

Molecular dissection of the roles of nucleotide binding and hydrolysis in dynein's AAA domains in *Saccharomyces cerevisiae*

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The motor protein cytoplasmic dynein is responsible for most of the minus-end-directed microtubule traffic within cells. Dynein contains four evolutionarily conserved AAA (ATPase associated with various cellular activities) domains that are thought to bind nucleotide; the role of nucleotide binding and hydrolysis in each of these four AAA domains has constituted an important and unresolved question in understanding dynein's mechanism. Using *Saccharomyces cerevisiae* cytoplasmic dynein as a model system, we mutagenized residues involved in nucleotide binding or hydrolysis in the four AAA domains and examined the ability of the mutant dyneins to mediate nuclear segregation *in vivo* and to bind microtubules *in vitro*. Our analysis shows that an AAA1 hydrolysis mutant blocks dynein function, whereas a triple AAA2/3/4 hydrolysis mutant does not, suggesting that nucleotide binding is required at only one site. We also show that nucleotide binding at AAA3, but not hydrolysis, is essential for motor activity *in vivo* and ATP-induced dissociation of dynein from microtubules, suggesting that this domain acts as a critical allosteric site. In contrast, mutations in AAA2 cause subtle defects in dynein function, whereas mutation in AAA4 produce no obvious defects. These results show that the four conserved dynein AAA domains have distinct functions in dynein's mechanochemical cycle.

Dynein motors power the beating of cilia and flagella as well as nearly all of the minus-end-directed microtubule transport in fungi and animal cells, including roles in nuclear segregation, mitosis, and the transport of membranes, virus, and signaling molecules (1, 2). When compared to the other cytoskeletal motor proteins, kinesin and myosin, very little is known about the mechanism of dynein-based motility, primarily because of the large size of dynein, which has hindered protein expression and mutagenesis studies.

Cytoplasmic dynein is a homodimer of two >500-kDa heavy chains and a number of smaller associated polypeptides (Fig. 1A) (1). The motor domain of the heavy chain contains six predicted AAA [ATPase associated with various cellular activities (3–5)] domains that are arranged in a ring with a microtubule binding stalk emerging between domains 4 and 5 (6, 7). Other AAA ATPases oligomerize into hexameric rings (4); however, dynein is unusual in creating a ring from distinct AAA domains that are concatenated within a single large polypeptide. Sequence analysis of the dynein heavy chain shows that the nucleotide binding regions in the first four AAA domains are highly conserved (Fig. 1B), suggesting that they bind and potentially hydrolyze nucleotide (5). AAA domains 5 and 6 are highly divergent from other AAA ATPases and do not contain signature sequences associated with nucleotide binding. Thus, AAA5 and AAA6 are likely to serve structural roles, perhaps being required to complete the ring (5). Consistent with these sequenced-based predictions, the dynein heavy chain has been shown to bind four molecules of ATP (8). Biochemical studies also have shown that UV-vanadate-induced polypeptide cleavage at AAA1 inactivates all dynein ATPase activity (9, 10), although these experiments do not rule out the possibility that the ATPase activity of other AAA domains became inactivated upon chain cleavage. The

roles of nucleotide binding to AAA1 and AAA3 have been investigated by inactivating point mutations, both of which resulted in a loss of dynein motor activity (6, 11). However, a comprehensive structure–function analysis of the roles of both nucleotide binding and hydrolysis in dynein's four nucleotide-binding AAA domains had not been made. Here, we have carried out such a study by introducing mutations into the sole nonessential genomic copy of the motor domain of the *Saccharomyces cerevisiae* cytoplasmic dynein heavy chain (*DYN1*) and then investigating the consequences of these mutations on dynein function *in vivo* and *in vitro*.

