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Dynein's Binding Ability to Microtubules in Budding Yeast Saccharomyces Cerevisiae

Gregory Lyle Waltz

DYNEIN'S BINDING ABILITY TO MICROTUBULES IN THE BUDDING YEAST *SACCHAROMYCES CEREVISIAE*

by

Gregory Lyle Waltz

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Gregory Lyle Waltz, M.S.

Western Michigan University, 2003

Cytoplasmic dynein is a minus-end directed microtubule motor that recently has been described as a member of the AAA^+ ATPase family. Dyn1p contains four ATP-binding consensus sequences. To understand the role of each ATP-binding site we have begun to systematically mutate the consensus sequence at each ATP-binding site. Thirty-two mutant alleles have been constructed and integrated into the yeast *Saccharomyces cerevisiae*. We have examined each mutant for ability to grow in rich media, to produce binucleate cells at 12°C, ability to grow in the absence of *CIN8*, and ability of Dyn1p to bind microtubules. Attempts to localize cytoplasmic dynein by direct immunofluorescence were not successful when expressed from its promoter. We have thus expressed Dyn1p from the galactose inducible promoter, *GAL1*. Dynein, microtubules and DNA were visualized using standard immunofluorescence techniques. Our results show that wild-type Dyn1p binds at the minus-end of microtubules. No Dyn1p staining is seen within the nucleus. A yeast strain carrying a mutant allele of dynein lacking the microtubule-binding site does not bind to microtubules. Strains mutated in each of the four ATP-binding sites show intermediate phenotypes between wild-type and the strains lacking the microtubule-binding site.

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CHAPTER 1

INTRODUCTION

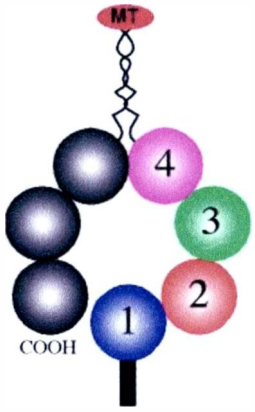
Cell division is one of the basic processes guaranteeing the continuation of life. Prior to cell division the cell duplicates DNA and forms two nuclei each with a complete set of DNA. A cell has to orchestrate the interaction of intracellular components to partition the two nuclei into separate daughter cells that are formed during cell division. Control of this process is critical because failure to properly segregate the duplicated DNA may allow both nuclei to remain within one daughter cell leading to the death of the other daughter cell. One component responsible for cell division is cytoplasmic dynein.

AAA$^+$ Domains and Organization of the Dynein Motor Unit

Proteins that have AAA$^+$ domains are defined as **A**TPases **a**ssociated with a variety of cellular **a**ctivities (Kunau 1993, Neuwald 1999). AAA$^+$ domains are modules that occur either individually or as repeats and this superfamily has members found in prokaryotes and eukaryotes (Neuwald 1999). Dynein is a member the of AAA$^+$ ATPase superfamily.

Several studies have revealed the structure of dyneins several AAA$^+$ domains and have suggested that within each domain there is a potential ATP-binding site,

which are called P-loops. One study using electron microscopy of negative stained images illustrates that the AAA^+ domains formed a complex in the dynein heavy chain containing an apparently central hollow core (Marchese-Ragona 1998). Electron microscopic images confirmed a seven domain circular complex as the head of the dynein heavy chain (Samso 1998, King 2000). After that it was established that dynein contains six AAA^+ domains containing P-loops with cellular activities and a seventh unrelated domain unit within the dynein heavy chain region (King 2000) as portrayed in figure 1.1. The P-loops inside the AAA^+ domains were sequenced in the 1980's.



Based on S. King, Journal of Cell Science 113:2521-2526

Figure 1.1. A drawing of the hexameric ring structure of the AAA domains in dynein. The domain labeled 1 contains a putative binding site were ATP hydrolysis takes place to provide dynein energy for translocation. The domains 2, 3 and 4 can also bind ATP but are doubted to hydrolyze it. The other three domains have similar structure to the first four domains but do not bind ATP. There is a noticeable gap between the 7th domain and the 1st domain indicating the are not connected. There is a coiled coil between the 4th and 5th domain that holds the microtubule-binding site.

In the early 1980's, J. E. Walker identified a very important region of high sequence conservation. This region, Walker A, has a stretch of small hydrophobic residues followed by (Gly/Ala-X-X-Gly-X-Gly-Lys-Thr/Ser), where X pertains to any amino acid residue. All three glycine residues form part of the loop that provides a large pocket (Walker 1982). Many ATP and GTP-binding proteins have a phosphate-binding loop (P-loop). The first P-loop of dynein is of great interest.

The highly conserved first P-loop sequence in dynein is common among a large number of other enzymes and is the principal candidate site for ATPase activity (Gee 1997, Asai; D. J. 2001). ATPase activity provides energy for movement, but the 2nd, 3rd, and 4th P-loops in dynein may also bind nucleotide, perhaps to regulate the dynein molecule (Gee 1998, Eshel 1995, Mocz 1996, Gee 1998). The other P-loops may induce conformational change in the dynein heavy chain that also shields that catalytic site from water (Neuwald 1999). New observations support current structural models in which P-loops adjacent the 1st P-loop interact to support the translocation of the dynein motor along the microtubule lattice (Silvanovich 2003). One interesting fact is that the amino acid sequence of cytoplasmic dynein's 3rd P-loop is fully conserved within different species. Dynein's 3rd P-loop is required for the ATP-induced release of dynein from microtubules. However, if the 3rd P-loop is disabled it does not stop the ability of the 1st P-loop to hydrolyze ATP (Silvanovich 2003). The 5th, 6th and 7th AAA^{+}- like domains lack intact P-loop motif and their domains P-loop are disrupted (Neuwald 1999). These last domains most likely add structural balance to the heavy chain head, but it is doubtful they bind ATP because

nonconserved residues are located in these P-loops (Gibbons 1991, Eshel 1995, Martin 1999). However, those nonfunctional domains may assist in MT binding.

Affects of Amino Acids on Proteins

To begin, the structure and function of a protein depends on its tertiary structure. For example, several types of associations between side chains hold the tertiary structure intact. Lysine (K) is an example of a charged side chain containing an amine (NH_3^+) group. In addition, there are the hydrophobic interactions of nonpolar carbon and hydrogen side chains of amino acids that include alanine (A), glycine (G) and proline (P) (Turner 1998). Hydrophilic interactions include amino acids similar to threonine (T), which has a polar uncharged hydroxyl side chain. Finally, the exchange of one amino acid with another type could affect the structure and function of a protein. Our interest lies with the dynein's heavy chain that has four P-loops with a conserved consensus of GXXXXGKT where X can be any amino acid. Our study focuses on the conserved G, K and T, which were substituted with A.

The amino acid exchange from K to A must have a dramatic effect on the function and conformation of the dynein protein or affect P-loops interaction. Lysine has a side chain consisting of three methylene groups in a series that terminates with a positively charged amino group ($CH_2 -CH_2 -CH_2 -NH_3^+$). Lysine replaced with alanine's non-polar methyl group (CH_3) could have multiple effects. First, the amino group impacts a positive charge on the dynein protein. This conserved lysine can form a salt bridge with any negatively charged group or especially one of the PO_3^-

groups of an ATP molecule. Second, lysine is basic and is not hydrophobic. The substitution of the basic lysine to alanine that is hydrophobic would deter H_2O molecules that are necessary for ATPase activity. The replacement of lysine with alanine in the first P-loop may lower dynein's ability to hydrolyze ATP. The same K to A amino acid substitution in P-loops 2, 3 or 4 may also hinder dyneins ability to localize to microtubules. Other mutations including multiple replacements or large deletions in the dynein heavy chain could greatly affect dyneins ability to utilize ATP or localize to microtubules. However, there is a possibility that double mutations or deletions could provide a fully functional dynein.

To be functionally effective dynein must attach to a microtubule (MT) to be able to travel on it. Dynein consists of a series of domains with the MT-binding region at the end of a stalk protruding from the main head (Gee 1997, Koonce 1997, King 2000). The MT-binding site is approximately 340 amino acid residues downstream from the fourth P-loop region and before the fifth AAA^+ domain (Gee 1997, Gee 1998, Koonce 1997, and King 2000). The MT-binding site is a coiled-coil that extends from the motor region, with a small globular domain at its tip allows for attachment to MT. Dynein attachment to MT is vital for mitotic spindle migration.

The Mitotic Spindle and Mitosis

One important component in higher eukaryotes is the microtubule-organizing center (MTOC) which forms the mitotic spindle. The centrosome is the dominant MTOC in animal cells. Most cells divide symmetrically and cleave at the middle of

the cell, producing two equal new cells (Helfant 2002). Asymmetric cells, such as budding yeast, must segregate the two nuclei between two cells that have unequal size and shape. The division plane of the nuclei relies on the position of the nascent bud (Endow 1997, Hunter 2000). The spindle pole body (SPB) is the MTOC in the yeast *Saccharomyces cerevisiae* (Bullitt 97). The MTOC and the SPB in yeast are different morphologically but equivalent in their function. One difference is that the SPB is laminar and embedded in the envelope of a nucleus that does not disassemble during mitosis (Hoyt 1996, Helfant 2002). However, the SPB nucleates MT similar to MTOC.

The SPB pair duplicates once per cell cycle and nucleates the polymerization of three types of microtubules. One type is the kinetochore MT and the second type are the non-kinetochore while the third type is called cytoplasmic MT. Because yeast have less than 50 MT compared to thousands in higher eukaryotes the genetic tools available make *S. cerevisiae* an excellent system for studying cell division. Moreover, *S. cerevisiae's* small set of MT motors has been helpful in determining their roles (Hoyt 1996). The roles of dynein and dynactin have been determined by directed mutations of each.

During mitosis compromised spindle orientation or nuclear migration can be an affect of mutations in the MT motor dynein or the associated dynactin complex (Stearns 1997). The role of dynein and dynactin was revealed in knock out studies in *Saccharomyces cerevisiae* (Eshel 1993, Li 1993, Clark 1994, Muhua 1994, Karki 1999). For instance, Bi-nucleate cells, although not lethal, are often more numerous

than the wild type with deletions of dynactin and dynein. Often the cause of bi-nucleate cells is caused from improper spindle orientation.

