interact to support the translocation of the dynein motor down the microtubule lattice.

INTRODUCTION

Cytoskeletal motor proteins participate in cell division, cell motility, and the establishment of cell polarity. The action of these motor enzymes in the intracellular transport of organelles and cytoplasmic constituents is dependent on their ATP-dependent translocation along either microtubules or actin filaments (Baker and Titus, 1998; Hirokawa *et al.*, 1998; Karki and Holzbaur, 1999). The cytoplasmic dyneins consist of two identical heavy chains and a complement of intermediate, light-intermediate, and light chain subunits (reviewed in King, 2000a). Similarly, the kinesin and myosin motors contain heavy chains and a more limited set of accessory subunits (Vale and Fletterick, 1997; Bresnick, 1999). For each

family of motors, the heavy chain subunit is the polypeptide that is responsible for ATP hydrolysis and force production (Hackney, 1996; Sweeney and Holzbaur, 1996). Inspection of the primary sequences of known myosin and kinesin heavy chains has revealed a single nucleotide triphosphate-binding or P-loop domain, the site of ATP binding and hydrolysis (Goodson *et al.*, 1994; Ruppel and Spudich, 1996a). Mutations in the unique ATP hydrolytic sites of myosin and kinesin heavy chains are known to inhibit motor function and/or interactions with the filament substrate (Meluh and Rose, 1990; Rasooly *et al.*, 1991; Nakata and Hirokawa, 1995; Ruppel and Spudich, 1996b; Sasaki and Sutoh, 1998).

For the dynein heavy chain, the original analysis of predicted amino acid sequences revealed a central cluster of four P-loop motifs predicted to bind and/or hydrolyze ATP (Gibbons *et al.*, 1991; Ogawa, 1991; Koonce *et al.*, 1992; Mikami *et al.*, 1993; Li *et al.*, 1994). The most N-terminal of the four P-loops, P1, is absolutely conserved among dyneins and is considered the principle site of ATP hydrolysis based on photocleavage experiments (Gibbons *et al.* 1987). The successive duplication of the single P-loop site of an ancient

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Abbreviations used: DHC, dynein heavy chain; P-loop, phosphate-binding motif; MAP, microtubule associated protein.

protodynein is proposed to account for the evolution of the additional P-loops (Gibbons, 1995), but the functional significance of ATP binding and/or hydrolysis at these sites is not established. Subsequent studies have further shown that unlike kinesin, the microtubule-binding domain in the dynein heavy chain is well separated from the site of ATP hydrolysis. How then does the energy of ATP hydrolysis regulate microtubule binding at a distant domain?

One explanation is suggested by the discovery from recent sequence alignments and electron microscopic studies that the dynein heavy chain structure is related to the structure of AAA oligomeric ATPases (Samso *et al.*, 1998; Neuwald *et al.*, 1999). The AAA domain that defines this protein family includes a nucleotide-binding site with the associated P-loop signature on a core α/β tertiary structure. Oligomeric assemblies of AAA domains form a hexameric organization that is characteristic of this family of proteins, including chaperones (Saibil, 2000), proteasomes (Rohrwild *et al.*, 1997; Navon and Goldberg, 2001), katanin (Hartman and Vale, 1999), N-ethylmaleimide-sensitive membrane fusion complex (NSF-D2; Hanson *et al.*, 1997), and the RuvB DNA helicase (Yu *et al.*, 1997). In these AAA oligomeric ATPases, nucleotide-binding and/or hydrolysis at one AAA domain is thought to produce a series of conformational changes that are passed around the hexameric ring of AAA domains and apparently mediates their action on bound ligands or substrates. The dynein heavy chain comprises a concatamer of six AAA modules, of which only the first four contain associated P-loops (Neuwald *et al.*, 1999). The six modules are assembled into an annular ring that is similar to the hexameric structure of oligomeric AAA ATPases (Mocz and Gibbons, 2001; King, 2000b). Thus a similar mechanism based on the cooperative interaction of AAA domains may couple ATP binding and/or hydrolysis at P1 through adjacent AAA domains to regulate the conformation and attachment state of the distant microtubule-binding site. The long-standing question of how nucleotide binding and/or hydrolysis at other P-loops affects dynein function remains to be determined, and the answer may lie in the proposed cooperative interaction between AAA domains.

In the present report we investigate the functional significance of the P-loops within the first and third AAA domains using site-directed mutagenesis of the residues involved in nucleotide binding. We provide the first evidence that P-loop 3 is essential for dynein function and that it can influence the microtubule binding properties of the dynein motor. Our results provide experimental support for a model in which the multiple P-loops in the dynein heavy chain function to regulate its interaction with the microtubule lattice.

MATERIALS AND METHODS

Drosophila Stocks and Genetic Crosses

The deficiency *Df(3L)10H*, *st e⁴* (64B10-12; 64C5-9), which removes the cytoplasmic *Dhc* gene, was obtained from J. Garbe (University of California, Berkeley). The stock used for transformation, *Df(1)w^{67c23}* (Lefebvre and Green, 1972), was provided by J. Tamkun (University of California, Santa Cruz). The $\Delta 2-3$ stock, providing a source of transposase (Robertson *et al.*, 1988), was obtained from the Bloomington Stock Center. The cytoplasmic DHC mutation *Dhc64C⁶⁻¹⁰* is a recessive lethal allele generated by EMS, described in Gepner *et al.*, 1996. The null allele *Dhc64C^{\gamma1463a}* was generated by γ -irradiation and fails to produce a detectable product (Robinson *et al.*, 1995). For

the purposes of this study, the identity of *Dhc64C^{\gamma1463a}* as a lethal *Dhc* allele is established by the rescue of the recessive lethality in the presence of the wild-type transgene, *WT-DHC*. Wild-type flies used in this study were *Drosophila melanogaster* Oregon R.