Materials and Methods

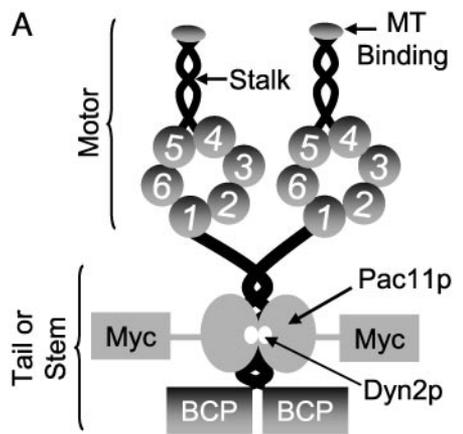
Creation of Dynein Mutants and Tagged Proteins. To create a high-affinity tag for detecting dynein by blots, we created a biotinylatable cassette by using the sequence encoding the carboxyl-terminal domain (amino acids 1080–1175) of the *Pichia pastoris* pyruvate carboxylase gene, *PYCI*. This domain, referred to as the biotin carrier protein (BCP) domain, is sufficient for efficient biotinylation *in vivo* and *in vitro* and folds into an independent domain (12). A similar approach has been used to tag the yeast kinesin, *CIN8*, with the BCP domain from *S. cerevisiae* *PYCI* (13). Because the BCP domains in the *S. cerevisiae* and *P. pastoris* *PYCI* genes are only 40% identical at the DNA level [but can functionally replace one another (14)] we used the *P. pastoris* sequence to facilitate specific tagging of the dynein heavy chain by homologous recombination. Plasmids containing 5' and 3' fusions of the BCP domain to the 5' and 3' ends, respectively of the *Kluyveromyces lactis* *URA3* gene were constructed as has been done for other tags (15). All tagging and mutations in *DYN1* and *PAC11* (the dynein intermediate chain) were made by using the long flanking homology method of homologous recombination at the endogenous locus (15) and confirmed by DNA sequencing.

Microtubule Binding and Release Assays. Yeast cells were grown to $OD_{600} = 1.2$, washed with H₂O, and resuspended in 1 packed cell volume of lysis buffer (35 mM Pipes, pH 7.2/2 mM MgCl₂/1 mM EGTA/1 mM DTT/1 mM Pefabloc/10 μg/ml each of pepstatin A, leupeptin, and aprotinin). The extract was then drop-frozen in liquid nitrogen, and the frozen pellets were ground in liquid nitrogen with a mortar and pestle for 1 min. An equal volume of lysis buffer plus 100 mM potassium acetate was added to the extract, which was spun at 290,000 × *g* for 20 min. Soluble dynein from this supernatant was used for microtubule binding and release experiments. For microtubule binding experiments, 0.1 mg/ml taxol-stabilized bovine brain microtubules was mixed with yeast protein extract (total protein concentration of ≈1 mg/ml) in the presence of one of the following: 5 mM MgATP,

Abbreviations: AAA, ATPase associated with various cellular activities; BCP, biotin carrier protein.

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B Walker A

Dyn1p AAA1	G	P	A	G	T	G	K	T	*
CDHC1 AAA1	G	P	A	G	T	G	K	T	
Dyn1p AAA2	G	K	A	G	C	G	K	T	
CDHC1 AAA2	G	P	S	G	S	G	K	S	
Dyn1p AAA3	G	P	P	G	S	G	K	T	
CDHC1 AAA3	G	P	P	G	S	G	K	T	
Dyn1p AAA4	G	A	S	R	T	G	K	T	
CDHC1 AAA4	G	V	S	G	A	G	K	T	
Vps4p	G	P	P	G	T	G	K	S	
NSF D1	G	P	P	G	C	G	K	T	
Katanin	G	P	P	G	T	G	K	T	

Walker B

Dyn1p AAA1	A	W	G	C	F	D	E	F	N	*
CDHC1 AAA1	A	W	G	C	F	D	E	F	N	
Dyn1p AAA2	I	W	V	V	F	D	S	D	L	
CDHC1 AAA2	Q	W	I	V	F	D	G	D	V	
Dyn1p AAA3	L	V	L	F	C	D	E	I	N	
CDHC1 AAA3	L	V	L	F	C	D	E	I	N	
Dyn1p AAA4	T	C	L	I	I	D	E	S	N	
CDHC1 AAA4	I	A	F	I	M	D	E	S	N	
Vps4p	S	I	I	F	I	D	E	V	D	
NSF D1	H	I	I	I	F	D	E	I	D	
Katanin	S	T	I	F	I	D	E	I	D	

