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Biochemistry

KAR3-encoded kinesin is a minus-end-directed motor that functions with centromere binding proteins (CBF3) on an *in vitro* yeast kinetochore

(chromosome segregation/microtubules/mitosis)

KIM MIDDLETON AND JOHN CARBON

Department of Biological Sciences, University of California, Santa Barbara, CA 93106

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ABSTRACT **We have used *in vitro* motility assays to investigate the mechanism of kinetochore function in the budding yeast *Saccharomyces cerevisiae*. Functional centromeric DNA plus a tripartite centromere binding protein complex, CBF3, was found to be necessary but not sufficient for *in vitro* kinetochore activity. A fourth required component was identified as the motor protein Kar3p, a previously reported yeast kinesin known to be involved in karyogamy and mitosis. Our data support genetic evidence suggesting that Kar3p is a kinetochore-associated motor and imply that CBF3 plays a regulatory role in kinetochore function.**

The subcellular structure known as the kinetochore is vital to eukaryotic chromosome segregation. During cell division, the kinetochore is responsible for attaching paired sister chromatids to microtubules of the mitotic spindle and likely plays an active role in subsequent events leading to completed chromosome segregation. In the budding yeast *Saccharomyces cerevisiae*, this structure is composed of centromeric DNA (the *CEN* locus) plus associated proteins (1, 2). The yeast centromere is specified in cis by only 125 bp of DNA (1, 2). *In vitro* mutagenesis studies have shown that a 28-bp region of the centromere, CDEIII, is critical for accurate mitotic chromosome segregation (3, 4). By using an *in vitro* DNA fragment mobility shift assay, CDEIII has been shown to bind proteins (4, 5). A point mutation in the CDEIII region (RN2011) that abolishes *in vivo* centromere function also prevents *in vitro* protein binding to the CDEIII region of *CEN3* DNA (4, 5). The CDEIII binding proteins (CBF3) have been purified using a *CEN3* DNA affinity column (5). The core CBF3 multisubunit complex contains three major proteins of 110 kDa (Cbf2p/Ndc10p) (6, 7), 64 kDa (Cbf3p) (8), and 58 kDa (Ctf13p) (9). The specificity of CBF3 for binding to the CDEIII sequence is apparent, since these proteins do not bind to an RN2011 DNA affinity column (ref. 5 and see Fig. 2*B*). CBF3 preparations normally contain several minor components, present in substoichiometric levels relative to the three major core subunits; however, these do not consistently show specificity for binding to wild-type *CEN3* DNA (refs. 5 and 10 and see Fig. 2*B*).

Because the CDEIII region is essential for chromosome segregation *in vivo*, we reasoned that the CDEIII–CBF3 complex might partially reconstitute kinetochore function *in vitro*. In experiments previously reported, we demonstrated that *CEN3* DNA-coated fluorescent microbeads bind the CBF3 complex (CBF3 beads) (11). When CBF3 beads were introduced into a perfusion chamber containing polarity-marked, fluorescently-labeled microtubules and viewed by fluorescence microscopy, we observed that CBF3 beads can bind to microtubules and, in the presence of MgATP, move toward the minus end of the microtubules with a velocity of 4–5 μm/min. Beads that were linked to RN2011 DNA showed significantly less microtubule binding and motor activity in this assay. In addition, proteins that were eluted from an RN2011 DNA column, thus lacking CBF3, were inactive when exposed to the wild-type *CEN3* DNA beads. Our interpretation of these results was that the *CEN3* DNA affinity-purified proteins include a motor activity that binds to functional centromeric DNA and, furthermore, that the CBF3 proteins play a major role in mediating this activity, with one or more of them possibly being the motor itself. This report is the result of our further investigations into the role played by CBF3 proteins in kinetochore activity *in vitro*.

