The Kinesin-related Protein Kip1p of *Saccharomyces cerevisiae* Is Bipolar*

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Kip1p is a mitotic spindle-associated kinesin-related protein in *Saccharomyces cerevisiae* that participates in spindle pole separation. Here, we define the domain arrangement and polypeptide composition of the Kip1p holoenzyme. Electron microscopy of rotary shadowed Kip1p molecules revealed two globular domains 14 nm in diameter connected by a 73-nm long stalk. When the Kip1p domain homologous to the kinesin motor domain was decorated with an unrelated protein, the diameter of the globular domains at both ends of the stalk increased, indicating that Kip1p is bipolar. Soluble Kip1p isolated from *S. cerevisiae* cells was homomeric, based on the similarity of the sedimentation coefficients of native Kip1p from *S. cerevisiae* and Kip1p which was purified after expression in insect cells. The holoenzyme molecular weight was estimated using the sedimentation coefficient and Stokes radius, and was most consistent with a tetrameric composition. Kip1p exhibited an ionic strength-dependent transition in its sedimentation coefficient, revealing a potential regulatory mechanism. The position of kinesin motor-related domains at each end of the stalk may allow Kip1p to cross-link either parallel or antiparallel microtubules during mitotic spindle assembly and pole separation.

Assembly of the mitotic spindle and separation of the spindle poles during anaphase depends on the function of several distinct members of the kinesin family of force-generating proteins. A major class of kinesins in higher and lower eukaryotes which associate with the spindle are members of the bimC subfamily. The bimC kinesins, like all kinesin subfamilies, have been grouped by sequence conservation within the kinesin-related motor domain (1). Conditional mutants have been used to show that the bimC kinesins Kip1p and Cin8p of *Saccharomyces cerevisiae*, bimC of *Aspergillus nidulans*, cut7 of *Schizosaccharomyces pombe*, and KLP61F (KRP130) of *Drosophila melanogaster* are required for separation of the duplicated spindle poles during mitotic spindle assembly (2–6). Antibody-mediated depletion or inactivation of bimC kinesin Eg5 of *Xenopus* and human reveal spindle pole separation defects as well as defects in spindle pole organization, suggesting some bimC kinesins also organize parallel microtubules into bundles (7–10). In addition, the bimC kinesins may have functions subsequent to spindle assembly. Kip1p and Cin8p are required for maintenance of pole separation during metaphase and have been implicated in anaphase B spindle elongation, and KLP61F has been localized by immunoelectron microscopy to the midzone of anaphase spindles (11–14). The simplest model that explains bimC function in pole separation is that the motor proteins cross-link microtubules emanating from opposite poles and slide them apart.

Although the conserved motor domain defines membership in the kinesin family, intracellular function is largely specified by sequences outside the motor domain which determine the oligomeric composition and the cargo. The best characterized holoenzyme structure is of “conventional” kinesin. Conventional kinesin from a number of tissue sources is found primarily as a heterotetramer composed of two identical heavy chains and two light chains (15–17). The heavy chains form a closely associated pair of NH₂-terminal globular motor domains followed by an extended stalk consisting of a parallel two-stranded α-helical coiled coil. The stalk links the motor domains to the vesicle cargo by interactions with the COOH-terminal globular domain of the heavy chains, and possibly with the light chains which appear to associate with the COOH-terminal end of the stalk by a coiled-coil interaction (18, 19). There is a wide diversity of holoenzyme compositions and domain arrangements among other members of the kinesin family. Kinesins have been characterized that contain a single motor subunit, four identical motor subunits or two distinct motor subunits, and a variety of non-motor accessory chains have been identified (reviewed in Ref. 20).

Knowledge of the oligomeric composition and domain arrangement of a kinesin holoenzyme can provide significant insight into its intracellular function and mechanism. The bimC kinesin KLP61F from *Drosophila* embryos is a homotetramer with a pair of motor domains at each end of a stalk (21, 22). This bipolar configuration apparently allows KLP61F to cross-link adjacent microtubules in interpolar regions of the mitotic spindle where microtubule orientation is parallel and in the spindle midzone where many microtubules are antiparallel (14). An outstanding question is whether all bimC kinesins will share this domain arrangement, particularly since the non-motor sequences which specify quaternary structure show little sequence conservation. The only sequence conserved in the non-motor region is the bimC box, a 40-amino acid region located near the COOH terminus with 25–50% sequence identity (6). However, bimC box function does not appear to be conserved. Phosphorylation of an invariant threonine in the bimC box of Eg5 and KLP61F targets the protein to the mitotic spindle, but mutation of the invariant threonine has no effect on cut7 spindle targeting or function, and the bimC box is absent in Kip1p and Cin8p (8, 14, 23, 24).