Transgenic lines were established by P-element transformation using standard methods (Karess and Rubin, 1984). In this text, the transformed lines will be referred to as follows: *WT-3HA*, expressing the wild-type *Dhc* transgene with the 3HA epitope tag; *P1-3HA*, the tagged transgene with a mutation in P-loop 1; *P3-3HA*, the tagged transgene with a mutation in P-loop 3; and *P1P3-3HA*, the tagged transgene with mutations in both P-loops 1 and 3. Stocks are maintained as homozygous for the transgene in a background of wild-type *Dhc64C*.

Mutagenized and/or tagged transgenes (described below) were tested for their ability to rescue the lethality of *Dhc64C* alleles as follows: *white (w)* males homozygous for the transgene (*P(Dhc)*) on the second chromosome (*w/y; P(Dhc)/P(Dhc); +/+*) were crossed to *w* virgin females heterozygous for the deficiency *Df(3L)10H e* and the balancer *TM6B, D, Hu e (w/tw; +/+; Df(3L) 10H e/TM6B, D, Hu e)*. Progeny males of the genotype *w/Y; P(Dhc)/+; Df(3L)10H e/+* were selected by the absence of the dominant marker mutations *Dichaete (D)* and *Humeral (Hu)*, and were crossed to virgin *w/w; +/+; Dhc64C^{allele e}/TM6B, D Hu e* females. Critical class progeny, those hemizygous for the *Dhc* allele (*Dhc64C^{allele e}/Df(3L)10H e*), are rescued only if the transgene they express is functional. The rescued critical class flies are recognized by the absence of the dominant markers *D* and *Hu* and the presence of the recessive marker *e*.

For those cases where the transgenic line being tested contained the insertion on the X chromosome, the rescue crosses were analogous to those described above. In the first cross, females expressing the transgene were crossed to *w* males with third chromosome *Df(3L)10H e/TM6B, D Hu e*. After the second cross, the rescued critical class is identified by the same markers described above; in addition, because the X-linked transgene is contributed by the male parent, only female progeny inherit the transgene and are rescued.

Site-directed Mutagenesis and Epitope-tagging of Dhc Transgenes

Genomic DNA containing the *Dhc64C* transcription unit was previously isolated (Li *et al.*, 1994) and cloned into the P-element vector pCaSpeR4 to make a functional *Dhc* transgene (Gepner *et al.*, 1996). For the current work, a 7-kb *SphI* fragment containing the four P-loops was removed from the transgene as a cassette, modified by mutagenesis or epitope-tagging or both, and reinserted to create modified *Dhc64C* transgenes.

The site-directed mutations in P-loops 1 and 3 were created using a PCR amplification-ligation technique (Michael, 1994). For the mutagenesis of P1, the mutagenic primer 5'-PO₄-CCTGCCGGTACTGGAATAGCAGAATTCGTCAAG-3' alters the wild-type P1 sequence from GPAGTG**KT** to GPAGTG**IA**. The analogous mutagenesis of P3 used the mutagenic primer 5'-PO₄-CCACCTG-GCTCTGGTATAGCTATGACCCTGTTCT-3' to change the wild-type P3 sequence GPPGSG**KT** to GPPGSG**IA**. Products were sequenced to verify no additional mutations had been introduced by PCR.

To detect protein expression from the transgenes, the influenza hemagglutinin epitope triple tag (3HA; Tyers *et al.*, 1992) was inserted ~600 base pairs upstream of P-loop 1, after residue 1713 in the *Dhc64C* peptide. After construction of the *SphI* cassettes that contained a 3HA epitope tag, or a 3HA tag plus a mutation in P-loop 1, P-loop 3, or both, complete transgenes were assembled in the P-element vector pCaSpeR4, which contains the mini-*white* gene.

Protein Preparations and Immunoblotting

Embryo and ovary extracts were made in PMEG buffer (100 mM PIPES, pH 6.9, 5 mM MgOAc, 5 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT, 0.9 M glycerol) plus protease inhibitors (10 μ g/ml aprotinin, 1

$\mu\text{g/ml}$ leupeptin and pepstatin, 0.1 $\mu\text{g/ml}$ each of soybean trypsin inhibitor, *n*-tosyl L-arginine methyl ester, and benzamidine). Experiments were carried out at 4°C unless otherwise noted.

Soluble ($125,000 \times g$) extracts of hand-dissected ovaries were sedimented through 5–20% sucrose density gradients, as described previously (McGrail *et al.*, 1995). Sedimentation standards were run in parallel on a separate gradient.

UV-vanadate cleavage experiments were carried out on ovary extracts, as described previously (Hays *et al.*, 1994). Briefly, soluble extracts were adjusted to 1 mM MgATP/100 μM sodium vanadate and split into equal volumes. One aliquot was irradiated with long wave UV (366 nm) from a distance of 1 cm for 45 min. The other sample was shielded from light and served as the nonirradiated control sample.

Microtubule-associated proteins (MAPs) were prepared from 0–24 h embryos by affinity to taxol-stabilized microtubules, as described previously (Hays *et al.*, 1994). Preparations were run in parallel, either 1) supplemented with MgATP to a final concentration of 5 mM or 2) depleted of endogenous ATP by the addition of hexokinase and glucose. For salt extraction experiments, the final pellet was washed sequentially in equal volumes of PMEG/taxol containing 0, 0.1, 0.2, 0.3, and 0.5 M NaCl.