Mitotic spindle orientation is one important task during cell division in all eukaryotes. The responsibility of orientation belongs to astral MT that extend away from the mitotic spindle at the poles. In yeast they are called the astral MT are called cytoplasmic MT. Their function is to provide a method for correct positioning and elongation of the mitotic spindle during cell division. Organisms like budding yeast require the mitotic spindle to be parallel to the mother-bud neck. This ensures that one side of the mitotic spindle has cytoplasmic MT directed selectively toward cortex sites in the bud.

First the orientation of the mitotic spindle depends on the capturing of cytoplasmic microtubule-plus ends to sites in the wall cortex (Beach 2000, Adames 2000, Segal 2000, Liakoploulos 2003). Second, MT motors localized on MT may also attach to the cell cortex or other cytoplasmic structures (Carminati 1997, Shaw 1997). And among these MT motors there is little doubt that dynein and dynactin complexes interact with polarized actin cytoskelton in yeast (Karki 1999). Then forces generated by the MT motors provide proper mitotic spindle orientation (Kilmartin 1984, Snyder 1991). Finally these motors provide directional force to shorten or lengthen microtubules, which then align, and furthermore, move the spindle into position (Stearns 1997, Korinek 2000). Furthermore, these genetic studies have revealed more than one pathway for spindle orientation.

Two redundant pathways orient the mitotic spindle (Miller 1998). The motors

involved in orientation are cytoplasmic dynein and Kip3p while the proteins involved in orientation are Kar9p, Bim1p, Bik1p and the dynein associated complex dynactin (Li 1993, Cottingham 1999, Kahana1998, Miller1998, Eshel 1993, Morris 2000). The first pathway involves the kinesin related cortical binding protein Kar9p and microtubule-binding protein Bim1p capturing microtubule plus-ends near the cortex of the bud. Kar9p participates in cortical capture (Korinek 2000, Lee 2000) while forming a complex with Bim1p. Bim1p also binds with cytoplasmic MT completing the connection of MT to cortical patches in the bud (Adames 2001). Moreover, Bim1p promotes MT disassembly that shortens MT. However, during MT disassembly Bim1p maintains contact with the MT (Yeh 2000). This connection allows the kinesin-related protein Kip3p MT motor to contribute in the orientation of the mitotic spindle. Kip3p either pulls on the MT or Kip3p regulates microtubule length at microtubule ends (Heil-Chapdelaine 1999). The other pathway was discovered during experiments with mutants of the proteins present during mitosis.

Experiments show that cells with Bim1p and Kar9p double mutants are viable (Korinek 2000, Lee 2000) indicating that another orientation mechanism exists. However, cells that have bim1p mutant proteins and exit mitosis commonly become binucleate. Bim1p mutants appear to have a defect in orientating pre-anaphase spindles (Lee L 2000). The most probable cause happens when cytoplasmic microtubules passing through the neck continue to probe the bud cortex and grow longer, often pushing the spindle further from the neck (Adames 2000). Other investigations with the Num1p/ Kar9p double mutants have been shown to behave the

same as dynein/Kar9p double mutants. Also Num1/ Kip3p double mutants behave the same as dynein Kip3p double mutants clearly indicating there are two orientation pathways (Farkasovsky 2001, Miller 1999, Yeh 2000). Finally, Kar9p/Kip3p double mutants were able to survive as were dynein/Kip2p double mutants in consequence indicates the other pathway relies on dynein, Num1p and Kip2p.

The second pathway that positions the mitotic spindle depends on the cortex associated protein Num1p (Heil-Chapdelaine 2000); the minus-end directed microtubule motor cytoplasmic dynein; the dynactin complex, the kinesin related motor Kip2p and the plus-end microtubule associated protein Bik1p (Miller 1998, Adames 2000, Farkasovsky 2001, Liakoploulos 2003). Based on genetic deletions or fluorescence labeling Num1p forms a cortical MT capture-site and colocalizes with dynactin and dynein at microtubules at the cortical sites (Sheeman 2002). Kip2p as part of the dynein-mediated pathway in nuclear migration promotes MT assembly to provide growth while promoting MT stability (Miller 1998). Bik1p is proposed to localize to the plus-end of MT and at cortical sites while recruiting dynein. Dynein mobility is enhanced by Num1p and dynactin contact and provides MT sliding (Sheeman 2002) by pulling on MT (Bloom 2001) helping orientate the mitotic spindle (Adames 2000). After the mitotic spindle is correctly orientated spindle elongation takes place during anaphase.

Spindle elongation depends on the cortical protein Num1p, cytoplasmic dynein and dynactin an actin-binding regulator (Saunders 1995, Yeh 1995, Heil-Chapdelaine 2000). To begin with, Num1p localizes to the cortex of the mother and

bud and is proposed to assist in capturing MT to the cortex (Farkasovsky 2001). Then the presence of dynein contributes to spindle elongation through the neck in budding yeast (Yeh 2000) and a growing quantity of evidence implies that dynein can manipulate microtubule polymerization dynamics (Hunter 2000). Furthermore, dynein and dynactin interact with microtubules at the bud cortex after the mitotic spindle has passed through the mother-bud neck to complete nuclear migration in the bud. These interactions include dynein binding to MT while dynactin binds dynein and the cortex linking the MT to the cortex (Adames 2000). This is accomplished because dynactin promotes dynein motor ability of translocating toward the minus-end of a MT (Sheeman 2002). Finally while being bound to the cortex the force of translocation would provide the force needed to pull the nucleus into the bud neck (Adames 2000).

However, in cells lacking functional dynein or dynactin, spindle movement into the neck can be delayed (Eshel1993, Li 1993, Yeh 1995).

Cells lacking functional dynein exhibited MT that remained at the cell cortex twice as long as wild type cells. For example, in the absence of cytoplasmic dynein, the nucleus does not move equally toward the yeast-bud neck as with wild-type cells. This primarily causes spindle elongation that is confined to the mother cell (Yeh 1995). Equally important, dynein mutants often had long curling MT that failed to display end-on interactions with the cortex (Carminati 1997). Consequently these MT still could pass through the neck and continue to probe the bud cortex and grow longer, sometimes ultimately moving the spindle further from the neck (Adames 2001).

The Molecular Motor Dynein and the Dynein Regulator Dynactin

There are two main types of dynein, axonemal and cytoplasmic. Cytoplasmic dynein was originally identified as a force-generating ATPase in *Tetrahymena cilia* (Gibbons 1965). Later discoveries provided evidence that cytoplasmic dynein powered minus-end directed motion in non-ciliated cells (Paschal 1987). Cytoplasmic dynein provides neurons the ability to migrate in the mammalian brain as well as assisting to translocate nuclei in fungi (Review by Bloom 2001). Equally important, dynein consists of many subunits.

Cytoplasmic dynein is a multi-unit protein-complex that is a MT motor (Vallee 1988, Schroer 1994). One subunit is the heavy chain Dyn1p that contains two protruding structures that house four ATP binding sites and a MT binding site as shown in Figure 1.2. One site is believed to be an ATP-hydrolytic site that provides the energy for dyneins movement along MT (Lye 1987). One less understood subunit is Dyn2p the putative light chain and is required for the complex of dynein to function. Studies indicate that dyneins subunit Pac1p is involved with recruitment of dynein to MT and regulation of motor activity (Coquelle 2002, Hoffmann B 2001, Tai C Y 2002). Data from two-hybrid assays indicated that the mammalian homolog of Pac1p, Lis1p bound two regions of the cytoplasmic Dyn1p. The Pac1p homolog Lis1p bound the first AAA repeat (P1 loop) implicated in motor activity while also binding to the N-terminal domain implicated in cargo binding (Sasaki, 2000, Tai, 2002). In addition, Pac1p associates with the protein Bik1p that also recruits dynein to cytoplasmic MT (Coquelle 2001). Bik1p appears to be in the same pathway as

dynein because Bik1p null mutants displayed greatly reduced or absence of dynein to cytoplasmic MT (Sheeman 2003). As well, another important dynein subunit is the intermediate chain Pac11p that is the scaffold for the whole dynein complex. This intermediate chain Pac11p can simultaneously bind to the dynein heavy chain Dyn1p as well as Dyn2p, the three light chain subunits (Vale 2003). Additionally, Pac11p also associates with MT and the Nip100p subunit of dynactin (Kahana 1998, Susalka 2002).

Although not constantly associated, the multi-subunit dynactin complex does assist cytoplasmic dynein (Hirokawa 1998). The role of dynactin appears to be regulatory since interruption of this complex impedes transport normally provided by cytoplasmic dynein (Burkhardt 1997). Phenotypes of the dynactin subunit Arp1p null mutant behave the same as dynein null mutants (Muhua 1998) implicating dynein and dynactin work in the same pathway. Dynactin is shown in Figure 1.2 and has three subunits that are best understood and are necessary to promote dynein processivity. Furthermore, they contributes to MT binding and cortical patch anchorage. Those subunits are Nip100p, Arp1p and Jnm1p. Nip100p not only associates with dyneins Pac11p but also to binds microtubules (Waterman-Storer 1995). The dynactin subunit Jnm1p is required for proper spindle partitioning during mitosis. Genetic depletion experiments indicate that the binding of Nip100p to Arp1p's homolog Act5p, the cortical binding subunit of dynactin, is dependent on the presence of Jnm1p (Kahana 1998). To summarize, the subunits of dynein and dynactin that have been described and are shown in Figure 1.2 are necessary for a timely and successful mitosis.