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MATERIALS AND METHODS

Preparation of Proteins. DNA affinity-purified proteins were prepared as described (5) except that the preparation of pBR322 DNA affinity-purified proteins was accomplished by passage of proteins purified 15-fold for the CBF3 complex over a pBR322 DNA affinity column, constructed as described in ref. 5 using the *Eco*RI/*Bam*HI pBR322 DNA fragment. In some cases (see Fig. 3), the *CEN3* DNA affinity-purified proteins were eluted from the *CEN3* DNA affinity columns by a salt gradient. The proteins were eluted at 4°C using a linear gradient from 230 to 500 mM KCl, over 5 ml, at a flow rate of 0.1 ml/min. Thirty-six fractions of 200 μl each were collected. Each protein fraction was assayed for *CEN3* DNA fragment mobility shift activity (*CEN3* DNA binding; Fig. 3), microtubule binding ability (MT sticking; Fig. 3), and microtubule gliding activity (MT gliding; Fig. 3). The first passage of proteins over the affinity column resulted in the coincident elution of shift and motor activity. A second passage of the peak activity fractions over the column was performed to separate motor and shift activity. In this passage, fractions 19–25 were mixed with 9 ml of original load protein (i.e., protein that had not gone over the first affinity column) and passed over the affinity matrix. The load proteins were included to provide a chaperone activity that is lost during passage over the column (5).

Whole-cell protein extracts were prepared as described (5) except that cytoplasmic and nuclear extracts were pooled. α-Factor arrest of *bar1* cells (strain G1906c) has been described (12).

Microtubule Gliding, Bead Sticking, and DNA Fragment Mobility Shift Assays. The microtubule gliding assay was based on the protocol of Hyman (13). A 4-μl glass perfusion chamber was coated with *CEN3* DNA binding proteins (5) and incubated for 10 min; casein (5 mg/ml) was added to block any unbound sites on the glass surface, and incubation was continued for 5 min. Approximately 10^8 polarity-marked microtubules were introduced into the chamber and incubated for 10 min, and then any unbound microtubules were washed out with antifade buffer (11) plus 10 mM taxol. Bound

Table 1. The microtubule binding and gliding properties of proteins isolated from different DNA affinity columns

Property	Microtubule gliding assay		
	CEN3 DNA column	RN2011 *CEN* DNA column	pBR322 DNA column
No. of MTs bound per field	22 ± 5	25 ± 8	28 ± 6
% of MT population bound	6 ± 0.2	6 ± 0.2	6 ± 0.2
% of MTs moving	34 ± 8	36 ± 8	38 ± 6
Direction of movement	Minus end directed	Minus end directed	Minus end directed
Average MT velocity, μm/min	5.7 ± 0.7	5.6 ± 1.2	5.8 ± 1.0

In all cases, the amounts of protein used were below the saturation level of the assay, hence ensuring meaningful quantitative comparisons of motor activity between preparations. Assays were carried out at 5 mM MgATP. See *Materials and Methods* for other assay details. MT, microtubule.

microtubules were observed with a Nikon Microphot-SA microscope using epifluorescence. To activate the motor protein, MgATP (5 mM in antifade buffer plus taxol) was introduced into the chamber. Gliding activity was observed and recorded onto an optical disk recorder by using a silicon-intensified tube camera and a time-lapse shutter system to minimize light exposure. The entire system was coordinated using IMAGE-I software (Universal Imaging, Media, PA). Polarity-marked microtubules were prepared as described (13). Bead assays were carried out as described (11), except MgATP was introduced directly into the perfusion chamber. Microtubule and bead velocities were determined using IMAGE-I software; at least 20 microtubules or beads were observed for each measurement. For microtubule or bead sticking, at least 10 fields were observed per experiment; the data shown is a summary of at least three experiments. Tubulin was made as described (14) and obtained from Cytoskeleton (Santa Barbara, CA).

CEN3 DNA fragment mobility shift assays were performed as described (4).

Western Blots and Protein Gels. Protein gels and silver staining were carried out as described (11). Western blots were performed using the ECL detection system according to the manufacturer's instructions (Amersham). The anti-Kar3p antibody (a generous gift from M. Rose, Princeton University) was raised (in a rabbit) against the carboxyl-terminal 208 amino acids of Kar3p (15); this antibody was not affinity purified. The general kinesin antibody was raised (in a rabbit) against the motor domain of the *Drosophila ncd*-encoded kinesin (16); this antibody was affinity purified (a generous gift from V. Gelfand, University of Illinois, Urbana). Anti-Cbf2p antibody was raised against the carboxyl-terminal 445 amino acids of Cbf2p and was affinity purified (6).