Sucrose gradient fractions enriched for dynein, above, were used to assay microtubule binding affinities of the transgenic proteins. Microtubules were assembled from purified 6S bovine tubulin (generously supplied by Susan Gilbert) in PEM buffer with 30 μM taxol. Varying concentrations of polymerized tubulin (0, 1, 2, 5, 10, 20 μM) were mixed with constant amounts of gradient-purified dynein and binding allowed to proceed for 30 min at room temperature. Reactions using the mutant lines were supplemented with 5 mM MgATP to eliminate binding of endogenous dynein to microtubules. Reactions were pelleted through a 20% sucrose cushion at $100,000 \times g$ for 30 min. Equal volumes of supernatants and pellets were analyzed on immunoblots, using anti-HA antibody to follow the binding behavior of the tagged dynein.

SDS-PAGE and immunoblotting were done using standard methods (Laemmli, 1970; Towbin *et al.*, 1979). Blots were processed using the Tropic chemiluminescence system (Applied Biosystems, Foster City, CA). The anti-HA monoclonal antibodies 12CA5 (Covance, Richmond, CA) and HA.11 (Covance) were diluted 1:2000. The anti-DHC mAb P1H4 (McGrail and Hays, 1997) was diluted 1:10,000. Alkaline phosphatase-conjugated secondary antibodies (Applied Biosystems) were diluted 1:10,000.

Immunocytochemistry

Ovaries were dissected, fixed, and prepared for immunocytochemistry as described previously (Li *et al.*, 1994). For experiments requiring colchicine treatment, 2-d-old females were starved 2 h and then fed 200 $\mu\text{g/ml}$ colchicine in yeast paste for 24 h before ovary dissection and fixation. The anti-HA mAb 12CA5 was diluted 1:250. Rabbit anti-HA polyclonal (Santa Cruz Biotechnology) was used at 1:200. Anti- α -tubulin directly conjugated to FITC (Sigma) was diluted 1:200. Anti-rabbit Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and anti-mouse Alexa-fluor 488 (Molecular Probes) were preabsorbed against fixed embryos and used at a final dilution of 1:100 and 1:400, respectively. Samples were mounted in a solution of 10% PBS/90% glycerol containing 1 mg/ml *p*-phenylenediamine (Sigma) and examined by confocal microscopy using a 40 $\times$ or 100 $\times$ plan apo objective on either a Bio-Rad 2000 lasersharpe confocal microscope or a Yokogawa spinning disk confocal with Ultraview software (Perkin Elmer-Cetus, Inc). Images were prepared using MetaMorph (Universal Imaging Corp.) and Adobe Photoshop.

Figure 1. Schematic representations of the cytoplasmic dynein heavy chain protein (DHC) and the *Dhc* transgenes used in these studies. The open bar represents the endogenous wild-type DHC, showing the relative positions of the four P-loop motifs (P1, P2, P3, P4) and the proposed location of the microtubule-binding domain (MTB; Gee *et al.*, 1997). In fly DHC, the residues 1895–1902, 2210–2217, 2580–2587, and 2922–2929 encode P-loops 1, 2, 3 and 4, respectively. The two α -helices in the fly DHC that correspond to those proposed in the rat MTB are located between residues 3186–3262 and 3398–3481. The shaded region near the N-terminus indicates the location (residues 182–476) of the epitope recognized by the anti-DHC antibody P1H4. A series of *Dhc* transgenes containing mutations (*) in P1, P3, or both were constructed with an epitope tag (3HA) as described in MATERIALS AND METHODS. The designations for each DHC product are shown at left.

RESULTS

Mutant P-loop Transgenes

To investigate the functional significance of nucleotide binding domains (P-loops) in the DHC, mutations introduced in P-loops 1 and 3 (P1 and P3) were analyzed in transgenic flies. Mutations that alter P1, P3, and both P-loops 1 and 3 were generated in vitro by site-directed mutagenesis of genomic fragments of the cytoplasmic *Dhc* gene (see MATERIALS AND METHODS; Figure 1). The conserved lysine and threonine residues within the P-loop were replaced with the comparably sized, uncharged residues isoleucine and alanine respectively (P1: GPAGTGKT > GPAGTGIA; P3: GPPGSGKT > GPPGSGIA). Our selection of the conserved lysine and threonine residues for mutagenesis was based on extensive investigations that establish an absolute requirement for these residues in nucleotide binding at P-loops (Story and Steitz, 1992; Logan and Knight, 1993; Shen *et al.*, 1994; Smirnova *et al.*, 1998). To detect the transgenic DHC products, each construct was tagged with a triple copy of the influenza hemagglutinin epitope (3HA; Figure 1). For each *Dhc* transgene, (wild-type [WT-3HA]; P1 mutant [P1-3HA]; P3 mutant [P3-3HA]; and P-loop 1 and 3 mutant [P1P3-3HA]), multiple independent transgenic lines were recovered. Genetic crosses to flies containing dominant markers were conducted to establish the chromosome linkage of inserted transgenes. To determine the number of transgene inserts, genomic DNA blots were hybridized with a probe that detects the endogenous *Dhc64C* gene, as well as a unique fragment associated with each transgene insertion. An mAb against the HA epitope tag was used in immunoblot analysis of extracts to confirm the expression of the transgenes. Based upon the initial characterization of more

Table 1. Transgene rescue of *Dhc64C* lethal mutation

Transgene	Line	Linkage	Progeny classes: number of surviving adults							
			Df/null		Df/bal		WT/null		WT/bal	
			+	–	+	–	+	–	+	–
WT-3HA	4.3	1	53	0	58	59	80	62	59	68
	5.1	2	49	0	42	38	68	73	61	71
	12.5 ^a	1	48	0	36	27	65	56	51	56
P1-3HA	18.1	2	0	0	103	105	163	138	134	120
	20.2 ^a	2	0	0	82	83	135	159	160	129
	22.1	2	0	0	100	83	147	134	115	125
	31.1	2	0	0	93	122	152	127	107	122
	32.1	1	0	0	33	39	69	80	59	80
	32.3	2	0	0	41	32	59	54	56	42
P3-3HA	3.2	2	0	0	18	21	33	35	26	33
	3.4	2	0	0	11	24	41	42	30	34
	3.5	2	0	0	12	17	18	29	21	29
	31.1 ^a	2	0	0	73	56	113	110	97	99
P1P3-3HA	5.2	2	0	0	16	22	18	23	30	22
	31.4	2	0	0	21	35	41	46	41	48
	62.1 ^a	1	0	0	24	17	32	32	40	32
	62.2	2	0	0	46	54	94	98	78	104