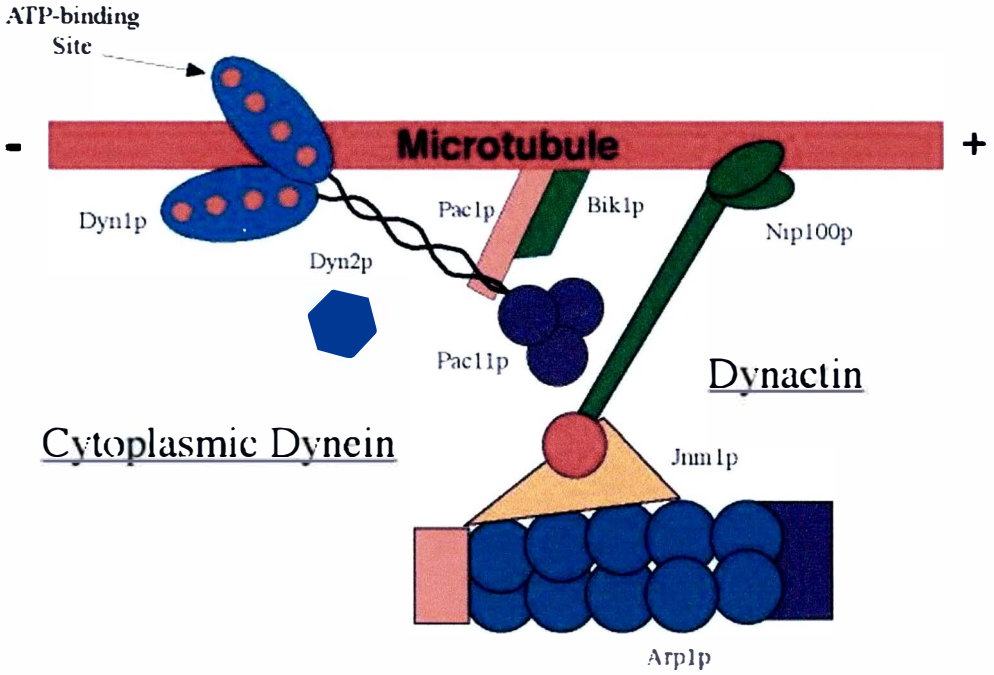


Figure 1.2 Model for cytoplasmic dynein and dynactin complexes. Dynein subunit Dyn1p has two globular heads each containing four ATP binding sites at one end of the heavy chain while Pac1p and the intermediate chains Pac11p bind the other end. Dyn2p, the putative light chain, is necessary for proper dynein function but the location of Dyn2p in the dynein complex is not clear. Bik1p, a dynein recruiter protein, simultaneously bind to Pac1p and MT. Both Pac1p and the globular heads associate with a microtubule while Pac11p Binds to Nip100p the subunit of dynactin. The subunit of dynactin Nip100p not only binds to dynein through pPac11p but Nip100p binds MT. The presence of Jnm1p is required for connecting Nip100p to Arp1p the cortical binding subunit of dynactin.

CHAPTER 2

RESULTS

Focus of Study

The theory behind this study involves two beliefs. First, dynein is a microtubule motor that binds to mitotic spindle's cytoplasmic microtubules. Second, if dynein has directed mutations in any of its four P-loops, then the dynein mutant could not bind microtubules. Then we wanted to observe dynein, microtubules and DNA in budding yeast cells during mitosis. Finally, our goal was to determine the if the dynein mutants could bind microtubules.

It has been established that dynein binds cytoplasmic microtubules of the mitotic spindle during mitosis (Yeh 1995). Then dynein hydrolyzes ATP to obtain energy to travel towards the minus-end of cytoplasmic microtubules (MT). Even if dynein travels toward the minus end of MT how does that contribute successful mitosis? One theory is that the dynein is recruited to the cell wall cortex and binds to the cortex by an interaction with the dynactin complex that is also at the same cortical site. Then dynein binds to MT that have been recruited toward the cortex site and pulls on those MT by attempting to move toward the minus-end of the microtubule (Heil-Chapdelaine 2000). However, our study investigates only the binding of dynein to MT.

Dynein Mutants

To achieve our objective Dr. John Geiser created 32 dynein mutants that contain mutations in one or several of the four putative ATP binding sites. These sites are also called P-loops because they may bind phosphate. Our study concentrates on the highly conserved glycine (G), lysine (K) and threonine (T) amino acids in a P-loop and they were substituted systematically with the amino acid alanine (A). In contrast, the entire P-loop sequence was removed in all four individual P-loops. In other instances a P-loop and the microtubule-binding site were both removed. To illustrate, Table 2.1 describes the four P-loops of the wild type dynein heavy chain

Table 2.1

The Amino Acid Sequence of P-loops

P-loop	Amino acids
P1	GPAGTGKT
P2	GKAGCGKT
P3	GPPGSGKT
P4	GASRTGKT
Consensus	GXXXXGKT

For the microtubule-binding mutant the P-loop sequences were left intact but the microtubule-binding site that follows after P-loop 4 has been removed. One example of a typical mutation would be the K1A mutant that only has the lysine replaced with alanine in the 1st P-loop: (GXXXXG**K**T) → (GXXXXGAT). However, the ΔP1 dynein mutant has had the entire 8 amino acid sequence of the P-loop removed.

Another example may be a double mutant such as the K12A dynein mutant that has had K changed to A in both P-loops one and two. However, a different type of double mutation would be the K1AΔMT dynein mutant, which has had K changed to A in the first P-loop, and the microtubule-binding site removed. Typically, the other dynein mutants were constructed in the same fashion.

The inserted dynein gene has a galactose promoter to promote gene expression approximately 100 fold over natural expression. Fluorescent microscopy was utilized to visualize dynein, microtubules and DNA during mitosis. The purpose of immunofluorescence is to detect where a protein is in the cell. In our study, we used antibodies for the *tub1* protein of the microtubules that are part of the mitotic spindle in yeast. In fact this antibody only recognizes the *tub1* protein of microtubules. Just as important, all yeast strains contain a genomic mutant insertion of the dynein heavy chain with a HA epitope used for antibody labeling. The cellular DNA was stained with 4',6-diamidino -2-phenylindole (DAPI).

To compare the dynein P-loop mutants we looked at wild type and a micro-tubule binding mutant that lacks microtubule-binding ability. We have recorded wild type and the 32 dynein mutants. Native expression levels of dynein could not be visualized but overexpression of dynein allowed images to be recorded. However, overexpression may exaggerate the ability of dynein to bind MT. In spite of this, we felt this method would establish if dynein mutants bind cytoplasmic microtubules. To demonstrate, images of wild type, the microtubule-binding mutant and dynein mutants are shown in the next nine figures.

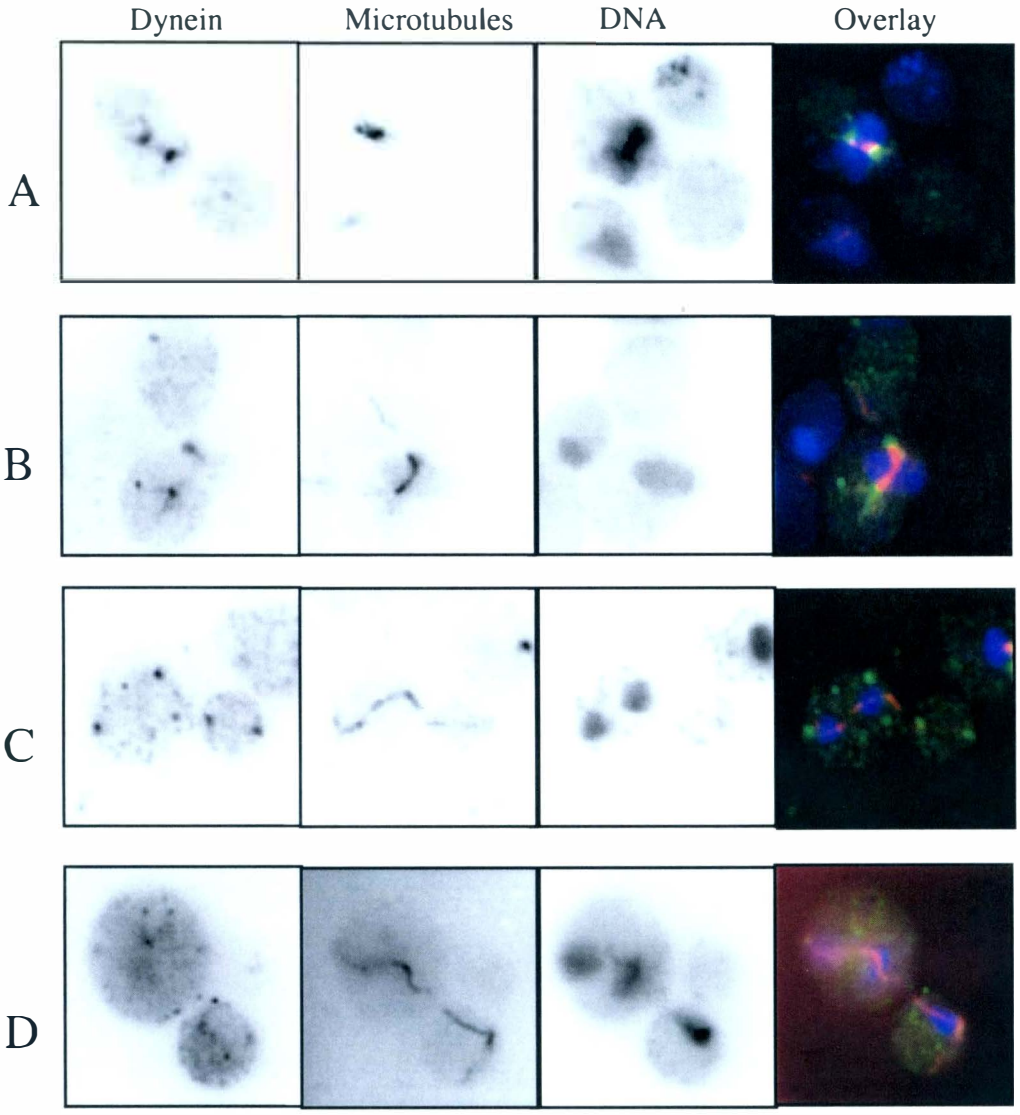


Figure 2.1. Wild type JGY234 and the MT-binding mutant JGY251. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae.* In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino -2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were falsec olored with dynein green, microtubules red and DNA blue. Wild type dynein is shown in rows A and B. The MT-binding mutant is shown in rows C and D.
(A,B) P_{GAL1}dyn1-HA (JGY234). (C,D) P_{GAL1} dyn1-ΔMT-HA (251).