Fig. 1. Overall dynein structure and mutations generated in the AAA domains. (A) Predicted domain structure of *S. cerevisiae* cytoplasmic dynein and the introduction of tags. Cytoplasmic dynein is composed of two heavy chains that contain six AAA domains within the motor domain, the first four of which are predicted to bind nucleotide. The light (Dyn2p) and intermediate chain (Pac11p) are associated with the tail domain. Dyn1p was tagged with a 96-aa biotinylatable domain from the *P. pastoris* Pyc1p protein. Pac11p was tagged with 13 copies of the Myc epitope. (B) Sequence alignment of the Walker A and Walker B motifs from *S. cerevisiae* (Dyn1p) and rat (CDHC1) cytoplasmic dynein and other members of the AAA superfamily (Vps4p, NSF, and katanin). The starred (*) amino acids are the amino acids that were mutated in Dyn1p (K changed to A; E changed to Q). In the AAA2 Walker B box, DSDL was mutated to NSDL.

5 mM MgADP, 5 mM MgATP γ S, 2 mM MgADP–3 mM Na-vanadate, 5 mM EDTA, or 6.6 units/ml apyrase. The extract plus microtubules were incubated at 22°C for 10 min and spun through a 40% (wt/vol) sucrose cushion (in lysis buffer supplemented with 10 μ M taxol) at 290,000 \times g for 15 min. Supernatant and pellet fractions were run on SDS/PAGE gels, and immunoblots for the myc-tagged dynein intermediate chain, Pac11p, were performed. The dynein intermediate chain was used for quantitating dynein levels because the electrophoretic transfer from gel to nitrocellulose was more complete and reproducible

than for the large dynein heavy chain. Image J was used for blot quantification. For microtubule release experiments, dynein was first bound to microtubules in the presence of 6.6 units/ml apyrase, as described above, and the microtubules were centrifuged through a 40% sucrose cushion onto a 60% (wt/vol) sucrose cushion (in lysis buffer supplemented with 10 μ M taxol). Dynein was then released from microtubules with 5 mM Mg-nucleotide in lysis buffer supplemented with 150 mM KCl, the microtubules were recentrifuged, and the supernatants and pellets were analyzed by SDS/PAGE and immunoblot.

Results and Discussion

The nucleotide binding pocket of AAA ATPases contains two defining sequences called the Walker A and Walker B motifs (4, 16, 17). Mutation of the conserved lysine in the Walker A (or P loop) motif (GX₄GKT) to an alanine results in loss of nucleotide binding, whereas mutation of a conserved glutamic acid in the Walker B motif (DEXX) to a glutamine results in loss of nucleotide hydrolysis (see examples in refs. 18–20). We therefore created a K to A mutation in the Walker A motif and an E to Q mutation in the Walker B motif in each of dynein’s conserved AAA domains. Based on these studies of other AAA ATPases, we predict that these mutations will block nucleotide binding and nucleotide hydrolysis, respectively in each dynein AAA domain. The Walker B glutamic acid is not present in AAA2; however, an aspartic acid that is amino-terminal to the position of the conserved glutamic acid is conserved and might function in ATP hydrolysis, so this residue was mutated to asparagine (Fig. 1B).