To investigate how Kip1p of *S. cerevisiae* functions in mitotic spindle assembly, we characterized the oligomeric composition and the positions of the kinesin-related domains in the Kip1p* This work was supported by National Institutes of Health Grant GM50884 (to D. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
holoenzyme. Using size exclusion chromatography and sucrose gradient centrifugation, we found that soluble Kip1p is most likely homomeric and consists of four Kip1p polypeptides. Electron microscopy of rotary shadowed Kip1p molecules demonstrated that native Kip1p has globular domains located at both ends of a stalk. Decoration of the kinesin-related domains with an unrelated protein increased the size of the globular domains at both ends of the stalk, demonstrating that Kip1p is bipolar. Thus Kip1p shares structural features with the bimC kinesin KLP61F1. While the bimC kinesins were previously grouped by sequence conservation in the motor domains and by similar roles in spindle assembly, our results here suggest that bimC kinesins also share a common oligomeric composition and domain arrangement. A Kip1p molecule with kinesin motor-related domains at opposite ends of a stalk could bundle and organize microtubules in the mitotic spindle which are parallel, as well as bundle antiparallel microtubules extending from opposite poles and slide them apart.

**EXPERIMENTAL PROCEDURES**

**Materials, Strains, and Growth Conditions**—The yeast strains used in this study are derivatives of S. cerevisiae 288C. Strains were used were DSY1 (leu2-3, 112, ade2-101, his3Δ200 hip1Δ1:His13 pMR231) for expression of HA-Kip1p. Strains were grown at 30 °C in YPD or, when necessary for plasmid selection, in synthetic selective media (25). Chicken skeletal muscle myosin was a gift from H. L. Sweeney (University of Pennsylvania, Philadelphia, PA). α- Factor peptide was synthesized by Research Genetics, Inc. (Huntsville, AL). Sepharose S-400 resin, hydroxyurea, nocodazole, protein size markers, and protease inhibitors were obtained from Sigma and ultra-pure sucrose from ICN (Costa Mesa, CA).

**Expression Vectors**—Kip1p tagged with three copies of the hemagglutinin epitope (HA-Kip1p) was expressed from pMR2310, which is a low copy number centromere-based plasmid described previously (3). To construct the baculovirus transfer vector for expression of His6-Kip1p in insect cells, a unique BamHI site was introduced at the NH2 terminus of KIP1 by polymerase chain reaction amplification, and a 4.5-kilobase BamHI-NcoI fragment containing the KIP1 gene was cloned into the BamHI-NcoI sites of pVL1393 (Invitrogen, Carlsbad, CA). A 22-base pair double-stranded oligonucleotide encoding a Met-His tag was introduced at the BamHI site, creating plasmid pRD688. The correct sequence of the tagged polymerase chain reaction-amplified region of KIP1 was verified by DNA sequencing. The resulting KIP1 gene encodes the following amino acid sequence at the NH2 terminus: MH-HHHHHHSIGSMARS... (the native KIP1 starting methionine is shown in bold). To test whether His6-Kip1p functions in yeast, the tagged yeast was subcloned into a centromere-based plasmid containing the ADH1 gene promoter, and complementation of a kip1Δ strain was tested using the plasmid shuffle technique, as described for HA-Kip1p by Roof et al. (3).