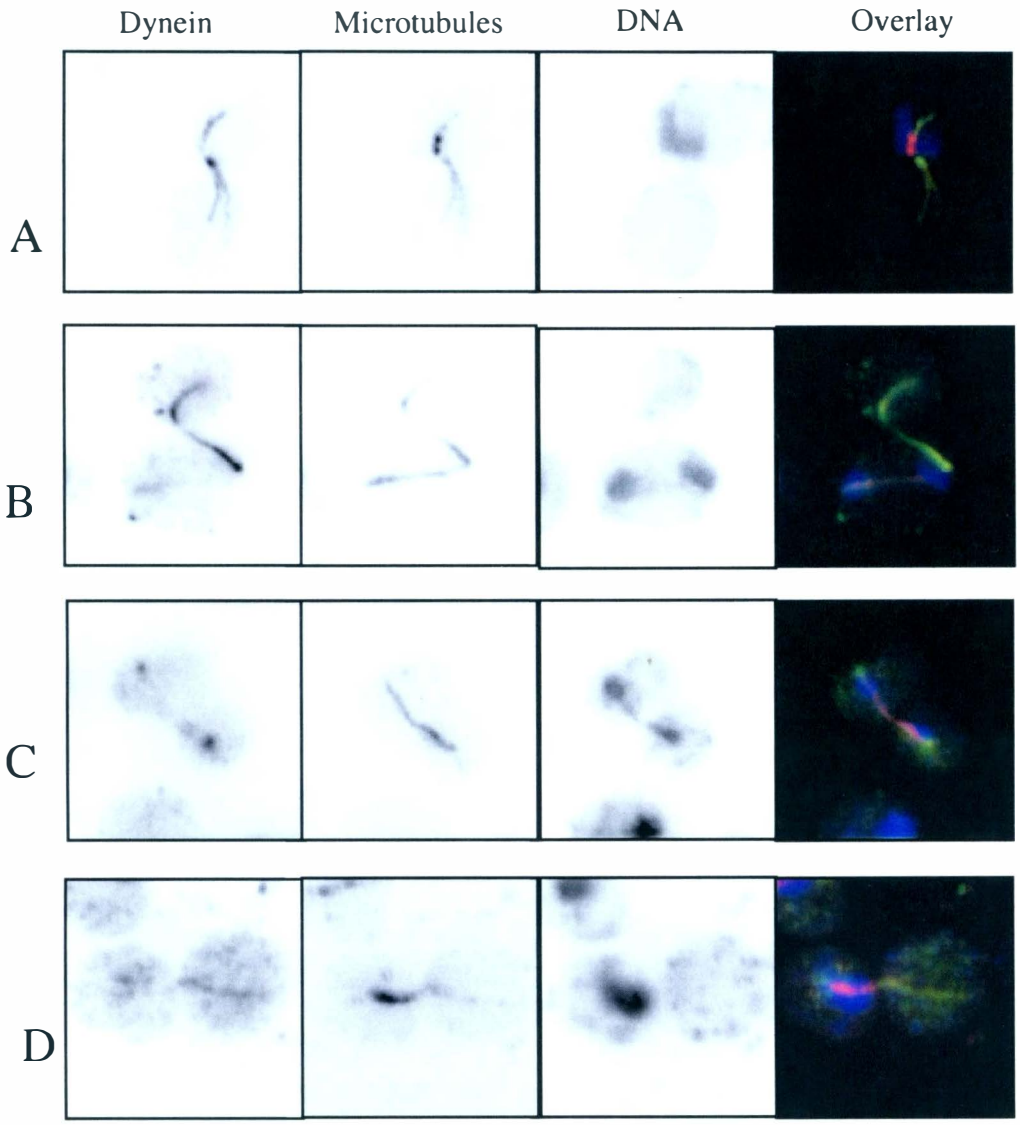


Figure 2.2. Mutant dyneins JGY242, JGY244, JGY246 and JGY248. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae.* In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were false colored with dynein green, microtubules red and DNA blue. Rows A, B, C and D have had K to A replacement in P-loops 1, 2, 3 and 4 respectivally. (A) P_{GAL1} dyn1-K1A-HA (JGY242). (B) P_{GAL1} dyn1-K2A-HA (JGY244). (C) P_{GAL1} dyn1-K3A-HA (JGY246). (D) P_{GAL1} dyn1-K4A-HA (JGY248).

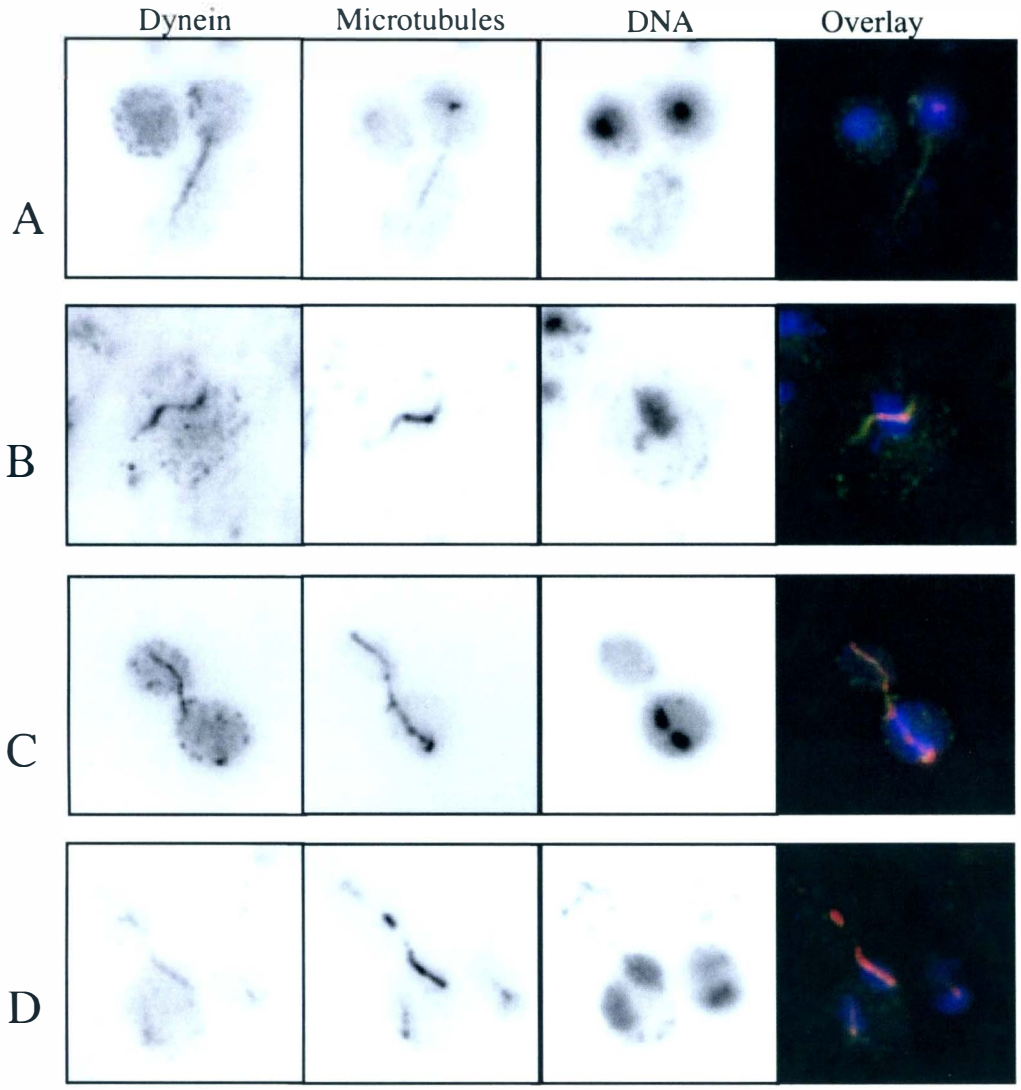


Figure 2.3. Mutant dyneins JGY 243, JGY245, JGY247 and JGY250. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were false colored with dynein green, microtubules red and DNA blue. Rows A, B, C and D show images of mutants that have had the entire P-loops 1, 2 3 and 4 removed respectivally.
(A) P_{GAL1} dyn1-ΔP1-HA (JGY243). (B) P_{GAL1} dyn1-ΔP2-HA (JGY245).
(C) P_{GAL1} dyn1-ΔP3-HA (JGY247). (D) P_{GAL1} dyn1-ΔP4-HA (JGY250).

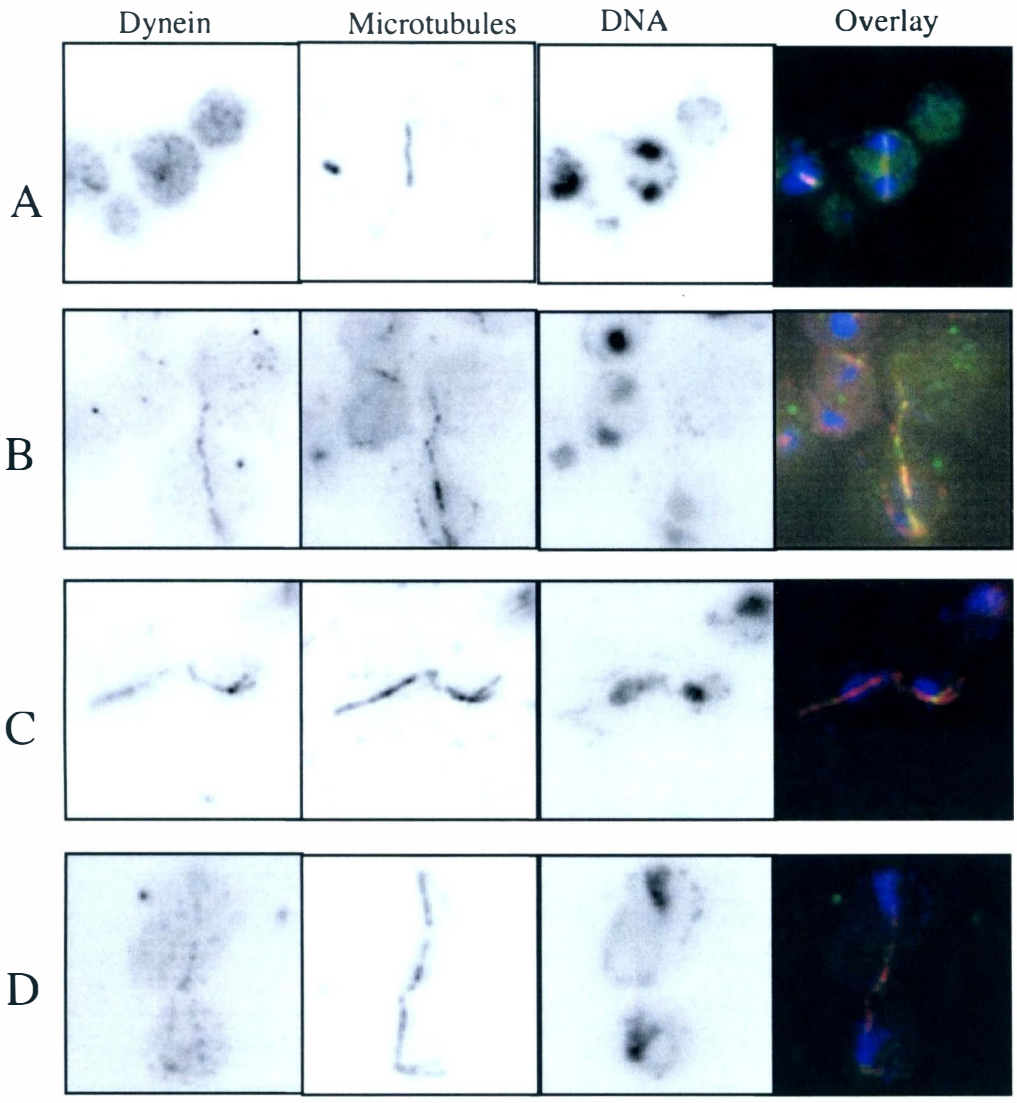


Figure 2.4. Mutant dyneins JGY241, JGY253, JGY255 and JGY256. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were false colored with dynein green, microtubules red and DNA blue. Rows A, B, C and D have had multuple P-loop K to A substutions.
(A) P_{GAL1}dyn1-K1234A-HA (JGY241). (B) P_{GAL1} dyn1-K124A-HA (JGY253).
(C) P_{GAL1}dyn1-K123A-HA (JGY255). (D) P_{GAL1}dyn1-K134A-HA (JGY256).

