Homotetrameric Form of Cin8p, a Saccharomyces cerevisiae Kinesin-5 Motor, Is Essential for Its in Vivo Function

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Kinesin-5 motor proteins are evolutionarily conserved and perform essential roles in mitotic spindle assembly and spindle elongation during anaphase. Previous studies demonstrated a specialized homotetrameric structure with two pairs of catalytic domains, one at each end of a dumbbell-shaped molecule. This suggests that they perform their spindle roles by cross-linking and sliding antiparallel spindle microtubules. However, the exact kinesin-5 sequence elements that are important for formation of the tetrameric complexes have not yet been identified. In addition, it has not been demonstrated that the homotetrameric form of these proteins is essential for their biological functions. Thus, we investigated a series of Saccharomyces cerevisiae Cin8p truncations and internal deletions, in order to identify structural elements in the Cin8p sequence that are required for Cin8p functionality, spindle localization, and multimerization. We found that all variants of Cin8p that are functional in vitro form tetrameric complexes. The first coiled-coil domain in the stalk of Cin8p, a feature that is shared by all kinesin-5 homologues, is required for its dimerization, and sequences in the last part of the stalk, specifically those likely involved in coiled-coil formation, are required for Cin8p tetramerization. We also found that dimeric forms of Cin8p that are nonfunctional in vivo can nonetheless bind to microtubules. These findings suggest that binding of microtubules is not sufficient for the functionality of Cin8p and that microtubule cross-linking by the tetrameric complex is essential for Cin8p mitotic functions.

Mitotic chromosome segregation is the mechanism by which duplicated genomic information is transmitted to daughter cells during cell division. This essential process is mediated by the mitotic spindle, a highly dynamic, microtubule-based structure that undergoes a distinct set of morphological changes. Many of these changes are achieved by the action of molecular motors from the kinesin-5 (BimC) family, which use ATP hydrolysis to unidirectionally move along microtubules. Members of the kinesin-5 family are conserved in the amino acid sequence of the motor (force-producing) domain and apparently perform similar roles in many different cell types (1–7). Kinesin-5 motors are required for bipolar spindle assembly, and elimination of their function blocks this essential early mitotic step in fungal, insect, and mammalian cells (8–11). The yeast Saccharomyces cerevisiae expresses two kinesin-5 motors that overlap in function, Cin8p and Kip1p. Although neither is individually essential, one of the pair is required for viability (2, 3, 12). Loss of KIP1 function causes less severe phenotypes than loss of CIN8, suggesting that Cin8p is more important for successful yeast spindle function (2). Aside from their essential role in spindle assembly, Cin8p and Kip1p are also required for the maintenance of spindle bipolarity following assembly and are responsible for producing most of the spindle-elongating force during anaphase (2, 13, 14).

Kinesin-5 motor proteins are unique molecular motors in that they function as homotetramers, with pairs of catalytic motor domains located on opposite sides of the active motor complex (15, 16). It is thought that this special architecture enables the kinesin-5 motors to cross-link and slide antiparallel microtubules originating from opposite spindle poles (8, 10). Similar to other members of the kinesin superfamily, the N-terminal catalytic domain of kinesin-5 motors is responsible for their motor activity, i.e. binding and moving along microtubules. The stalk and the tail regions are believed to contain structural elements that are responsible for multimerization of the kinesin-5 protein complex. Although it has been demonstrated that truncated forms of kinesin-5 polypeptides that contain the catalytic domain and parts of the stalk form dimers (17), the precise structural elements in the stalk and tail that are essential for their dimerization and tetramerization have not as yet been identified. In addition, although it has been recently demonstrated that the tetrameric kinesin-5 HsEg5 can slide antiparallel microtubules in vitro (18), it is not clear whether formation of this homotetrameric complex structure is essential for kinesin-5 intracellular functions.

In this study we investigated the in vivo and in vitro properties of a series of Cin8p truncations and internal deletions, in order to identify structural elements in the Cin8p sequence that are required for Cin8p functionality, spindle localization, and multimerization.