**Generation of Kip1p-specific Antibodies and Immunoblotting**—Kip1p polyclonal antibodies were generated in rabbits using bacterially expressed and glutathione-agarose purified GST-Kip1p (residues 493–758) and GST-Kip1p (residues 757–1111) fusion proteins. Antigen columns were made from the above purified proteins by coupling to CNBr-activated Sepharose 4B beads according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Kip1p-specific antibodies were isolated from total rabbit antisera using the Kip1p antigen columns followed by protein A concentration (26). Native Kip1p was detected by immunoblotting using an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) with a mixture of the two affinity purified Kip1p polyclonal antibodies followed by anti-rabbit hors eradise peroxidase-conjugated secondary antibody. HA-tagged Kip1p was detected by immunoblotting using the enhanced chemiluminescence detection system with the ICA15 monoclonal antibody followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. The incubation buffer was 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.2% Tween 20 supplemented with 2% bovine serum albumin for the blocking and primary antibody incubation steps or supple-

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1 The abbreviations used are: HA, hemagglutinin; His6, a tag of six consecutive histidine residues; Ni-NTA agarose, Ni2+-nitriloacetic acid agarose; GST, glutathione S-transferase.
The native molecular weight was calculated from the sedimentation coefficient as a function of the Perrin parameter as described (32). The axial ratio, assuming Kip1p is a prolate ellipsoid, was estimated from the calibration curve generated by plotting the sedimentation coefficient of protein standards with an axial ratio, Perrin parameter, and native molecular weight for the Kip1p complex. To determine the sedimentation coefficient as a function of ionic strength, Kip1p and the standard proteins were analyzed in buffer supplemented with NaCl, as described (31).

Calculation of Molecular Weight, Diffusion Coefficient, and Axial Ratio—The native molecular weight was calculated from the sedimentation and diffusion coefficient using the Svedberg equation with \( M_w = \frac{RT}{D_s\rho} \) and \( r = \frac{8.31 \times 10^7 \text{erg deg}^{-1} \text{mol}^{-1}}{\text{the ideal gas constant}, T = 293 \text{ K and } \rho = 0.9982 \text{ g cm}^{-3} \text{ the density of water at 293 K}.} \) A partial specific volume \( (\phi) \) of 0.725 cm\(^3\) g\(^{-1}\) was used. The diffusion coefficient was calculated from the Stokes radius \( (R_s) \) according to the equation \( D_s\rho = \frac{RT}{6\pi\eta R_s} \), with \( N = \text{Avogadro number}. \) The axial ratio, assuming Kip1p is a prolate ellipsoid, was estimated from the Perrin parameter as described (32).

Rotary Shadowing and Electron Microscopy—His\(_6\)-Kip1p isolated as described above from baculovirus-infected insect cells at a concentration of \(~2.5 \mu\text{g/ml}\) was applied to freshly cleaved mica and shadowed for \(2.5 \mu\text{g/ml}\) was applied to freshly cleaved mica and shadowed for 15–20 drops of 100 mM ammonium acetate, treated with 1% aqueous uranyl acetate for 30 s, and rinsed with 30% methanol. Excess methanol was removed leaving only a thin film behind and the specimen was frozen in liquid nitrogen. After freeze-drying in a Balzer's freeze-fracture machine, the specimen was shadowed with platinum at a 15° angle and replicated with carbon. Samples were viewed with a Philips electron microscope at 60 kV.

RESULTS

Size and Subunit Composition of the Kip1 Protein Complex—The predicted amino acid sequence of Kip1p contains an NH\(_2\)-terminal domain of 425 amino acids with high sequence similarity to the force generating domain of kinesin, followed by an additional 686 amino acids which do not exhibit close similarity to other members of the kinesin family or other known proteins (Fig. 1). The 686-amino acid region contains several segments predicted to form coiled coils, and it is therefore likely that Kip1p forms a multimeric complex containing several copies of the Kip1p peptide and possibly additional yeast proteins. To address the subunit composition of the Kip1p complex, we compared the hydrodynamic properties of Kip1p isolated from yeast to Kip1p isolated from insect cells in which the KIP1 gene was expressed from a baculovirus vector. In yeast, the KIP1 gene was expressed from its native promoter to maintain the native protein level and preserve stoichiometry with any heterologous subunits, and experiments were performed with soluble extracts. For baculovirus-mediated expression, the KIP1 gene was tagged with six consecutive histidine residues at the NH\(_2\)-terminus and the protein was partially purified by isolation of the nuclear fraction, followed by Ni-NTA affinity chromatography. The wild type Kip1p from yeast and His-tagged Kip1p from baculovirus expression were detected by immunoblotting with antibody raised against the COOH-terminal 619 amino acids of Kip1p. When necessary for more sensitive detection, the KIP1 gene containing the hemagglutinin (HA) epitope sequence was used with the 12CA5 antibody. The HA-tagged and His-tagged KIP1 genes were functional in yeast, as tested in a complementation assay (3).