RESULTS

The CBF3 Proteins Are Necessary but Not Sufficient for *in Vitro* Kinetochore Function. Our original *in vitro* motility assay used *CEN3* DNA-coated fluorescent beads and depended upon the ability of *CEN3* DNA affinity-purified protein complexes to bind to both *CEN3* DNA and to microtubules (11). We reasoned, however, that a standard microtubule gliding assay should free the motor from the DNA dependence inherent in the bead assay and thus simplify the identification of the mechanochemical protein(s). The gliding assay we employed is described in *Materials and Methods*. As predicted, this assay is more efficient (≈4 times) than the bead assay at measuring motor activity of *CEN3* DNA affinity-purified proteins (compare Tables 1 and 2). We are confident that both assays identify the same motor activity, as the direction of movement is minus end directed in both cases (Fig. 1) and the average velocities of microtubule gliding and CBF3 bead movement are identical (Tables 1 and 2) [average velocity varies between 3 and 8 μm/min depending upon the MgATP concentrations used (data not shown)].

Surprisingly, we found that proteins purified by affinity chromatography off an RN2011 mutant *CEN3* column contain gliding activity identical to that observed with the wild-type *CEN3* DNA affinity-purified proteins (Table 1). Furthermore, proteins purified over a column containing a 375-bp pBR322 DNA fragment also exhibited the same gliding activity (Table 1). Clearly, the RN2011 and pBR322 DNA affinity-purified proteins do not include the CBF3 core complex, as determined by silver staining (Fig. 2 *A* and *B*) and by Western blot analysis using the anti-Cbf2p antibody (ref. 6 and Fig. 2*F*). It would appear, then, that the CBF3 core complex is not required for the motor to be active in the absence of a *CEN* DNA cargo. These results also suggest that the motor is a general DNA-binding protein.

It seemed reasonable, therefore, that the motor should bind to beads containing the 375-bp pBR322 DNA fragment. We mixed pBR322 DNA beads with *CEN3* DNA affinity-purified proteins and looked for microtubule binding and motor activity. The results, shown in Table 2, indicate that the pBR322 beads are unable to sustain microtubule binding and motor activity. However, in agreement with previously reported results (11), *CEN3* DNA beads mixed with *CEN3* DNA affinity-purified proteins are able to carry out the motor function (Fig. 1 *B* and *C* and Table 2). These results indicate that, in order to partially reconstitute kinetochore function *in vitro*, both motor and CBF3 proteins are necessary. It

Table 2. Summary of the ability of *CEN3* DNA affinity-purified proteins to support microtubule binding and motor activity of *CEN3* and pBR322 DNA beads

Property	Bead assay	
	CEN3 DNA beads	pBR322 DNA beads
MT-attached beads per field	7.7 ± 1.7	0.2 ± 0.1
% of bead population attached to MTs	1.5 ± 0.3	0.04 ± 0.02
% of beads moving	22 ± 4.5	0.0
Direction of movement	Minus end directed	No movement
Average bead velocity, μm/min	5.0 ± 1.1	0.0

Bead assays were carried out in the presence of 5 mM MgATP. See *Materials and Methods* for other assay details. MT, microtubule.

