Biochemical Studies of the *Saccharomyces cerevisiae* Kinetochore

by

Matthias Benno Jaffé

B.S., Chemistry (1992)

Universidad Simón Bolívar

Submitted to the Department of Biology
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Signature of Author.....
Department of Biology
April 25 1996

Certified by.....
Peter K. Sorger
Assistant Professor of Biology
Thesis Supervisor

Accepted by.....
Frank Solomon
Chairman, Graduate Student Committee

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ABSTRACT:

Kinetochores are structures that assemble on centromeric DNA and mediate the attachment of chromosomes to the microtubules of the mitotic spindle. The components of the kinetochore are poorly understood, but the simplicity of the *S. cerevisiae* kinetochore makes it an attractive candidate for molecular dissection. We used an *in vitro* microtubule binding assay to characterize two potential kinetochore components. One such component is present in a protein-free, low molecular weight fraction. Whole-cell extracts lose their microtubule binding activity upon dialysis. We found that the addition of a semi-purified low molecular weight fraction from whole cell extracts could restore the activity of dialyzed extracts. This low molecular weight fraction appears to be species-specific as it is absent in *K. lactis* and *S. pombe*. The activity also proved to be resistant to enzymatic treatments aimed at destroying peptides and oligonucleotides. Chromatographic purification identified two major moieties with a molecular weight under 1100 kDa.

The gene product of MIF2 was the second factor studied. Overexpression of MIF2 is associated with increased levels of chromosome loss (Brown et al., 1993), and mutations in MIF2 are synthetically lethal with mutations of NDC10 and CTF13 (Meluh and Koshland, 1995). We show here that extracts from strains carrying temperature sensitive alleles of MIF2 display reduced microtubule binding activity. This finding supports the idea that MIF2 is involved in kinetochore function.

Thesis Supervisor: Peter K. Sorger
Title: Assistant Professor of Biology

INTRODUCTION:

The Kinetochore of *S. cerevisiae:*

Kinetochores are complex protein-DNA structures that mediate the binding and movement of chromosomes along the spindle microtubules during mitosis (Mitchison 1988). Very little is known about other aspects of mitosis such as motility, checkpoint control and cell cycle regulation that ensure proper chromosome segregation. Before these phenomena can be approached from a molecular view point, the basic underlying mechanisms of DNA and microtubule binding need to be better understood. With this objective in mind, I undertook a series of experiments aimed at identifying the proteins and other factors required for the *in vitro* reconstitution of the DNA and microtubule binding activities of whole cell extracts.

I chose to carry out these studies making use of the budding yeast *Saccharomyces cerevisiae.* This system offers not only the potential for relatively simple genetic manipulation, but also the advantage of a centromere limited to only 125 bp per chromosome. In contrast, even the closely related fission yeast *Schizosaccharomyces pombe* proves to be much more complex, carrying several 50-100 kb centromere sequences per chromosome. Additionally, each chromosome in S. cerevisiae binds to a single microtubule, while higher cells attach several microtubules to each chromosome. One obvious limitation of *S. cerevisiae* results from the poor cytology of budding yeast cells which make it impossible to view chromosomes in real time. Nevertheless, studies using electron microscopy (Byers & Goetsch 1975, 1975) and recent fluorescence in situ hybridization (FISH) support the view that mitosis in budding yeast is fundamentally similar to mitosis in other organisms.

Centromeres:

Cetromeric DNA sequences were originally identified and cloned due to their potential to increase the rate of plasmid maintenance during cell division (Clarke & Carbon 1980). All centromeres from *S. cerevisiae* studied so far contain three conserved Centromeric DNA Elements, called

CDEI, CDEII and CDEIII (Hegemann & Fleig 1993). A region spaning 125 bp and encompassing these three elements has proven to be necessary and sufficient for CEN activity in both mitosis and meiosis (Cottarel et al 1989). CDEI is an imperfect palindrome spaning 8 bp to which the protein CBF1 binds. Though not essential, the deletion of CDEI increases the rate of chromosome loss by 10 fold (Niedenthal et al 1991). The adenine and thymine rich CDEII is the least conserved of the three and spans between 78-86 bp in the different centromeres. Even though small deletions in CDEII have only minimal effect on CEN activity, the deletion of the entire CDEII region results in loss of centromeric activity (Gaudet & Fitzgerald-Hayes 1987). CDEIII is a highly conserved stretch of 25 bp that binds to the CBF3 complex of proteins (Ng & Carbon 1987). Like CDEI, CDEIII is an imperfect palindrome and is considered the "core centromere region" since single point mutations within this sequence can destroy all CEN function (McGrew et al 1986). CDEIII is also sensitive to its proper orientation with respect to CDEI and CDEII (Murphy et al 1991).

Centromere Binding Proteins:

A series of Centromere Binding Factors (CBFs), have been isolated by DNA-affinity chromatography using wild-type CEN sequences. These proteins have shown to specifically bind centromeres *in vitro* as judged from bandshift and footprinting assays. CBF1, is one of these protein complexes isolated using CDEI DNA (Bram & Kornberg 1987). A member of the helix-loop-helix family of proteins, CBF1 binds to CDEI DNA as a homodimer protecting a 12-15 bp region in DNaseI footprinting experiments (Jiang & Philippsen 1989). Strains carrying a deletion of CBF1, although still viable, display a ten-fold higher rate of chromosome loss than do wild-type strains (Niedenthal et al 1991). CBF1 appears to have a dual role, acting as a transcription factor (Thomas et al 1992) in addition to binding CDEI. It's function as a transcriptional activator may be separable from its role as a kinetochore protein since point mutations that affect each activity have been separately isolated (Mellor et al 1991)

CBF3 is a CDEIII binding complex also isolated by DNA affinity chromatography. Purified CBF3 is reported to contain three major proteins

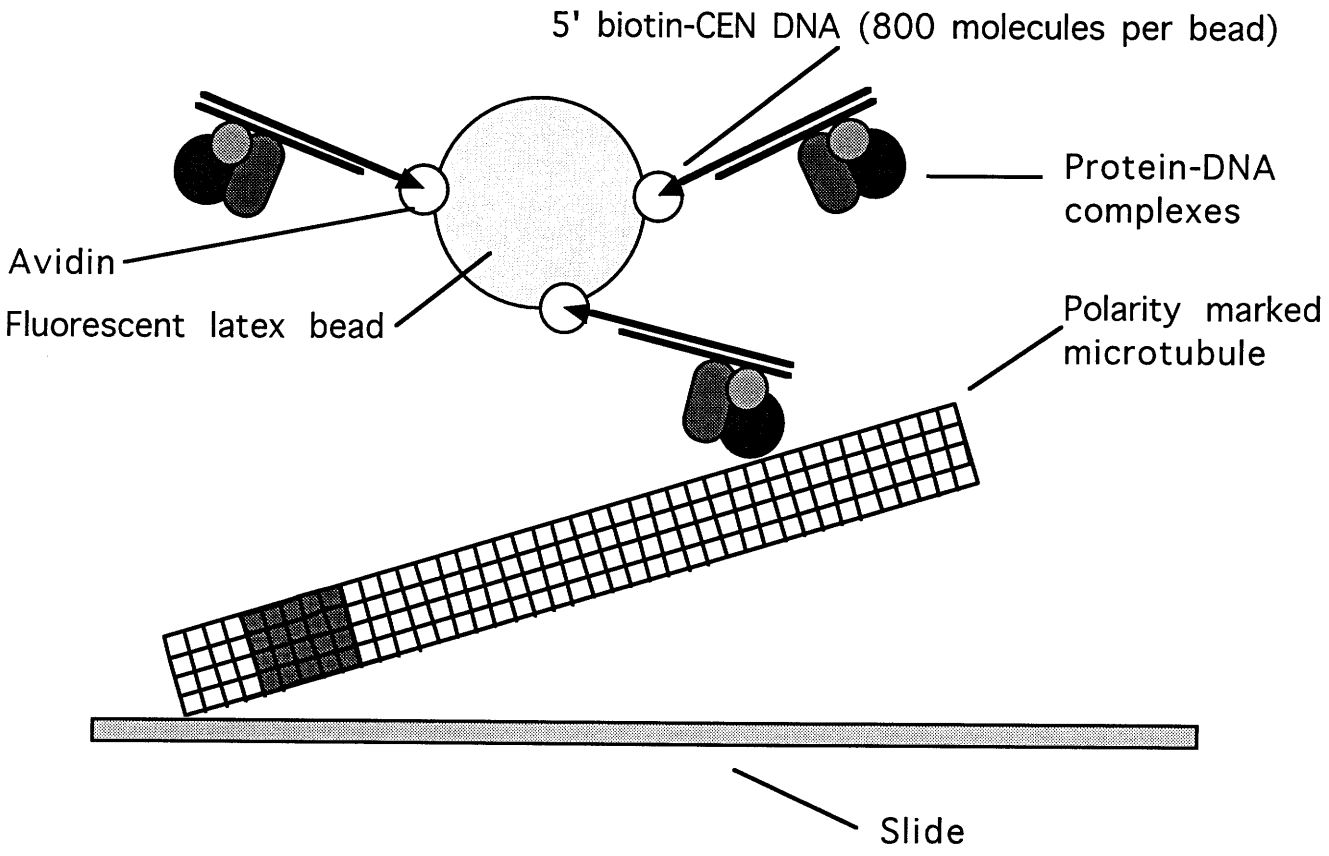
of 58, 64 and 110 kDa (Lechner & Carbon 1991). Gel filtration data suggests a native molecular weight of 240 kDa for the complex, suggesting that it is a homotrimer consisting of one of each of the three proteins. The CBF3 DNaseI footprint is much larger than that reported for CBF1, spaning a total of 56 bp. The genes coding for all three CBF3 proteins have been cloned, in some instances independently by several groups involved in isolating *trans*-acting mutations that affect chromosome stability. In order to avoid confusion I will simply refer to these genes as NDC10, CEP3 and CTF13 for the centromere binding proteins CBF3-p110, CBF3-p64 and CBF3-p58 respectively. Recently a 23 kDa protein encoded by the SKP1 gene was isolated as a high copy suppressor of a particular allele of CTF13 (P. Hieter, unpublished). Antibodies directed against the SKP1 gene product are capable of supershifting the band corresponding to CBF3 in bandshift gels, suggesting that this protein is also part of the DNA binding complex. All four proteins are essential for growth, and mutations in any one of them increase the rate of chromosome loss.

In vitro Microtubule Binding Assay:

Initial observations suggested that CBF3 was not only essential but also sufficient for microtubule binding *in vitro* (Hyman et al., 1992), even though *in vivo* results argued otherwise (Clarke, 1990). This issue was further studied using an *in vitro* reconstitution assay capable of generating kinetochores containing different sets of components and to measure the microtubule binding activity of the so formed complexes (Sorger et al., 1994). In this assay, biotinylated CEN DNA sequences are attached to fluorescently labeled latex beads coated with streptavidin. Microtubules that are taxol-stabilized and rhodamine labeled are adsorbed to the glass surface of a perfusion chamber constructed on a microscope slide. Beads that are pre-incubated with wild-type yeast extract will bind to these microtubules when perfused into the chamber. The binding can be observed and quantified by fluorescence microscopy (see figure 1). This binding is specific, as beads coated with mutant CEN sequences that are inactive *in vivo*, display no binding *in vitro*.

Figure 1

In vitro microtubule binding assay: In this assay, biotinylated CEN DNA sequences are attached to fluorescently labeled latex beads coated with streptavidin. Microtubules that are taxol-stabilized and rhodamine labeled are adsorbed to the glass surface of a perfusion chamber constructed on a microscope slide. Beads that are pre-incubated with wild-type yeast extract will bind to these microtubules when perfused into the chamber. The binding can be observed and quantified by fluorescence microscopy.


5' biotin-CEN DNA (800 molecules per bead)
Protein-DNA complexes
Avidin
Fluorescent latex bead
Polarity marked microtubule
Slide

Using the assay described above a clear requirement for functional CBF3 was made evident. It was shown that extracts from cells carrying temperature sensitive mutations in different CBF3 components, displayed low levels of microtubule binding while the congenic strains showed normal levels. Functional CBF3 complexes could be reconstituted by mixing extracts deficient in two different CBF3 components, resulting in restoration of wild-type microtubule binding levels (Sorger et al., 1994). However, it was also shown that CBF3 alone is not sufficient for microtubule binding. When CBF3 purified up to 60-fold by ion-exchange chromatography was assayed for microtubule binding, it displayed levels that were 200-fold lower than wild type extracts. When the same extracts from temperature sensitive mutations in CBF3 described previously were added to purified CBF3, microtubule binding activity was reconstituted. This argues that additional factors associated with CBF3 are required for microtubule binding activity. These factors are the ones I am interested in identifying as part of my work. The approach taken towards their identification was the fractionation of whole cell yeast extracts while following the microtubule binding activity by the *in vitro* microtubule binding assay described above.

Other Kinetochore Proteins:

Additional kinetochore components have been suggested form genetic studies. Most of these candidates were isolated as mutations that affect chromosome stability. Proper mitotic chromosome replication and segregation requires the function of many gene products, several of which can potentially result in chromosome missegregation when mutated while not being directly related to proper kinetochore function. Therefore not all of these candidates are expected to interact directly with the centromere, CBF1 or CBF3. Nonetheless, some of these proposed candidates definitely merit closer attention. One such mutant is *mif2* (for mitotic fidelity of chromosome transmission).

MIF2

In 1986 Hartwell and Meeks-Wagner reasoned that some gene products would be required in precise amounts in order to attain the appropriate protein stoichiometries in such multi-component structures as the kinetochore. Abnormal stoichiometries of these products might decrease the fidelity of mitotic chromosome transmission. Two such genes were identified by screening yeast genomic libraries made in high copy vectors that caused high frequency of chromosome loss (Meeks-Wagner et al 1986). While both MIF1 and MIF2 were unique sequences, only MIF2 proved to be essential, as disruption of the genomic MIF2 locus was lethal to cells. When MIF2 was introduced on a multi-copy vector it was able to induce a 150 fold increase in chromosome loss in a standard sectoring assay. No effect was observed on CEN plasmids.