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**TABLE 1**

**Yeast strains and plasmids used in this study**

| Genotype |
|----------|
| **Yeast strains** | |
| BS334 | α pep4-3 reg1-501 leu2-3,112 gal1 pth1-112 ura3-52 (43) |
| MAY2057 | a his3::Δ200 leu2-3,112 lys2-801 ura3-52 cin8::LEU2 |
| MAY2063 | a ade2-101 his3::Δ200 leu2-3,112 lys2-801 ura3-52 cin8::URA3 |
| MAY2077 | a his3::Δ200 leu2-3,112 lys2-801 ura3-52 kip1::HIS3 |
| MAY3789 | a ade2-101 his3::Δ200 leu2-3,112 lys2-801 ura3-52 cyh2 cin8::HIS3 kip1::HIS3 pMA1208 |
| MAY5590 | a ade2-101 his3::Δ200 leu2-3,112 lys2-801 ura3-52 cyh2 cin8::HIS3 kip1::HIS3 pTK97 |
| MAY6752 | a his3::Δ200 leu2-3,112 lys2-801 ura3-52 cyh2 cin8::HIS3 dyn1-Δ3::HIS3 pEH24 |
| **Plasmids** | |
| pEH24 | CIN8 URA3 CYH2 CEN |
| pEH50 | 6MYC-CIN8-BCP LYS2 CEN |
| pEH54 | PGAL1::GAL1 > 6MYC-CIN8-BCP URA3 CEN |
| pEH55 | PGAL1 > CIN8-3HA LEU2 CEN |
| pEH73 | Δmotor-cin8-3HA HIS3 CEN |
| pEH113 | CIN8 LEU2 CEN |
| pEH72 | 6MYC-NLS-CIN8 LEU2 2μ |
| pMA1125 | CIN8 URA3 CEN |
| pMA1208 | CIN8 LEU2 CYH2 CEN |
| pMA1260 | CIN8 LYS2 CEN |
| pRS315 | (vector) LEU2 CEN |
| pRS317 | (vector) LYS2 CEN |
| pSM218 | (vector) LEU2 2μ |
| pTK49 | CIN8-3HA LYS2 CEN |
| pTK97 | KIP1 URA3 CEN |
| pTK103 | 6MYC-CIN8 LYS2 CEN |
| pTK138 | 6MYC-CIN8 LEU2 2μ |
| pTK167 | CIN8-3HA HIS3 CEN |

**EXPERIMENTAL PROCEDURES**

Microscopy, gel electrophoresis, and immunoblotting were performed as described previously (19).

**Yeast Strains and DNA Manipulations—**Rich media (YPD) and synthetic media (SD) were as described by Sherman et al. (20). Hydroxyurea (Sigma) was added to 0.1 M in pH 5.8 liquid synthetic media. S. cerevisiae strains are listed in Table 1. All strains were derivatives of S288C with the exception of the strain BS334, which was used for overexpression of Cin8p from the PGAL1 promoter. In this case galactose was added to 0.5%.

Standard techniques were utilized for DNA manipulations (22). CIN8 truncation and deletion alleles were constructed by either restriction enzyme cleavage and religation or oligonucleotide-directed mutagenesis using either the USE mutagenesis method (23) or megaprimer mutagenesis (24). Specific details about construction of mutants are available as a supplemental method (23) or megaprimer mutagenesis (24). Specific details about construction of mutants are available as a supplemental method (23) or megaprimer mutagenesis (24).

**Microscopy**—Sections were mounted in 50% glycerol, 50% DMSO, and 25% PBS (pH 7.4). Coverslips were coated with Cryo-Mount (Biocare Medical) and used for cryo-immuno-EM.

**Immunoprecipitation and Hydrodynamic Studies**—All immunoprecipitations were performed using strain MAY2063 carrying two centromeric plasmids, one encoding an N-terminal 6Myc-tagged Cin8p variant (variants of pTK103) and the other encoding full-length Cin8p with a C-terminal 3HA tag (pTK167). The “no tag” control strain carried pMA1260 and pTK167. For examining the Δmotor mutant, cells co-expressed full-length 6myc-Cin8p (pTK103) and Δmotor-Cin8p-3HA (pEH73). Each immunoprecipitation used 35 ml of a 100 Klett (mid-log phase) culture grown in synthetic selective media. Extracts were prepared by liquid nitrogen grinding as described for the hydrodynamic studies (see below) except the buffer contained 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml each antipain, aprotinin, and leupeptin. A small portion of each extract was set aside to check expression levels of the Cin8p mutants. Extracts were diluted 3.5-fold and then clarified by three 10-min microcentrifuge spins at 4 °C. Monoclonal anti-Myc antibody, 9E10 (Santa Cruz Biotechnology), was added to 1 μg/ml and incubated for 4 h at 4 °C. Protein G-Sepharose beads (Sigma) in buffer containing 0.2 mg/ml BSA were added to a final concentration of 10% v/v and incubation was continued for 1 h. Beads were washed four times in buffer with BSA, resuspended in protein sample buffer, and boiled for analysis by SDS-PAGE and immunoblotting.