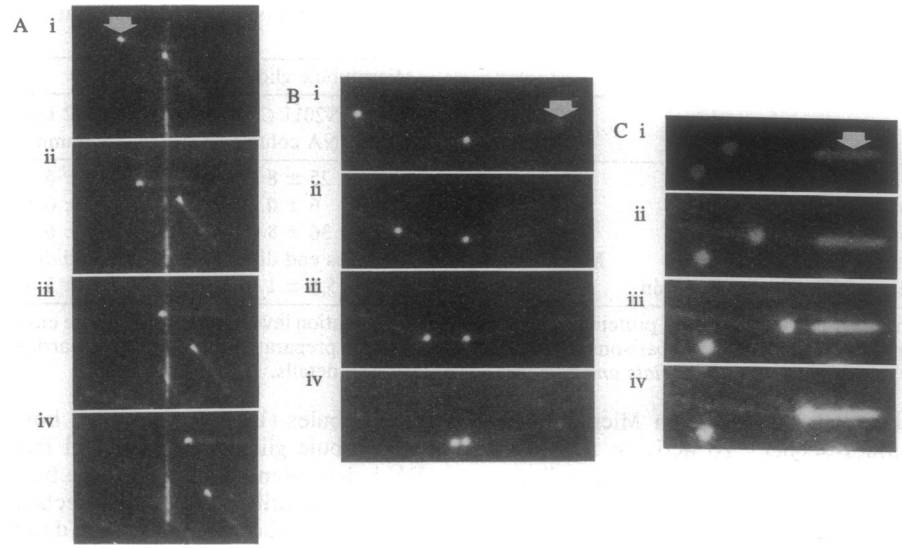

FIG. 1. Analysis of microtubule-based motor activity. (*A*) Gliding assay. Fluorescently labeled microtubules, polarity marked with their minus ends identifiable by the brightly labeled seeds (arrow), are shown. The microtubules are bound to motor proteins that are coating the surface of a glass perfusion chamber. MgATP is added to the chamber to activate motor proteins; motor activity is detected as gliding of microtubules. Microtubules can be seen to glide in a minus-end-directed manner (from i to iv), with their brightly labeled seeds trailing. (*B*) Bead assay. A single fluorescently labeled microtubule is shown (arrow marks the bright seed). The two very bright spots located on the microtubule are *CEN3* beads. In the presence of MgATP, the bead most distal to the microtubule seed moves in a minus-end direction (from i to iv) along the microtubule until it collides with the stationary bead that is bound near the center of the microtubule. (*C*) A *CEN3* bead is shown moving, in a minus-end direction, toward the brightly labeled seed (arrow) of a microtubule.

appears, then, that the role of CBF3 in the *in vitro* kinetochore system is to mediate motor activity in some way.

The CBF3-Associated Motor Is Identified as Kar3p. We have previously shown that the CBF3-associated motor exhibits kinesin-like properties (11). Western blots of our DNA affinity-purified proteins, using a high-titer antibody that recognizes a broad range of kinesins (16), detect a single band of ≈80 kDa, present in all of the affinity-purified preparations (Fig. 2*E*). Because the kinesin-like protein Kar3p, which based on genetic evidence has been proposed to be involved in both karyogamy and mitosis (15, 17), is about this size, we probed our blots with anti-Kar3p antibody