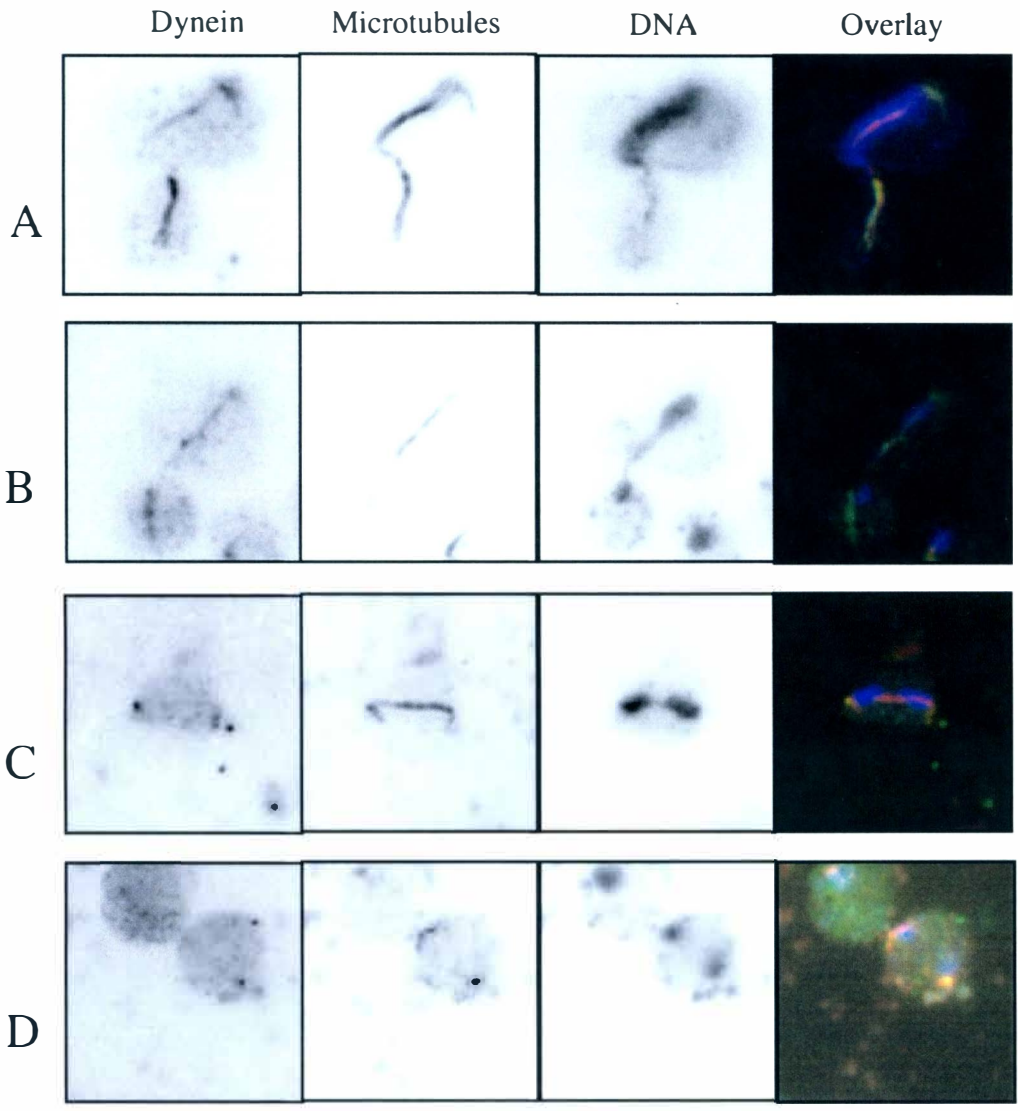


Figure 2.5. Mutant dyneins JGY237, JGY238, JGY239 and JGY240. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were false colored with dynein green, microtubules red and DNA blue. Rows A, B, C and D have had multuple P-loop K to A substutions.
(A) P_{GAL1} dyn1-K23A-HA (JGY237). (B) P_{GAL1} dyn1-K24A-HA (JGY238).
(C) P_{GAL1} dyn1-K34A-HA (JGY239). (D) P_{GAL1} dyn1-K234A-HA (JGY240).

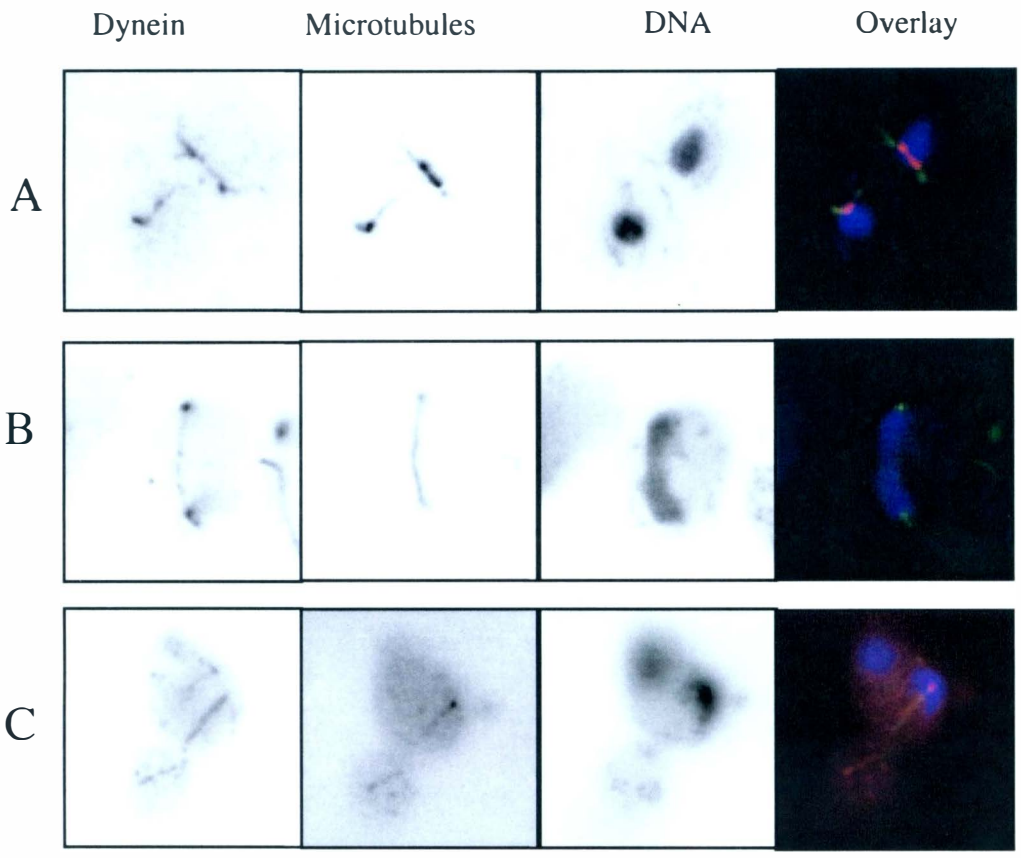


Figure 2.6. Mutant dyneins JGY235, JGY236 and JGY257. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibodyYOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images werefalse colored with dynein green, microtubules red and DNA blue. Rows A, B, and C have had multuple P-loop K to A substutions.
(A) P_{GAL1} dyn1-K12A-HA (JGY235). (B) P_{GAL1} dyn1-K13A-HA (JGY236).
(C) P_{GAL1} dyn1-K14A-HA (JGY257).