Upon cloning (Brown et al., 1993), MIF2 was localized to chromosome XI. Its DNA sequence codes for a 549 aa long protein with a predicted molecular weight of 62.5 kDa and an above average content of acidic residues. Surprisingly, it was shown that while the sequence pulled out in the original screen by Meeks-Wagner only coded for the 364 amino-terminal residues of MIF2, cells displayed much higher rates of chromosome loss when overexpressing this truncated version as opposed to the full length protein.

Temperature sensitive mutants of MIF2 isolated by *in vitro* mutagenesis delayed cell cycle progression when grown at the restrictive temperature. These mutants also displayed increased levels of chromosome loss, supersensitivity to microtubule destabilizing drugs such as Methyl benzimidazole-2-yl-carbamate (MBC) and morphologically aberrant spindles (Brown et al., 1993). Mutant spindles in these strains arrest normal growth immediately prior to anaphase spindle elongation and frequently break apart inappropriately into two short, disconnected and often missoriented spindles (Brown et al., 1993).

It is not presently clear how MIF2 contributes to chromosome segregation and spindle integrity, however a 12 aa sequence of high homology

between the MIF2 protein and the A/T rich DNA binding motifs (Churchill and Travers, 1991) of *Drosophila* D1 (Ashley et al., 1989) and HMGI(Y) (Reeves and Niessen, 1990) suggest that pMIF2 could be acting directly on CDEII. It was recently reported (Brown 1995) that there are two extended regions of homology between MIF2 and the mammalian centromere protein CENP-C. The autoantigen Centromere Protein C (CENP-C) localizes to the inner kinetochore plate of mammalian cells, and was the first component of the kinetochore to be identified in humans (Saitoh et al., 1992). The homology of MIF2 is highest to the C-terminal end of CENP-C where a stretch of 27 amino acids displays 40% identity between the two sequences. This C-terminal region also exhibits the highest homology between the mouse and human CENP-C sequences. Mutations in the MIF2 homology domain of CENP-C impair the ability of CENP-C to assemble at the kinetochore in mammalian cells (Lanini and McKeon, 1995). Finally, the two strongest temperature sensitive alleles, mif2-2 and mif2-3 revealed amino acid substitutions in this region of the protein.

Other genetic evidences support the idea that MIF2 is a centromere binding protein. MIF2 stabilizes dicentric mini-chromosomes and confers high instability to chromosomes bearing mutations in CDEI (Meluh & Koshland 1995). Similarly synthetic phenotypes between mutations in MIF2 and three components of CBF3 (p110, p64 and p58) were reported (Meluh & Koshland 1995).

This series of findings led me to use the *in vitro* microtubule binding assay described earlier in order to further study and ascertain the role of MIF2 from a biochemical perspective. To this respect I used strains carrying temperature sensitive alleles of MIF2 to prepare whole cell extracts, which could be analyzed for microtubule binding activity. We expected that the involvement of the MIF2 protein product in the kinetochore complex should be reflected by reduced microtubule binding levels of these MIF2-compromised extracts. This proved the be the case and will be described in detail later.

EXPERIMENTAL MATERIALS AND METHODS

Yeast strains:

All extracts used for the low molecular weight factor experiments and purification were made from the strain PS886: leu2, trp1, ura3-52, prb1122, pep4-3, prc1-407, GAL, Mat a/α unless otherwise specified. Strain PS612 is leu2, trp1, ura3-52, MAt a.

The strains carrying the temperature sensitive alleles of MIF2 are: PS995 (mif2-2), and PS997 (mif2-3). PS995 is ura3-52, mif2-2, Mat a. PS997 is ura3-52, mif2-3, Mat a. PS993 is the congenic wild-type strain.

Plasmids Construction:

pMJ001 was constructed by inserting a 2.3-kb *Pst*I/*Bgl*II fragment from pMB024 (Brown et al., 1993) containing the MIF2 gene into a *Pst*I/*Bam*HI cut YCplac33 vector (Gietz and Sugino, 1988). pMJ004 was obtained by removing a 38 bp *Sna*BI-*Bam*HI fragment from pMJ001. This fragment contains a sequence coding for 12 amino acids, including the 8 amino acid sequence RPRGRPKK believed to be a potential A/T rich DNA binding motif (Brown, 1993).

Preparation of Low Molecular Weight fraction from whole cell extracts:

Boiled Low Molecular Weight Fraction: 200 µl of extract were boiled in eppendorf tubes for 10 min. The boiled material was then spun at 50K rpm for 30 min. in a TLA-100 rotor (g=108K). The clear supernatant was removed and stored at -20°C.

Filtered Low Molecular Weight Fraction: 200 µl of extract were placed in a microcon-3 spin-filter (Amicon, Beverly, MA) which has a MW cut-off limit of 3 kDa. The tubes were spun at 14000 g in a table top micro-centrifuge

for 120 min. This method can be scaled up by using centricon-3 spin filters which are spun at 7330 rpm in a JA-20 rotor (6500 g). All centrifugations are done at 20°C.

Preparation of Beads Carrying Centromeric DNA:

Wild-type *CEN3* DNA was derived from pRN505 (Ng and Carbon, 1987). The CDEIII-3bpΔ, CDEI-2bpΔ, and CDEIII-only were originally constructed using oligonucleotide directed mutagenesis (Sorger et al., 1994). The DNA sequences to be coupled to beads were PCR-amplified using a biotinylated right hand primer. Purified DNA was coupled to fluorescent latex beads (Molecular Probes, Eugene, OR) as described elsewhere (Sorger et al., 1994). To make beads with different numbers of active DNA centromeric DNA molecules, the total amount of DNA per bead was kept constant at 800 molecules per bead by mixing active wild-type, CDEI-2bpΔ or CDEIII-only DNA and inactive CDEIII-3bpΔ DNA. Overall DNA-coupling efficiency was ascertained by measuring band intensities after electrophoresis on agarose stained gels. Unbound CEN DNA and serially diluted CEN DNA of known concentration were compared to a standard amount of the molecular mass marker ΦX174. In a typical measurement, the ethidium bromide stained agarose gel was recorded on a GelPrint 2000i digitizer (Biophotonics, Ann Arbor, MI) and the bands of interest quantified using the program IPLab Gel 1.5 Beta 1 (Molecular Dynamics, Sunnyvale, CA). DNA-coupled bead concentration was quantified by fluorimetry against a calibration curve of commercial fluorescent beads of known concentration (Molecular Probes, Eugene, OR). Fluorimetric measurements (Ex 530 nm/Em 560 nm) were performed on a Hitachi F-4500 Fluorescence Spectrometer (Hitachi, San Jose, CA) and verified by counting beads at an appropriate dilution under the fluorescence microscope using a flied of known size.

Microtubule Binding Assay:

Centromere dependent microtubule binding activity was measured by the method of Sorger et al., 1994. Protein concentrations in the range of 45-90

µg/rxn were typically used, unless otherwise indicated. When testing for the low molecular weight fraction effect, 1 µl of either the boiled or spin-filtered low molecular weight fraction was added to each reaction. Breakage buffer (30 mM Potassium Phosphate pH=7, 60 mM β-Glycero-Phosphate, 150 mM KCl, 6 mM EDTA, 6 mM EGTA, 6 mM NaF, 10% Glycerol) was used as control.

Purification of the Low Molecular Weight Fraction:

a) C18 Sep-Pak cartridges: A 2 ml aliquot of the spin-filtered low molecular weight fraction was diluted 1:1 with a 1% aqueous TFA solution in order to lower pH to 3.5. The dry resin was conditioned with 10 ml of 0.1% TFA in acetonitrile that were passed through a Sep-Pak Plus C18 cartridge (Waters, Milford, MA) using a glass syringe. Prior to loading the diluted sample, the cartridge was flushed with 10 ml of H_2O containing 0.1% TFA. The loaded sample was then washed with 4 ml H_2O + 0.1% TFA, and eluted with acetonitrile + 0.1% TFA. Fractions of approximately 500 µl each were collected manually. After vacuum-drying and resuspending in 100 µl water, the eluted fractions were analyzed for their stimulatory activity and pooled.

b) VYDAC C18 Reverse Phase HPLC Chromatography: 270 µl of pooled Sep-Pak purified material were mixed with 30 µl 1% TFA to bring the overall TFA concentration to 0.1%. A VYDAC C18 type 218TP54 HPLC column (VYDAC, Hesperia, CA) was equilibrated for 100 minutes using a 0.1% TFA solution at a flow rate of 0.1 ml/min. The sample was then injected, washed for 40 min. with 0.1% TFA and eluted following a shallow gradient which took the acetonitrile concentration from 0-100% in 100 minutes. Elution from the column was monitored at 280, 260 and 214 nm. Fractions were collected at 1 min. intervals, vacuum-dried, resuspended in 100 µl H_2O and tested for stimulatory activity as described above.

c) Gel Filtration Chromatography: 5 µl of 1M TEAA buffer (triethylamine/ acetic acid pH=6.5) were added to fractions #50 and #61 from the VYDAC C18 column (see figure 8). A TSK2000 SWXL gel filtration column

(Posotosohaas, Philadelphia, PA) was equilibrated for 40 minutes using 50 mM TEAA buffer at a flow rate of 0.8 ml/min. The samples were injected independently and 500 µl fractions were collected. 50 µl of a 1 mg/ml solution of Vitamin B12 (MW=1355 Da) and Insulin A chain (MW=2530 Da) were used as molecular weight calibration standards. Collected fractions were vacuum-dried, resuspended in 50 µl H_2O and tested for stimulatory activity as described above.

Preparation of Extracts:

Extracts were prepared by mechanical disruption of cells in liquid nitrogen (Sorger et al., 1988). The solid cell-powder was mixed 1:1 with 2x breakage buffer to yield a final extract concentration of 30 mM Potassium Phosphate pH=7, 60 mM β-Glycero-Phosphate, 150 mM KCl, 6 mM EDTA, 6 mM EGTA, 6 mM NaF, 10% Glycerol, and 1 mM PMSF. All extracts were flash-frozen in liquid nitrogen and stored at -80°C. *E. coli* extracts were prepared by sonicating the bacterial cells and centrifuged in order to obtain a cytosolic soluble fraction.

Preparation of Cell Cycle Arrested Extracts:

α-Factor Arrest: An overnight culture of PS612 cells was used to inoculate 1L of YEPD to an OD_{600}=0.1. Cells were grown at 30°C to an OD_{600}=0.5 at which point the culture was split in two. One half was treated 0.1 mg/L of α-factor peptide, and both cultures were grown for another 60 min. At that point cells were harvested and processed into extract as described above. All buffers used during the harvest and to make extract contained 0.1 mg/l alpha factor.

Stationary Phase: Cells were grown with agitation overnight at 30°C. The overnight cultures were then placed at room temperature for a period of 10 days. Cells were harvested and processed into extract as usual.

Preparation of Extracts form MIF2 Temperature Sensitive Strains:

Overnight cultures of wild-type (PS993), mif2-2 (PS995) and mif2-3 (PS997) strains were used to inoculate YEPD media to an OD_{600} = 0.1. These cultures were grown at room temperature to a cell count of $2x10^7$ cells/ml. All cell number determinations were made by counting on a hemocytometer. Each culture was then split in two, one half was kept growing at room temperature while the other half was shifted to 37°C by placing it in a water bath shaker at that temperature. Each culture was independently harvested. The cultures grown at room temperature were collected when they reached a cell count of $5x10^7$ cells/ml, while the cultures growing at 37°C were harvested after 3 Hrs. Harvested cells were then resuspended in 1x breakage buffer (30 mM Potassium Phosphate pH=7, 60 mM β-Glycero-Phosphate, 150 mM KCl, 6 mM EDTA, 6 mM EGTA, 6 mM NaF, 10% Glycerol, and 1 mM PMSF), flash-frozen in liquid nitrogen and mechanically disrupted by the method described above. The resulting extracts were normalized for protein concentration and assayed using the standard bead binding assay for *in vitro* microtubule binding.

Bandshift Gels:

CDEIII binding was measured on non-denaturing acrylamide (bandshift) gels using an 88 bp CDEIII-containing probe as described (Sorger et all., 1994). CDEI binding was similarly measured but using a 153 bp CDEI-containing probe. Gels where quantified on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Treatment of the Low Molecular Weight Fraction with Proteinase-K, RNase A, DNase 1, Micrococcal Nuclease, and Alkaline phosphatase.

Two reactions, one containing 10 µl and another containing 90 µl of Sep-Pak-purified low molecular weight fraction, were treated with 50 µg/ml of

Proteinase-K (Amresco, Solo, OH) in a total reaction volume of 100 µ. Two similar samples were prepared, which in addition to the above contained 2 mM PMSF (Sigma, St. Louis, MO). All reactions were incubated in a water-bath at 37°C for 6 hours. After the incubation period, 5 µ of 10 mg/ml BSA where added to each reaction tube and the mixture filtered in a microcon-3 spin-filter (Amicon, Beverly, MA) at 14000 g for 120 min. The resulting enzyme-free material was tested for stimulation of dialyzed whole-cell extract's microtubule binding activity as described previously.

Similar experiments were carried out where the Sep-Pak purified low molecular weight fractions were treated with 2 U/rxn Calf Intestinal Phosphatase (Boehringer Mannheim, Indianapolis, IN) in commercial 1X CIP buffer, or 1.2 U/rxn RNase A (Boehringer Mannheim, Indianapolis, IN), 19.5 U/rxn DNase 1 (Worthington, Freehold, NJ) or 48 U/rxn Micrococcal Nuclease (Worthington, Freehold, NJ) in 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 50 mM TRIS pH=7.9. In every case, the reactions where incubated for a 6 hour period, and then spin filtered in order to remove the particular enzymes.

Proteinase K, a very promiscuous peptidase that cleaves peptide bonds primarily after the carboxyl group of hydrophobic amino acids, would destroy the activity if the activity corresponded to a peptide or small protein. RNase A, a pyrimidine-specific endoribonuclease that acts on single-stranded RNA, would destroy the activity in the unlikely event it was due to an RNA molecule. DNase 1 is a double-strand specific endonuclease that would destroy any small DNA sequences. Finally, Alkaline Phosphatase which selectively cleaves terminal phosphate groups, could potentially identify a phosphorylated moiety. In all four cases an aliquot of the filtered low molecular weight fraction was treated with the particular enzyme in a suitable buffer for a one hour period at 37°C. Controls were carried out for those enzymes to which inhibitors were available. In these cases, the enzyme was pre-incubated with the inhibitor for 10 min. prior to contact with the filtered low molecular weight fraction. After the 37°C incubation period, the mixture was spin-filtered in the same fashion as used when preparing the fractions from crude extract. This procedure would assure the removal of the particular enzyme which would

otherwise have interfered with the bead binding assay. The resulting filtered material was assayed for stimulation of the microtubule binding activity of dialyzed extract.