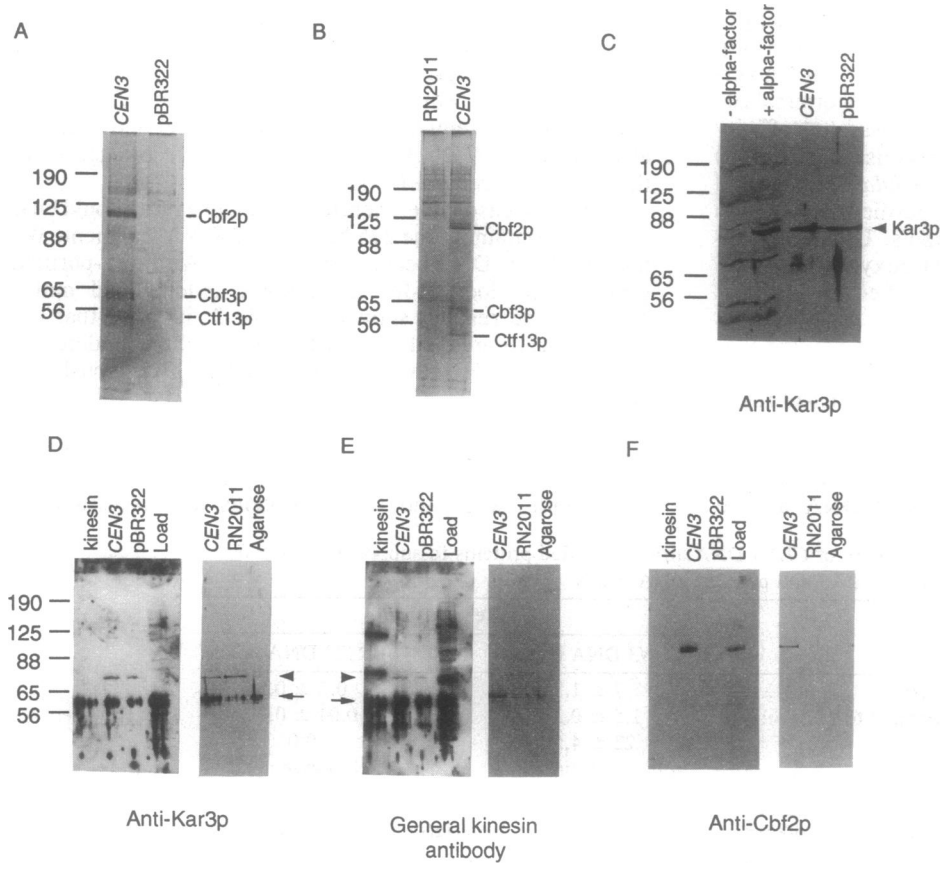

FIG. 2. (*A*) Silver-stained gel showing the proteins eluted from *CEN3* and pBR322 DNA affinity columns. (*B*) Silver-stained gel showing the proteins eluted from RN2011 DNA and *CEN3* DNA affinity columns. In both *A* and *B*, ≈0.2 μg of protein has been loaded in each lane. The three proteins constituting the CBF3 complex are indicated as Cbf2p (110 kDa), Cbf3p (64 kDa), and Ctf13p (58 kDa). (*C*) Western blot of *S. cerevisiae* proteins using an anti-Kar3p antibody. Lanes: − alpha-factor, 50 μg of whole-cell extract protein; + alpha-factor, 50 μg of whole-cell extract protein from cells induced with α factor; *CEN3*, as in *A*; pBR322, as in *A*. The Kar3p band is indicated (arrowhead). (*D*) Western blot using an anti-Kar3p antibody. Lanes: kinesin, ≈0.2 μg of squid kinesin (generous gift from J. Scholey, University of California, Davis); *CEN3*, as in *A*; pBR322, as in *A*; Load, ≈50 μg of yeast protein as loaded onto the affinity column; *CEN3*, as in *B*; RN2011, as in *B*; Agarose, proteins eluted from an agarose affinity column without bound DNA. The diffuse, dark band at ≈63 kDa (arrow) is due to a nonspecific reaction with contaminating keratin protein. The position of Kar3p is shown (arrowhead). (*E*) The same blot as in *D* but reprobed with a general kinesin antibody. (*F*) The same blot as in *D* but reprobed with anti-Cbf2p antibody.