To estimate the sedimentation coefficients of the Kip1p complexes, we used sucrose gradient centrifugation. The sedimentation coefficients were measured by comparing the position of Kip1p in the gradient with the positions of a series of proteins with known sedimentation coefficients (Fig. 3). The concentration of the partially purified Kip1p from baculovirus expression was adjusted to give a signal in immunoblots approximately equal to that obtained for Kip1p from yeast. The sedimentation coefficient of wild type Kip1p from yeast was 8.2 ± 0.4 S (\( n = 6 \)) and His-tagged Kip1p expressed from baculovirus was 7.9 ± 0.2 S (\( n = 6 \)). The difference between these values is not significant (t test, \( p = 0.16 \)). Since the sedimentation coefficient of Kip1p from yeast was close to that of the homomeric Kip1p complex from insect cells, we infer that soluble Kip1p in yeast extracts is most likely homomeric as well. When the 5.5-kDa HA epitope tag was present on the Kip1p expressed in yeast,
the sedimentation coefficient increased to 8.6 ± 0.2 (n = 3).

The Stokes radius and diffusion coefficient of the Kip1p complexes were measured after size exclusion chromatography (Fig. 4). The Stokes radius of HA-tagged Kip1p from yeast was 13.4 nm and the Stokes radius of His-tagged Kip1p expressed from baculovirus was 12.5 nm. We were unable to use untagged Kip1p from yeast in size exclusion chromatography because adsorption of Kip1p to the column reduced its concentration below the level of detection with the polyclonal anti-Kip1p antibodies. The mean sedimentation coefficient and Stokes radius values were used to calculate the native molecular masses using the method of Siegel and Monty (30) and the results are summarized in Table I. The HA-tagged Kip1p complex from yeast was about 474 kDa and the His-tagged Kip1p complex obtained from insect cells was about 406 kDa. Comparison of the monomer molecular weight predicted from the amino acid sequence to the native molecular weight gave a subunit-to-complex ratio of 3.2 to 1 for the baculovirus-expressed protein and 3.6 to 1 for the yeast-expressed protein. The subunit composition which is consistent with the molecular weight estimates and the symmetrical bipolar structure observed by electron microscopy described below is that Kip1p, from yeast or expressed from baculovirus, is a homotetramer.

Kip1p Is Bipolar—To investigate the structure of Kip1p, we performed electron microscopy of rotary shadowed Kip1p. Kip1p expressed from baculovirus was purified by isolation of the nuclear fraction of the insect cells, size exclusion chromatography, and Ni-NTA affinity chromatography, with a final purity estimated by densitometry of Coomassie-stained gels of 90% (Fig. 2). Kip1p was applied to freshly cleaved mica and processed for rotary shadowing electron microscopy using the technique of Loesser and Franzini-Armstrong (33).

Most molecules consisted of two globular domains of approximately equal diameter separated by a thin stalk (Fig. 5A). The dimensions of the domains were measured and the size distributions are presented in Fig. 5B. The overall length was 104.7 ± 12.0 nm (n = 106), which is longer than the overall length of 80.4 ± 8.1 nm reported for conventional kinesin (34), and the overall length of 95.6 ± 9.8 nm reported for the kinesin-related Drosophila KLP61F protein (22). The central stalk of Kip1p was 73.1 ± 12.5 nm (n = 106) long. An α-helix of this length would contain 493 amino acids, closely corresponding to the 489-amino acid length of the central region of Kip1p which is delimited by segments predicted to form a coiled coil (Fig. 1).

Thus the coiled-coil domains of the Kip1p polypeptides most likely are adjacent along their entire length.