RESULTS:

A) CHARACTERIZATION OF A LOW MOLECULAR WEIGHT FACTOR INVOLVED IN THE MICROTUBULE BINDING ACTIVITY OF WHOLE CELL EXTRACTS:

As has been previously mentioned in the introduction, several proteins have been shown to specifically bind centromeric DNA sequences in *S. cerevisiae*. It is now clear that these complexes known as CBF1 and CBF3, do not mediate microtubule binding by themselves when assayed *in vitro,* implying that other kinetochore proteins are yet to be identified. The isolation of these additional components is one of the major objectives of our group, and I will describe some attempts to further study the composition of the budding yeast kinetochore.

Many protein purifications utilize as an initial concentrating step, a precipitation achieved by salting-out proteins using almost saturating concentrations of ammonium sulfate. The resulting protein-precipitate is then cleared of salt by subjecting to dialysis. When this protocol was attempted by the Sorger and Hyman groups for the purification of microtubule binding components, it was observed that wild-type whole cell extracts lost virtually all microtubule binding activity. DNA-binding activity was, however, not affected.

The coupled precipitation-dialysis procedure can potentially remove a number of species from the extract. During the ammonium sulfate cut, a small but significant fraction of proteins can be lost, as well as lipids and other macro-molecules. The dialysis step in turn, allows all soluble low molecular weight factors to diffuse through the membrane. It is therefore not clear which species is responsible for the loss of microtubule binding activity when absent. The restoration of the microtubule binding activity of the ammonium sulfate precipitated proteins was attempted by adding back different fractions obtained from whole cell extract. These fractions where enriched in particular components which could have potentially been lost during the precipitation step.

1) A protein-free fraction can restore microtubule binding of a dialyzed ammonium sulfate cut of wild-type whole cell extract:

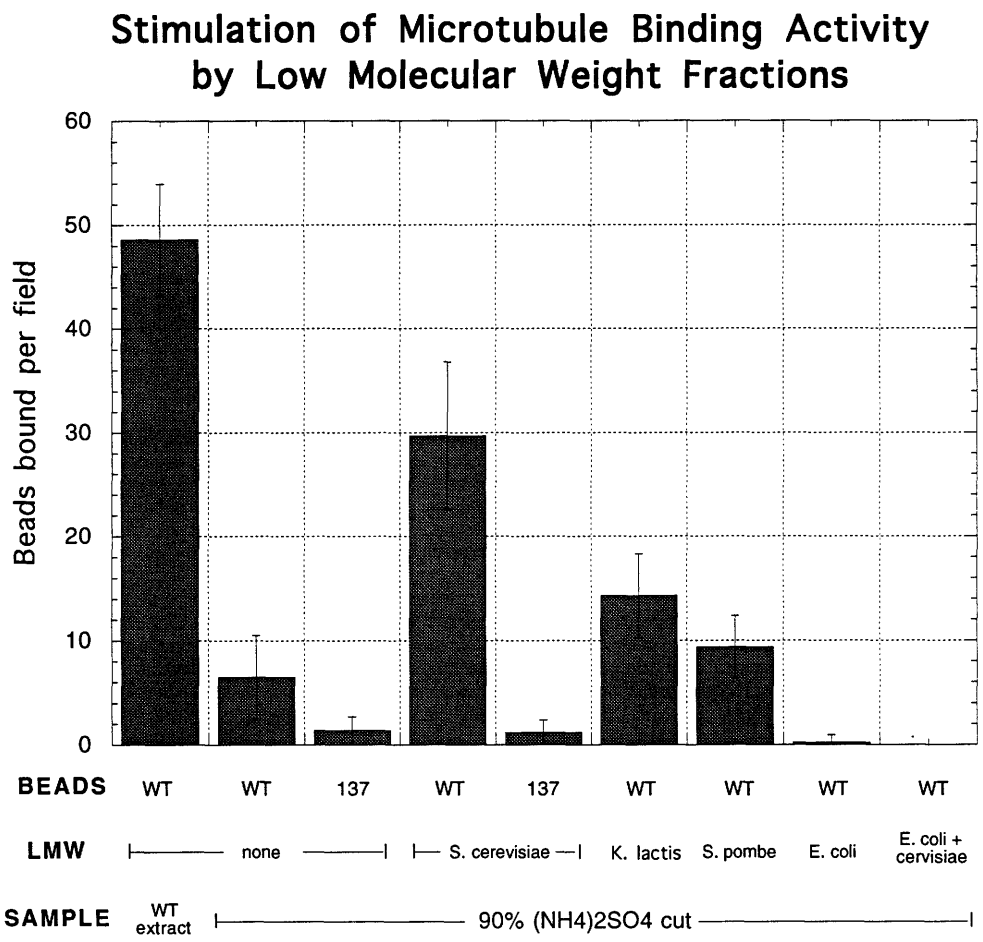
Wild-type whole cell extract was boiled for 10 min. in order to denature proteins, which were subsequently pelleted by spinning for 30 min. at high speed. This procedure cleared the extracts of protein by at least 98% as judged by a Bio-Rad Bradford-type protein concentration assay. The resulting supernatant was then added back to the dialyzed ammonium sulfate cut and tested for microtubule binding activity using the standard bead binding assay. An 8 to 12 fold stimulation of microtubule binding activity could be observed in those samples to which the boiled supernatant was added back as compared to control. This stimulation proved to be specific in nature, as CDEIII(-) CEN bearing beads were not stimulated and showed only a low background binding with or without the boiled extract fraction. This finding reinforces our suspicion that the protein fraction, inactive by itself, requires the presence of additional soluble factors not precipitated during the ammonium sulfate cut. The boiled low molecular weight fraction had no effect on DNA-binding activity as judged by bandshift gels using a ^{32}P-labeled CDEIII probe (data not shown).

2) The restoring activity is species-specific:

In order to further study the nature of the restoring activity, we attempted to obtain similar protein-free fractions from other organisms. Whole cell extracts were prepared from *E. coli*, *K. lactis* and *S. pombe* cell cultures. All extracts were normalized for total protein concentration as measured by Bradford and subsequently treated by the same boiling/centrifugation method as described above. When these fractions were added back to the 90% ammonium sulfate cut of whole cell extracts, only the fraction obtained from the *S. cerevisiae* cultures restored microtubule binding activity to a significant extent, although not completely to wild-type levels (Figure 2). The *K. lactis* fraction achieved close to half the stimulation level as compared to that of *S. cerevisiae* , while *S. Pombe* showed no significant

Figure 2

Stimulation of the microtubule binding activity of an ammonium sulfate cut from *S. cerevisiae* whole-cell extract by low molecular weight fractions obtained from different organisms. The 90% $(NH_4)_2SO_4$ cut displays 10 fold lower microtubule binding activity than the original extract from which it was obtained. The low molecular weight fraction from *S. cerevisiae* extract stimulates the microtubule binding activity of this ammonium sulfate cut by 6 fold. This stimulation is only observed when using beads carrying wild-type CEN sequences, but not for beads carrying a mutation in CDEIII (137 beads). The low molecular weight fraction from *K. lactis*, stimulates the ammonium sulfate cut but to a lesser extent. Low molecular weight fractions from *S. pombe* and *E. coli* do not stimulate the microtubule binding activity to a significant degree.


Stimulation of Microtubule Binding Activity
by Low Molecular Weight Fractions
Beads bound per field
60
50
40
30
20
10
0
BEADS
WT
WT
137
WT
137
WT
WT
WT
WT
LMW
none
S. cerevisiae
K. lactis
S. pombe
E. coli
E. coli + cervisiae
SAMPLE
WT extract
90% (NH4)2SO4 cut

stimulation. The *E. coli* fractions inhibited microtubule binding activity. We hypothesize that residual levels of DNases in the bacterial extract could potentially survive the boiling step and degrade the centromeric DNA sequences attached to the fluorecently labeled beads.

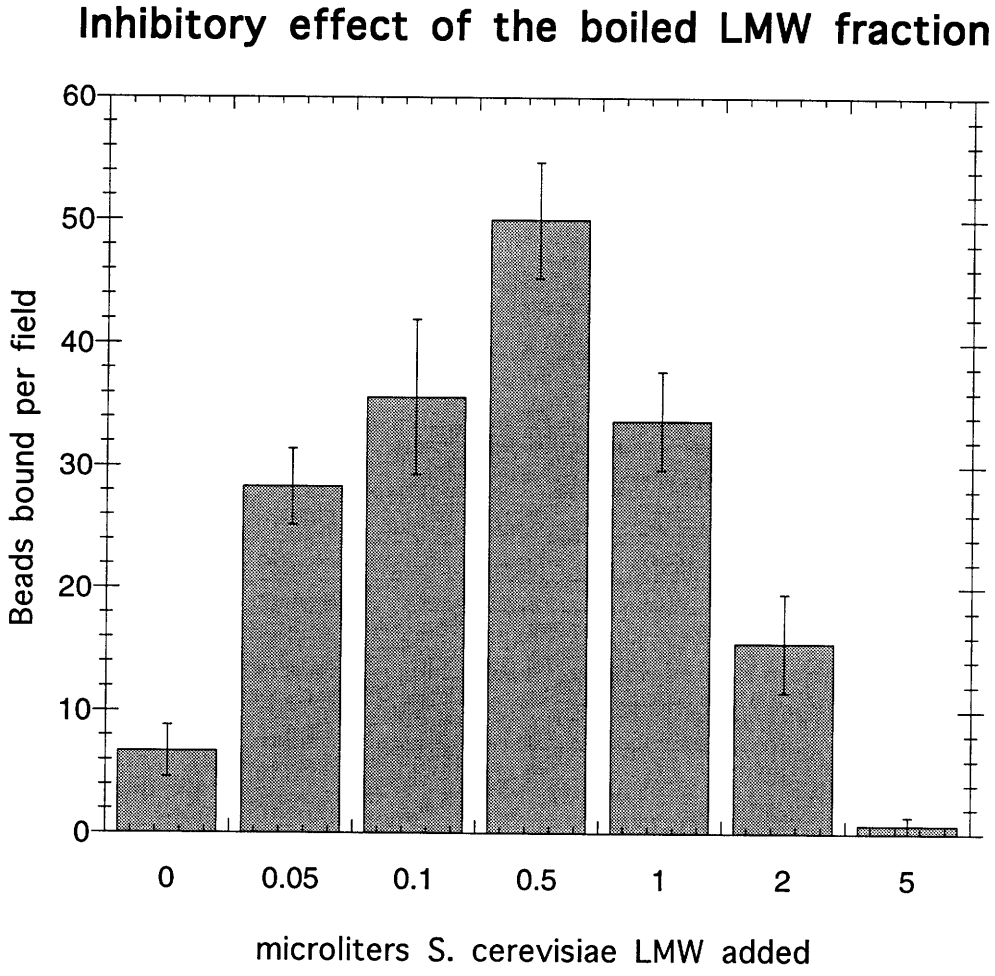
This finding suggests that the restoring activity is only present in the extract from budding yeast, and to a much lesser extent in the extract of the closely related *K. lactis* . Even though no conclusion can be drawn from the *E. coli* fractions, the total absence of activity in the *S. pombe* fractions suggests species specificity. This species specificity further implies that the activity responsible for restoring microtubule binding is most likely not a ion, or another common metabolite which should be found in similar levels in the other species.

3) The boiled fraction can be inhibitory to the microtubule binding activity at higher concentrations:

In an attempt to increase restoration of microtubule binding activity above the observed 60-70% of wild-type levels, larger amounts of the boiled low molecular weight fraction were added back to the ammonium sulfate cut. Surprisingly, even though a linear relationship between stimulation of microtubule binding activity and volume of boiled fraction added was observed over a range of concentrations, this behavior peaked at 0.5 µl per reaction. Volumes greater than 0.5 µl of boiled low molecular weight fraction displayed an inhibitory effect (Figure 3). The inhibitory effect reduced microtubule binding activity to less than background levels at higher volumes. This result raises the possibility that even though all extracts in the previous inter-species experiment were normalized for initial protein concentration, they did not contain equal levels of other specific factors responsible for the stimulatory and/or inhibitory activities.

Figure 3

Titration of the stimulatory activity of a low molecular weight fraction from *S. cerevisiae*. Decreasing amounts of a low molecular weight fraction obtained from boiled whole-cell extracts were added back to ammonium sulfate cut fractions of wild-type extract. The microtubule binding activity was measured as number of beads bound per field of known size.


Inhibitory effect of the boiled LMW fraction
Beads bound per field
60
50
40
30
20
10
0
0
0.05
0.1
0.5
1
2
5
microliters S. cerevisiae LMW added

In order to rule out the possibility that some of the boiled fractions which did not display stimulation of microtubule binding activity in the previous inter-species experiment were being used at levels where the inhibitory effect overcame the stimulatory activity, a titration of all fractions was performed. Even when the fractions were diluted 1000 fold, the overall behavior observed initially was maintained, that is, only the fraction obtained from *S. cerevisiae* showed a significant stimulatory activity (Figure 4).

4) Dialyzed whole cell extract and micro-filtered fractions behave equivalently to the 90% ammonium sulfate cut and boiled low molecular weight fraction.

Reasoning that any components not precipitated during the ammonium sulfate precipitation step are probably low molecular weight components, 3 ml of whole cell extract from *S. cerevisiae* were dialyzed over night against three changes of 800 ml 1X breakage buffer, using a dialysis membrane with a 12-14 kDa cut-off limit. The dialyzed material obtained in this fashion displayed a lower microtubule binding activity than the ammonium sulfate cut, but could be stimulated to even higher levels than the latter by adding back the boiled fraction (Figure 5A). As with the ammonium sulfate precipitated material, the dialyzed extract displayed the same inhibitory effect when the boiled fractions were used at levels higher than 0.5 µl per reaction (data not shown). This material is relatively easier to obtain and introduces a lower salt concentration to the bead binding assay. All subsequent experiments described were carried out using this type of dialyzed extract.