We sought to study the role of nucleotide binding and hydrolysis on dynein function in the context of the native holoenzyme expressed at endogenous levels from the dynein promoter. Therefore, we introduced the Walker A and B mutations into the sole nonessential genomic copy of *DYN1* preserving the dynein promoter (see *Materials and Methods*). To measure and compare the protein levels of WT and mutant cytoplasmic dynein, we tagged Dyn1p at the amino terminus (the nonmotor domain of dynein) with a 96-aa region of the *P. pastoris* pyruvate carboxylase protein, Pyc1p, which is a substrate for *in vivo* biotinylation (see *Materials and Methods*). The biotin-tagged dynein could be visualized on blots by using streptavidin and biotin-horseradish peroxidase. We also tagged the carboxyl terminus of the endogenous copy of the dynein intermediate chain, Pac11p, with 13 copies of the Myc epitope tag, which provided the most reproducible method for quantitating the association of dynein with microtubules in yeast extracts by immunoblots (see *Materials and Methods*). These tags did not affect *in vivo* dynein function, as assayed by nuclear segregation (Fig. 2).

Nucleotide Binding and Hydrolysis at AAA1 and Nucleotide Binding at AAA3 Are Required for Nuclear Segregation. Deletion of the dynein heavy chain causes nuclear segregation defects that result in binucleated cells (21, 22). Thus, to analyze the effects of the Walker A and B mutations for dynein function *in vivo*, we scored the percentage of binucleate cells in the population after growth at 16°C, which exacerbates the dynein nuclear segregation phenotype (21, 22).

Our analysis shows that nuclear segregation occurred normally in the putative AAA2 and AAA3 hydrolysis mutants and in both AAA4 mutants (Fig. 2), indicating that dynein function was preserved. A minor nuclear segregation defect was observed in the strain bearing a mutation predicted to block nucleotide binding at AAA2. In contrast, strains containing mutations predicted to block either nucleotide binding or hydrolysis at AAA1 or nucleotide binding at AAA3 displayed nuclear segregation defects that were more severe than deletion of the heavy chain (dyn1 Δ , Fig. 2), suggesting that these mutations imparted a deleterious gain-of-function phenotype. We hypothesized that

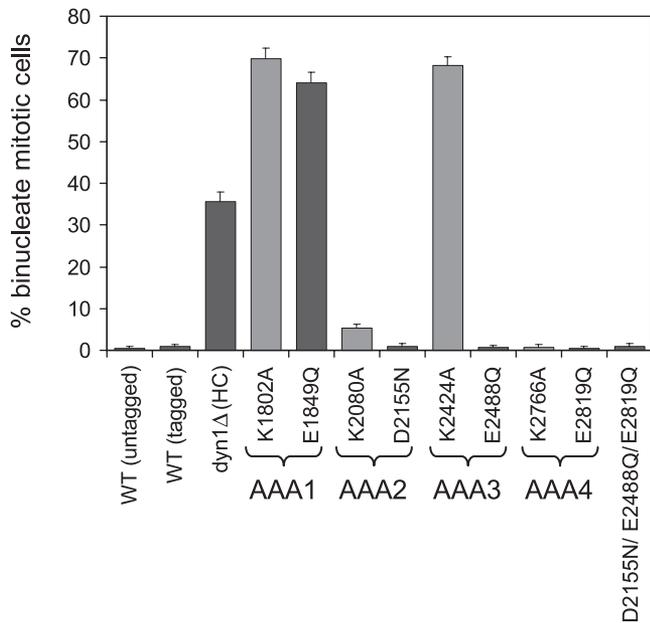


Fig. 2. Mutations in AAA1 and AAA3 cause defects in nuclear segregation. To monitor nuclear segregation, the number of binucleate mitotic cells was determined after growth at 16°C for 16 h. The mean and standard error of the proportion are shown ($n > 200$). Walker A mutants (predicted to block nucleotide binding) are shaded with gray, whereas Walker B mutants (predicted to block nucleotide hydrolysis) are shaded with black.

this phenotype could be caused by rigor binding of the mutant dynein molecules to microtubules, a possibility that was explored in the microtubule binding experiments described below.

Mutation of AAA Domains Has Little Effect on Dynein Microtubule Binding.