**Hydrodynamic Analysis**—To determine the size of Cin8p complexes in crude extracts, a combination of gel filtration and sucrose density gradient centrifugation was used. Cultures were grown in 100 ml of appropriate synthetic media to mid-log phase (100 Klett units). Cells were harvested, resuspended in a small volume of 25% glycerol, and frozen at −80 °C until use. To prepare extracts, cells were washed in TNED buffer (50 mM triethanolamine-NaOH, pH 7.75, 500 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 3 The abbreviations used are: HA, hemagglutinin; 5-FOA, 5-fluoro-orotic acid; NLS, nuclear localization sequence; BSA, bovine serum albumin.)
and 0.5 μg/ml each pepstatin A, antipain, leupeptin, and apro- tinin) and resuspended in 0.25 ml of the same buffer. This mixture was frozen dropwise in liquid nitrogen. Cells were disrupted by grinding for 200 strokes under liquid nitrogen using a pre-chilled mortar and pestle. 0.2 ml of additional buffer was added. Cell debris was removed by two 10-min spins in a micro-centrifuge at 4 °C followed by 30 min at room temperature in a Beckman Airfuge at 130,000 × g. As a final clarification step, extracts were filtered through 0.2-μm spin filters (U. S. Biologicals). Gel filtration was performed by applying 0.2 ml of clarified extract to a Superose 6 (Amersham Biosciences) column pre-equilibrated with TNED buffer. 0.5-ml fractions were collected and then supplemented with 10 μg of BSA as a control for protein recovery following precipitation with methanol/chloroform (4:1). Protein pellets were resuspended in 1× Sample buffer, and the fractions were analyzed by SDS-PAGE and immunoblot (22). The Stokes radius of Cin8p was determined using the plot of the Stokes radius of standard proteins (chicken muscle myosin, 19.45 nm; thyroglobulin, 8.5 nm; apoferritin, 6.35 nm; β-amylase, 5.4 nm; catalase, 5.22 nm; alcohol dehydrogenase, 4.58 nm; BSA, 3.55 nm; and carbonic anhydrase, 2.01 nm) versus square root (−logKw) and (Kd)1/3 as described previously (25). The two methods were then averaged to arrive at an Rv value for Cin8p. Briefly, Ksw = (Ve − Vo)/(Vr − Ve) and Kd = (Ve − Vo)/(Vb − Ve). Vb is the theoretical total volume of the column plus its tubing. Plasmid DNA and acetone were used to determine the void volume (V0) and total measured volume (Vr), respectively, of the Superose 6 column.

For sucrose gradients and determination of the sedimentation coefficient, extracts were prepared in the same manner as for gel filtration. 0.5 ml of the clarified extract was applied to an 11-ml 5–25% sucrose gradient in TNED buffer. Other ranges of sucrose gradient were also tested in some cases. The gradient was centrifuged in an SW41 rotor at 39,000 rpm for 30 h. Fractions were collected from the bottom of the gradient and concentrated as described above. The sedimentation coefficient of Cin8p was determined by plotting the elution volume versus the sedimentation coefficient of standard proteins (carbonic anhydrase, 3.13 S; BSA 4.43 S; alcohol dehydrogenase 5.5 S; β-amylase 8.9 S, catalase 11.3 S; and apoferritin 17.6 S).

The molecular weight of Cin8p complexes was calculated from the Stokes radius (σ) and sedimentation coefficient (s) using the formula M = 6πηνσ/(1 − νp). The constants used were viscosity (ŋ) = 1.002 g m⁻¹ s⁻¹; density of medium (p) = 0.9992 g cm⁻³; partial specific volume of protein (νp) = 0.736 cm³ g⁻¹; and Avogadro’s number (N). The frictional ratio was calculated using the formula χf = a/(3σM/4πN)1/3. The axial ratio was determined by assuming a prolate ellipsoid shape as described previously (26). To determine the stoichiometry of the variants for which an S-value was not measured, we used the plot of Rv versus stoichiometric molecular weight (see supplemental figure) for Cin8p forms where the S-value was measured (see Table 3). These data show that there is a linear relationship between Rv and Mv for Cin8p tetramers, trimers, dimers, and monomers. The Rv value of the remaining Cin8p forms was used to extrapolate a Mv from the graph, and from this a stoichiometry was calculated.

RESULTS

Functional Analysis of Cin8p—Cin8p consists of an N-terminal motor domain (amino acids 74–513), followed by a 411-amino acid stalk (amino acids 534–945) and a 92-amino acid tail (amino acids 946–1038) at the C terminus (Fig. 1). The stalk contains four regions of heptad repeat sequences predicted by the method of Lupas et al. (27) to form α-helical coiled-coil. Other prediction methods revealed similar regions of potential coiled coils, including the C-terminal portion of the stalk for which the method of Wolf et al. (28) indicates a high probability of multimeric coiled coils (not shown). The C-terminal tail harbors a 29-amino acid destruction signal sequence required for APC/Cdh1-mediated degradation, and an NLS, which contributes to optimal nuclear localization (19).

To assess the importance of the different domains for Cin8p function, we constructed a series of truncated and internally deleted variants. We first assessed the ability of these mutants to function as the sole source of kinesin-5 activity and thus support cell viability. cin8Δ is synthetically lethal with both kip1Δ and dyn1Δ (encodes the heavy chain of dynein) (2, 13). Therefore, we transformed low copy centromeric plasmids carrying Cin8p alleles into two strains, cin8Δ kip1Δ and cin8Δ dyn1Δ. These normally inviable genotypes are supported by a plasmid-borne copy of KIP1 (cin8Δ kip1Δ) or CIN8 (cin8Δ dyn1Δ) that also carried the URA3 gene. These strains can only survive on 5-fluoroorotic acid (5FOA)-containing media if they are transformed with an active CIN8 allele. The cin8Δ mutants were assayed for their ability to permit loss of the KIP1-URA3 (or CIN8-URA3) plasmid and thus grow on 5FOA. Several truncated forms in which much of the 92-amino acid tail region was removed were still functional (truncated after amino acid 1031, 26006 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 36 • SEPTEMBER 8, 2006
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Internal deletions in the tail and stalk revealed that all four predicted coiled-coil regions were essential for function (Fig. 2A). Interestingly, removal of the predicted noncoiled region between coils 1 and 2 (Δ668–745) did not destroy functionality in cin8Δ dyn1Δ cells but significantly reduced growth of cin8Δ dyn1Δ cells. On the other hand, the noncoiled region between coils 3 and 4 (842–885) was essential in both backgrounds. In the N-terminal portion of the protein, the highly conserved motor domain and neck regions were essential for function (Δmotor and Δneck). Regions that are not conserved among kinesin-5 family members such as the N-terminal 73 amino acids and a 100 amino acid insertion in the center of the motor domain (2) were not essential (ΔN-70 and Δmotor-insert, respectively).