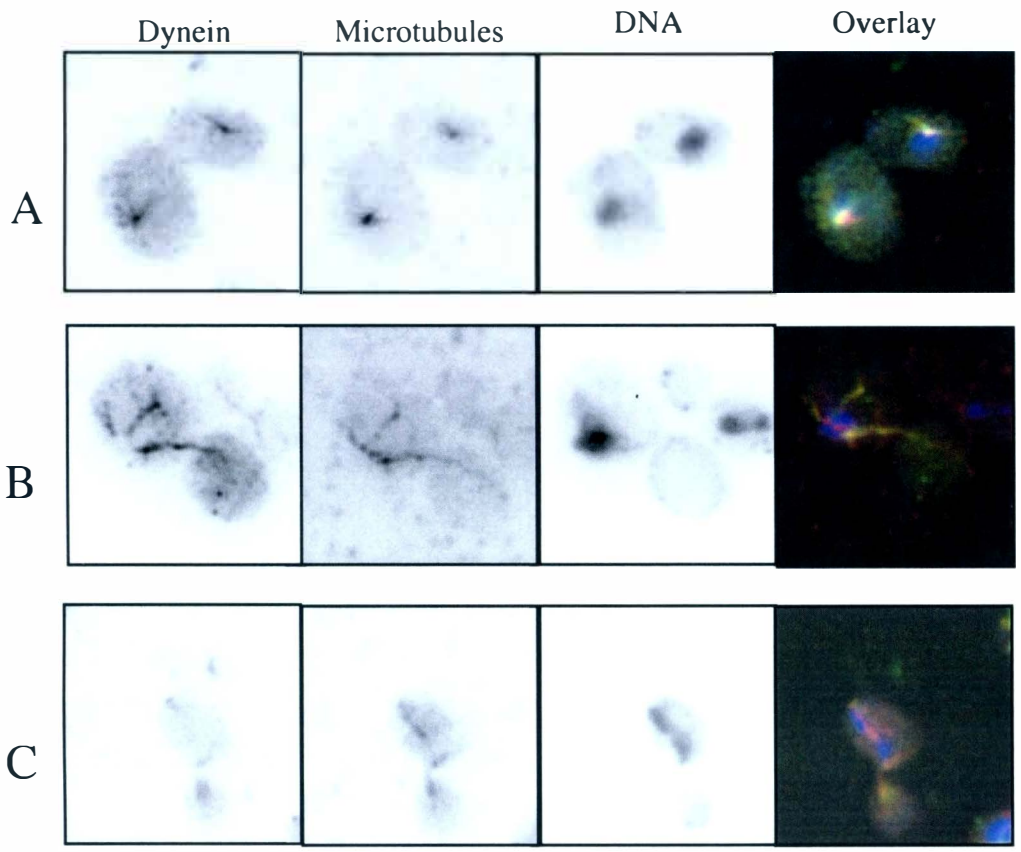


Figure 2.7. Mutant dyneins JGY697, JGY698 and JGY252. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and poly-clonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole(DAPI) andimaged with a DAPI filter set. Overlay of all three images wserefalse colored withdynein green, microtubules red and DNA blue. Row A has had T replaced with A while row B has had GKT all replaced with A and row C had deletions of both P-loops 2 and 3.
(A) P_{GAL1}dyn1-T4A-HA (JGY697). (B) P_{GAL1}dyn1-GKT2-HA (JGY698).
(C) P_{GAL1}dyn1-ΔP23-HA (JGY252).

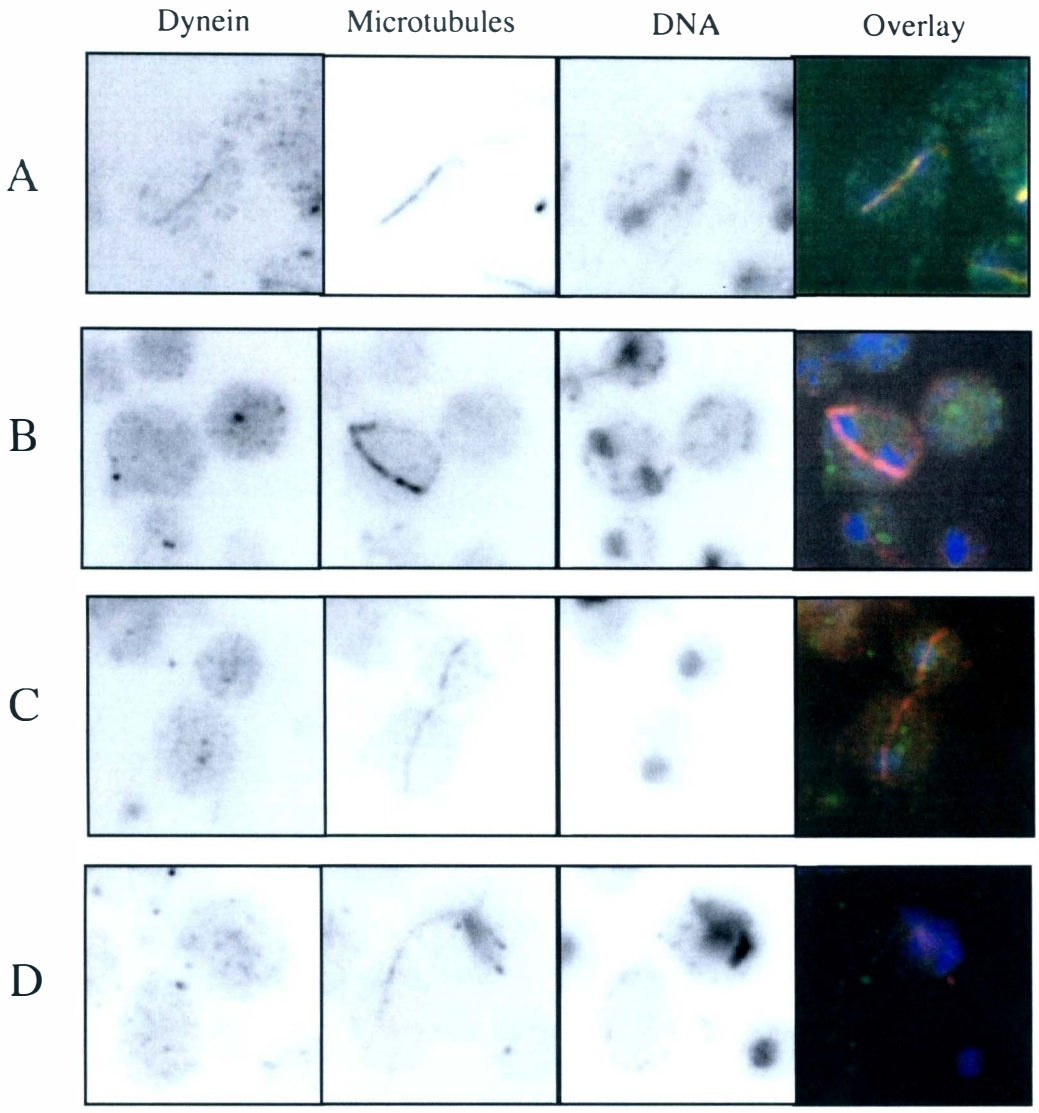


Figure 2.8. Mutant dyneins JGY699, JGY695, JGY694 and JGY696. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In The Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images werefalse colored with dynein green, microtubules red and DNA blue. Row A, B, C and D have had the MT-binding site removed and the K to A P-loop replacement in P-loops 1, 2, 3 and 4 respectivally.
(A) P_{GAL1}dyn1-K1AΔMT–HA (JGY699). (B) P_{GAL1}dyn1-K2AΔMT–HA(JGY695).
(C) P_{GAL1}dyn1-K3AΔMT–HA (JGY694). (D) P_{GAL1}dyn1-K3AΔMT–HA (JGY696).

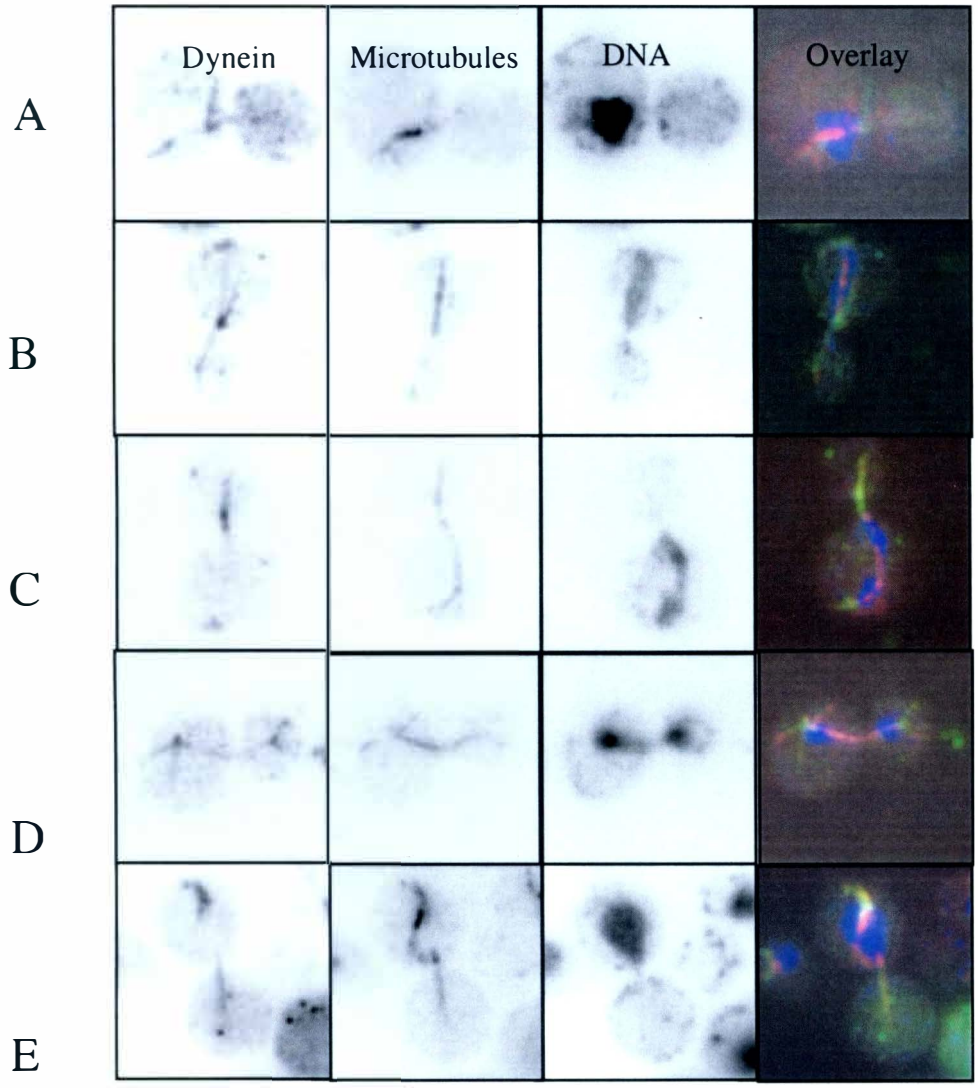


Figure 2.9. Mutant dyneins JGY700, JGY710, JGY711, JGY712 and JGY713. Immunofluorescence imaging of dynein, microtubules and DNA in *S. cerevisiae*. In the Dynein column, Dyn1 was labeled with mouse monoclonal anti-HA and CY2 conjugated polyclonal goat anti-mouse IgG and imaged with a FITC filter. In the Microtubule column, tubulin is labeled with rat monoclonal anti-tubulin antibody YOL1/34 and polyclonal Texas Red conjugated goat anti-rat IgG and imaged with a Texas Red filter. In the DNA column, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged with a DAPI filter set. Overlay of all three images were false colored with dynein green, microtubules red and DNA blue. Rows A, B, C, D and E had amino acids replaced with A in different combinations.
(A) P_{GAL1} dyn1-GKT4-HA (JGY700). (B) P_{GAL1} dyn1-G3A1-HA (JGY710).
(C) P_{GAL1} dyn1-T2A-HA (JGY711). (D) P_{GAL1} dyn1-T3A-HA (JGY712).
(E) P_{GAL1} dyn1-KT2AA-HA (JGY713).