The results of rescue crosses show that none of the transgenes with mutations in P-loops 1 and/or 3 rescue the lethality of the DHC null genotype, *Dhc64C^{γ4163a}/Df(3L)10H*. Only the wild type tagged transgene (WT-3HA) is able to rescue the critical class of progeny (Df/null). The critical class is not recovered in the absence of the WT-3HA transgene. Shown are the number of surviving progeny of each class resulting from the rescue cross scheme described in MATERIALS AND METHODS. The third chromosome genotypes are abbreviated as follows: *Df* = *Df(3L)10H*, which removes the *Dhc* gene; null = the null allele *Dhc64C^{γ4163a}*; bal = the balancer chromosome *TM6B, D Hu e*; WT = wild type. The presence or absence of the transgene is indicated by (+) or (–), respectively.

^aThese lines are used for the analyses in Figures 2–8.

than 30 transformants, representative transgenic lines were selected that contained single insertions on the X or 2nd chromosome. These representative lines were used to test the functional significance of P-loop 1 and P-loop 3.

Transgenes with Mutations in P-loops 1 or 3 Fail to Rescue Lethal Mutations in the Dynein Heavy Chain

To test the significance of P-loop function in vivo, we asked whether the expression of transgenes encoding DHCs with mutant P-loops can rescue the recessive lethality of previously characterized *Dhc64C* alleles. Genetic crosses were performed to separately introduce each representative transgene, WT-3HA, P1-3HA, P3-3HA, and P1P3-3HA, into flies that also carried a strong or weak allele of the endogenous *Dhc64C* gene. Flies of genotype *Dhc64C^{γ4163a}/Df(3L)10H* carry a *Dhc64C* null allele and a small deficiency that removes the *Dhc64C* gene. These animals die during the first larval instar. In the presence of the WT-3HA transgene, this lethality is rescued; adult flies eclose and are viable. In contrast, we found that none of the transgenes encoding mutant P-loops rescued this lethality. Moreover, multiple copies of the mutant transgenes also failed to rescue lethality of the dynein null background. The results of rescue experiments for the null genotype are summarized for several independent transgenic lines in Table 1.

As a less stringent test for the function of the mutant DHCs, we conducted rescue experiments using the hypo-

morphic allele, *Dhc64C^{6–10}*. In contrast to the DHC null mutation, the partial function of this weak *Dhc* allele is revealed by its delayed lethal phase during pupariation, and its ability to complement certain other *Dhc64C* alleles (Gepner *et al.*, 1996). If any of the mutant P-loop transgene products retained low levels of function, then we might expect them to rescue the weak *Dhc* allele. However, just as for the null allele, the lethality of the weak allele was rescued only by the wild-type tagged transgene. Furthermore, the failure to rescue is not due to the presence of the HA epitope tag. In parallel experiments, we obtained the same outcomes with a set of *Dhc* transgenes containing P-loop mutations identical to those described above, but lacking the tag. Our results support the conclusion that both P1 and P3 are essential for dynein function during *Drosophila* development.

P-loop Mutant Heavy Chains Assemble into Dynein Motor Complexes

In vivo, two cytoplasmic DHCs assemble into a motor complex containing additional intermediate, light-intermediate, and light chain polypeptides. The complex has a native molecular weight of >10⁶ daltons and migrates on sucrose gradients as a 19–20S particle. To investigate whether the protein products of the mutant *Dhc* transgenes assemble into a motor complex, soluble extracts of adult ovaries from each representative line were analyzed by sucrose density gradient centrifugation and immunoblotting (Figure 2). Because

Figure 2. P-loop mutant DHC proteins are components of a 19S complex. Sucrose gradient sedimentation profiles of protein products from the mutated *Dhc* transgenes are similar to each other, and to endogenous dynein. Samples spanning the entire gradient were immunoblotted and probed with antibodies recognizing the dynein heavy chain, or, in replicate blots, the HA epitope. Profiles shown are (A) WT-3HA; (B) P1-3HA; (C) P3-3HA; and (D) P1P3-3HA. Lane E, equal total protein from each starting extract was loaded onto gels (15 μ g). Lane P, sample of pellet after density gradient fractionation. Lanes 1–23, samples of fractions taken across the gradient, where 1 is from the bottom of the gradient (20% sucrose). S values indicated are derived from the mobilities of standards.

of the inability of the mutant proteins to rescue the lethality of the DHC null, all assays were done in a background of wild-type (endogenous) dynein. The behavior of the transgenically expressed protein can be followed via the epitope tag. Replicate immunoblots were probed with either an anti-HA mAb to specifically detect the tagged transgene product or with an anti-DHC mAb that detects both the endogenous and transgenic DHC. A comparison of the blots reveals that all the transgenic DHCs, mutant and wild-type, exhibit sedimentation profiles similar to those of the endogenous DHCs. Furthermore, comparison of the profiles with known standards reveals that the transgenic products, like the native DHC, migrate on sucrose density gradients as a 19S complex. As discussed above, the HA-tagged wild-type transgene acts to rescue lethal dynein mutations, supporting the interpretation that the transgenic product assembles into a bona fide dynein complex. Collectively, these results strongly suggest that the addition of the triple HA epitope tag and mutations in P-loop 1 and/or 3 do not prevent the incorporation of the *Dhc* transgene products into a 19S dynein motor complex.