We tested the nonfunctional alleles of Cin8p for dominant negative effects using a kip1Δ strain. If a mutant form of Cin8p interferes with the endogenous Cin8p activity, it would create a situation that mimics the deleterious cin8Δ kip1Δ genotype. We found three alleles (CIN8 – 871, Δ872–885, and Δ842–885) that exhibited a strong dominant negative effect at 37 °C (Fig. 2A) and a weaker effect at 26 °C (not shown). Thus, a region including amino acids 872–885 is necessary to prevent this dominant negative effect. Four other mutants (955, 885, 842, and Δcoil4) exhibited weak dominant negative effects when expressed at high copy (data not shown). Immunoblot analysis of tagged versions of the Cin8p mutants revealed that dominant negative effects were not because of differences in expression levels (Fig. 4 and data not shown). Negative growth defects were not observed when these truncated alleles were transformed into cin8Δ KIP1 strains, suggesting that the negative effect depends upon an interaction with the endogenous copy of Cin8p. In support, the cin8Δ Δcoil1,871 double mutant, missing the region critical for Cin8p-Cin8p interactions (see below) was not dominant negative (Fig. 2B, Δcoil 1,871). The dominant negative effect was also abolished by motor domain mutants combined in cis with the CIN8 – 871 mutation (e.g. G171E,871; F467A,871, and R394A,H396A,871). These results suggest that the dominant negative alleles interact with endogenous Cin8p and cause aberrant outcomes in a manner that is dependent upon the activity of the catalytic motor domain.

Cin8p Spindle Localization Depends upon the C-terminal Tail and Stalk Coil 1—N-terminal 6Myc epitope-tagged versions of select Cin8p mutants were examined for their ability to localize to the mitotic spindle in a cin8Δ genetic background. To enhance the immunofluorescence signal, constructs were expressed from high copy 2-µm plasmids. 6myc-Cin8p could be detected on nuclear spindle microtubules (Fig. 3) as was observed previously for Cin8p tagged with HA at its C terminus (2). Truncating into the C terminus (1031, 990, and 972), and removing the previously identified nuclear localization sequence (C-terminal seven amino acids) led to diffuse cytoplasmic staining (Fig. 3) as was observed previously for Cin8p tagged with HA at its C terminus (2). Truncating into the C terminus (1031, 990, and 972), and removing the previously identified nuclear localization sequence (C-terminal seven amino acids) led to diffuse cytoplasmic staining (Fig. 3) as was observed previously for Cin8p tagged with HA at its C terminus (2).
showed the same staining pattern as the independent mutants. Furthermore, the combined mutant was phenotypically no worse than either single mutant in functionality tests (data not shown). These results suggest these sequences may be part of a single nuclear import motif with similarities to a bipartite NLS (KKHAIEDEKSSENVDNEGSRKMKL) but with an

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**Figure 3. Cin8p truncated forms decorate nuclear and cytoplasmic microtubules.** The indicated 6Myc-tagged (A) or 6myc-SV40-NLS-tagged (B) CIN8 forms were expressed from high copy plasmids in cin8Δ cells (MAY5590). Cultures were treated with the DNA synthesis inhibitor hydroxyurea to cause the accumulation of cells containing a short pre-anaphase bipolar spindle. The cells were fixed and stained for Cin8p (anti-Myc), tubulin (anti-tubulin), and DNA (4,6-diamidino-2-phenylindole (DAPI)). wt, wild type.

**Table 2**

Localization of Cin8p mutants in cin8Δ strain

| Cin8p form | 6myc-Cin8p | 6myc-SV40-NLS-Cin8p |
|------------|------------|----------------------|
| 1038 (wild type) | Spindle | Spindle |
| 1031* | Spindle and cytoplasm | Spindle |
| 990 | Spindle and cytoplasm | Spindle |
| 972 | Spindle and cytoplasm | Spindle |
| 955 | cMT > spindle | cMT > spindle |
| 885 | Cytoplasm > cMT | Spindle and nucleus |
| 871 | Weak cytoplasm | Spindle and nucleus |
| 779 | Weak cytoplasm | Spindle and nucleus |
| 706 | | |
| 590 | Cytoplasm (some nucleus) | Nucleus |
| Δ956–1030 | cMT and spindle | ND |
| Δcol 4 | Spindle | ND |
| Δ872–885 | Spindle and nucleus | ND |
| Δ842–885 | Spindle and nucleus | ND |
| Δcol 1 | Nucleus | ND |
| K1033D, M1034L | Spindle and cytoplasm | Spindle |
| K1012A, K1013A | Spindle and cytoplasm | Spindle |
| K1012A, K1013A, K1033D, M1034L | Spindle and cytoplasm | Spindle |
| S1010A | Spindle | ND |
| S1010A | Spindle | ND |

*Data are from Ref. 19.