Comparisons between Dynein Mutants Binding to MT

The differences between wild type and the MT-binding mutant are seen in Figure 2.1. Rows A and B are wild type displays heavy binding of dynein to MT, which serves as the positive control. In contrast, rows C and D show the dynein MT-binding mutant did not bind to MT but dynein was seen elsewhere in the cell. The MT-binding mutant serves as the negative control.

A comparison of the dynein mutants with K to A mutations in P-loops 1 through 4 are shown in Figure 2.2. The dynein mutant K1A appeared to bind strongly to MT but the MT appeared longer than wild type. The K2A dynein mutant appeared to bind strongly to MT and the MT are longer than the wild type. The K3A dynein mutant appeared to bind strongly to short MT. The K4A dynein mutant appeared to bind only lightly to MT. The K4A mutant also had long MT and dynein was seen elsewhere in the cell.

A comparison of the dynein mutants with complete deletion of all eight amino acids in P-loops 1 through 4 are shown in Figure 2.3 The dynein mutant ΔP1 appeared to bind strongly to microtubules but the MT appeared longer than wild type. The ΔP2 dynein mutant appeared to bind strongly to MT. The ΔP3 mutant appeared to bind to long MT but dynein was also seen elsewhere in the cell. The ΔP4 dynein mutant appeared to only bind lightly to MT. The ΔP4 mutant also had long MT and dynein was seen elsewhere in the cell.

Three different degrees of MT binding were observed in images shown in Figures 2.4, 2.5, 2.6, 2.7 and 2.9. First, the dynein mutants that displayed strong MT

binding were GKT4, G3A1, T2A, KT2A, T4A, GKT2, K12A, K23A, K123A, K124A and L1234A. Second, the dynein mutants that displayed intermediate MT binding were K24A, K34A, K234A, K14A and the ΔP23 mutant. Finally, the dynein mutant K134A displayed slight MT binding.

A comparison of the dynein mutants having the MT-binding site removed and lysine changed to alanine in P-loops 1 through 4 are shown in Figure 2.8 The K1AΔMT dynein mutant appeared not to bind to MT but the MT appeared longer than wild type. The K2AΔMT dynein mutant appears not to bind to MT. When observing the K3AΔMT dynein mutant it was noticed that there was slight binding to MT. Dynein was also seen elsewhere in the cell. The K4AΔMT dynein mutant appears not to bind to MT but dynein was seen elsewhere in the cell.

Degree of MT Binding

Wild type dynein and any mutant dynein that heavily localized to cytoplasmic microtubules were scored (+) as shown Table 2.2. Whereas any mutant dynein that did not localize to cytoplasmic microtubules resembling the dynein microtubule-binding mutant was scored (-). Two other scores in this study were the intermediate localization that was scored as (+/-), and the light localization that was scored as (-/+). All of the images had DNA, microtubules and dynein present. Microtubules were always present and relatively close to any DNA present in the cell. Most of the cells displayed dynein localized to microtubules. However, the degree of dynein localization to microtubules varied for several mutants.

The images that rate (+) because of heavy localization to MT were: Wild type, mutants K1A, ΔP1, K2A, GKT2, ΔP2, K3A, ΔP3, T4A, K12A, K13A, K23A, K123A, K124A, K1234A, GKT4, G3A1, T2A, T3A and KT2AA. The images that rate (+/-) because of intermediate localization to MT were K14A, K24A, K34A, K234A and ΔP23. The images that rate (-/+) because light localization to microtubules were K3AΔMT, K4A, ΔP4 and K134A.

The images that rate (-) because of no localization to MT were ΔMT, K1AΔMT, K2AΔMT and K4AΔMT.

Table 2.2

Various Binding Levels of Dynein Mutants to MT

DYN1 Allele	Binding	*DYN1* Allele	Binding	*DYN1* Allele	Binding
WT	+			ΔMT	-
K1A	+	ΔP1	+	K1AΔMT	-
K2A	+	ΔP2	+	K2AΔMT	-
K3A	+	ΔP3	+	K3AΔMT	-/+
K4A	-/+	ΔP4	-/+	K4AΔMT	-
GKT4	+	K12A	+	K14A	+/-
G3A1	+	K13A	+	K24A	+/-
T2A	+	K23A	+	K34A	+/-
T3A	+	K123A	+	K234A	+/-
KT2A	+	K124A	+	ΔP23	+/-
T4A	+	K1234A	+		
GKT2	+			K134A	-/+

Data from another study (Geiser 1997) displayed in Table 2.3 shows double mutants of dynein mutants and the microtubule motor Cin8p. The table also indicates the sensitivity of the mutants to temperature. Twenty-two of the twenty-five dynein mutants in this study perished when expressed in the absence of Cin8p. In addition, it was observed that when grown at 12 degrees Celsius all but three dynein mutants had a high ratio of binucleate cells. Also the scores of the binding to MT of dynein mutants and controls are listed for to assist in correlation of effects.

Table 2.3

Double Mutants *DYN1* and *CIN8*

DYN1 Allele	Growth	% Binuc at 12°C	Δ cin8	GAL growth	loc
WT	+	1	+	+	+
ΔMT	+	37	-	+	-
K1A	+	38	-	-	+
K1AΔMT	+	26	-	+	-
ΔP1			-	+/-	+
K2A	+	6	-	+	+
K2AΔMT	+	52	-	+	-
GKT2	+	47	-	+/-	+
ΔP2	+	51	-	+/-	+
K3A	+	67	-	-/+	+
K3AΔMT	+	36	-	+	-/+
ΔP3	+	46	-	+	+
K4A	+	2	+	+	-/+
K4AΔMT	+	37	-	+	-
T4A	+	4	+	+	+
ΔP4	+	38	-	+	-/+
K12A	+	38	-	+	+
K13A	+	31	-	+	+
K14A	+	51	-	+	+/-

K23A	+	42	-	+	+
K24A	+	14	-/+	+	+/-
K34A	+	39	-	+	+/-
K123A	+	46	-	+	+
K124A	+	37	-	+	+
K134A	+	32		+	-/+
K234A	+	42		+	+/-
K1234A	+	40	-	+	+

Table 2.3. Amino acid changes in the P-loop consensus (GXXXXGKT) appear in the *DYN1* column. Normal growth is shown in the Growth column compared to growth in the GAL column and the percentage of binucleate cells is shown in the % Binuc column. Dynein mutant survival data without cin8p is shown in the Δcin8 column and the rating for localization to microtubules is shown in the loc column. Source: Dr John Geiser 1997.

CHAPTER 3

DISCUSSION

Cytoplasmic Dynein in Yeast

Dynein is located normally on the cytoplasmic microtubules (MT's) in yeast. Wild type dynein appears symmetrically distributed along the length of both mother and daughter cytoplasmic MT's (Bloom 2001). The sole function of cytoplasmic MT's in growing yeast cells is to localize and orient the mitotic spindle (Huffaker 1988). A yeast strain carrying a mutant allele of dynein lacking the microtubule-binding site serves this study as the negative control. We have observed that the positive control wild type dynein localizes heavily to cytoplasmic MT's and was scored as (+) while the microtubules-binding mutant does not localize to MT's and was scored (-).

Most of our dynein mutants were observed to be localized to cytoplasmic microtubules minus-end. And then some dynein mutants localized to MT's but also displayed dynein elsewhere in the cell. These mutant strains were K4A, ΔP4, K14A, K1234A, K134A, K234 GKT4 and all of the double mutants containing the ΔMT mutation.

Nineteen dynein ATP binding-site mutants display wild type and scored (+)

localization to MT's. Three other ATP binding-site mutants appear similar to the microtubule-binding mutant and did not localize to MT's and were scored (-). In contrast, several mutants display in-between localization to MT's. Nine of the dynein mutants appear to localize to MT's somewhere between (+) or (-). These nine are divided into two collections where one collection of five localizes intermediate (+/-) and the other collection of four localizes lightly (-/+). However, many of the dynein mutants observed do have dynein localized in the vicinity of the mitotic spindle.

Dyneins Relationship with Temperature and *CIN8*

Data from another study (Geiser 1997) demonstrates that most dynein P-loop mutants are not viable unless the microtubule motor Cin8p is present in the cell. However, wild type dynein is viable in the absence of Cin8p, which establishes dyneins importance in being able to localize to MT's. Table 2.3 shows that twenty-two out of twenty-five dynein mutants perished when expressed in the absence of Cin8p. In addition, it was observed that when grown at 12 degrees Celsius all but three dynein mutants had a high ratio of binucleate cells (Geiser 1997). This demonstrates the importance of localization and binding of dynein to MT's. Scores of the localization to MT's of dynein mutants and controls are also listed on Table 2.3.

Conclusions about the Hypothesis

Dynein's heavy chain is an assembly of 6 P-loop domains forming a hexameric ring (Silvanovich 2003). The first 4 domains contain a consensus of nucleotide tri-

phosphate-binding motifs. These arrangements may cause a mutation in one P-loop to effect the other P-loops or the microtubule-binding site located after P-loop 4. The results collected and interpreted helped us answer the hypothesis we set out to address.

The hypothesis "If mutations are made in conserved residues important for binding microtubules, then dynein will not localize to microtubules." was proven false because all of the dynein mutants bound to microtubules. However, some of the mutants bound to a much lesser degree than wild type. Furthermore, because of overexpression of the dynein mutants there could be artifacts that could cause incorrect conclusion as to whether dynein was indeed bound to microtubules. One other odd observance was the MT-binding double mutant K3AΔMT that appeared to bind lightly to MT. Whether the mutation caused MT binding or selective recruitment to MT needs to be addressed with the K3AΔMT mutant and a nonfunctional Bik1p that is a dynein recruiter protein. Another double mutant candidate would be dynactin's subunit Nip100p that binds dynein and MT.

However, we are interested in the binding of dynein to microtubules and that makes microtubule stability dynamics important to this experiment. But because dynein was over-expressed approximately 100-fold using the inducible galactose promoter, it is possible that the images contained some artifacts.

In fact other studies indicate that dynein, besides localizing to the minus-end of MT's, has also been observed to accumulate at the plus-end or polymerizing ends of MT's (Xiang 2000) and at the cell wall cortex. Nonetheless, these observations

may have been results of overexpression. Thus, the ability to collect data from native expression of cellular proteins would clarify dyneins location in the cell and amount of dynein binding to MT.