To assay dynein binding to microtubules, microtubule cosedimentation assays were performed with yeast extracts and taxol-stabilized bovine brain microtubules, and the amount of dynein in the supernatant and microtubule pellet was determined by immunoblot analysis of the myc-tagged dynein intermediate chain. In yeast strains in which the dynein heavy chain had been deleted, the intermediate chain did not bind to microtubules by itself (Fig. 3*A* and *B*). First, we investigated the nucleotide dependence and optimal conditions for dynein binding to microtubules in yeast extracts. Of several conditions tested [MgATP, MgADP, EDTA, apyrase, MgATP γ S (a nonhydrolyzable or slowly hydrolyzable analogue), and MgADP-vanadate (a transition state analogue)], maximal microtubule binding occurred in the absence of either nucleotide (apyrase) or magnesium (EDTA, Fig. 3*B*). Thus, as has been reported for cytoplasmic and axonemal dynein in other systems (23, 24), yeast dynein binds tightly to microtubules in the absence of magnesium-complexed nucleotide.

We then tested the ability of dynein-bearing AAA domain mutations to cosediment with microtubules in the absence of nucleotide (the apyrase condition, Fig. 3*C*). The Walker A mutation in AAA2 produced the largest ($\approx 50\%$) decrease in dynein binding to microtubules. The Walker A and B AAA1 mutants and the Walker A AAA3 mutant also showed small ($< 25\%$), but statistically significant ($P < 0.005$) decreases in microtubule binding. The other mutations produced no effect. The diminished microtubule cosedimentation seen in a subset of the mutant dyneins was not caused by lower protein levels, because quantitation of the amount of heavy chain using streptavidin and biotin-horseradish peroxidase blots revealed normal Dyn1p expression levels (data not shown). Thus, in conclusion, nucleotide site mutations did not dramatically affect dynein