The complementing fraction to the dialyzed material described above needs to be the protein-free cytosol, lost through the pores of the dialysis membrane. In order to verify this idea, 150 µl of whole cell extract was centrifuged in a spin filter with a 3 kDa molecular weight cut off limit which should retain most proteins while allowing all low molecular weight molecules to pass through. The resulting material which contained no measurable protein as judged by Bradford, behaved comparably to the

Figure 4

Comparative titration of low molecular weight fractions obtained from *S. cerevisiae* (solid circle) , *K. lactis* (solid triangle) and *S. pombe* (solid diamond). The three separate fractions were diluted up to 1000 fold and tested for the stimulation of the microtubule binding activity of the 90% $(NH_4)_2SO_4$ cut. Microtubule binding activity is expressed as beads bound per field.

Comparison of LMWs from Different Organisms

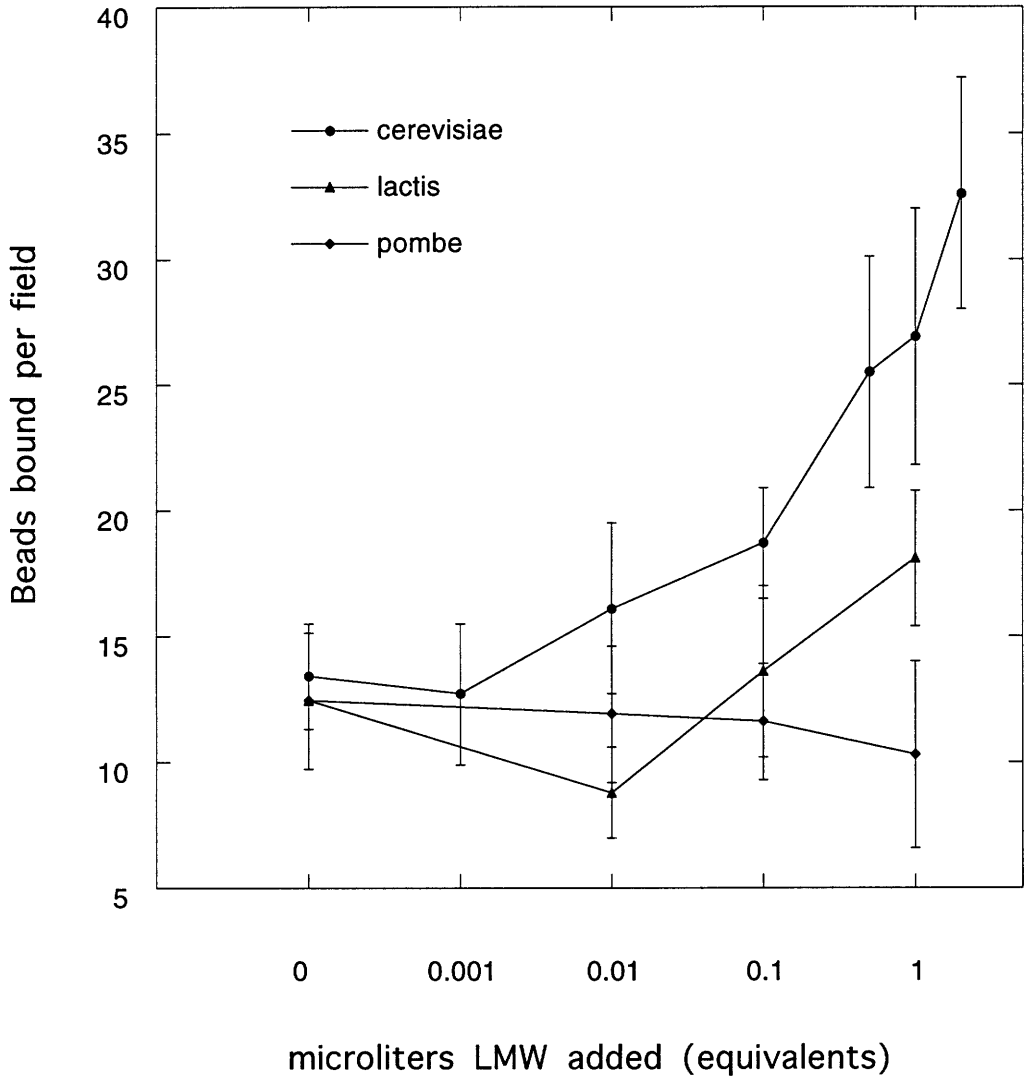


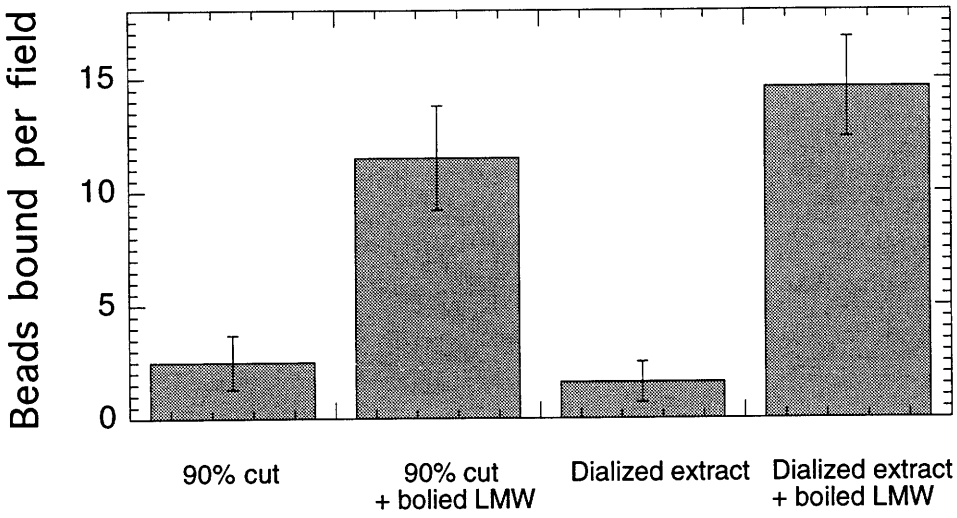
Figure 5

A) Comparison of dialyzed extract vs. the 90% (NH4)2SO4 cut. Wild-type extract dialyzed over night against breakage buffer (see materials and methods) was compared for microtubule binding activity to the previously described ammonium sulfate cut. In both cases background microtubule binding levels are low, but can be stimulated up to 5 fold by the addition of a low molecular weight fraction.

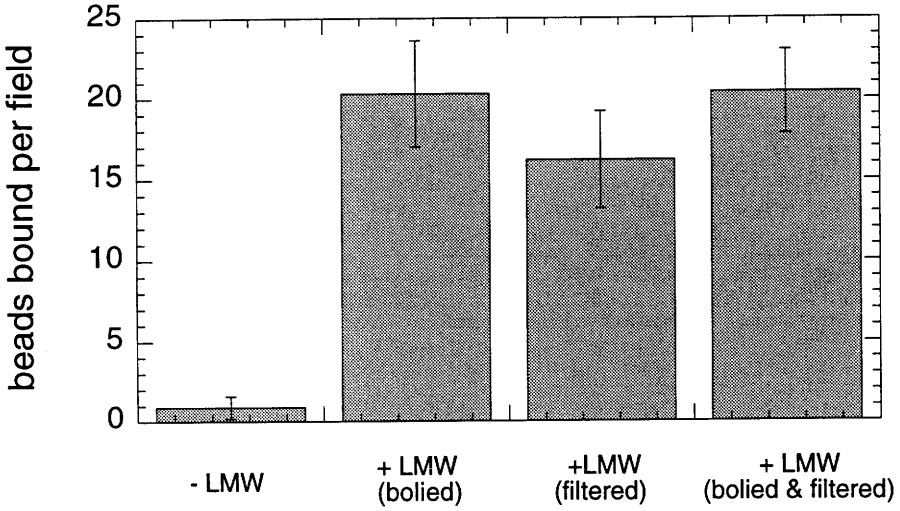
B) Low molecular weight fractions obtained either by boiling or through filtration (see materials and methods) were compared for their stimulation of dialyzed whole cell extract.

C) Titration of the stimulatory activity of a filtered low molecular weight fraction from *S. cerevisiae*. Increasing amounts of a low molecular weight fraction obtained by filtration of whole-cell extracts were added back to dialyzed wild-type extract. The microtubule binding activity was measured as number of beads bound per field of known size.

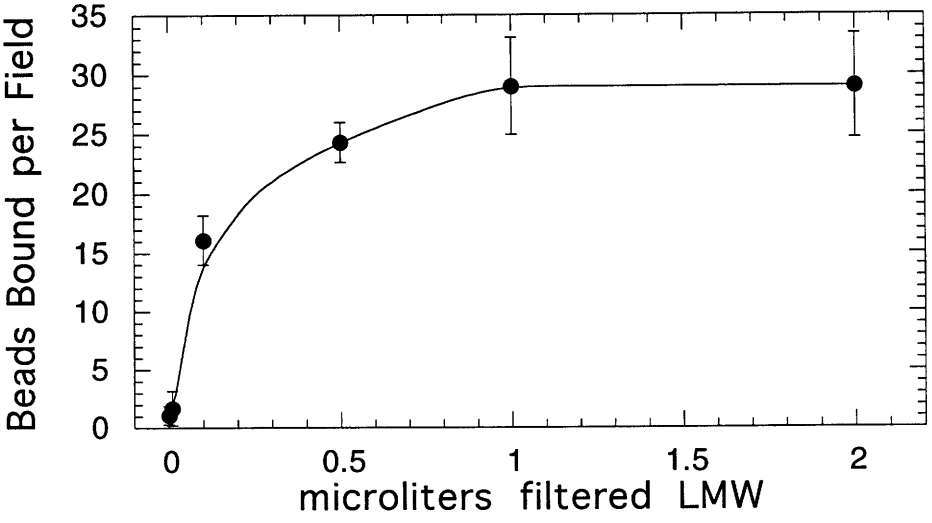
A


Beads bound per field
0
5
10
15
90% cut
90% cut
+ bolied LMW
Dialized extract
Dialized extract
+ boiled LMW

B


beads bound per field
0
5
10
15
20
25
- LMW
+ LMW
(bolied)
+LMW
(filtered)
+ LMW
(bolied & filtered)

C


Beads Bound per Field
0
5
10
15
20
25
30
35
0
0.5
1
1.5
2
microliters filtered LMW

boiled fractions stimulating the microtubule binding activity of dialyzed extract by roughly 10 fold (Figure 5B). However, the filtered material did not display an inhibitory effect when used at higher concentrations, simply reached a plateau at around a 10 fold stimulation and leveled off, irrelevant of volume added (Figure 5C). No cooperative or cumulative effect could be observed when using the boiled and filtered materials together in the same reaction (data not shown), which leads us to believe that it contains the same stimulatory activity, but lacks the inhibitory activity that is present in the boiled material.

5) Comparing the microtubule binding stimulatory effect of the filtered LMW factor on dialyzed extract when using beads with increasing number of wild-type centromeric sequences bound.

The sensitivity of the bead binding assay can be fine-tuned by using beads with decreasing number of wild-type CEN sequences attached, while keeping the overall bead DNA density constant by compensating with all-negative CEN (CDEIII-) sequences. This can be achieved by linking wild-type and mutant (CDEIII-) CEN DNA sequences to beads in varying ratios so that the total number of CEN sequences on each bead is 800.

To test whether the low molecular weight fraction effect could be further magnified by using a particular bead type, a full bead series was constructed. Beads containing 0, 4, 16, 32, 64, 128, 256 and 800 WT CEN sequences per bead while maintaining the total number of DNA molecules attached constant at 800 were tested (Figure 6). As expected, the 0 WT or all-negative CEN beads displayed only background binding with no significant stimulation upon the addition of the filtered material. The beads carrying 32 wild-type CEN sequences performed best by displaying the largest fold stimulation of microtubule binding activity by low molecular weight fraction. The fact that the 800 WT or all-wild-type beads displayed a lower fold stimulation can be explained by arguing that the LMW effect is subtle and can be best observed in conditions were the overall microtubule binding has been slightly compromised.

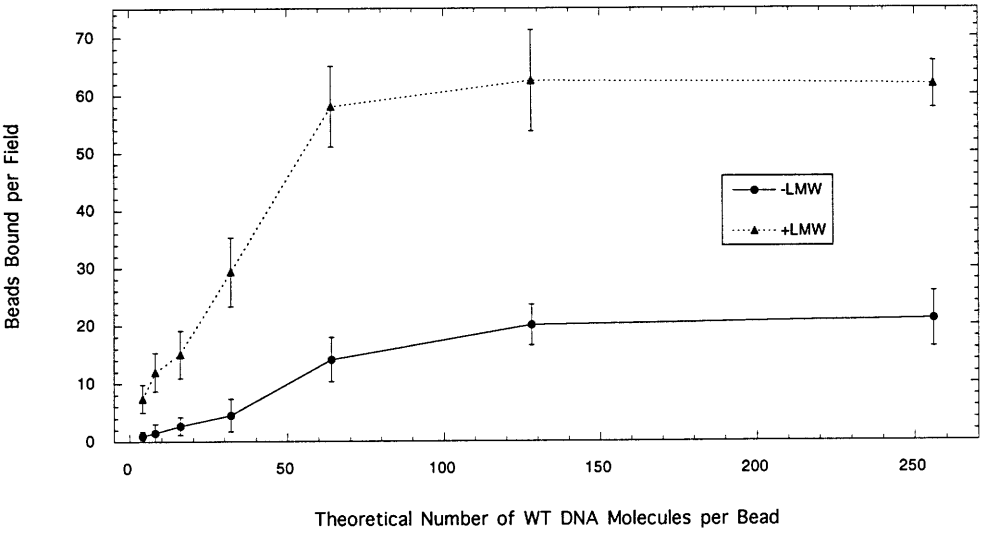
Figure 6

Microtubule binding of beads carrying varying numbers of WT CEN DNA molecules per bead. Wild-type and mutant (CDEIII-) CEN DNA sequences were linked to beads in varying ratios so that the total number of CEN sequences on each bead was 800. Microtubule binding activity of dialyzed extract in the presence of the low molecular weight fraction and dialyzed extract alone was assayed. Microtubule binding is expressed as beads bound per field.

