

A kinesin-like calmodulin-binding protein in *Chlamydomonas*: evidence for a role in cell division and flagellar functions

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Summary

Kinesin-like calmodulin-binding protein, KCBP, is a novel member of the C-kinesin superfamily first discovered in flowering plants. This minus-end-directed kinesin exhibits Ca^{2+} -calmodulin-sensitive motor activity *in vitro* and has been implicated in trichome morphogenesis and cell division. A homologue of KCBP is also found in the unicellular, biflagellate green alga *Chlamydomonas reinhardtii* (CrKCBP). Unlike plant cells, *Chlamydomonas* cells do not form trichomes and do not assemble a phragmoplast before cell division. To test whether CrKCBP is involved in additional microtubule-based processes not observed in plants, we generated antibodies against the putative calmodulin-binding domain and used these antibodies in biochemical and localization studies. In

interphase cells CrKCBP primarily localizes near the base of the flagella, although surprisingly, a small fraction also localizes along the length of the flagella. CrKCBP is bound to isolated axonemes in an ATP-dependent fashion and is not a component of the dynein arms, radial spokes or central apparatus. During mitosis, CrKCBP appears concentrated at the centrosomes during prophase and metaphase. However, during telophase and cytokinesis CrKCBP co-localizes with the microtubules associated with the phycoplast. These studies implicate CrKCBP in flagellar functions as well as cell division.

Key words: Kinesin, Flagella, Calmodulin, Centrosome, *Chlamydomonas reinhardtii*

Introduction

Since the initial discovery of kinesin in extracts of squid axoplasm, members of the kinesin superfamily of microtubule-based motors have been identified as key players in a variety of cellular processes including organelle, mRNA and vesicle transport, spindle microtubule dynamics, and chromosome segregation (for a review, see Miki et al., 2005). Whereas the majority of family members within the kinesin superfamily of motors are plus-end directed motors, one of the 14 families comprises kinesins that are minus-end directed motors. This family includes a unique kinesin with a calmodulin-binding domain referred to as kinesin-like calmodulin-binding protein (KCBP).

KCBP was first identified and characterized in *Arabidopsis* (AtKCBP) (Reddy et al., 1996) and is classified as a member of the Kinesin-14 family. Whereas KCBP shares several common domains with many other kinesins including a motor domain, a stalk and a tail domain, KCBP also has a myosin tail homology region 4 domain (MyTH4) and a talin-like region found in some myosins (Reddy and Reddy, 1999). In addition, KCBP has a unique calmodulin-binding domain (CBD) at the C terminus adjacent to the motor domain. It was originally thought that KCBP was only found in angiosperms, however, in recent database searches KCBP homologues have been identified in gymnosperms (*Picea abies*) as well as green algae (*Chlamydomonas reinhardtii* and *Stichococcus bacillaris*). A calmodulin-binding kinesin has also been reported in sea urchin (*Strongylocentrotus purpuratus*) (Rogers et al., 1999),

however this kinesin (SpKinC) lacks the MyTH4 and talin-like domains. To date, KCBP homologues have not been identified in the genome sequences of mammals, fungi, *Drosophila* or *C. elegans* (Abdel-Ghany et al., 2005). Therefore, true KCBP homologues appear to be unique to plants and green algae.

In vitro functional assays using the bacterially expressed head domain of KCBP demonstrated that KCBP binds microtubules in an ATP-dependent manner and is indeed a minus-end-directed motor (Song et al., 1997). These studies have also revealed that KCBP is negatively regulated by Ca^{2+} -calmodulin; studies using bacterially expressed KCBP indicate that both ATPase activity and microtubule interactions are inhibited by the presence of Ca^{2+} -calmodulin *in vitro* (Kao et al., 2000; Narasimhulu et al., 1997; Narasimhulu and Reddy, 1998; Song et al., 1997). In addition, production of chimeric kinesins containing the calmodulin-binding domain have shown that this domain can act as a regulatory module in animal chimeric kinesins including N-kinesins that are plus-end-directed motors (Reddy and Reddy, 2002).