* Cin8p staining is detected on the spindle microtubules and in the cytoplasm.

* cMT indicates cytoplasmic microtubules.

* Cytoplasmic staining of Cin8p is brighter than that of the spindle microtubules.

* Cytoplasmic staining of Cin8p is brighter than that of cytoplasmic microtubules.

* Cin8p staining is detected on spindle microtubules and in the nucleus.

* Equal Cin8p staining on the spindle and cytoplasmic microtubules.

* ND indicates not determined.
The above findings indicate that Cin8p mutants have varying abilities to bind to microtubules and can even bind cytoplasmic microtubules when nuclear entry is impaired. To better evaluate the microtubule binding capabilities of these mutants without regard to localization, we placed an SV40-NLS after the 6Myc tag at the N terminus. We previously reported that the SV40-NLS could restore wild-type like localization to the cin8-1031 mutant missing the C-terminal NLS (19). The SV40-NLS had no effect on the staining pattern of wild-type Cin8p (Fig. 3). Truncation mutants up to amino acid 885 showed spindle staining very similar to wild type. Cin8p-871, the dominant negative allele, showed markedly reduced spindle staining and diffuse nuclear staining. A similar result was seen for the dominant negative internal deletion alleles (Δ842–885, Δ872–885) that have Cin8p’s own NLS but no SV40-NLS. The SV40-NLS version of truncation 590 showed diffuse nuclear staining but no spindle staining.

Taken together, our localization results suggest that coil 1 is essential for microtubule binding by Cin8p, and the region required for the dominant negative effect, amino acids 872–885, enhances microtubule binding.

**Stalk Coil 1 Is Essential for Cin8p-Cin8p Interactions**—Because other kinesin-5 homologues form homotetramers (15, 16), and because we identified dominant negative CIN8 alleles, we expect that Cin8p interacts with itself in a complex. To test this, we looked for the ability of two epitope-tagged versions of Cin8p to co-immunoprecipitate from yeast extracts. Either a triple hemagglutinin tag (3HA) was added to the C terminus or a 6Myc tag was added to the N terminus of Cin8p. Expression of either CIN8-3HA or 6myc-CIN8 was able to suppress the lethality of cin8Δ kip1Δ indicating that the tagged gene products are functional. The tagged Cin8ps were co-expressed in a cin8Δ strain, and protein extracts immunoprecipitated with anti-Myc antibody. Cin8p-3HA was present in immunoprecipitates from extracts co-expressing 6myc-Cin8p (Fig. 4, 1038) but not untagged Cin8p (Fig. 4, 1038 untagged). The co-immunoprecipitation was not microtubule-dependent because treatment of extracts with the microtubule depolymerizing drug nocodazole did not affect the interaction (data not shown).

The 6Myc-tagged Cin8p truncated forms were tested for their ability to interact with full-length Cin8p-3HA (Fig. 4). Truncated forms up to amino acid 842 co-precipitated with Cin8p-3HA just as well as the full-length Cin8p (1038). Truncated Cin8p-779 and Cin8p-706 showed a reproducibly weaker interaction. Truncation at amino acid 590, removing most of coil 1, eliminated the interaction. An internal deletion of coil 1 was also unable to co-precipitate, suggesting that coil 1 is essential for the Cin8p-Cin8p interaction. Based upon experiments using other internal deletion mutants (Δcoil2–3, Δcoil4, Δ668–

**FIGURE 4.** Co-immunoprecipitation demonstrates that Cin8p-Cin8p interactions require coil 1 in the stalk region. Protein extracts were generated from cells co-expressing two forms of Cin8p from separate low copy plasmids in a cin8Δ strain (MAY2063). The first plasmid expresses full-length Cin8p with a C-terminal 3HA tag (pTK167). The second plasmid expresses the indicated truncation or deletion mutant with an N-terminal 6Myc tag (derived from pTK103) except for the untagged control (pMA1260). The Δmotor-Cin8p mutant has a C-terminal 3HA tag (pEH73) and was co-expressed with full-length 6myc-Cin8p (pTK103). Extracts were treated with 9E10 antibody and protein G beads to precipitate the 6Myc-tagged forms of Cin8p. The amount of Cin8p-3HA that co-immunoprecipitates (co-IP) is shown on the top panel. The bottom panel shows the expression level of 6myc-Cin8p versions prior to immunoprecipitation. Equal amounts of extract were applied to each lane.

unusually long 18-amino acid spacer separating the two clusters of basic amino acids (29). As with many bipartite NLS sequences, there is a potential cdc2 phosphorylation site adjacent to the putative bipartite NLS in Cin8p. However, mutation of the serine to alanine (S1010A) had no effect on Cin8p localization, suggesting that phosphorylation at this site does not regulate localization. Additionally, this region of the Cin8p tail is not identified in algorithms that recognize typical nuclear localization sequences. Because the mislocalized tail mutants are still functional, there must be enough Cin8p entering the nucleus to carry out essential nuclear functions for Cin8p. Therefore, the tail of Cin8p has regulatory sequences that include a long segment of basic amino acids that contribute to nuclear localization, but additional determinants for nuclear import are likely in other parts of the protein.