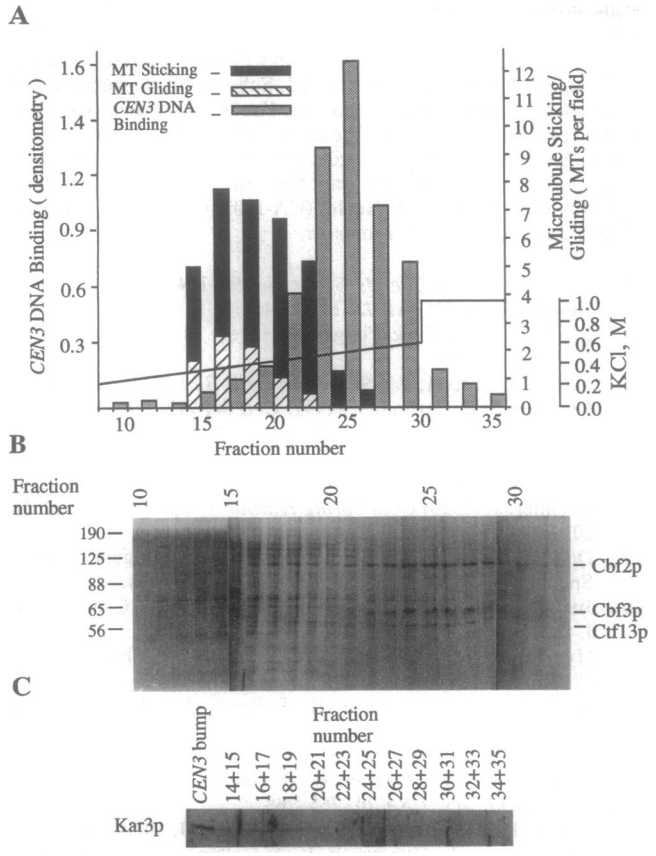

FIG. 3. Separation of microtubule (MT) gliding and *CEN3* DNA-binding activity by *CEN3* DNA affinity column chromatography. (*A*) *CEN3* DNA affinity column binding protein elution profile after a second passage over a *CEN3* DNA affinity column, using salt gradient elution (see *Materials and Methods*). Each fraction was assayed for *CEN3* DNA fragment mobility shift activity (*CEN3* DNA binding), microtubule binding (MT sticking), and microtubule gliding (MT gliding). (*B*) Silver-stained SDS/PAGE gel of fractions from the *CEN3* DNA affinity column shown in *A*; fractions 10–32 are shown (20 μl of protein per lane). (*C*) Western blot of an SDS/PAGE separation of the *CEN3* DNA affinity column binding proteins (fractions 14–36), using an anti-Kar3p antibody (≈200 μl of protein per lane). The lane marked *CEN3* bump contains ≈0.2 μg of *CEN3* DNA affinity-purified proteins eluted by a standard 500 mM KCl step elution (8). The peak of Kar3p content occurs in fractions 16–19, coincident with the peak of motor activity (*A*).

(15). The results (Fig. 2*D*) indicate that this antibody reacts strongly with the 80-kDa protein in our preparations and, unlike the general kinesin antibody, does not recognize squid kinesin protein (compare kinesin lanes in Fig. 2 *D* and *E*). In addition, Kar3p is highly induced in cells treated with α factor (15), and the 80-kDa cross-reacting band in our DNA affinity-purified protein preparations is exactly coincident with an induced Kar3p band (Fig. 2*C*). We thus conclude that Kar3p is present in our protein preparations.

Kar3p is the only detectable motor in our DNA affinity-purified proteins. Western blots using two different anti-dynein antibodies (18, 19) gave no signal with these preparations (data not shown). It is therefore highly likely that Kar3p is responsible for the observed motor activity. One would thus predict that Kar3p will always cofractionate with motor activity. In a purification strategy that used a shallow salt gradient to elute CBF3 proteins from a *CEN3* DNA affinity column, we observed that motor activity, as assayed by microtubule gliding, can be separated from *CEN3* DNA fragment shift activity (Fig. 3*A*). The CBF3 complex fractionates with fragment shift activity (Fig. 3*B*). As predicted,

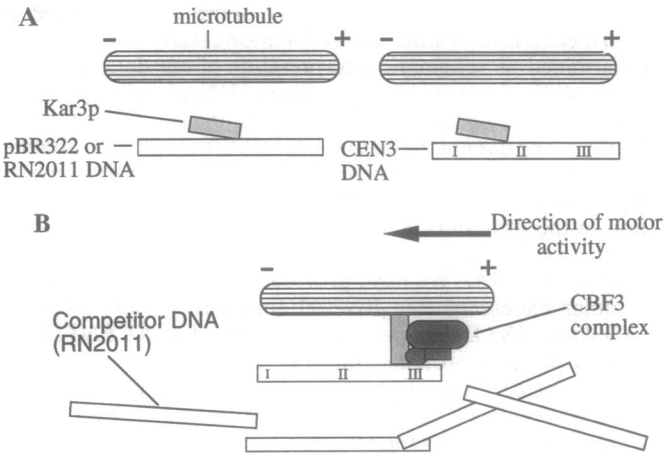

FIG. 4. Schematic interpretation of our *in vitro* results. (*A*) In the absence of CBF3, Kar3p binds nonselectively to DNA, but it is inactive in mediating the attachment of the DNA to microtubules. (*B*) Kar3p binds more tightly to the *CEN3* DNA–CBF3 complex than to mutant *CEN3* DNA (RN2011). Furthermore, Kar3p is activated for microtubule binding and motor activity in the presence of bound CBF3.

the peak of microtubule gliding activity is coincident with the presence of Kar3p (Fig. 3*C*).