Several studies have suggested that KCBP plays a role in diverse microtubule-based cellular processes, including trichome morphogenesis as well as mitosis and cell division. In *Arabidopsis*, mutants that lack KCBP (*zwi*) produce abnormal trichomes. Wild-type *Arabidopsis* trichomes are composed of a single large epidermal cell with a protruding stalk and three or four branches, which are microtubule-based structures; however, *zwi* mutants produce trichomes with a shortened stalk and only two branches (Oppenheimer et al.,

1997). Injection of antibodies into *Tradescantia virginiana* stamen cells, which constitutively activate KCBP, has provided evidence for a positive role for KCBP in spindle formation and a negative role for KCBP in phragmoplast formation (Vos et al., 2000). In dividing cells of *Arabidopsis* and tobacco, KCBP localizes to the preprophase band of microtubules, the mitotic spindle microtubules and the phragmoplast, further supporting a role for KCBP in mitosis and cell division (Bowser and Reddy, 1997).

The predicted *Chlamydomonas* KCBP (CrKCBP) amino acid sequence is about 50% identical and 65% similar to plant KCBPs, and like AtKCBP has a C-terminal conserved motor domain with adjacent calmodulin-binding domain, a coiled-coil stalk and both the MyTH4 and talin-like regions (Abdel-Ghany et al., 2005). However, unlike plant cells, *Chlamydomonas* cells do not have trichomes and do not assemble a phragmoplast before cell division. Interphase cells of *Chlamydomonas* assemble four- and two-membered rootlet microtubules in the cytoplasm as well as the nine doublet and two singlet microtubules comprising the flagellar axoneme (Gaffal and el-Gammal, 1990; Goodenough and Weiss, 1978; Johnson and Porter, 1968; Weiss, 1984). During cell division, *Chlamydomonas* cells assemble a phycoplast before cytokinesis. The key difference between the phycoplast and the phragmoplast is the orientation of the microtubules that comprise these structures relative to the plane of cell division. The microtubules assemble parallel to the plane of cleavage in the phycoplast whereas microtubules assemble perpendicular to the plane of cleavage in the phragmoplast (Gaffal and el-Gammal, 1990; Johnson and Porter, 1968). Based on these differences in microtubule organization, we hypothesized that KCBP in *Chlamydomonas* may be involved in additional cellular processes not observed in plant cells.

To test this hypothesis, we confirmed the predicted CrKCBP amino acid sequence by RT-PCR, characterized CrKCBP transcript levels in response to deflagellation and flagellar resorption, and generated antibodies against the calmodulin-binding domain of CrKCBP for localization studies. Our data are consistent with a role for CrKCBP in flagellar assembly/function as well as cell division.

Results

CrKCBP sequence analysis

Abdel-Ghany et al. (Abdel-Ghany et al., 2005) reported functional domains within *Chlamydomonas* KCBP (CrKCBP) based on the protein sequence generated from analyses of the *Chlamydomonas* genome using splice site prediction programs. We have now confirmed the complete coding sequence of CrKCBP by RT-PCR using RNA isolated from wild-type cells as template. The amino acid sequence predicted from our cDNA agrees with the predicted protein sequence available at the JGI v3.0 *Chlamydomonas* genome database with a few minor exceptions. (Note: in the JGI v3.0 *Chlamydomonas* genomic sequence, the gene encoding CrKCBP has been named e_gwH.38.2.1 and is located on scaffold 38:824280-831809.) First, results from our 5'RACE analysis predict 41 additional amino acids at the amino terminus. In addition, two splice junctions were incorrectly predicted. These splice junction sites are between exons 10 and 11 and between exons 12 and 13. The complete coding and predicted amino acid sequences are available in GenBank,

accession DQ499010. CrKCBP has a predicted molecular mass of 140 kDa and pI of 5.78; as noted by Abdel-Ghany et al. (Abdel-Ghany et al., 2005), CrKCBP is a C-kinesin with both motor-and calmodulin-binding domains located at the C terminus of the protein as well as an amino-terminal myosin tail homology 4 (MyTH4) domain (Fig. 1A). Searches of the NCBI protein database reveal the presence of a B41 domain corresponding to the region described as the talin-like domain by Abdel-Ghany et al. (Abdel-Ghany et al., 2005). The B41 domain is present in a number of proteins that interface between the plasma membrane and cytoskeleton such as myosin, ezrin, moesin and radixin.