One or both of the globular domains observed in the Kip1p complex must contain the sequences which are homologous to the globular force-generating domain of kinesin. If four Kip1p polypeptides are arranged in the antiparallel orientation shown in Fig. 5C, then there would be a pair of kinesin-related domains at each end of the stalk. Consistent with this arrangement, the size and shape of the globular domains at opposite ends of the stalk appeared similar by electron microscopy, and the diameter of the domains was consistent with the expected size of two closely associated kinesin motor domains. The Kip1p globular domains were 14.3 ± 2.9 nm (n = 212) in diameter (Fig. 5). In comparison, the motor domains of kinesin are visible by rotary shadow electron microscopy as two individual domains 8–10 nm in diameter (34, 35), and the two motor domains at each end of the KLP61F stalk are visible as a single globular domain of 20 nm diameter (22). In addition to the kinesin-related domain and the stalk, Kip1p contains 196 amino acids at its COOH terminus which are not predicted to form an extended α-helix and would probably form a globular structure. In the antiparallel arrangement of four Kip1p polypeptides shown in Fig. 5C, the COOH-terminal region may contribute to the size of the globular domains. A possible alternate arrangement of the Kip1p polypeptides is a parallel association, which would place all of the kinesin-related domains at one end of the stalk and all of the COOH-terminal domains at the other end.

To unambiguously determine the position of the kinesin-related domains within the Kip1p complex, we used rotary shadow electron microscopy of Kip1p which was decorated at the kinesin-related domain. Decoration was accomplished using an alkaline phosphatase-Ni-NTA conjugate to bind to the histidine tag present at the NH2 terminus of Kip1p, followed by sucrose density gradient sedimentation to isolate decorated Kip1p. Rotary shadowed Kip1p molecules were visible by electron microscopy as a stalk domain connecting two globular domains, with each of the globular domains enlarged compared with underdecorated Kip1p (Fig. 6). The diameter of the decorated globular domains was 24.5 ± 4.3 nm (n = 58). In comparison, underdecorated Kip1p from the same preparation which was shadowed in parallel had a globular domain diameter of 15.4 ± 3.5 nm (n = 14). In contrast to the enlarged globular domains, the stalk domain of the decorated Kip1p had a width and length characteristic of underdecorated Kip1p. The decorated Kip1p stalk length was 68.6 ± 9.8 nm (n = 29), compared with an underdecorated Kip1p stalk length of 74.7 ± 7.6 nm (n = 7). The diameter of nearly all of the globular domains was increased, indicating that labeling with Ni-NTA alkaline phosphatase and sedimentation purification was an efficient means of decora-
tion. Since the globular domains at both ends of the stalk were decorated, we conclude that Kip1p is bipolar.

Kip1p Exhibits an Ionic Strength-induced Transition in Its Sedimentation Coefficient—Conventional kinesin, kinesin-II, and several myosin II molecules can exist in compact conformations due to one or more bends in the coiled-coil stalks (31, 36–38). For instance, during sucrose density centrifugation,Drosophila and bovine brain conventional kinesin heavy chains are in an extended conformation at high ionic strength, but convert to a compact conformation at low ionic strength with a corresponding increase in mobility from \( 5 \text{S} \) to \( 7 \text{S} \) (31, 38).

To address whether Kip1p exhibits a similar transition, we performed sucrose density gradient centrifugation of Kip1p and standard proteins at a series of ionic strengths (Fig. 7). At the NaCl concentration of 150 mM used in the hydrodynamic and electron microscopy experiments described above, the sedimentation coefficient was 8.2 S. The sedimentation coefficient remained essentially constant at NaCl concentrations up to 500 mM NaCl, indicating the tetrameric complex is stable in high ionic strength buffer. As the NaCl concentration was decreased below 150 mM, Kip1p exhibited an increased mobility relative to the standard proteins, reaching a maximum sedimentation coefficient of 10 S at 50 mM NaCl. The increased mobility of Kip1p at low ionic strength is consistent with a conformational transition from an extended to a compact form.

Microtubule Binding Is Not Required for Kip1p Complex Assembly—Kip1p localizes to the microtubules of the mitotic spindle (3). To investigate the requirements for assembly of Kip1p into the tetrameric complex, we tested whether complex formation depends on association with the mitotic spindle. Kip1p was synthesized in the presence or absence of microtubules and the sedimentation coefficients were measured.