Is the observed DNA binding a unique property of Kar3p, or is this a general property of kinesin family members? The Western blot shown in Fig. 2*D* indicates that the amount of Kar3p in the sample loaded onto the DNA affinity column (50 μg of total protein) is below the level of detection by the ECL Western assay (there is no 80-kDa Kar3p band visible in the lane marked load). However, all of the affinity-purified samples (>0.2 μg of total protein per lane) show a clear Kar3p signal (Fig. 2*D*, lanes marked CEN3, pBR322, and RN2011). This indicates that Kar3p binds tightly to the DNA column and is highly enriched in the eluent. In contrast, Kar3p does not bind to the agarose support beads in the DNA affinity columns (Fig. 2 *D* and *E*). It is apparent, therefore, that Kar3p is a DNA-binding protein. The major component that cross-reacts with the general kinesin antibody is slightly smaller than Kar3p (Fig. 2*E*, lane marked load) and does not react with the anti-Kar3p antibody (Fig. 2*D*, lane marked load). Furthermore, although this protein is clearly present in the column load mixture, it does not bind to the DNA affinity columns. Thus, the ability to bind DNA is not a general property of the kinesins in our preparations and appears to be unique to Kar3p.

DISCUSSION

Kar3p is known to function in both nuclear fusion (karyogamy) and mitosis (15, 17). Genetic evidence strongly suggests that Kar3p is acting as a minus-end-directed, microtubule-based motor in both of these events (15, 17). In contrast to classical kinesin, a plus-end-directed motor (20), Kar3p has its motor domain located at the carboxyl terminus (15). It is noteworthy that the motor domain of the *Drosophila ncd* kinesin, a known minus-end-directed motor, is also located at the carboxyl terminus of the protein (21, 22). Our *in vitro* results now confirm that Kar3p is in fact a minus-end-directed motor. Recently, it has been shown that Kar3p, prepared by overexpression from the cloned *KAR3* gene in *Escherichia coli*, can function as a plus-to-minus motor in a microtubule gliding assay (23).

It has been suggested, again through genetic evidence, that Kar3p could be localized at the mitotic kinetochore (17). Our results support this hypothesis and imply that Kar3p may provide some of the force needed for chromosome-to-pole

movement. However, the relevance of our findings to the *in vivo* mechanism of kinetochore function clearly remains to be determined. A schematic interpretation of our data is shown in Fig. 4; Kar3p appears to be a general DNA-binding protein; however, DNA bead assays (this study and ref. 11) show that Kar3p alone cannot mediate *CEN* DNA/microtubule attachment (i.e., *in vitro* kinetochore activity). Apparently, when Kar3p alone is bound to DNA the conformation or orientation of the protein is such that it cannot carry out functions such as microtubule binding or microtubule-based motor activity (Fig. 4*A*). We have previously shown (11) that when a molar excess of free RN2011 (mutated *CEN3*) DNA is added to *CEN3* DNA beads and then mixed with *CEN3* DNA affinity-purified proteins, there is no effect upon the efficiency of *CEN3* bead microtubule binding or motor activity. If, however, free *CEN3* DNA is used as a competitor, bead activity is almost completely obliterated (11). Our interpretation of this result is shown schematically in Fig. 4*B*; because RN2011 DNA cannot compete with *CEN3* DNA beads, we conclude that Kar3p has a much higher affinity for the *CEN* DNA–CBF3 complex. Significantly, because Kar3p can only mediate *CEN* DNA–microtubule binding in the presence of CBF3, we conclude that the CBF3 complex can somehow alter (regulate?) Kar3p to enable it to bind to and move on microtubules with its *CEN* DNA cargo. This could occur, as shown in Fig. 4*B*, as an alteration in the conformation of Kar3p or by some other mechanism (e.g., by direct binding of Kar3p to the CBF3 complex).

The three genes specifying the CBF3 proteins are essential in yeast (refs. 6, 7, and 9; J. Lechner, personal communication). In contrast, although *kar3* null mutants show a pronounced mitotic phenotype, Kar3p is not essential for mitosis (15). It would thus appear that the general theme of functional redundancy observed for several motor proteins (17, 24) can be extended to their role at the kinetochore. The functional redundancy of Kar3p raises some interesting questions as to the role of motors at the *S. cerevisiae* kinetochore. Is there more than one motor at the kinetochore? Are motors essential for anaphase A? Is anaphase A functionally redundant in *S. cerevisiae* mitosis? Finally, it seems quite likely that additional *S. cerevisiae* kinetochore proteins are yet to be identified. In this regard other groups have reported microtubule binding activity inherent in yeast *CEN*-bearing plasmid chromatin (25, 26) and in crude yeast extracts (27). The correlation between these and our *in vitro* kinetochore reconstitution activities remains to be determined.

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