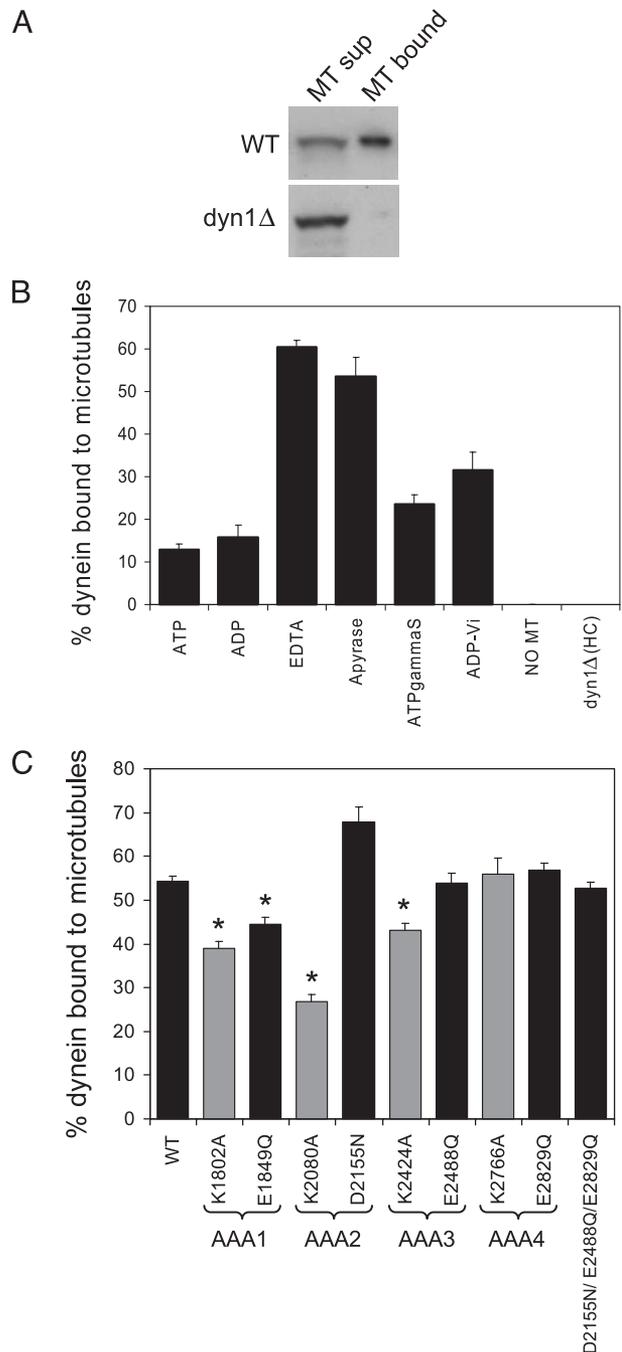


Fig. 3. Mutations in dynein AAA domains minimally affect dynein microtubule (MT) binding. (A) Anti-myc immunoblots of WT and dyn1 Δ microtubule supernatant and pellet fractions loaded stoichiometrically on the gel, showing that the dynein intermediate chain does not associate with microtubules in the absence of the heavy chain. (B) Yeast extract containing WT dynein or no dynein (extract from dyn1 Δ) was mixed with taxol-stabilized bovine brain microtubules in the presence or absence of the indicated Mg-nucleotides (5 mM). The dynein intermediate chain present in the microtubule pellet fractions was plotted as the percentage of total dynein (supernatant plus pellet fraction), and the mean and SEM are shown ($n = 4$). Because these experiments were performed in extracts containing adenylate kinase, we cannot exclude conversion of some ADP to ATP. (C) Yeast extract from WT or AAA mutant cells was mixed with bovine brain microtubule in the presence of apyrase. The percentage of dynein intermediate chain present in the microtubule pellet fractions was plotted as the percentage of total dynein, and the mean and SEM are shown ($n = 4-14$). The starred (*) means are statistically different from the WT mean ($P < 0.005$, *t* test). Walker A mutants (predicted to block nucleotide binding) are shaded with gray, whereas Walker B mutants (predicted to block nucleotide hydrolysis) are shaded with black.