### Table I

Biophysical properties of Kip1p

| Property                     | S. cerevisiae Kip1p | Baculovirus-expressed His6-Kip1p | S. cerevisiae HA-Kip1p |
|------------------------------|---------------------|----------------------------------|------------------------|
| Sedimentation coefficient (S) | 8.2 ± 0.4 (n = 6)    | 7.9 ± 0.2 (n = 6)                | 8.6 ± 0.2 (n = 3)      |
| Stokes radius (nm)           | ND                  | 12.5 ± 1.3 (n = 3)               | 13.4 ± 0.4 (n = 2)     |
| Axial ratio                  | ~30                 | ~30                              | ~30                    |
| Polypeptide molecular mass (Da) | 125,716             | 126,927                          | 131,253               |
| Native molecular mass (Da)   | ND                  | 406,000                          | 474,000               |

* The sedimentation coefficients and Stokes radii are expressed as the mean ± S.D. with the sample size (n) shown. The axial ratios and native molecular mass were calculated as described under “Experimental Procedures” and the polypeptide molecular weights were calculated from the predicted amino acid sequences.

* ND, not determined.

![Figure 5](image-url)

**A** gallery of electron micrographs of rotary shadowed Kip1p molecules. His6-Kip1p was partially purified as shown in Fig. 2 by isolation of the nuclear fraction, size exclusion chromatography, and Ni-NTA-agarose chromatography. Kip1p was applied to mica and shadowed with platinum at a 15° angle by the technique of Loesser and Franzini-Armstrong (33) as described under “Experimental Procedures.” The bar represents 100 nm.

**B** morphometric analysis of Kip1p. The overall length, stalk length, and head diameters were measured and the size distributions, values of the mean ± S.D., and sample size (n) are shown.

**C** schematic model of the predicted tetrameric structure of Kip1p, based on electron microscopy and hydrodynamic analysis. Two polypeptides are shown in black and two in gray. The large spheres represent the NH2-terminal kinesin-related domains and the small spheres represent the COOH-terminal regions. The actual location of the COOH-terminal domains is unknown. The stalk is likely to contain four strands, and is represented here as an antiparallel four-stranded a-helical coiled coil. See “Discussion” for details.
deplete the existing pool of Kip1p which was synthesized in the presence of microtubules, we took advantage of the finding that all Kip1p in the cell is degraded at the end of mitosis and in G1. Cell cultures were arrested in G1 with the mating pheromone a-factor to allow Kip1p degradation, released from the a-factor arrest and rearrested with either hydroxyurea or the microtubule depolymerizing drug nocodazole. In the hydroxyurea arrest, Kip1p was synthesized in the presence of microtubules of the mitotic spindle, and in the nocodazole arrest, Kip1p was synthesized in the absence of microtubules. Immuno-fluorescence microscopy was used to verify that the cultures were arrested at the expected stage, and that there were no detectable microtubules in the nocodazole-treated culture. The sedimentation coefficients were identical for both arrest regimes; 8.0 S (Fig. 8), indicating that microtubules are not required for assembly of Kip1p complex.

FIG. 6. Decoration of the Kip1p kinesin-related domains increased the size of the globular domains at both ends of the stalk. A. gallery of Kip1p molecules after decoration. His6-Kip1p which carried six consecutive histidine residues at the NH2 terminus was partially purified by Ni-NTA-agarose chromatography, incubated with Ni-NTA alkaline phosphatase conjugate, and the decorated Kip1p was purified by sucrose gradient centrifugation followed by rotary shadowing at a 15° angle and electron microscopy. The bar represents 100 nm. B, gallery of undecorated Kip1p molecules. The Kip1p was from the same preparation used in A except it was not incubated with the Ni-NTA alkaline phosphatase conjugate. The Kip1p was purified by sucrose gradient centrifugation followed by rotary shadowing simultaneously with the decorated Kip1p. C, morphometric analysis of decorated Kip1p. The size distribution of the globular domains is shown, and the mean diameter and standard deviation is indicated. Two alkaline phosphatase molecules (140 kDa each) binding to two kinesin-related domains (47 kDa each) would increase the total volume ~4-fold and, assuming the decorated and undecorated domains are spherical, the diameter would increase from 14 to ~22 nm.

FIG. 7. Kip1p exhibits an ionic strength-induced transition in its sedimentation coefficient. A soluble extract of strain DS138 containing wild type Kip1p expressed from its native promoter was centrifuged through 5–20% sucrose gradients in buffer containing different concentrations of NaCl. Each sedimentation coefficient was estimated using a calibration curve of S versus distance migrated of standard proteins centrifuged in parallel at the same NaCl concentration. The line intersects the mean S value at each NaCl concentration except the 200 and 350 mM points. The midpoint of the transition was about 70 mM NaCl.