UV-Vanadate Cleavage of the DHC Is Blocked by Mutation of P-loop 1, But Not P-loop 3

A characteristic feature of DHCs is their susceptibility to cleavage in the presence of the phosphate analog orthovana-

Figure 3. Mutations in P1, but not P3, inhibit UV-vanadate cleavage of DHC. (A) Western blot probed with antidynein heavy chain antibody. All UV-vanadate treated (+) lanes show a cleaved fragment (LUV, open arrow) absent from the untreated (–) lanes. This fragment results from cleavage of endogenous DHC (shaded arrow). In addition, the samples of WT-3HA and P3-3HA DHC yield doublets of LUV fragments (dots), which comprise the cleavage products derived from the tagged and endogenous DHC proteins. (B) The same blot was stripped and reprobed with antibody against the HA epitope. Only extracts from the WT-3HA and P3-3HA lines yield the LUV fragment after UV exposure.

date and UV light (reviewed in Gibbons and Mocz, 1991). In the presence of MgATP, vanadate replaces the P_i derived from ATP hydrolysis and mediates the UV-specific photolysis of the DHC peptide backbone, yielding two fragments termed HUV and LUV (high- and low-molecular-weight UV fragments). Thus, if our mutations in P1 or P3 alter the capacity of the DHC to bind or hydrolyze MgATP, then the DHC should be resistant to UV-vanadate cleavage. We tested this possibility on ovary extracts from the transgenic lines and the control background strain used for transformation. After UV-vanadate treatment, extracts were immunoblotted and probed with an anti-DHC mAb that detects an epitope contained within the LUV fragment of both the wild-type and tagged proteins. As shown in Figure 3A, all samples treated with UV and vanadate yield a LUV cleavage product of ~200 kDa, which derives from endogenous dynein and is absent in the untreated control lanes. Subsequently, the blot was stripped and reprobed with an anti-HA mAb to identify the tagged transgene products. If the HA-tagged DHCs were cleaved, the resulting LUV fragments would contain the HA epitope. For the transgenic lines tested, we find that neither the P1-3HA nor P1P3-3HA DHCs yield a cleavage product that is detected with the anti-HA antibody. In contrast, both the WT-3HA and P3-3HA DHCs are cleaved by UV-vanadate treatment (Figure 3B).

The LUV cleavage fragments recognized by the anti-DHC antibody are resolved as a doublet of polypeptides in the extracts from WT-3HA and P3-3HA ovaries. Superimposing the blots shown in Figure 3, A and B, reveals that the LUV band of slower mobility is detected by both the anti-HA and anti-DHC antibodies. Therefore, the doublet of polypeptides

(+) of 5 mM MgATP, or under conditions of ATP depletion (-). (B) Microtubule pellets from A were washed once in buffer (W), then sequentially with increasing concentrations of salt (0.1–0.5 M NaCl, as indicated). The release profiles of the tagged proteins resemble those seen by the anti-DHC antibody, which recognizes both tagged and endogenous dynein. All the mutant proteins exhibit similar binding behavior.

corresponds to the distinct LUV fragments derived from endogenous and HA-tagged DHCs. Inspection of the doublets reveals that the amounts of WT-3HA and P3-3HA products appear slightly reduced relative to the endogenous wild-type dynein heavy chain. No doublet is observed with the anti-DHC antibody in the lanes containing P1-3HA and P1P3-3HA extracts, another indication that these mutant DHCs are not cleaved by UV-vanadate treatment.

Mutations in P-loops 1 or 3 Result in ATP-insensitive Microtubule Binding

The dynein motor complex characteristically binds to microtubules in an ATP-sensitive manner. We previously demonstrated that *Drosophila* cytoplasmic dynein binds to taxol-stabilized microtubules in the absence of MgATP and is released from microtubules by the addition of MgATP (Hays *et al.*, 1994). The mutations introduced into P1 and P3 in this study replace highly conserved lysine and threonine residues. Based on the analysis of other P-loops, these mutations are predicted to disrupt the interaction of the P-loop with the phosphate groups of ATP. To determine whether the mutations affect the ATP-sensitive microtubule-binding property of dynein, we prepared embryo MAPs from the representative transgenic lines and monitored the products by immunoblotting. Although all of the transgenic DHC products copelleted with assembled microtubules after ATP depletion, only the wild-type DHCs released from microtubules upon subsequent addition of MgATP. To further test the binding properties of the mutants, we modified the preparation by adding MgATP to the soluble extracts at the time of microtubule assembly. As predicted, under these conditions the WT-3HA DHC has a low affinity for microtubules (Figure 4A). In contrast, mutations in P1 or P3, or both, cause the DHCs to bind to microtubules even in the presence of 5 mM MgATP. Furthermore, once bound to the taxol-stabilized microtubules, the DHCs containing mutant

Figure 4. (A) DHCs with mutant P-loops bind to microtubules in the presence of ATP. MAPs prepared from the representative transgenic lines were blotted and probed with anti-HA antibody. When ATP is depleted, WT-3HA dynein pellets with microtubules (lane P) identically to endogenous dynein, as detected by anti-DHC antibody in control OregonR extracts. In the presence of ATP, WT-3HA dynein does not significantly pellet with microtubules, as expected from the known behavior of endogenous dynein. In contrast, mutations in either P1 or P3, or both, result in copelleting of the corresponding DHC with microtubules. Lane E, initial soluble extract; lanes P, S, and W, taxol stabilized microtubule pellet, supernatant, and wash (respectively) in the presence

P-loop domains remained bound even when subsequently resuspended in the presence of 10 mM MgATP (unpublished data). These results demonstrate that disruptions in P1 or P3 confer ATP-independent binding of DHC to microtubules.