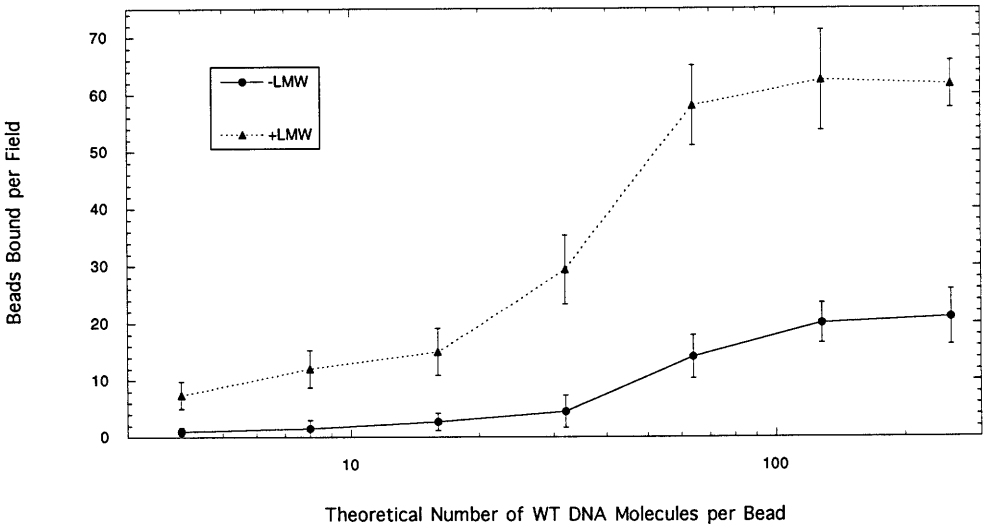
A) Linear plot

B) Logarithmic plot

A


Beads Bound per Field
70
60
50
40
30
20
10
0
0
50
100
150
200
250
-LMW
+LMW
Theoretical Number of WT DNA Molecules per Bead

B


Beads Bound per Field
70
60
50
40
30
20
10
0
10
100
-LMW
+LMW
Theoretical Number of WT DNA Molecules per Bead

6) The LMW factor rescuing activity is absent in stationary phase cells but present in G1 arrested cultures:

Low molecular weight fraction was isolated from cells grown to stationary phase. No stimulatory activity could be detected when adding back the low molecular weight fraction from stationary phase cells to dialyzed extract obtained from congenic exponentially growing cells. It was further observed that the undialyzed extracts from stationary phase cultures displayed no microtubule binding activity by themselves. We reasoned that the lack of active low molecular weight fraction could be responsible for the absence of microtubule binding activity in stationary phase extracts. Adding back LMW fraction obtained from exponentially growing cell cultures, did not stimulate the microtubule binding activity of stationary cell extracts. This leads us to believe that although the LMW activity is absent in the stationary phase, other factors involved in the microtubule binding activity are absent or down-regulated. When stationary phase cells were re-stimulated by diluting into fresh media, both the microtubule binding activity, and the stimulatory activity of the filtered LMW fraction were restored, indicating that the behavior described above is specific to stationary phase.

LMW factor obtained from cells arrested in either G1 by α-factor displayed a similar stimulatory effect when added back to dialyzed extracts. The absence of the LMW activity at only certain stages of the cell cycle but not others, suggests that the stimulatory activity could be under some kind of cell cycle control.

7) The stimulatory activity is not CDEII specific:

It has been reported that the absence of CDEII has only a modulatory effect on microtubule binding activity of whole cell extracts when assayed *in vitro* (Sorger et al., 1994). We were interested in studying the requirement of CDEII for the low molecular weight stimulation of microtubule binding. A series of beads containing various amounts of CDEII(-) CEN sequences where the CDEII and CDEI element had been

replaced with random vector DNA, were used to analyze the stimulatory behavior of the low molecular weight activity.

At high CEN DNA density points (800 molecules/bead), these all-CDEII(-) /CDEIII(+) beads bound microtubules equally well as wild -type beads. However, a significant difference is observed when the overall density of each DNA type decreases. Under these conditions an up to ten fold lower level of bead binding can be observed for the CDEII deficient beads as compared to the wild-type CEN bearing beads. This difference is smaller than what has been reported, but we believe that this can be attributed to the fact that Sorger et al where using 5 times higher protein concentrations per reaction than what we used in this set of experiments.

When the LMW activity was assayed using the wild-type and CDEII(-) /CDEIII(+) bead series, it was shown that the overall fold stimulation at each point of the series was similar, nevertheless the overall microtubule binding activity reflected the normal behavior described above, being much lower for the CDEII(-) beads than for the wild-types at low density points. This result suggests that the microtubule binding activity stimulated by the low molecular weight factor does not depend on a CDEII specific interaction (data not shown).

8) Low molecular weight activity is insensitive to Proteinase K, RNase A, DNase 1, and Alkaline phosphatase.

To test the nature of the compound responsible for the stimulatory activity, the filtered low molecular weight fraction was exposed to several enzymatic treatments to asses the effect on microtubule binding stimulation. In all cases, the enzyme-treated material retained its full stimulatory activity. The same result was obtained even after overnight incubations with higher enzyme concentrations and at different temperatures, leading us to believe that the activity is not the result of the presence of either RNA, DNA, a peptide, or a phosphorylated moiety.

B) CHROMATOGRAPHIC PURIFICATION:

Having established the presence of a discrete low molecular weight activity, we attempted to purify it by chromatographic means.

9) C-18 reverse phase chromatography:

A preparative scale purification was carried out using a C-18 Sep-Pak cartridge. The filtered whole cell extract fraction was diluted 1:1 in 1% TFA in order to lower pH to 3.5. This mixture was passed through a C-18 Sep-Pak cartridge, washed with water, and eluted with acetonitrile. After vacuum-drying and resuspending in water, the eluted fractions were analyzed for their stimulatory activity. A single peak which stimulated the microtubule binding activity of dialyzed extracts by 15-20 fold eluted right after the acetonitrile step, while both the flow through and H_2O washes showed no detectable levels of stimulation (Figure 7). The eluted activity was specific, as only beads bearing wild-type CEN sequences, but not all-negative CEN bearing beads could be stimulated to bind microtubules (data not shown).

The low molecular weight factor did not bind to anion (Mono-Q and HQ) or cation exchange (Mono-S and HS) resins which were examined under a range of pH and salt conditions as potential second steps in the purification scheme. Therefore, the next step was an attempt to resolve the Sep-Pak purified fraction over a VYDAC C-18 HPLC column using a shallow gradient for maximum resolution. Under these conditions six peaks bearing microtubule binding stimulatory activity were eluted. Two fraction in the wash phase (fractions #26 and #34), and the remaining four (fractions #50, #54, #58 and #61) at increasing concentrations of acetonitrile over a range spanning from 10-30% acetonitrie:water (Figure 8A). In order to quantify the specific activity of these four fractions, aliquots of each were diluted several fold and assayed in parallel for stimulation of the microtubule activity of dialyzed extract. This titration showed that two peaks (fractions #50 and #61) out of six showed at least a 10 fold higher specific activity than the rest (Figure 8B). All further studies were focused on these two fractions.

Figure 7

One step elution profile of the low molecular weight fraction from a Sep-Pac C-18 cartridge. Loading, wash and elution with acetonitrile steps are shown with arrows. Fractions collected at different points were assayed for stimulation of the microtubule binding activity of dialyzed extract. Microtubule binding activity is expressed as beads bound per field.

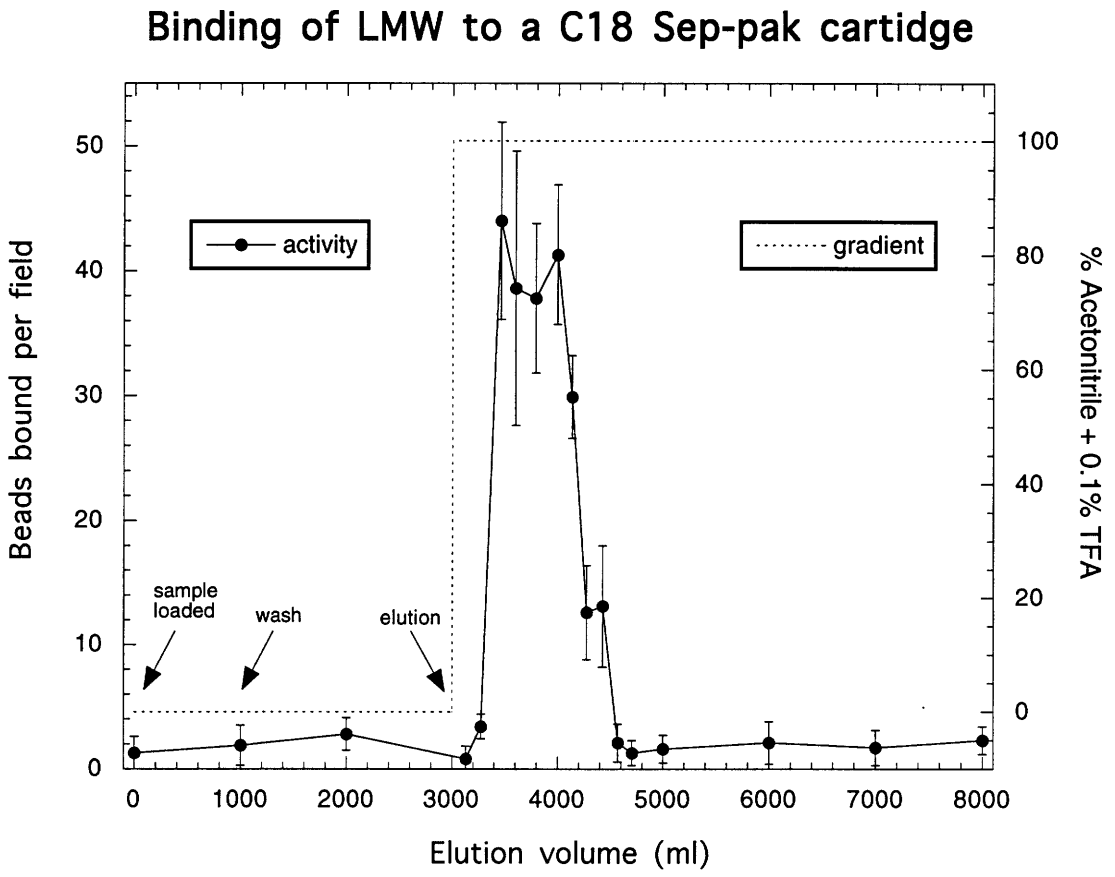
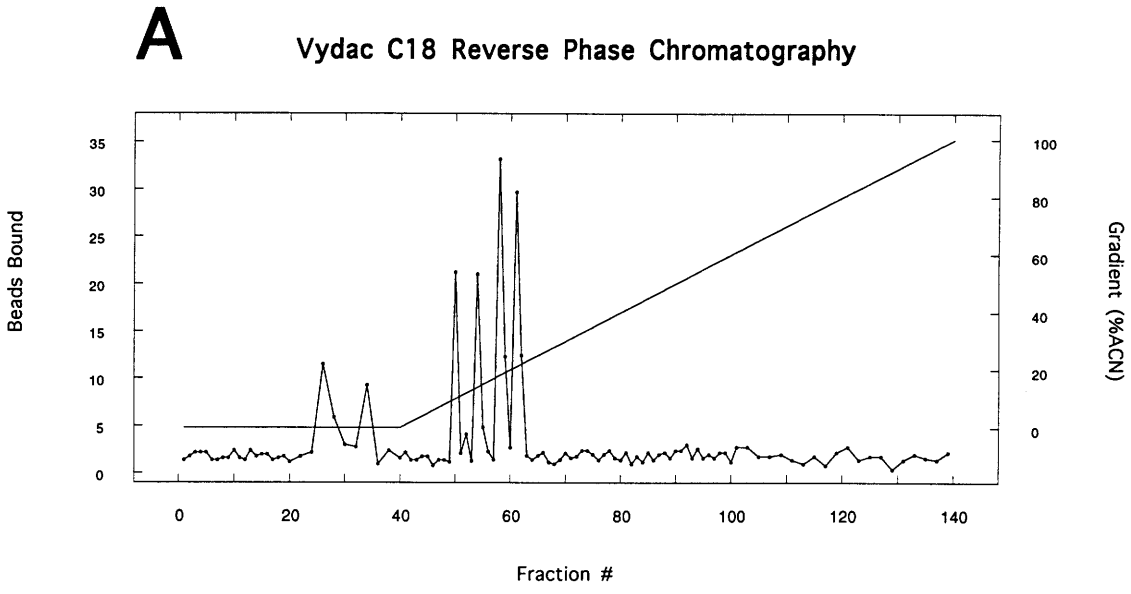
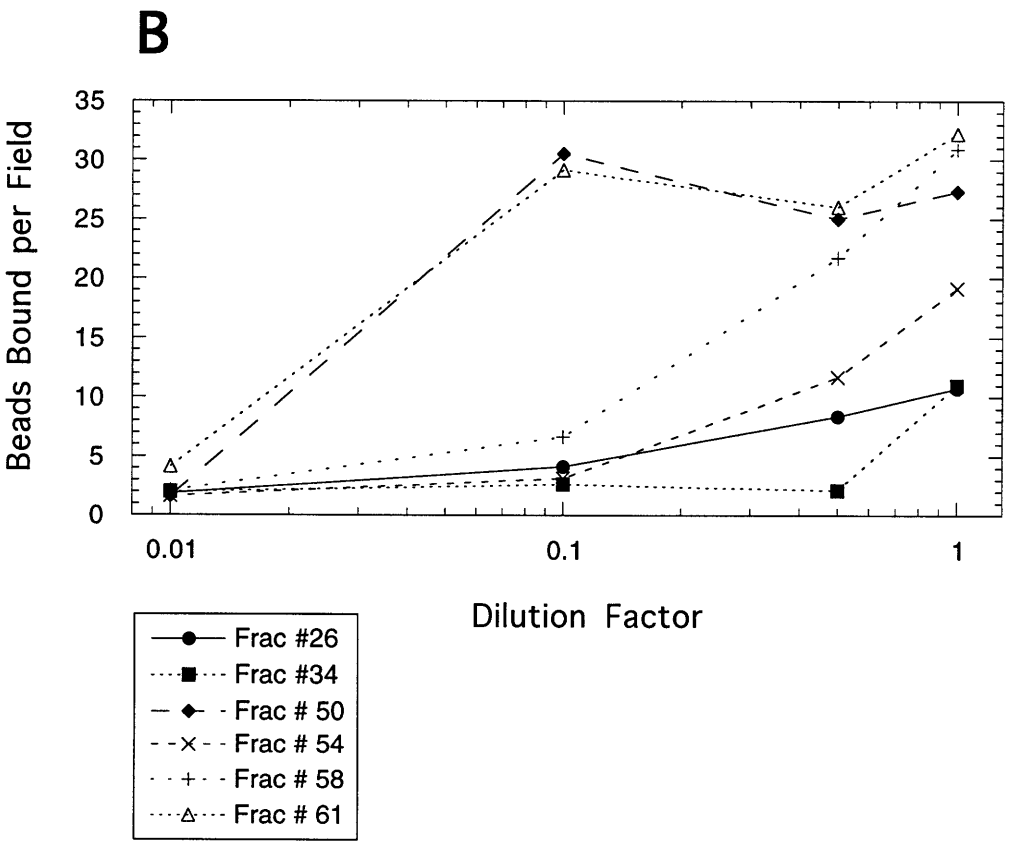