Future Directions

In complex systems, various components interact with each other in complex ways. By changing one component of the system, the results may deliver an unintended outcome. It is far from an easy task to be sure that changing the system did not deliver slanted results. Therefore, we plan to confirm our present results by a method of labeling cellular components in live cells using Green Fluorescent Protein (GFP). Dynein mutants labeled with GFP expressed at native levels (a more natural environment) will eliminate artifacts created by overexpression of dynein which may be long MT's. Further testing of P-loop 4 needs to be done with mutants that would cause an effect on localization to microtubules. These could include one of dynactin's subunits or other dynein subunits besides the heavy chain or microtubule-binding site. Images of GFP-dynein localizing to MT's in a pilot study were obtained with success. To enhance observation at native levels, images were obtained at different depths of view called z-slices and were overlaid. The results were single images of the cell(s) under investigation capturing z-slices and overlaying them into a two-dimensional image.

Other plans include other mutant subunit dyneins along with dynactin mutants. These double mutant studies will confirm that single mutant results were not from indirect influences.

CHAPTER 4

METHODS

Yeast Strains

The mutant strains used in this study are described in Table 4.1

Table 4.1

Yeast Strains

Strain	Genotype	Plasmid
JGY234	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-DYN1-HA	pJG341
JGY 235	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K12A-HA	pJG341
JGY236	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 $_{GAL1}$-dyn1-K13A-HA	pJG341
JGY237	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K23A-HA	pJG341
JGY238	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K24A-HA	pJG341
JGY239	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K34A-HA	pJG341
JGY240	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K234A-HA	pJG341
JGY241	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K1234A-HA	pJG341
JGY242	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K1A-HA	pJG341
JGY243	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K13A-HA	pJG341
JGY244	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-ΔP1-HA	pJG341
JGY245	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-ΔP2-HA	pJG341
JGY246	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K3A-HA	pJG341
JGY247	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1	pJG341

	P_{GAL1}-dyn1-ΔP3-HA	
JGY248	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K4A-HA	pJG341
JGY250	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-ΔP4-HA	pJG341
JGY251	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-ΔMT-HA	pJG341
JGY252	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-ΔP23-HA	pJG341
JGY253	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K124A-HA	pJG341
JGY255	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K123A-HA	pJG341
JGY256	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K134A-HA	pJG341
JGY257	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-K14A-HA	pJG341
JGY694	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-P3ΔMT-HA	pJG341
JGY695	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-P2ΔMT-HA	pJG341
JGY696	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-P4ΔMT-HA	pJG341
JGY697	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-T4A-HA	pJG341
JGY698	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-GKT2-HA	pJG341
JGY699	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-P1ΔMT-HA	pJG341
JGY700	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-GKTP4AAA-HA	pJG341
JGY710	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-G3A1-HA	pJG341
JGY712	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-T3A-HA	pJG341
JGY713	MATa lys2-801 his3-Δ200 leu2-3,112 ura3-52 pac11-Δ1 P_{GAL1}-dyn1-KT2AA-HA	pJG341
Plasmid	Plasmid Markers	Parent
pJG341	CEN6 ARSH4 LEU2 P_{GAL}1-PAC11-$CYC1_{term}$	p415 GAL1

Media

Wild type and mutant yeast were grown on Yeast Peptone Dextrose Agar. To prepare cells for experimental treatments the yeast cells were grown in growth

synthetic defined (SD) broth supplemented with a 1X amino acid mix without leucine, 50 μg of adenine per ml and 25 μg of uracil per ml. The medium was also prepared to initially contain 2% raffinose as the yeast's carbon source. Galactose was added to obtain a 2% concentration in the broth after reaching the logarithmic growth phase.

Growth of Wild Type and Mutant Dyneins

Wild type and mutant strains of the yeast *Saccharomyces cerevisiae* were grown to mid-log, 60 Klett (K) ~10^8 cells/ml, in a shaker bath at 30°C. The next day the dynein strains were diluted back to 30 K and allowed then to grow to 60 K in a shaker bath at 30°C. The broth media was then adjusted to have a 2% galactose concentration to induce the galactose promoter. Then the yeast cells were allowed to grow for 3 hours. After the 3 hours of incubation the cells were prepared for formaldehyde fixation.

First the dynein mutants were produced by site directed mutagenesis utilizing plasmid insertion for homologous recombination of the dynein heavy chain gene *DYN1*. Additionally the wild type and dynein mutant strains also have a plasmid that allows growth in media lacking leucine. Equally important, the inserted dynein mutant gene has an inducible galactose promoter.

Yeast Cell Fixation Procedures

First the cells were fixed by adding of 37% formaldehyde to broth media for a final 3.7% concentration. Second the yeast cells were incubated at room temperature for 30 minutes. Following that the cells were centrifuged at high-speed, 1400rpm, for

30 seconds. Then the cells were resuspended in liquid a phosphate buffer solution containing 40mM K_2HPO_4, 10mM KH_2PO_4 and 0.5M NaCl was used. Just before use this solution had bovine serum albumin added to a final concentration of 0.04% (PBS/BSA) to serve as a protein blocker during antibody treatment. The cells were then resuspended in PBS/BSA and stored at 4° C.

Spheroplasting

The yeast cells were first sonicated with a rod sonicater on level 3 for five seconds to separate the buds from mother cells. Then the cells were centrifuged in a microcentrufuge at high speed, 1400 rpm, into a pellet. A zymolyase solution was freshly prepared containing 3.0ml 1.2M Sorbitol, 1.0ml 0.1M KPO_4 at pH 7.5, 8.7µl 25 mM *beta*-mercaptoethanol, 12.5µl 20µg/ml zymolyase 20T and 1 ml H_2O. To permeabilize the yeast cells they were resuspended in 0.5 ml of the zymolyase solution. The cells were incubated at 30°C for one hour at 30 rpm in a rotating incubator. After the zymolyase treatment, the cells were centrifuged into a pellet on slow speed, 300 rpm, for 30 seconds in a microcentrifuge. The cells were resuspended and concentrated by adding PBS/BSA to equal 10% of the original volume. The cells were gently mixed by tritation in buffer and kept on ice until needed.

Polylysine Coating of Slides and Addition of Cells

All manipulations were done in a dark moisture chamber. Each well on the immunofluorescence slide received 20µl of 0.1% polylysine that was dissolved in

distilled H_2O. The polylysine in the wells was allowed to dry at 37°C. The slide was then washed vigorously with distilledH_2O and allowed to dry at 37°C. The slide wells were washed three times with PBS/BSA.

The wells were aspirated and 20µl of cells were added to each well. The slide was incubated in a moisture chamber at room temperature for 5 minutes. The wells were aspirated and washed three times with PBS/BSA. The wells were aspirated and immediately immersed in –20°C methanol for 6 minutes, then immediately immersed in –20°C acetone for 20 seconds. The slide was then allowed to air dry outside of the moisture chamber and was washed three times with PBS/BSA. The wells on the slide were keep wet from this point on in a moisture chamber.

Labeling Cellular Components

Primary and secondary antibodies were used to label the dynein heavy chain inserted HA epitope and microtubules alpha tubulin. The primary antibody was anti-HA (12CA5) mouse derived monoclonal antibody diluted 1:1000. The secondary antibody was a fluorescent-labeled polyclonal goat anti-mouse (CY2) diluted 1:100. The primary antibody was rat derived monoclonal anti-alpha tubulin (YOL1/34) diluted 1:400 and the secondary antibody was fluorescent labeled polyclonal anti-rat Rodamine diluted 1:400. The antibodies were diluted with PBS/BSA.

First, the addition of 20µl of the primary antibody dilution was put into the wells of the prepared immunofluorescence slide containing the yeast cells. The cells were incubated for 2 hours at room temperature or overnight at 4°C. After incubation,

the wells of the slide were washed three times with PBS/BSA. Next, the secondary antibody dilution was added. The slide was incubated for 2 hours at room temperature or overnight at 4°C. After incubation, the wells were washed three times with PBS/BSA. This procedure was followed for both the microtubule and the dynein antibody sets.

To stain the DNA, each well received 20µl of 1µg per ml of 4',6-Diamidino-2-phenylindole (DAPI) and incubated at room temperature for 3 minutes and washed three times with PBS/BSA. An anti-bleaching mounting solution was prepared with 100mg p-diphenyldiamine, 10 ml PBS and the pH adjusted 9.0 with 1M KOH and mixed into 90 ml of glycerol. Next, the wells were aspirated and the mounting solution was added to each well. A coverslip was placed on the slide and pressed between paper towels to remove excess mounting solution. The slide was placed under a heavy object for 30 minutes. Finally the edges were cleaned and sealed with nail polish. The fully prepared slides were stored at –20°C in the dark and were viewed with a fluorescent microscope within two weeks.

Epi-fluorescence Microscope and Digital Camera Setup

Haploid yeast cells during the anaphase stage of mitosis were imaged using fluorescent microscopy. Each selected cell was imaged using the FITC frequency for dynein and then imaged using the Rhodamine frequency for microtubules. Then the cell was imaged using the ultra-violet frequency for DNA. Each of three images of the same cell was false colored with green for the dynein signal, red for the

microtubule signal and blue for the DNA signal. These three colored images were overlaid to make a fourth composite image showing the location of each labeled component and their location relative to each other. However, the original image collected of dynein, MT or DNA was also saved to a file as an inverted black and white image. These images were used for comparison to the two other black and white images and the color overlay. Finally, to record the binding of dynein mutants to microtubules and the DNA in the cell we utilized the following instruments and tools.

Our light microscopy equipment includes a Nikon Microphot FXA with complete optics for bright field, differential interference contrast and epi-fluorescence microscopy. A Nikon super high-pressure mercury lamp, model HB, was used for fluorescence excitation. A Sutter instrument control box from Omega Optics to controlled fluorescence filters. The filters are the 61 (or triple band) series which allows simultaneous viewing for all three flourochromes or individually. (Filter set 61002SBX-DAPI/FITC/Texas Red with single band exciters)

The objective lens is a Nikon DIC oil immersion with 1.25 N/A.

We used a CCD Hamamatsu C4742-95 cooled camera that has high resolution and low light (sub-visual) capability. This device is coupled to a Datastor pentium III processor through a Mutech Imaging board. Digital acquisition and processing is done utilizing Metamorph software from Universal Imaging Corporation. Further image enhancement was done with Adobe Photoshop 6.0 and images were processed last with Adobe PageMaker 7.0.

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