Two nonfunctional Cin8p mutants (955 and 885) localized to both cytoplasmic and nuclear microtubules (Fig. 3 and Table 2). The density of *S. cerevisiae* microtubules is greater in the nucleus than the cytoplasm. This is reflected by the increased brightness of the nuclear microtubules when stained with tubulin antibodies (Fig. 3). When the microtubules were decorated with Cin8p-955 or Cin8p-885, however, cytoplasmic microtubule staining appeared as bright as or brighter than nuclear microtubule staining (Fig. 3). This may indicate that these truncated forms localize to the cytoplasmic microtubules at a higher density than they localize to the nuclear microtubules.

The dominant negative allele CIN8–871 showed diffuse cytoplasmic staining with rare microtubule staining. Shorter truncated forms (842, 779, and 706) showed similar but weaker staining patterns overall. Cin8p-590 showed only diffuse cytoplasmic staining with no microtubule staining. Deletion of the two highest probability coiled-coil regions caused different localization patterns. Cin8p-Δcoil1 stained the nucleus but not spindle microtubules, whereas the Cin8p-Δcoil4 mutant gave strong spindle staining (Table 2).
Multimerization State of Cin8p Variants

To determine whether a homotetrameric state is essential for Cin8p function, we performed experiments to assess the multimerization state of the different Cin8p variants. First, to examine whether Cin8p forms an elongated homotetrameric complex like other kinesin-5 homologues (15, 16, 30), we measured the hydrodynamic properties of Cin8p from whole yeast extracts. Epitope-tagged Cin8p was expressed at low copy in a cin8Δ/H9004 strain. Yeast extracts were prepared under high ionic strength conditions that minimized nonspecific Cin8p aggregation, and were then applied to a sucrose gradient to determine the sedimentation coefficient and a Superose 6 gel filtration column to determine the Stokes radius (Fig. 5 and Table 3). The peak Cin8p-containing fractions were identified by immunoblotting and compared with a set of standard proteins with known hydrodynamic properties. 6myc-Cin8p was found to have a Stokes radius of 14.0 nm and a sedimentation coefficient of 8.3 S. Based on these measurements we calculate the Cin8p complex has a native molecular mass of 498 kilodaltons and an axial ratio of 39:1. Similar results were obtained for Cin8p-3HA (Table 3). Because the molecular mass of a single 6myc-Cin8p polypeptide chain is 129 kilodaltons, our measurements are consistent with an elongated tetrameric complex for Cin8p, as has been found for other kinesin-5 motors.

Cin8p that was highly overexpressed from the GAL1 promoter and then partially purified by 20–40% ammonium sulfate precipitation or ion exchange chromatography prior to loading on the Superose 6 column had a similar Stokes radius (~14 nm) (Table 3). This suggests that the measurements we made of Cin8p from crude extracts are not affected by contaminating proteins. We believe that under the in vitro conditions of the gel filtration column it is unlikely that Cin8p is associated with other polypeptides in stoichiometric amounts. However, because it was difficult to purify significant quantities of Cin8p complex from yeast, the possibility of Cin8p forming complexes with other polypeptides cannot be ruled out.

To determine which regions of the stalk and tail of Cin8p contribute to tetramer formation, we measured the hydrodynamic properties of some of the truncation and deletion mutants described above. All the versions of Cin8p that were functional in vivo had similar Stokes radius values (13.1–14.2 nm; see Table 3). For instance, 6myc-Cin8pΔ668–745, the functional deletion mutant missing the region between coils 1 and 2, had a Stokes radius value of 13.1 nm. This is slightly smaller than the value for full-length Cin8p as one would expect for a complex of similar size and shape but made of smaller polypeptides.

Three nonfunctional truncated forms (Cin8pΔ871, -779, and -706) had Stokes radii and sedimentation coefficients consistent with homodimers rather than tetramers (Fig. 5 and Table 3). The proposed dimeric forms of Cin8p still had elongated structures, but the axial ratios (21:1, 20:1, and 19:1, respectively) were smaller than the tetrameric forms. This dimeric structure is consistent with our observation that these nonfunctional Cin8p forms maintained the ability to co-immunoprecipitate with full-length Cin8p (Fig. 4). The shortest Cin8p truncation, eliminating most of coil1 (6myc-Cin8pΔ590), had a considerably smaller Stokes radius (4.9 nm) and sedimentation coefficient (4.2 S) consistent with a monomer.
Three of the nonfunctional mutants, 6myc-Cin8p-955, 6myc-Cin8p-855, and 6myc-Cin8p-Δcoil4 (Table 3), gave ambiguous hydrodynamic results. When the center of the peak is used to determine the hydrodynamic values, the calculated stoichiometry for both of these forms is a trimer. However, the broader sedimentation and gel filtration profiles suggest that these forms may contain mixed populations of complexes. One possibility is a mixture of dimers and tetramers, whereas another is a mixture of elongated and compact forms.

To summarize, examination of the hydrodynamic properties of Cin8p variants revealed that all functional forms of Cin8p formed tetrameric complexes. Truncated forms at amino acid 990 (or longer) were tetramers, whereas the form truncated at amino acid 590 forms a monomer. Forms truncated between amino acids 706 and 871 were dimers. We also found that coil 4 is important for formation of the tetrameric Cin8p complex.