In this paper we show that the bimC kinesin Kip1p of S. cerevisiae is bipolar, suggesting that microtubule cross-linking is a conserved feature of the bimC subfamily. The bimC subfamily of kinesins has been studied in a wide variety of eukaryotes and it has been established that subfamily members possess a conserved motor domain and usually perform similar functions in spindle pole organization and separation (39, 40). The mechanism by which one bimC kinesin generates movement was suggested by structural characterization of the Drosophila KLP61F holoenzyme. KLP61F possesses two pairs of motor domains at opposite ends of a long stalk, and this arrangement allows it to cross-link adjacent microtubules in the mitotic spindle (14, 22). The general applicability of this model was previously limited because the non-motor domains of bimC kinesins, which determine oligomeric composition and subunit arrangement, show little sequence conservation. Our demonstration that a fungal bimC kinesin is also bipolar strongly suggests that bipolarity is a common feature of the bimC subfamily.

Electron microscopy of rotary shadowed Kip1p molecules was used to investigate the arrangement of domains in the Kip1p holoenzyme. We show that Kip1p molecules consist of globular domains (14.3 ± 2.9 nm in diameter) at opposite ends of a 73.1 ± 12.5-nm long stalk. The reported size of kinesin motor domains observed by rotary shadowing ranges from 8 nm for a single kinesin motor domain to 20 nm for a pair of motor domains in KLP61F. The apparent diameters may be influenced by the affinity of the two motor domains for each other, by differences in the absorption of the proteins to mica, and the angle of rotary shadowing. To determine the location(s) of the

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kinesin-related domains in the Kip1p molecule, we decorated the kinesin-related domain with an unrelated protein, and measured the diameter of the domains in electron micrographs. The globular domains at each end of the stalk increased in size, indicating that Kip1p is bipolar. For decoration, we used a Ni-NTA alkaline phosphatase conjugate which binds with very high affinity to the polyhistidine tag at the NH2 terminus of Kip1p. Since polyhistidine tags are frequently added to recombinant proteins to aid purification and do not usually perturb function, this method may prove useful for domain analysis of other proteins.

Our hydrodynamic analysis of Kip1p suggested that the holoenzyme consists of four Kip1p polypeptides. Kip1p isolated from yeast and Kip1p which was expressed by baculovirus in insect cells had the same sedimentation coefficient. Since highly expressed Kip1p from insect cells would not be stoichiometrically associated with heterologous proteins, we infer from the similar sedimentation coefficients that soluble Kip1p from yeast is also homomeric. We do not eliminate the possibility that Kip1p in yeast associates with other proteins in low affinity or transient interactions, or with proteins whose binding does not significantly alter the sedimentation velocity of the holoenzyme. Size exclusion chromatography of Kip1p was used to measure the Stokes radius and in combination with the sedimentation coefficient, to estimate the holoenzyme molecular weight. The estimated molecular weights corresponded to 3.2 Kip1p polypeptides in the complex from insect cell expression and 3.6 polypeptides in the complex from yeast. The difference between the calculated native molecular weight of the isolated Kip1p complex and the predicted molecular weight of four Kip1p polypeptides could have been caused by using in the calculations a value of the partial specific volume that was too low (a value of 0.725 cm3 g−1 was used) or by a weak interaction between the Kip1p complex and the size exclusion matrix resulting in an overestimate of the diffusion coefficient. Nevertheless, a tetrameric composition is the simplest explanation for the bipolar structure of Kip1p observed by electron microscopy, where the globular kinesin-related domains at opposite ends of the stalk were relatively uniform in size.