Binding of LMW to a C18 Sep-pak cartidge
Beads bound per field
% Acetonitrile + 0.1% TFA
activity
gradient
sample loaded
wash
elution
0
10
20
30
40
50
0
20
40
60
80
100
0
1000
2000
3000
4000
5000
6000
7000
8000
Elution volume (ml)

Figure 8

A) Reverse phase chromatography elution profile of semi-pure low molecular weight fraction from a VYDAC C-18 HPLC column. Sep-Pac purified material was loaded and then eluted from the column with a shallow acetonitrile gradient. Fractions collected and assayed for activity, identify a total of 6 activity bearing peaks.

B) Activity comparison of peaks identified in A. The six peaks obtained form the elution of the VYDAC column were pooled and diluted 1:1, 1:10 and 1:100. The resulting dilutions were assayed for stimulatory activity of dialyzed extract. Microtubule binding activity of dialyzed extract was measured as beads bound per field.


A
Vydac C18 Reverse Phase Chromatography
Beads Bound
Gradient (%ACN)
Fraction #
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140


B
Beads Bound per Field
Dilution Factor
0.01
0.1
1
Frac #26
Frac #34
Frac # 50
Frac # 54
Frac # 58
Frac # 61

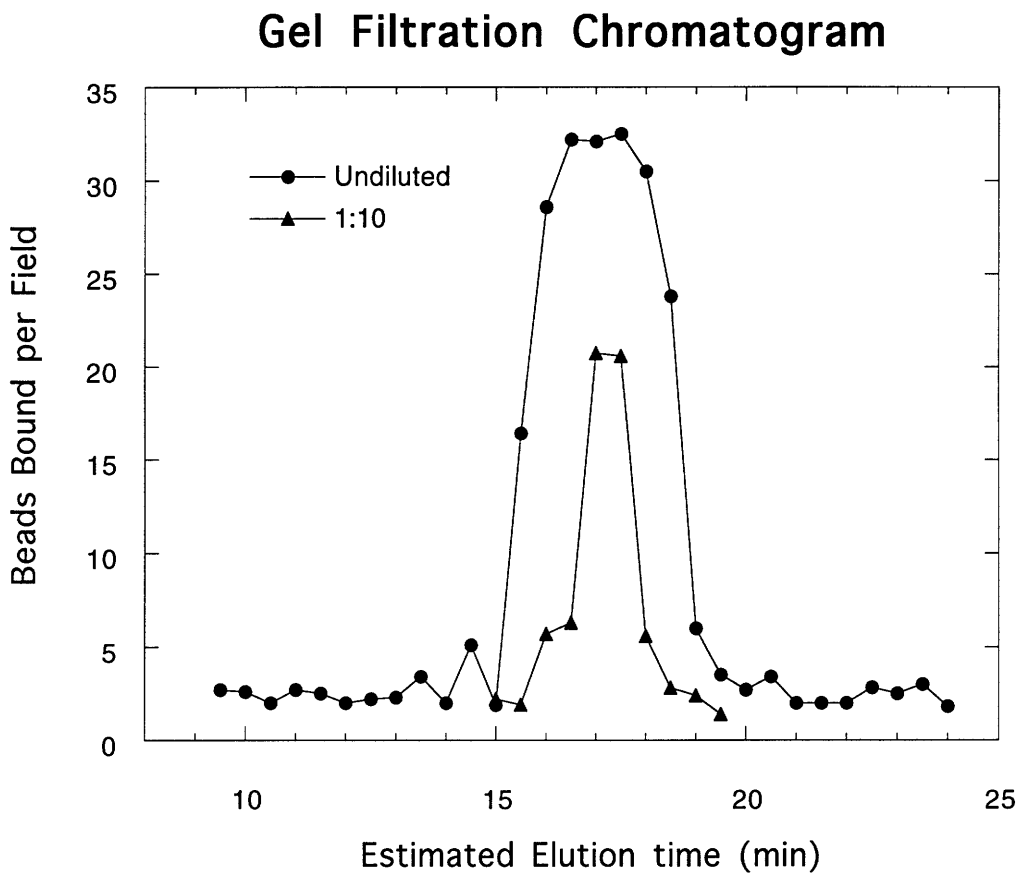
10) Gel filtration and mass spectroscopy suggest a molecular weight under 1000 Da associated the stimulatory activity.

Fractions #50 and #61 collected from the VYDAC C-18 column were further purified by subjecting to gel filtration chromatography on a TSK 2000SWx1 sizing column. Vitamin B12 (MW=1355) and Insulin αchain (MW=2530) were used for the molecular weight calibration. Both fractions #50 and #61 displayed an elution profile in which the stimulatory activity was spread over a broad range of elution volumes. In order to decrease the sensitivity of the low molecular weight assay, the eluted fractions were diluted 10 fold. A narrower activity profile appeared when analyzing the diluted fractions, identifying an activity peak with an elution time of 17 min. (Figure 9). It can be calculated from the calibration standards that such an elution point corresponds to a molecular weight of 1100 Da. This molecular weight is at the limit of the resolution range for this column, so at best, we can set the upper limit of the activity bearing compound's molecular weight at 1100 Da.

When the gel filtration purified fractions were subjected to mass spectroscopy, a single peak at 600 Da was reproducibly observed. Nevertheless due to insufficient material, and the fact that this molecular weight falls into the range where the sample-support resin fragments, no firm conclusions can be drawn from these observations.

Figure 9

Gel filtration chromatography elution profile of semi-pure low molecular weight fraction from a TSK2000 SWXL sizing column. Elution time is given in minutes, and microtubule binding activity is expressed as beads bound per field. Undiluted fractions (full circles) and fractions diluted 1:10 (full triangles) to decrease the sensitivity of the assay are shown.


Gel Filtration Chromatogram
Undiluted
1:10
Beads Bound per Field
Estimated Elution time (min)
0
5
10
15
20
25
30
35
10
15
20
25

C) BIOCHEMICAL CHARACTERIZATION OF WHOLE CELL EXTRACTS FROM WILD TYPE AND Mif2^{TS} STRAINS:

The MIF2 gene product has been implicated by several groups (Meeks-Wagner et al 1986, Brown et al., 1993) in the process of proper chromosome segregation. Genetic evidence from a recent report (Meluh & Koshland 1995), suggest that pMif2 may be a centromere binding protein and/or a component of kinetochore protein complex. So far there is no biochemical data supporting these claims besides a limited homology of two regions (Regions I and II) of the MIF2 sequence to the C-terminal end of the autoantigen Centromere Protein C or CENP-C (Brown 1995). CENP-C is known to localize to the inner kinetochore plate of mammalian cells. There is also a good homology of a 12 aa domain of MIF2 to the A/T rich DNA binding motifs (Churchil and Travers 1991) of *Drosophila* D1 (Ashley et al., 1989) and HMGI(Y) (Reeves and Niessen, 1990). This last homology has been used to postulate that pMif2 binds directly to the centromeric sequence CDEII.

In this part of my work I approach the issue of whether pMIF2 is involved in kinetochore function by taking a biochemical approach. Whole cell extracts obtained from strains containing temperature sensitive alleles of MIF2 grown at the permissive and restrictive temperatures were assayed for their *in vitro* microtubule binding activity using the microtubule binding assay described in the introduction. In most of the experiments I focused on a particular temperature sensitive allele of MIF2 called mif2-3. This heat sensitive mutation was obtained by *in vitro* mutagnesis, and isolated as a complementing sequence of the lethal MIF2 disruption in a standard plasmid shuffle scheme (Brown et al., 1993,). Of the initial 20 temperature sensitive alleles isolated, mif2-3 proved to have the strongest phenotype at the restrictive temperature of 37°C. mif2-3 is a single point mutation resulting in the substitution amino acid of 505 from Pro to Leu. This mutation lies in the second homology block of MIF2 to CENP-C.

1) Whole cell extracts from Mif2^{TS} grown at the restrictive temperature display reduced microtubule binding activity *in vitro*:

Overnight cultures of wild-type and mif2-3 strains were used to inoculate YEPD media to an OD_{600} = 0.1. These cultures were grown at room temperature to a cell count of $2x10^7$ cells/ml. Each culture was split in two, one half was kept growing at room temperature while the other half was shifted to 37°C by placing it in a water bath shaker. Each culture was independently harvested. The cultures grown at room temperature were collected when they reached a cell count of $5x10^7$ cells/ml, while the cultures growing at 37°C were harvested after 3 Hrs. This time point results in the tightest arrest for mif2-3 cells, expressed by the percentage of cells having a large-budded morphology and judged by light microscope examination (Brown et al., 1993). Extracts obtained from these cultures were normalized for protein concentration and assayed using the standard microscopy assay for *in vitro* microtubule binding.

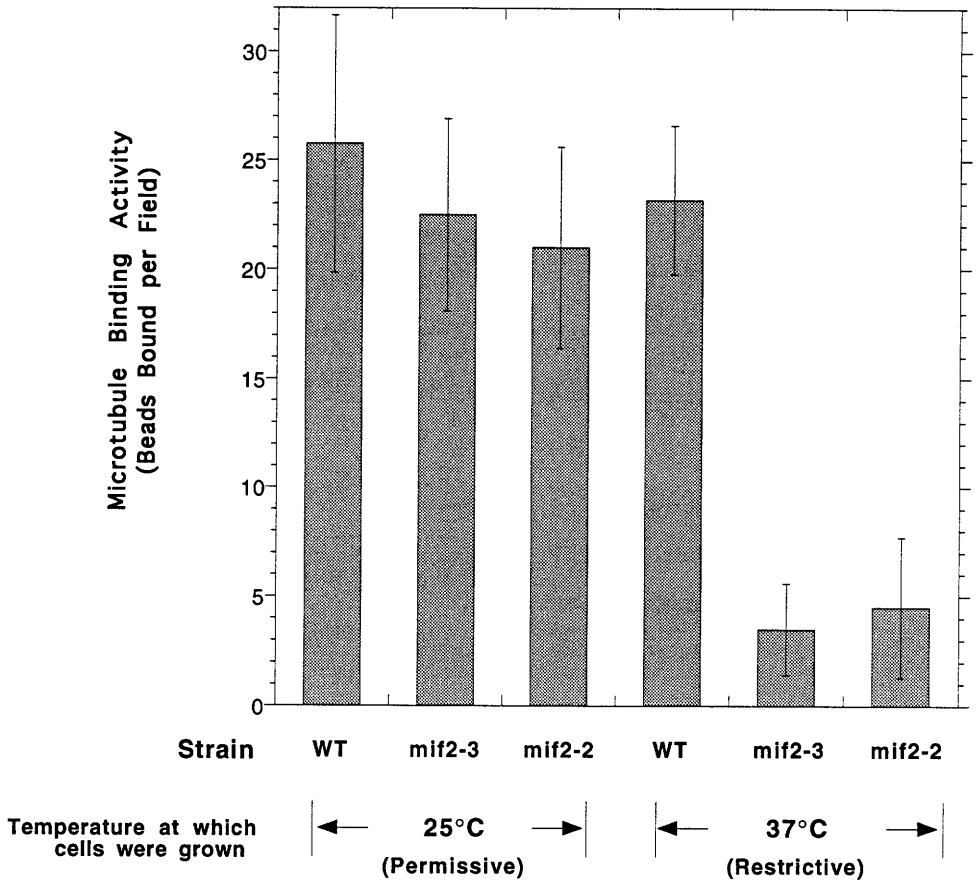
Observations from a total of five independent sets of extracts reproducibly shows that extracts from the mif2-3 strain at the restrictive temperature of 37°C displayed a microtubule binding activity which on the average was 4-6 fold lower than wild type levels. A limited number of experiments carried out with another Mif2 allele, mif2-2, also displayed a similar behavior (figure 10).

When the activity was assayed at lower protein concentrations (60 and 40 µg total protein) it was occasionally observed that even the mif2-3 extracts grown at the permissive temperature had a slightly lower specific activity that the wild type extracts. This difference, was never greater than 15%, as opposed to the reproducible 4-6 fold difference seen for mif2-3 at the restrictive temperature.

The experience with the low molecular weight fraction (see previous section) had suggested that beads with a lower density of wild-type CEN sequences could increase the sensitivity of that assay. We were interested in using such a bead series to compare the mif2-3 to the wild-type

Figure 10

Microtubule binding activity of temperature sensitive alleles of mif2. Cultures of mif2-2 and mif2-3, two different temperature sensitive alleles of MIF2 were grown at the permissive (25°C) and restrictive (37°C) temperatures. Harvested cells were processed into extracts, and analyzed for microtubule binding activity. Microtubule binding activity is expressed as the number of beads bound per field.