### DISCUSSION

**Homotetrameric Structure Is Essential for Cin8p Function in Vivo**—In this study we analyzed the in vivo function and hydrodynamic properties of Cin8p truncations and internal deletions in order to identify regions of the stalk and tail that are important for its multimerization and functionality. We found that all forms of Cin8p that were functional, including the different epitope-tagged versions and mutants, expressed in low copy or overexpressed, form complexes with a Stokes radius of 13–14 nm. The full-length versions of Cin8p have an axial ratio above 30 (Table 3). This high axial ratio indicates that the Cin8p complex is elongated, which is consistent with the complex shape demonstrated by rotary shadow microscopy for kinesin-5 homologues from *Xenopus* (Eg5) and *S. cerevisiae* (Kip1p) (15, 16) as well as other molecular motors from the kinesin (31) and myosin (32) superfamilies. A combination of gel filtration and sucrose gradient data indicated that the Cin8p complex is tetrameric and does not contain additional subunits in stoichiometric amounts. The sedimentation coefficient and Stokes radius values obtained for Cin8p (Table 3) are very similar to those reported for the other *S. cerevisiae* kinesin-5 protein Kip1p (16). These data indicate that similar to Kip1p and the homologous *Drosophila melanogaster* klp61F (15), Cin8p is a bipolar kinesin, with two pairs of motor domains located on opposite sides of the complex. This architecture enables Cin8p to cross-link and slide antiparallel spindle microtubules (21) and thereby to perform its mitotic roles (2).

Experiments presented here clearly indicate that to produce in vivo function, Cin8p has to form a properly folded tetrameric complex. All forms of Cin8p whose hydrodynamic properties indicated that they are dimeric or monomeric were not functional in vivo. Some forms of Cin8p have hydrodynamic properties that are consistent with a formation of a trimeric complex. However, because of the broad elution peaks from sucrose gradient and gel filtration columns, these forms could also be misfolded tetramers (Table 3). Although the exact nature of these "trimeric" complexes is not clear, the fact that these forms are not functional in vivo (Fig. 2) supports the notion that the homotetrameric and probably bipolar Cin8p complex is essential for its functionality.

Our data also indicate that the ability of Cin8p mutants to bind microtubules in vivo is not sufficient to accomplish the function of Cin8p. Mutant forms Cin8p-955, -885, -871, and Δcoil4 exhibited various degrees of binding to cytoplasmic and spindle microtubules (Fig. 3 and Table 2). Addition of a nuclear localization signal to the N terminus of these mutants increased their spindle microtubule binding (Fig. 3 and Table 2). However, none of these mutants could render viability when they were the sole source of kinesin-5 activity in the cells even with

### Table 3

Summary of hydrodynamic properties of Cin8p variants

| Protein       | In vivo function | $R_0$ | $n^0$ | $S$-value | Sequence mass | Native $M_r$ | Stoichiometry | $f_{0}^*$ | Axial ratio |
|---------------|-----------------|-------|-------|----------|--------------|--------------|--------------|----------|------------|
| Cin8p-3HA     | Yes             | 13.3  | 5     | 7.6      | 122,452      | 435,487      | 4            | 2.5      | 36         |
| 6myc-Cin8p-955| No              | 11.1  | 4     | 7.5      | 119,397      | 358,548      | 3            | 2.4      | 30         |
| 6myc-Cin8p-885| No              | 9.8   | 2     | 8.2      | 110,124      | 328,306      | 2            | 2.0      | 21         |
| 6myc-Cin8p-871| No              | 8.2   | 2     | 6.5      | 98,751       | 200,601      | 2            | 2.0      | 20         |
| 6myc-Cin8p-779| No              | 7.6   | 2     | 6.1      | 90,210       | 175,444      | 2            | 1.9      | 19         |
| 6myc-Cin8p-926| No              | 6.0   | 2     | 5.2      | 76,570       | 87,056       | 2            | 1.7      | 14         |
| Cin8p-3HA ox  | Yes             | 13.2  | 4     | 4.2      | 111,540      | 343,162      | 3            | 2.1      | 23         |
| 6myc-Cin8p-BCP| Yes             | 14.2  | 4     | 4.4      | 144,4        | 497,911      | 4            | 2.6      | 39         |
| 6myc-Cin8p-BCP ox ASP | Yes | 14.8 | 1     | ND       | 128,665      | 435,487      | 4            | 2.5      | 36         |
| 6myc-Cin8p-1031| No              | 8.5  | 4     | 6.5      | 98,751       | 200,601      | 2            | 2.0      | 20         |
| 6myc-Cin8p-900| No              | 14.4  | 4     | ND       | 668–745      | 358,548      | 4            | 2.5      | 36         |
| 6myc-Cin8p-Δcoil4| No        | 13.1  | 4     | ND       | 10.7–0.2     | 2            | 3            | 1.9      | 19         |
| 6myc-Cin8p-842| No              | 8.2   | 1     | ND       | 668–745      | 358,548      | 4            | 2.5      | 36         |
| Cin8p-ΔNect-3HA| No             | 12.9  | 1     | ND       | 0          | 4            | 3            | 1.9      | 19         |
| Cin8p-Δcoil4-3HA| No        | 9.9   | 0.4  | ND       | 0          | 4            | 3            | 1.9      | 19         |