The structure of the stalk is of interest since it positions the kinesin-related domains and potentially determines the geometry by which Kip1p interacts with microtubules. The observed stalk length of 73 ± 13 nm would correspond to 493 ± 9 amino acids in an α-helical secondary structure, using a value of 1.48 Å length/amino acid (41). This length closely corresponds to the 489-amino acid length of the central region of Kip1p, which contains segments predicted to form α-helical coiled coils (Fig. 1B). Thus the four polypeptides probably interact along the entire length of the central domain, with two strands oriented in one direction and two in the opposite direction, forming a four-stranded bundle (Fig. 5C). The precise arrangement of the strands in the bundle is unknown, and could consist of a pair of two-stranded coiled coil dimers which interact laterally as previously suggested (22, 39), or as a single four-helix coiled coil as shown in Fig. 5C. Antiparallel four-stranded coiled coils have been characterized by x-ray crystallography in the ROP and Lac repressor proteins (42, 43). We speculate that the stalk contains four-helix coiled coils for two reasons. First, the current methods for prediction of two- and three-stranded coiled coils do not predict a continuous coiled coil in Kip1p sufficiently long to account for the observed Kip1p stalk length (Fig. 1B), suggesting that this domain does not consist entirely of two- or three-stranded coiled coils. The sequences of four-stranded coiled coils may not produce a high coiled coil formation score because they differ from two- and three-stranded coiled coils in packing geometry and superhelical pitch, radius, and residues per turn, and exhibit different preferences for amino acids (41).

Second, sedimentation analysis of Kip1p over a salt concentration range of 200 to 500 mM did not reveal the large transition expected if two dimers were to dissociate (Fig. 7). Resistance to high salt is a characteristic of coiled coil interactions (19). A final possibility that should be considered is that some of the stalk may not be an α-helical coiled coil, which would be consistent with the regions of low predicted coiled coil probability and with the presence of two helix destabilizing proline residues (amino acids 847 and 873).

Kip1p associates with the microtubules of the mitotic spindle, and this binding potentially regulates Kip1p activity, structure, and stability. Conventional kinesin can undergo an ionic strength-induced conformation change which produces a compact form with a reduced microtubule-stimulated ATPase activity (31, 38). We investigated whether Kip1p can undergo an ionic strength-induced transition using sucrose gradient centrifugation and found that Kip1p exhibited a similar transition (Fig. 7). We speculate that the observed ionic strength induced transition mimics a similar transition that regulates Kip1p activity in vivo. Microtubule binding could also regulate Kip1p by controlling assembly of Kip1p polypeptides into a tetramer, and in addition, either microtubule binding or tetramerization could influence the rate at which Kip1p is degraded. We investigated these possibilities by using sucrose gradient centrifugation to assay assembly of Kip1p into the holoenzyme in the presence and absence of microtubules. Kip1p which was newly synthesized in either the presence of absence of microtubules accumulated to the same concentration and exhibited the same sedimentation coefficient (Fig. 8). Thus Kip1p assembly and stability is largely independent of microtubule binding.

The bipolar structure of Kip1p has significant implications to the interpretation of Kip1p localization and mutant phenotypes. Loss of Kip1p function combined with loss of function of the partially redundant homolog Cin8p results in accumulation of cells with unseparated spindle poles, an inability to maintain pole separation in the metaphase spindle, and decreases in the rate of anaphase pole separation (2, 3, 11, 12). Models to explain these results often propose that Kip1p and Cin8p cross-link pairs of antiparallel microtubules and slide them apart, a.
task the bipolar structure described here would be well suited for. However, immunolocalization of Kip1p (2, 3) and localization of Kip1p- and Cin8p-green fluorescent protein fusions3 reveals that these proteins are more concentrated near the spindle poles than in the midzone where the antiparallel microtubules are located. We suggest, in addition to a possible role in the motility events of spindle pole separation, that Kip1p focuses microtubules in the direction of the opposing spindle pole by bundling parallel microtubules. Consistent with such an activity, a three-dimensional ultrastructural analysis of the S. cerevisiae mitotic spindle has shown that microtubules emanating from the spindle poles are contained within a cylindrical area 200–300 nm in diameter (44). Moreover, the Kip1p homolog Cin8p of S. cerevisiae can bundle microtubules in vitro and mutational analysis suggests that bundling is more important in vivo than the plus-end directed motility of Cin8p (45). Microtubule bundling and mitotic spindle pole organization activities have also been proposed for Eg5 (7–10). These dual roles of Kip1p in the mitotic spindle suggest that the Kip1p complex is capable of interacting with both parallel and antiparallel microtubules, a concept that awaits further investigation.

Acknowledgments—We thank C. Franzini-Armstrong (University of Pennsylvania) for performing the rotary shadowing and W. Wunner (Wistar Institute) for insect cell culture services.

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3 D. M. Roof, unpublished data.