We examined whether the ATP-independent binding behavior of the P3 mutant protein differed from that of the P1 mutant. First, microtubule pellets were washed with increasing concentrations of salt. The resulting Western blot is shown in Figure 4B. The salt-dependent release profile appears similar for all the proteins; no dramatic difference is seen in the binding behavior of the P1 mutant protein as compared with P3. In addition, we find no evidence that the microtubule binding properties of dynactin, a dynein regulatory complex that copurifies with dynein in a MAP prep, are contributing differentially to the rigor binding behavior of the P1 and P3 mutant polypeptides. When the above blot is probed with an antibody against the p150 (Glued) subunit of dynactin, its salt-dependent release profile is analogous to that of the DHC and appears the same for all the mutant lines (unpublished data). In another *in vitro* assay of the microtubule binding affinities of the mutant dynein proteins, we monitored the extent of DHC binding as a function of the concentration of microtubules. The amount of tubulin required to cosediment equivalent proportions of the mutant DHCs appears to be similar for the P1 and P3 mutant lines. However, our analysis does not eliminate the possibility that a more quantitative study of binding affinities may reveal some difference between the P1 and P3 mutant heavy chains.

Mutations in P-loops 1 or 3 Alter the Localization Pattern of DHC during Oogenesis

We previously showed that cytoplasmic dynein is required for oocyte formation and has a distinct pattern of localization during *Drosophila* oogenesis (Li *et al.*, 1994; McGrail and

Hays, 1997). Shortly after formation of the 16-cell germline cyst, the DHC accumulates in the single cell destined to develop as the oocyte. Subsequently, dynein remains concentrated in the presumptive oocyte and exhibits a fairly uniform cytoplasmic distribution. In later stage 9 egg chambers, dynein becomes concentrated at the posterior pole of the developing oocyte. To further examine the functional consequences of P-loop mutations, we characterized the distribution of the *Dhc* transgene products in situ during oogenesis. If the localization of dynein during oogenesis depends on microtubule-based translocation, then P-loop mutations that alter the microtubule binding properties of the DHC should result in its mislocalization. As with the biochemical assays, because of the requirement for DHC function, our cytological analyses of the mutant DHC transgenes were conducted in the background of the wild-type endogenous DHC gene. The distributions of the transgenic polypeptides were determined using the anti-HA mAb. We find that throughout oogenesis, the control WT-3HA DHC displays a localization pattern that parallels that of the endogenous DHC (Li *et al.*, 1994; McGrail *et al.*, 1995). In stages 2–8, WT-3HA DHC accumulates uniformly in the cytoplasm of the oocyte and appears enriched on the oocyte nuclear envelope (Figure 5A). In stage 9, the WT-3HA DHC staining is concentrated at the posterior of the oocyte and at the apical margin of the follicle cells that surround the egg chamber (Figure 6A).

In contrast, the DHCs containing the P-loop mutations show abnormal distributions within the egg chamber. Consistent with the aberrant microtubule binding properties of the mutant dynein polypeptides observed in vitro, we find that the dynein complexes containing P1 or P3 mutant polypeptides are mislocalized during oogenesis. At early stages (stages 2–8), the mutant DHCs are enriched in the oocyte similar to wild-type dynein, but the staining pattern is less uniform and more fibrous in appearance (Figure 5, B–E). A fibrous staining pattern of the mutant DHC products is also present within the nurse cell cytoplasm, in both early and later stages. In the stage 9 oocyte, no mutant DHC is localized at the posterior; rather, there is a fibrous distribution of the mutant DHCs at the anterior margin (Figure 6B). These mislocalized DHCs could comprise either a homodimer of mutant heavy chains or heterodimers containing one mutant and one wild-type dynein heavy chain. At the same time, homodimers of endogenous wild-type dynein are assembled and localize in a normal manner (Figure 6D). The rigor-like binding to microtubules exhibited by the mutant DHCs in vitro suggested that the fibrous pattern represented labeling of microtubules in situ. Figure 6B shows double-labeling of a P1-3HA mutant egg chamber with antitubulin and anti-HA. The microtubules decorated with mutant DHC appear to originate from the anterior oocyte membrane in the region adjacent to the overlying follicle cells. Treatment with colchicine eliminates the fibrous appearance of the DHC as well as the microtubules, at all stages (Figure 6C shows stage 9). Similar results are obtained with the P3 and P1P3 mutant lines. Not surprisingly, the mutant proteins also colocalize with microtubules associated with the membranous fusome organelle during early stages of egg chamber formation (Grieder *et al.*, 2000).

Figure 5. P-loop mutant dyneins display novel localization patterns in egg chambers. Fixed ovaries from the representative lines were probed with the anti-HA antibody. All images shown are oriented with anterior to the left. WT-3HA DHC has a localization pattern that parallels endogenous wild-type DHC (Li *et al.*, 1994). Bar, 20 μ m. (A) WT-3HA DHC is diffuse throughout the oocyte and shows a bright ring of staining surrounding the oocyte nucleus during this previtellogenic stage. (B–D) P1-3HA, P3-3HA and P1P3-3HA DHC, respectively, display fibrous staining in the nurse cell and oocyte compartments, as well as localization along the apical membrane of follicle cells surrounding the egg chamber. (E) Enlarged views of the fibrous staining in the nurse cell (left) and oocyte (right) shown in B.