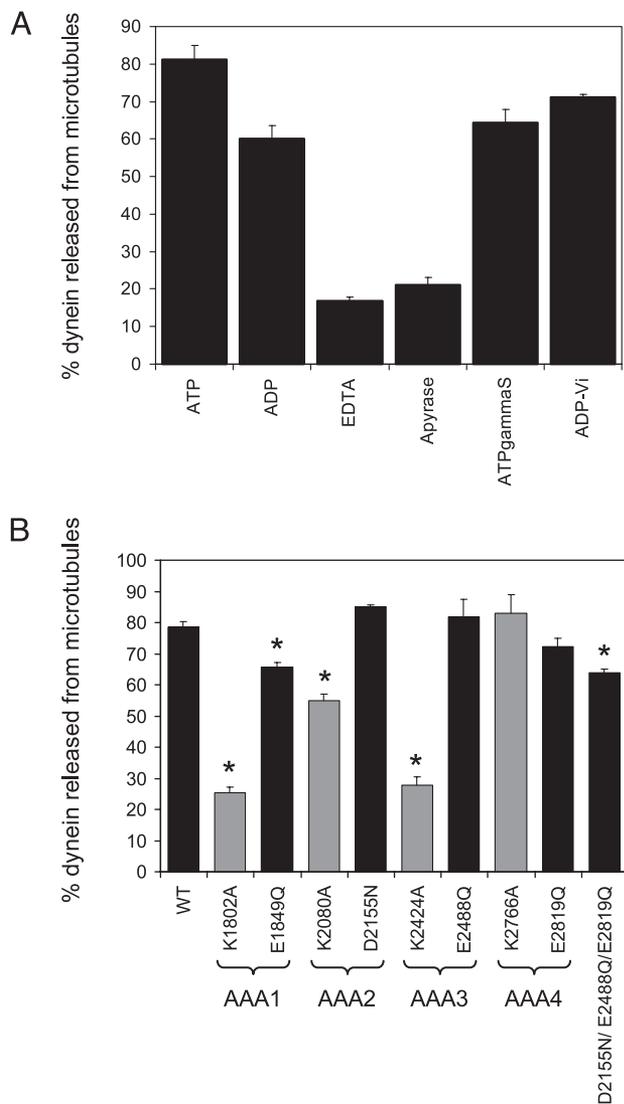


Fig. 4. Nucleotide binding at AAA1 and AAA3 is required for ATP-dependent dynein microtubule release. (A) Yeast extract containing WT dynein was bound to microtubules in the presence of apyrase and then released from microtubules by using different nucleotide conditions. The dynein intermediate chain released from microtubules was plotted as the percentage of total dynein (supernatant plus pellet fraction), and the mean and the SEM are shown ($n = 4$). (B) Yeast extract containing WT or mutant dynein protein was first bound to microtubules in the presence of apyrase and then released from microtubules with ATP. The dynein intermediate chain released from microtubules was plotted as the percentage of total dynein (supernatant plus pellet fraction), and the mean and the SEM are shown ($n = 3-17$). The starred (*) means are statistically different from the WT mean ($P < 0.005$, t test). Walker A mutants (predicted to block nucleotide binding) are shaded with gray, whereas Walker B mutants (predicted to block nucleotide hydrolysis) are shaded with black.

binding to microtubules, which also indicates that these mutations do not cause gross problems in protein folding.

Mutations in AAA1 and AAA3 Block ATP-Stimulated Release from Microtubules. To monitor WT dynein release from microtubules, dynein was first bound to microtubules in the absence of nucleotide (the apyrase condition) and then the dissociation of dynein from microtubules was assayed under various nucleotide conditions (Fig. 4A). Dynein was most effectively released from microtubules in the presence of MgADP or MgATP, and partial release was observed with MgATP γ S and MgADP-vanadate.

KCl (150 mM) was required for efficient release, but 150 mM KCl alone in the absence of nucleotide resulted in little dynein dissociation from microtubules (data not shown). These results are generally comparable to what has been reported for dynein in other systems (23–25).

We next tested the release of WT and mutant dyneins from microtubules in the presence of ATP (Fig. 4B). The Walker A mutations (predicted to block nucleotide binding) in AAA1 and AAA3 almost completely blocked ATP-stimulated release of dynein from microtubules. Smaller but significant ($P < 0.005$) defects in microtubule release were observed for the Walker B AAA1 and Walker A AAA2 mutants. The Walker B mutation of AAA3 and both AAA4 mutations had no effect on the ability of dynein to release from microtubules.

A Triple Walker B Mutation Suggests that AAA1 is the Sole Site of Nucleotide Hydrolysis. As discussed above, the mutation predicted to block nucleotide hydrolysis at AAA1 displayed a strong nuclear segregation defect, while the analogous mutations in AAA2, AAA3, or AAA4 had no effect. To confirm that AAA1 is indeed the sole site of nucleotide hydrolysis required for function, we constructed a triple mutant in which the Walker B hydrolysis mutation was made in AAA2, AAA3, and AAA4 (D2155N/E2488Q/E1819Q). As was the case for the single mutants in AAA2, AAA3, or AAA4, the triple mutant displayed normal nuclear segregation (Fig. 2) and microtubule binding (Fig. 3B), and only a very slight defect in microtubule release (Fig. 4B). These results strongly suggest that dynein motor activity requires only a single operational site for ATP hydrolysis.

Conclusions and Comparison to Other Findings. Previous biochemical studies have shown that dynein can bind four molecules of ATP per heavy chain (8), which raised the possibility that nucleotide binding and/or hydrolysis may be required at multiple AAA domains. Our analysis reveals that dynein function requires nucleotide binding only at AAA1 and AAA3 and nucleotide hydrolysis only at AAA1. Nucleotide binding at AAA2 also appears to play a minor role, as the Walker A mutation in this domain causes a defect in microtubule binding *in vitro* and a slight loss of dynein activity *in vivo*. Mutations in AAA4, on the other hand, produce no obvious defects. Thus, it appears that nucleotide binding and hydrolysis at AAA2 and AAA4 are not essential for dynein function *in vivo*. However, it is possible that these mutations might alter dynein motility in ways that would become manifest with *in vitro* motility assays. Direct measurements of the ATPase cycle using biochemical quantities of purified dynein mutants also could be used to confirm and extend our present studies.