Microtubule Binding Activity
(Beads Bound per Field)
30
25
20
15
10
5
0
Strain
WT
mif2-3
mif2-2
WT
mif2-3
mif2-2
Temperature at which cells were grown
25°C
(Permissive)
37°C
(Restrictive)

extracts. This experiment confirmed that the room temperature mif2-3 extract paralleled the overall behavior of the wild type extracts over the entire bead series (Figure 11). All three extracts reached a maximum activity plateau at the 32 wild-type CEN/bead point, and were consistently 5 fold more active than the 37°C degree mif2-3 extract.

We were interested to confirm that the observed drop in microtubule binding activity of the mif2-3 extracts was actually due to the absence of functional pMIF2 was not a secondary effect of the compromised TS strains. The wild-type MIF2 gene was subcloned from the original plasmid described by Brown (Brown et al., 1993), into the YCplac33 vector. It was shown that the resulting pMJ001 plasmid was able to rescue the temperature sensitive phenotype of the mif2-3 strain. Extracts from mif2-3 mutants carrying a copy of pMJ001 when grown at 37°C showed increased levels of microtubule binding activity as compared to mif2-3 strains carrying YCplac33 as the control plasmid. Although the wild-type copy of MIF2 could stimulate the microtubule binding activity of mif2-3 by 3-4 fold, it was not able to completely restore wild-type activity levels (figure 12).

2) Low microtubule binding levels of mif2-3 extracts appear to be CBF3 independent:

Having established a cause-and-effect relationship between the lack of functional pMIF2 and the low microtubule binding activity of mif2-3 extracts, we were now interested in investigating any potential connection between MIF2 and CBF3 function. CBF3 function was examined by measuring the DNA binding activity of the same extracts analyzed above for microtubule binding activity. Extracts were tested for their ability to bandshift a ^{32}P-labeled CDEIII probe on acrylamide gels. The resulting protein-DNA bands were quantified on a phosphoimager. Of the five series of independently made extracts, all displayed a lower than wild-type DNA binding activity in the mif2-3 strains, both at the permissive and the restrictive temperatures. Although there was significant fluctuation between the individual extract series, room-temperature-grown mif2-3

Figure 11

Microtubule binding activities of beads carrying varying numbers of WT CEN DNA molecules per bead. Wild-type and mutant (CDEIII-) CEN DNA sequences were linked to beads in varying ratios so that the total number of CEN sequences on each bead was 800. mif2-3 extracts form cells grown at the permissive (solid squares) and restrictive temperatures (open squares) are compared to congenic wild-type strains grown at the permissive (solid circles) and restrictive temperatures (open circles). Microtubule binding activity is expressed as the number of beads bound per field.

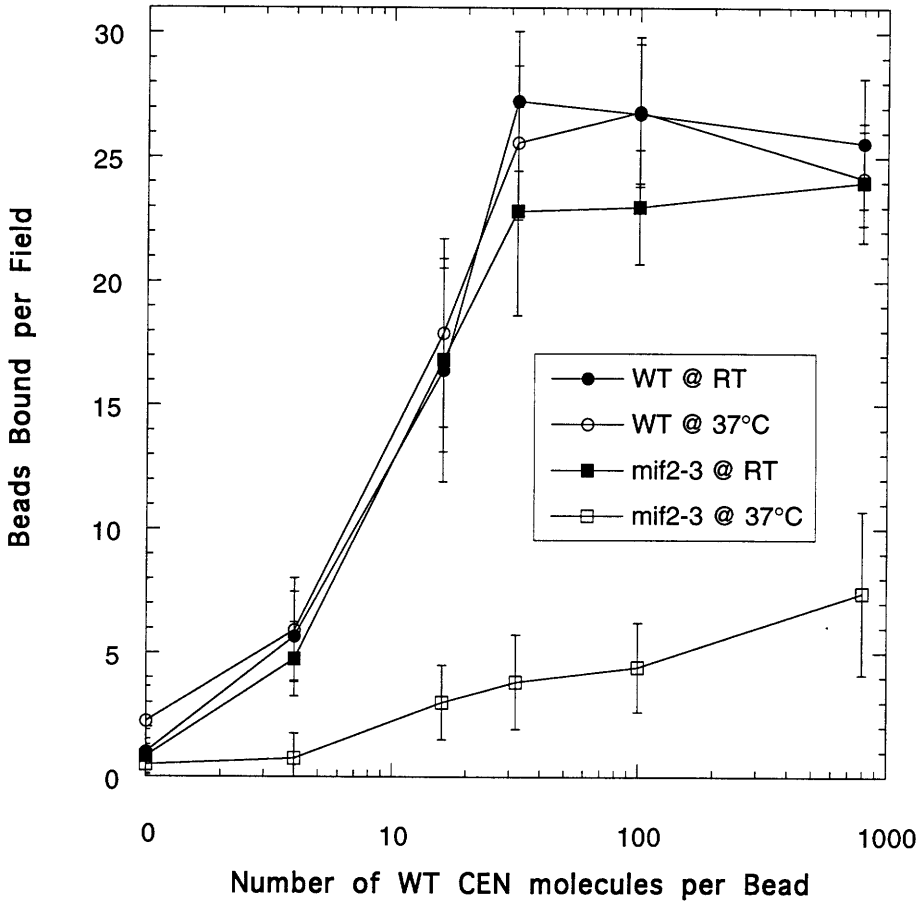
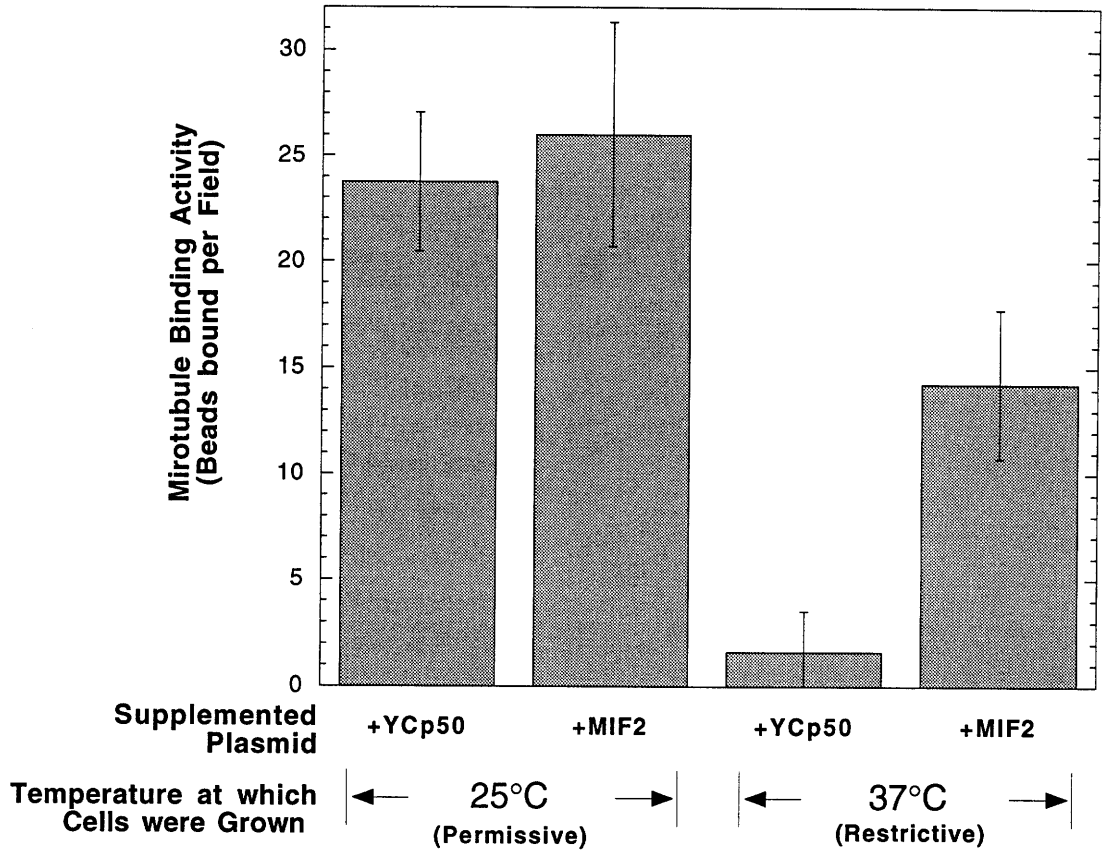

Beads Bound per Field
30
25
20
15
10
5
0
0
10
100
1000
Number of WT CEN molecules per Bead
WT @ RT
WT @ 37°C
mif2-3 @ RT
mif2-3 @ 37°C

Figure 12

Stimulation of the microtubule binding activity of mif2-3 strains by a plasmid carrying a wild-type copy of the MIF2 gene. mif2-3 cells were transformed with either a plasmid carrying the wild-type MIF2 gene (plasmid pMJ001) or a vector plasmid (YCp50). The resulting strains were grown at the permissive (25°C) and restrictive (37°C) temperatures and processed into extract. Microtubule binding activity of the extracts is expressed as beads bound per field.


Mirotubule Binding Activity
(Beads bound per Field)
30
25
20
15
10
5
0
Supplemented
Plasmid
+YCp50
+MIF2
+YCp50
+MIF2
Temperature at which
Cells were Grown
25°C
(Permissive)
37°C
(Restrictive)

extracts were down between 20-30% while 37°C-grown mif2-3 extracts were down by 30-40% as compared to wild-type extracts levels. Nevertheless, the difference between the mif2-3 extracts at the permissive and restrictive temperatures was never greater that 20%. It should be noted that wild-type extracts grown at 37°C also displayed DNA binding levels which were up to 30% lower than the respective room temperature levels. It is not clear why this difference arises.

Even though the lower DNA binding levels for the mif2-3 extracts is surprising, the comparison that is most valuable for our purposes is between mif2-3 extracts at the restrictive and permissive temperatures. In other words, is it possible that a difference of 20% in DNA binding activity between room-temperature-grown and 37°C-grown mif2-3 extracts is responsible for a five fold lower microtubule binding activity?

Mif2-3 extracts were normalized for CBF3 levels and the microtubule binding activity of room-temperature and 37°C-grown extracts compared side-by-side. We found that even under conditions where the overall protein concentration of the CBF3-normalized permissive extracts exceeded that of the restrictive extracts by 50%, the latter still displayed higher microtubule binding levels. We can therefore conclude that the low microtubule binding activity of the mif2-3 extracts grown at the restrictive temperature can not be explained on the basis of lower CBF3 levels.

3) The lower microtubule binding activity of mif2-3 extracts is not affected by the absence of CDEI:

It has been reported that mutations in MIF2 can exacerbate the rate of loss of minichromosomes bearing mutations in CDEI (Meluh & Koshland 1995). This suggests that MIF2 acts via CDEI in attaching CEN DNA to microtubules. We addressed this possibility by examining the microtubule binding ability of mif2-3 extracts when using beads bearing CEN DNA carrying a two bp deletion in CDEI. Although this is not the same mutation used in the mini-chromosome loss experiments (Meluh & Koshland 1995), it is known to be a strong mutation which abolishes CDEI function.

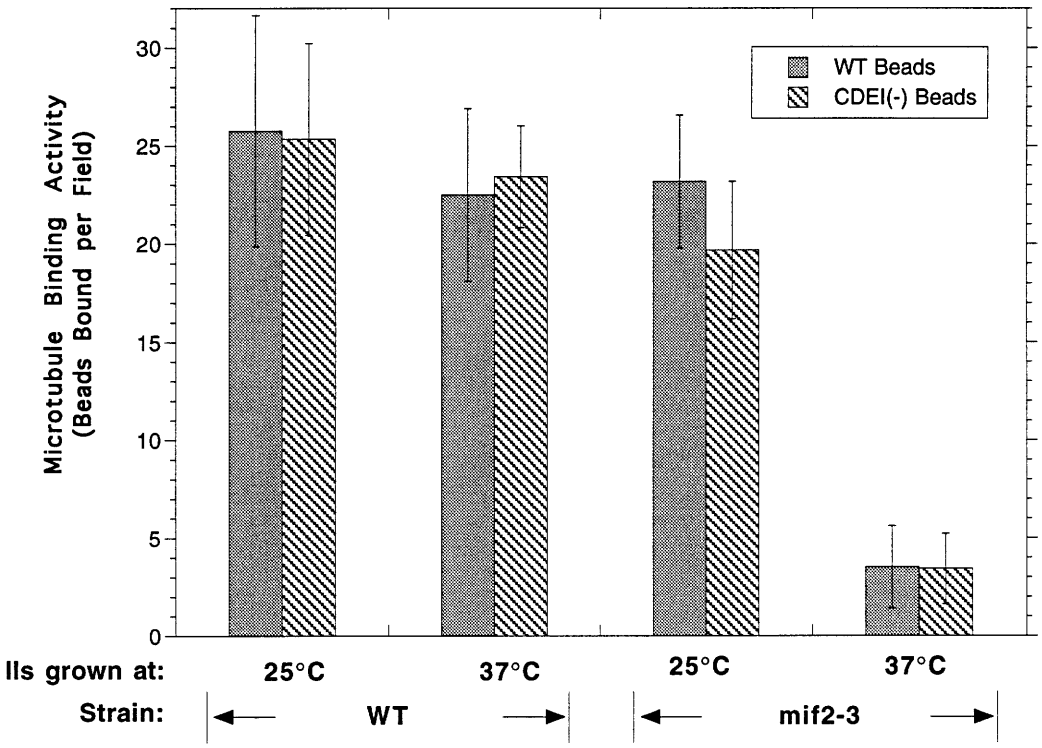
No significant difference in microtubule binding activity could be observed for the mif2-3 extracts or the wild-type extracts over the entire CDEI(-) bead series as compared to the wild-type bead series (figure 13. Only all-wild-type and all-CDEI(-) beads shown). It should be pointed out however that the only true CDEI(-) beads are the all-CDEI(-) beads where all 800 CEN molecules are CDEI(-). As has been explained earlier, a bead series is composed of beads with a decreasing number of the particular CEN sequences of interest (CDEI(-)/CDEIII(+) in this case), whereas the overall number of CEN sequences is kept constant with "mutant" CEN sequences which have a 3 bp deletion in CDEIII rendering them inactive. Since these mutant CEN sequences still carry a wild-type CDEI sequence, the lower density CDEI(-) beads carry increasing numbers of wild-type CDEI sequences which could potentially act in *trans* to the CDEI(-)/CDEIII(+) CEN molecules. A better design would make use of CDEI(-)/CDEIII(-) CEN sequences in balancing out the total DNA in lower density beads.