* $R_0$ indicates Stokes radius. Values indicate average ± S.D.
* $n$ indicates number of experiments.
* Mass is based on amino acid sequence, including epitope tags.
* Native $M_r$, $f_{0}^*$, and axial ratios were calculated from $R_0$ and S-values, see under "Experimental Procedures."
the additional NLS (Fig. 2A and data not shown). It has been shown by a number of groups that dimeric and monomeric forms of kinesin-5 homologues hydrolyze ATP (17, 33–35) and move microtubules in vitro (17, 33, 35). Therefore, it is likely that the Cin8p-955, 885, 871 and Δcoil4 retain some of their motor activity. It has also been shown that unlike the full-length Cin8p, the dimeric and nonfunctional Cin8p-871 form (Figs. 2 and 5A, Table 3) is unable to bundle microtubules in vitro (21), indicating that Cin8p-871 cannot cross-link microtubules. Taken together, these results indicate that microtubule cross-linking is necessary for Cin8p and probably other kinesin-5 family members to produce their intracellular functions. The bipolar tetrameric complex, which enables kinesin-5 homologues not only to bind and move but also to cross-link microtubules, is therefore absolutely essential for successful performance of their mitotic functions.

Functional Domains in the Cin8p Stalk and Tail—It seems likely that like Cin8p, a tetrameric structure is required for kinesin-5 motors to accomplish spindle function. Comparison of the stalk-tail regions of all known kinesin-5 homologues reveals that the main conserved feature is a long, high probability coiled-coil region located immediately after the neck region (coil 1 in Cin8p sequence; Fig. 6). The shortest length of coil 1 is 107 amino acids in the Aspergillus nidulans BimC sequence; the longest is 209 amino acids in the Drosophila melanogaster Klp61F sequence. After this coil, all kinesin-5 homologues have an extended region of high and moderate coiled-coil probability with numerous potential “breaks” in coiled-coil probability.

We found that Cin8p coil 1 is essential for self-interaction and sufficient for dimer formation. Because all kinesin-5 homologues share the coil 1 structure, it is very likely that this coil is required for dimerization of all other members of the kinesin-5 family.

In addition to coil 1, most kinesin-5 homologues have a shorter 30–60 amino acid-long, high probability coiled-coil in the last third of the non-motor part of the molecule (coil 4 in the Cin8p sequence). We found that sequences in the last third of the Cin8p stalk, which include coil 4, are required for its tetramerization, because the truncated form ending at amino acid 871 has hydrodynamic properties consistent with a properly folded dimer (Fig. 5 and Table 3). Our findings demonstrate that coil 4 and additional sequences in the region of amino acids 871–990 are essential for formation of a tetramer that can provide Cin8p activity. Because most kinesin-5 homologues contain at the end of their stalks a domain similar to Cin8p coil 4, we propose that this coil and sequences in its vicinity are essential for the formation of properly folded tetramers of other kinesin-5 homologues as well.

Finally, our analysis revealed one further region required for Cin8p functionality beyond the motor and those required for tetramerization. Deletion of the sequence between the end of the motor domain and the beginning of coil 1 (Cin8p-Δneck, amino acids 513–534) formed a tetramer (Table 3) but provided no function in vivo (Fig. 2). In kinesin-1 (conventional kinesin) proteins, the non-coil-coiled neck region adjacent to the catalytic motor domain was proposed to modulate the motor activity influencing processivity and directionality (36–40). This probably arises from specific interactions between amino acids in the neck region with sites in the catalytic motor domain (41). The finding that deletion of the non-coil-coiled neck region of Cin8p results in a nonfunctional protein suggests that the Cin8p neck may play a role in regulating its motor activity, perhaps similar to that suggested for other kinesin-related proteins.

Possible Regulatory Domain in Cin8p Stalk—We found that truncation of Cin8p at amino acid 871 creates a dominant negative effect in cells deleted for the function of Kip1p, but carrying an intact chromosomal copy of Cin8. This dominant negative effect is dependent on the ability of the mutant Cin8p to interact with the full-length Cin8p, and the effect requires the activity of the Cin8p motor domain in cis. We found that that the region between amino acids 871 and 885 in...
the Cin8p stalk attenuates the dominant negative effect suggesting that this region may regulate Cin8p activity. In support is the finding that Cut7, a Schizosaccharomyces pombe kinesin-5 homologue, may also contain a regulatory region in its stalk (42). Drummond and Hagan (42) have shown that the last 198 amino acids of Cut7 could not be deleted in a diploid strain. In contrast, a more extensive deletion of the majority of the cut7 sequence (deletion of amino acids 111–1085) could be obtained. Therefore, similar to Cin8p-871, this Cut7 truncation created a dominant negative effect in cells that contained a wild-type copy of the cut7 gene. This suggests that the regulatory function in the Cin8p stalk detected in this study may be a common feature of the kinesin-5 family.

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