Our results agree with and extend previous mutagenesis studies of cytoplasmic dynein AAA domains. Previous studies have shown that Walker A mutations in AAA1 and AAA3 of *Drosophila* and mammalian cytoplasmic dynein block ATP-dependent release of dynein from microtubules (6, 11). However, the mutant dynein molecules in both of these studies were expressed in the background of endogenous WT dynein, so homodimeric mutant and heterodimeric mutant-WT dyneins were being assayed in such experiments. AAA2 and AAA4 mutations and Walker B mutations in AAA1 and AAA3 and were not examined in these studies. Previous mutagenesis of the Walker A motif in AAA2 of *S. cerevisiae* DYN1 also revealed a nuclear segregation phenotype equivalent to deletion of the dynein heavy chain (26), which differs from the much milder defect reported here for the Walker A AAA2 point mutant. A possible explanation for this difference is that the entire P loop was deleted in the Sheeman *et al.* study (26), which may have resulted in a protein folding defect that caused a more global perturbation of dynein function.

Implications for Mechanism. Our analysis reveals that dynein operates through a mechanism distinct from other known AAA ATPases. Most AAA proteins have been shown to or are hypothesized to function as homooligomers or heterooligomers in which all or a majority of the subunits are active ATPases (3, 27). The AAA ATPase subunits in these complexes are thought to hydrolyze ATP cooperatively, and ATPase inactivation of a single subunit usually inactivates the complex as a whole (28–32). Dynein, on the other hand, is unique from other AAA ATPase machines in that its function requires only a single hydrolysis site. A second unusual aspect of dynein function is that one of the AAA domains (AAA3) requires nucleotide binding but not hydrolysis for function. This represents the only known example where nucleotide binding alone plays a regulatory role within a hydrolytically competent ring of AAA subunits.

Our results raise questions of how a hydrolytic site (AAA1) and a regulatory nucleotide binding site (AAA3) communicate with each other and with the microtubule binding domain during the mechanochemical cycle. One possible mechanism is that nucleotide hydrolysis and product release at AAA1 causes a power stroke (33), and subsequent dynein dissociation from the microtubule in this poststroke state requires nucleotide binding (or ADP-ATP exchange) at AAA3. These data are consistent with our results that nucleotide binding at AAA3 (but not hydrolysis) is necessary for motor release from the microtubule. This model would predict that the Walker A or B mutations in AAA1 would “freeze” dynein in its prestroke conformation,

whereas the Walker A mutation in AAA3 would freeze the dynein in its poststroke conformation on the microtubule. A second possible mechanism is that nucleotide binding at AAA3 is required to transmit the hydrolysis event at AAA1 to the microtubule binding domain. In this model, nucleotide exchange would not need to occur every cycle and the Walker A AAA3 mutation would be predicted to freeze dynein in its prepower stroke conformation. Moreover, the nucleotide binding state of AAA3 may serve a regulatory function. Suggestive of such an idea, ADP has been shown to stimulate both dynein ATPase activity and dynein motility (34–36), in contrast to kinesin and myosin where it is inhibitory. Perhaps ADP binding at AAA3 constitutes the mechanism of this observed regulatory effect. Both models still raise the question of how AAA1 and AAA3 communicate with one another and the microtubule binding stalk whereas the intervening AAA2 and AAA4 act more passively rather than as nucleotide-active switches as in other AAA ATPase rings (33). Examination of dynein motors bearing mutations in AAA1 and AAA3 by high-resolution electron microscopy will likely provide insight into communication between AAA domains and the microtubule binding domain.

We thank Nicole Mahoney, Andrew Carter, and Kevin Slep for critical reading of the manuscript and Niels Bradshaw for helpful discussions. This work was supported by a National Institutes of Health Program Project Grant P01-AR42895-10 (to R.D.V.). S.L.R.-P. was supported by National Institutes of Health Postdoctoral Fellowship GM67403-01.

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