4) The putative AT rich DNA binding motif appears not to be essential for MIF2 function.

As was mentioned at the beginning of this section, the homology of an 8 amino acid sequence to the known AT rich DNA binding motifs of the HMGI and *Drosophila* D1 proteins has been used as a strong argument supporting the idea that MIF2 binds directly to or interacts with the centromeric DNA element CDEII. In an attempt to investigate the importance of this sequence, we removed a total of 12 residues containing the consensus 8 amino acid sequence RPRGRPKK believed to be the potential A/T rich DNA binding motif (Brown, 1993). The resulting plasmid was transformed into strains PS997 (mif2-3) and PS993 (congenic wild-type) and transformants grown on selective plates at 25°C (permissive temperature) and 37°C (restrictive temperature). It was shown that the AT binding domain drop-out plasmid successfully rescued the temperature sensitive mutant strains at 37°C indistinguishable from the plasmid containing the wild-type MIF2 gene.

Figure 13

Microtubule binding of mif2-3 extracts to wild-type and CDEI deficient CEN DNA bearing beads. Cells from the mif2-3 strain and a congenic wild-type strain were grown at the permissive (25°C) and restrictive (37°C) temperatures, and processed into whole cell extract. Microtubule binding activity of beads bearing wild-type CEN DNA (solid columns) or CDEI deficient CEN DNA (striped columns) in presence of the two extracts was measured. Microtubule binding activity is expressed as the number of beads bound per field.


Microtubule Binding Activity
(Beads Bound per Field)
30
25
20
15
10
5
0
WT Beads
CDEI(-) Beads
Ils grown at:
25°C
37°C
25°C
37°C
Strain:
WT
mif2-3

DISCUSSION:

A) LOW MOLECULAR WEIGHT FRACTION REQUIRED FOR PROPER MICROTUBULE BINDING:

We have proven the presence of a low molecular weight fraction in whole-cell yeast extracts that is required for proper microtubule binding activity as judged by an *in vitro* assay. This molecule, or group of molecules, can be lost by dialysis or when extract proteins are precipitated by an ammonium sulfate cut step. The microtubule binding activity of the resulting inactive dialyzed extracts or precipitated protein fractions, can be re-stimulated 8-12 fold upon the addition of the low molecular weight fraction. This stimulatory protein-free fraction can be obtained either by filtering or boiling wild-type extracts in order to remove all proteins, leaving behind only cytosolic, soluble, low molecular weight material.

An inhibitory effect observed for higher concentrations of boiled low molecular weight fractions is absent in the spin-filtered fractions. The selective separation of the two effects upon filtration suggests that two different activities are in play. It also makes clear that the crude low molecular weight fractions are complex mixtures that require further purification in order to isolate the stimulatory activity.

There appears to be a certain level of species-specificity to this low molecular weight fraction since equivalent material obtained from *Schizosaccharomyces pombe* extracts displayed no stimulatory activity of the dialyzed *Saccharomyces cerevisiae* extract . Low molecular weight fractions from the more closely related *Kluveromyces lactis* exhibit a lower but detectable degree of stimulation. This clear species-specificity suggests that the factors responsible for the loss of microtubule binding activity of dialyzed extracts are most likely not an ion or common metabolite which should be found in similar concentrations in the three different microorganisms. This idea is further supported by the experiment where the three low molecular weight fractions are serially diluted and compared side-by-side for their stimulatory activity. The budding yeast fraction is 100 fold more active than the equivalent one

obtained from fission yeast and at least 10 fold more active than the fraction from *K lactis*. It would be extremely interesting perform the reverse experiment where beads coated with *K. lactis* CEN DNA, are used to test microtubule binding of dialyzed *K. lactis* whole cell extract. In this case we would predict that a low molecular weight fraction from *S. cerevisiae* would display a lower stimulatory effect than a *K. lactis* low molecular weight fraction. The absence of cloned K. lactis centromeres prevented us from carrying out this experiment.

The low molecular weight activity appears to be extraordinarily stable. Experiments aimed at destroying it with proteinases, ribonucleases and deoxyribonucleases where unsuccessful. It is therefore unlikely that the compound(s) responsible for the stimulatory effect is a peptide, or a short sequence of RNA or DNA. The resistance to the treatment with alkaline phosphatase further suggests that phosphorylation is not necessary for low molecular weight fraction activity. Nevertheless the clear absence of low molecular weight activity is stationary phase cell extracts indicates, that at least at a cellular level, the factor or factors responsible for activity can be degraded or down-regulated.

Using a bead series containing decreasing amounts of wild-type DNA per bead, it was discovered that the stimulation of dialyzed extract's microtubule binding activity by the low molecular weight fraction could be maximized when using beads carrying 32 wild-type CEN DNA sequences per bead. The fact that these beads displayed a larger stimulatory effect than the all-wild-type beads carrying all 800 WT CEN sequences per bead suggest that the low molecular weight effect is subtle in nature, and can be best observed when overall bead binding activity is slightly compromised.

It is not clear what the mechanism of action for the low molecular weight fraction is. Nevertheless, the persistence of the microtubule binding stimulatory effect when using the CDEII/CDEI deficient bead series suggests that the low molecular weight fraction probably acts in a CDEIII specific manner. In this respect, the absence of any detectable effect on DNA-binding activity excludes the possibility that the low molecular weight factor(s) are acting upon the CDEIII/CBF3 interaction. Nevertheless,

before ruling out any CDEI or CDEII specific interactions, it should be noted that the beads carrying lower amounts of CDEII/CDEI deficient CEN DNA contained increasing numbers wild-type CDEI and CDEII containing CEN DNAs. These inactive CEN sequences used to keep the overall number of DNA molecules per bead at 800, bear a 3 bp deletion in their CDEIII element, but are otherwise wild-type for CDEI and CDEII. In these artificially high CEN DNA densities encountered on a bead, the CDEI and CDEII elements on one DNA molecule could act in *trans* on neighboring CDEI and CDEII deficient CEN sequences. In order to rule out this possibility, balancing DNA sequences should be constructed which in addition to the 3 bp deletion in CDEIII are also deficient for CDEI and CDEII.

The failure to bind the stimulatory activity to any of several different ion-exchange columns under a number of pH conditions supports the idea that the factor responsible for the stimulatory effect is not an ion and is most likely uncharged. The successful binding to the C18 resin of the Sep-Pak cartridges via hydrophobic interactions corroborates this notion. The attempted reverse phase chromatography purification scheme suggests that there are more than one active species within the low molecular weight fraction responsible for the stimulation of the microtubule binding activity of dialyzed extract. It is not clear whether the two major and four minor activity peaks that eluted from the VYDAC C18 column correspond to degradation products of a single species, or if they constitute independent entities. It is clear however, that two (fraction #50 and #61) of the six activity peaks were at least 10 fold more active than the rest. The elution profile of these two fractions form the gel filtration column could not offer additional insights since the apparent molecular weight was close or under the exclusion limit of the resin. In any case the molecular weights of the species involved appear to be smaller than the Vitamin B12 (MW=1355) used as one of the molecular weight sizing standards.

Much to our regret, we have recently been unable to get the low molecular weight assay to reproduce the high stimulation levels shown in this set of results which were the product of experiments performed over a period of 9 months. We nevertheless feel confident that the data presented here for

the ammonium sulfate cut and dialyzed extracts represent a clear, strong, and specific effect. We obviously however still don't fully understand all the variables involved in this phenomenon, since different batches of dialyzed extract appear to display varying degrees of susceptibility to the low molecular weight fraction. Different batches of low molecular weight fractions do behave consistently though. Limited observations not discussed here, lead us to believe that the salt concentration in the dialysis buffer plays a key role in the loss of microtubule binding activity of wild-type extracts. Other variables such as cell cycle stage and precise cell density of the cultures could also be important but have not been studied so far.

B) MIF2 APPEARS TO BE INVOLVED IN MICROTUBULE BINDING:

We have show that two temperature sensitive alleles of MIF2 (mif2-2 and mif2-3) compromise microtubule binding activity as assayed *in vitro*. Both alleles have point mutations in the carboxy-terminal region of MIF2 which displays the highest degree of homology to mammalian centromere binding protein CENP-C. The fact that a plasmid carrying the wild-type MIF2 gene under its endogenous promoter rescues the microtubule binding levels of mif2-3 extracts suggests a direct role for MIF2 in mediating microtubule binding activity. An even more striking demonstration of the involvement of MIF2 in mediating CEN DNA and microtubule binding would have been the addition of MIF2 protein to the inactive cell extracts. The experiments aimed at tagging and overproducing MIF2 are underway, and we expect to conduct this experiments in the near future.

One concern arising from reduced microtubule binding levels is whether this drop originates from a defect in microtubule binding or DNA binding. Although it is hard to draw a precise line separating the two functions it is believed that these are two discrete activities since the widely accepted CDEIII binding complex, CBF3, is required but not sufficient for microtubule binding (Sorger et al., 1994). It is also clear that any mutation affecting DNA binding will be epistatic to the microtubule binding activity. Upon examination of CDEIII DNA binding levels, it became apparent that the mif2-3 extracts display lower DNA binding activities both at the restrictive and permissive temperatures than the congenic wild-type strain. Surprisingly the DNA binding activity of the extracts from the wild-type strain itself were also lower at 37°C than at room temperature. It is not clear why this difference arises, but the lack of protease deficient strains could result in accelerated protein breakdown at the higher temperatures even in the presence of protease inhibitors. Comparison of the mif2-3 extracts at both temperatures suggests a maximum difference in CBF3 activity of 20% as judged from CDEIII binding in bandshift gels. Side-by-side comparison of microtubule binding activity proved, however, that even an increase of 50% total protein concentration of 37°C-grown mif2-3 extracts still displayed significantly lower microtubule binding

levels as compared to the room temperature-grown extracts. Therefore, the reduced levels of DNA binding activity can not fully explain the lower microtubule binding activity of the mif2-3 extracts grown at the restrictive temperature. We therefore a propose that MIF2 follows a CBF3-independent mechanism of action in executing its role in microtubule binding. A plausible model could be that MIF2 bridges the CBF3 complex with specific microtubule binding proteins

We were not able to correlate any increased defects in microtubule binding activity between mif2-3 extracts and defects in the CDEI element. As was mentioned in the introduction it has been reported that the chromosome-loss phenotype of MIF2 mutants can be exacerbated by mutations in either CDEI or CBF1. Specifically MIF2 stabilizes dicentric mini-chromosomes and confers high instability to chromosomes bearing mutations in CDEI or CBF1 (Meluh & Koshland 1995). Our experiments using beads deficient in CDEI and CDEII did not display lower microtubule binding activities for the mif2-3 extracts than when using wild-type beads. As was pointed out for the low molecular weight fraction experiments, it may be advisable to construct beads with lower number of active CEN sequences mutant for CDEI, where the inactive CEN sequences used to keep the overall number of DNAs per bead constant are not only defective in CDEIII but are also CDEI and CDEII deficient. Such beads were unavailable for the experiments presented here, but could uncover a CDEI dependence of the MIF2 effect by increasing the sensitivity of the assay.

The putative A/T rich-DNA binding motif has been used to suggest that the MIF2 gene product could be binding the A/T rich CDEII element (Brown 1995; Meluh & Koshland 1995). Our findings using a plasmid lacking a 12 amino acid stretch including the conserved 8 amino acid sequence RPRGRPKK do not support this view. We found that a plasmid lacking the putative A/T binding motif was able to rescue the temperature sensitive phenotype of mif2-3 cells. It would be interesting to examine this plasmid using a standard plasmid-shuffle scheme rather than using it to cover the TS allele. A MIF2 swapper strain is currently being constructed and will be available soon for this type of test. We will also be able to use this strain to make extracts from cells expressing an A/T rich binding domain

deficient version of MIF2. These extracts can then be used to examine microtubule binding and DNA binding activities. It is plausible that strains compromised by decreased levels of microtubule or DNA binding activity are still viable. It therefore remains a possibility that the A/T rich DNA binding domain plays a non-essential role in kinetochore function which was not detected in this experiment.

Our finding suggesting the lack of a requirement for the A/T rich-DNA binding motif in MIF2 clearly contradict reported results (Brown 1995). This particular paper reports that several conditional lethal alleles of MIF2 reveal mutations in the A/T binding motif. Close sequence analysis however, shows that mif2-7, one of two of such alleles, contains three different mutations resulting in substitutions at amino acids 325, 353 and 399. Only one of the three mutations is claimed to fall into the A/T binding domain. However, the sequence defined by Brown as the A/T binding domain in this report is larger than the sequence she defined previously (Brown et al., 1993). This "extended A/T binding domain" incorporates additional residues which are homologous to HMGI but not to *Drosophila* D1 or any other A/T binding protein. Analysis of several such A/T rich-DNA binding proteins (Churchill and Travers, 1991) reveals that the only true consensus sequence among all members of the family is Gly-Arg-Pro, or GRP. Based on this, the closest mutation of mif2-7 is 4 residues N-terminal of the A/T binding domain as it was originally defined, and 7 residues upstream from the GRP sequence. Considering that any of the other two mutations could also have an effect on MIF2 function, we don't agree with the conclusion that mif2-7 affects the putative DNA binding domain. The other allele mentioned by Brown is mif2-9. This cold sensitive allele consists of two mutations, the closest to the area of interest being 8 residues N-terminal of mif2-7, and therefore not within the A/T binding domain as we see it.

It is also worth remarking that most proteins known to bind A/T rich-DNA contain several such DNA binding motifs (Yeast Datin, HMGI and HMGY contains 3 each while Drosophila D1 contains 11) while MIF2 displays only one.

Finally, the total lack of an A/T binding domain in CENP-C, which of all known proteins is the closest in sequence homology to MIF2, and is known to be kinetochore protein encourages us to believe that the putative A/T rich-DNA binding domain is not essential for MIF2 function.

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