DISCUSSION

We report here the novel finding that P-loop 3 in the third AAA domain functions in the ATP-induced release of DHC from microtubules. In analyzing the microtubule-binding

Figure 6. Novel localization of mutant P-loop dyneins appears to represent binding to microtubules. Fixed ovaries were double-stained against alpha-tubulin (green) and the HA epitope tag (red). Shown are oocytes within stage 9 egg chambers. Bar, 20 μ m. (A) Staining pattern of WT-3HA DHC parallels that of endogenous DHC, with accumulation at the posterior of the oocyte. A meshwork of microtubules is concentrated at the oocyte anterior. (B) Fibrous P1-3HA DHC staining is observed at the anterior margin of the oocyte, coincident with microtubules in the region nearest the follicle cells. An accumulation of P1-3HA DHC is also seen in the apical cytoplasm of the overlying follicle cells. Similar staining patterns were seen with the other P-loop mutant lines. (C) After treatment with colchicine, microtubules are no longer evident within the oocyte, and the fibrous pattern of the mutant dynein is eliminated. P1-3HA oocyte is shown; identical results were obtained for P1, P3, and P1P3 mutant DHC. (D) In ovaries expressing the P-loop mutant transgenes, a pool of endogenous wild-type dynein is properly localized at the posterior of the late stage oocyte, as detected by the anti-DHC antibody. Mutant dynein complexes at the oocyte anterior are also recognized by this antibody. None of the HA-tagged mutant polypeptides are detected at the oocyte posterior (see B above). Shown is a P1-3HA oocyte.

properties of P3-3HA DHC, we find the results are similar to those obtained for P1-3HA DHC. Mutation of the conserved lysine and glycine residues of P3, as well as P1, generates rigor-like, ATP-insensitive microtubule binding of the mutant DHC product. However, the P3 mutations apparently do not eliminate hydrolytic activity in the first AAA domain.

The functional role of P-loop 1 as the primary hydrolytic site in dynein is well established (Gibbons *et al.*, 1991; Ogawa, 1991; Gibbons, 1995). This was first demonstrated by vanadate photocleavage experiments in which loss of ATPase activity correlated with the proportion of DHC disrupted at the P1 site (Gibbons *et al.*, 1987; Mocz and Gibbons, 1990). A more recent study showed that mutation of the conserved lysine in P-loop1 of rat cytoplasmic DHC inhibited vanadate photocleavage (Gee *et al.*, 1997). When transiently expressed in cultured cells, this mutant DHC was observed to colocalize with microtubules. Consistent with these results, our vanadate photocleavage experiments indicate that the mutations in P1 eliminate the capacity of the DHC to bind or hydrolyze ATP. Moreover, experiments carried out in parallel demonstrate that the P3 mutant heavy chain is cleaved by UV-vanadate treatment, showing that mutation of P3 alone does not block ATP hydrolysis at P1. Therefore, the insensitivity of the mutant P1-3HA

and P1P3-3HA DHCs toward UV-vanadate cleavage is a direct result of the mutation in P1.

Our results extend the analysis of P1 function, providing additional biochemical demonstration that loss of hydrolytic activity in P1 is accompanied by the predicted ATP-insensitive binding of the mutant DHC to microtubules. Wild-type DHC exhibits a cyclical interaction with microtubules in the presence of MgATP (Johnson *et al.*, 1984; Hackney, 1996). The kinetic analysis of axonemal dynein has indicated that ATP binding induces rapid detachment of the DHC from the microtubule, followed by subsequent ATP hydrolysis and phosphate release (Porter and Johnson, 1983a; 1983b). The motor domain then rebinds the microtubule, and force production is coupled with ADP product release (Holzbaur and Johnson, 1989). Previous analyses of nucleotide binding at P-loops predict that our mutation of the lysine and threonine residues of P1 will inhibit the binding of MgATP. Consensus sequence analysis, structural analysis and saturation mutagenesis of P-loops have all demonstrated the requirement for the lysine and threonine residues in mediating interactions with the β and γ phosphate residues of ATP (Story and Steitz, 1992; Shen *et al.*, 1994; Smirnova *et al.*, 1998). The rigor-like binding of the P1 mutant DHC to microtubules in the presence of MgATP most likely reflects the

inability of ATP to bind and thereby induce the rapid detachment of the DHC.

Previous measurements of nucleotide binding affinities at multiple sites in axonemal dynein are consistent with the proposal that additional AAA modules are important for dynein function (Gee *et al.*, 1997; Koonce, 1997; Samso *et al.*, 1998). Although kinetic analyses have indicated that only one molecule of ATP is hydrolyzed per heavy chain per mechanochemical cycle (Shimizu and Johnson, 1983), a phase partition analysis of nucleotide binding to dynein indicates that each DHC may contain four different ATP-binding sites (Mocz and Gibbons, 1996). Furthermore, experiments using a fluorescent analog to study ATP binding to dynein have shown that each DHC contains a minimum of two distinct binding sites of high and low affinity (Mocz *et al.*, 1998). These biochemical studies are consistent with the presence of a single high-affinity catalytic site and lower affinity regulatory sites within the DHC. Similarly, the reversible inhibition of axonemal motility in vitro by high concentrations of ATP supports a model for regulatory ATP-binding sites of lower affinities (Kinoshita *et al.*, 1995). The integrity of an extended C-terminal dynein fragment that encompasses all six AAA domains is required for dynein's microtubule stimulated ATPase activity (Gee *et al.*, 1997), further supporting a regulatory role for the multiple AAA modules.

One mechanism of action for regulatory P-loops might be their modulation of ATP hydrolysis at P1 (Mocz and Gibbons, 1996). For example, ATP bound at P3 might be required for hydrolysis to occur at P1. In this case, the ATP-insensitive microtubule binding of the different P-loop mutant proteins might be produced by inhibition of the same step in the ATP hydrolytic cycle. However, our experiments show that vanadate-mediated cleavage at P1 does occur in the P3-3HA mutant polypeptide. This implies that ATP hydrolysis at P1 can occur in the absence of ATP binding at P3. However, our results do not rule out a potential effect on the rate of ATP hydrolysis at P1 by the mutations in P3.

We favor an alternative possibility, that nucleotide binding at P3 is required to induce a conformational change in the DHC that is necessary for the proper execution of a later step in the mechanochemical cycle (Mocz and Gibbons, 1996). Our photocleavage results are consistent with a regulatory mechanism in which nucleotide binding at P3 is significant in a step subsequent to ATP hydrolysis at P1. A structural transition mediated by P3 could be propagated through the DHC to regulate the proposed microtubule-binding site, which lies more than 500 residues C-terminal from P3 (Vallee and Gee, 1998). By inference, the binding of ATP at P-loops 2 and 4 could serve similar functions in regulating the binding of DHC to microtubules. It is also possible that the proposed conformational changes associated with ATP binding at regulatory P-loops of one DHC could alter the progress of the mechanochemical cycle in the partner DHC of a homodimeric complex. We have evidence that supports such cooperative interactions between the partner DHC motor domains and microtubules (Iyadurai *et al.*, 1999). Although some analyses of axonemal dynein suggest a nonprocessive mechanism of movement (Johnson, 1985; Hackney, 1996; Howard, 1997), for the cytoplasmic dynein motor, processive movement along the microtubule is presumed to be important for productive transport of organelles and other cargoes. By causing a conformational change that controls the rate of product release from the hydrolytic ATP site or that affects microtubule release from the binding do-

main, perhaps the regulatory P-loops contribute to a mechanism that ensures that dynein stays attached to the microtubule as it translocates.

In view of the ATP-insensitive microtubule binding of the mutant P-loop DHCs, our genetic results that the P1 and P3 mutant transgenes fail to rescue the lethality of mutations in the endogenous dynein gene are not surprising. The predicted loss of function for mutation of P1 has been previously reported for *Saccharomyces cerevisiae* cytoplasmic DHC (Eshel, 1995). The observed failure to rescue dynein mutants could result from reduced expression and/or stability of the mutant P-loop transgenic products. We do not favor this interpretation because the amount of the P3-3HA mutant transgene product is similar to that observed for the wild-type transgene, WT-3HA (see Figures 2-4), yet P3-3HA fails to rescue strong or weak DHC alleles (Table 1). Comparing the expression levels of all transgenic products, the level of P1-3HA and P1P3-3HA appear reduced relative to that of P3-3HA and WT-3HA (e.g., Figure 2). Would higher levels of P1-3HA or P1P3-3HA provide for rescue of DHC alleles? Three lines of evidence argue against this possibility. First, as discussed above, the level of P3-3HA is approximately equivalent to WT-3HA but does not rescue DHC mutant alleles. Second, regardless of the number of copies of the mutant transgenes or the strength of the *Dhc64C* allele to be rescued, we observed no rescue of lethal dynein mutations with any of the P-loop mutant transgenes. In contrast, a single copy of the wild-type functional transgene, WT-3HA, rescues the lethality of *Dhc64C* alleles (null and weak alleles). Third, in further experiments, we mobilized the DHC transgenes to different positions in the fly genome. Although we recovered new transgenic lines of WT-3HA with elevated levels of expression, we never recovered transgenic animals that expressed higher levels of the P-loop mutant DHC products. We believe the inability to recover such lines reflects the detrimental or dominant negative effect of the P-loop mutants on essential dynein functions. Our results indicate that both P1 and P3 are essential for dynein heavy chain function and suggest that the ATP-insensitive binding of the P-loop mutant dyneins would be detrimental if expressed at high levels. Consistent with this interpretation, at lower levels of transgene expression the presence of the endogenous wild-type dynein allows the recovery of adult females. Oocytes derived from these females exhibit a dominant mislocalization of dynein complexes that contain the P-loop mutant proteins, whereas the endogenous wild-type complexes are normally localized within oocytes.

The most striking aspect of mislocalization is the distribution of the P-loop mutant DHCs along a fibrous network of microtubules within the egg chamber and oocytes. A comparable observation has been made for rat brain DHC in tissue culture; expression of dynein heavy chain with a mutation in the same conserved lysine of P-loop 1 generates a rigor-like association of the DHC with microtubules in situ (Gee *et al.*, 1997). In egg chambers, the distribution of the transgenic mutant DHCs along microtubules is distinct from that of wild-type. For both the WT-3HA and endogenous DHC, the pattern of staining in early egg chambers is never fibrous but rather more diffuse. This presumably reflects a steady-state cytoplasmic pool of wild-type DHC that is not bound to microtubules. In later stage 9 oocytes, the aberrant accumulation of the mutant P-loop DHCs at the anterior cortex of the oocyte correlates with the previously characterized elevation in microtubule number at

the anterior (Theurkauf, 1994). Our results suggest that the normal localization of dynein at the posterior of the oocyte requires ATP-sensitive microtubule binding. One possibility is that dynein motor function and microtubule translocation are required for the normal localization of dynein. On the other hand, the rigor-like binding of the P-loop mutant DHCs to microtubules at the anterior may prevent the transport of dynein to the posterior pole by a separate means. For example, a mechanism based on kinesin-mediated cytoplasmic flow may play an important role in the localization of molecules within the oocyte (Cha *et al.*, 2002).

The cooperative function of adjacent AAA domains has been characterized for other AAA oligomeric proteins in their capacity to remodel substrates (reviewed in Vale, 2000). Using site-directed mutagenesis, we have demonstrated that P-loop 3 within the third AAA domain of the *Drosophila* cytoplasmic dynein heavy chain is functionally significant. The motor-microtubule cosedimentation data indicate that P3, in addition to P1, is required for the ATP-sensitive release of dynein from microtubules. Our results support the hypothesis that nucleotide binding at P3 is required to couple the ATP hydrolytic cycle at P1 to the binding and release of dynein from the microtubule.

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