A fraction of CrKCBP is associated with flagella

To begin investigating a possible role for KCBP in the assembly and/or function of *Chlamydomonas* flagella, we examined transcript levels of CrKCBP before and after flagellar deflagellation and flagellar resorption. To detect CrKCBP transcripts we used a DNA fragment corresponding to the N-terminal coding sequence of CrKCBP (nucleotides 239-701 of the coding sequence; Fig. 1A) as a hybridization probe on blots of RNA isolated from wild-type cells as described in the Materials and Methods. Because the amino terminus is the least conserved region of CrKCBP, we predicted that this probe would not hybridize to other kinesin superfamily members. Our probe recognized a single transcript of approximately 4.4 kb, which correlates well with the 4451 bp cDNA we generated using RT-PCR. CrKCBP transcript

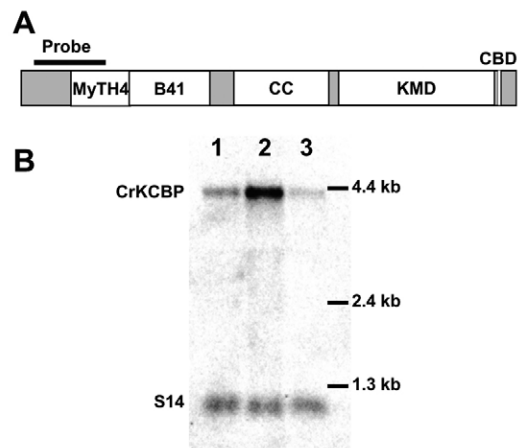


Fig. 1. (A) Diagram of domains within complete coding sequence of CrKCBP. CrKCBP contains a myosin tail homology domain (MyTH4), a band 4.1 domain (B41), present in a number of proteins that interface between the plasma membrane and cytoskeleton, a coiled-coil neck region (CC), a kinesin motor domain (KMD), and a calmodulin-binding domain (CBD). (B) RNA blot. 10 μ g of poly(A)⁺ selected RNA prepared from control cells (lane 1), 45 minutes after deflagellation (lane 2) or 45 minutes after induction of flagella resorption (lane 3) were loaded into each lane. The blot was probed with a DNA fragment at the 5' end of the CrKCBP coding sequence (labeled Probe in A) as well as the S14 gene encoding ribosomal S14 protein to serve as a loading control. The band of approximately 1 kb represents the S14 transcript. The band at approximately 4.4 kb represents the CrKCBP transcript. This transcript increases in abundance following deflagellation and decreases slightly in abundance during flagellar resorption.

levels increase in abundance following deflagellation and slightly decrease in abundance following flagellar resorption (Fig. 1B). These changes in transcript abundance are one hallmark of virtually all genes that encode structural proteins of flagella or proteins involved with flagellar assembly and structural maintenance (reviewed by Lefebvre and Rosenbaum, 1986) (see also Pazour et al., 1999; Walther et al., 1994).

Based on the finding that the changes in transcript levels of CrKCBP are consistent with the hypothesis that CrKCBP plays a role in flagellar assembly or function, we investigated whether CrKCBP protein localizes to flagella. We generated polyclonal antibodies against a peptide representing the predicted calmodulin-binding domain of CrKCBP. These antibodies are specific and recognize a single band of predicted molecular mass on immunoblots of isolated flagella (Fig. 2). In addition, CrKCBP is found in flagella of all *Chlamydomonas* mutants examined to date, including those with flagella that lack radial spokes (*pf14*) and the central pair (*pf18*), as well as a mutant that lacks the outer dynein arms and the II inner dynein arms (*pf30pf28*) (data not shown). Therefore, CrKCBP most likely does not localize to any of these axonemal structures.

To further investigate the association of CrKCBP with flagella, we examined whether CrKCBP remained associated with the axoneme, using various buffer conditions (Fig. 2). Upon demembration of isolated flagella with nonionic detergent, CrKCBP remains associated with isolated axonemes, however, CrKCBP is extracted from the axonemes using buffers with 0.6 M NaCl. Surprisingly, roughly 90% of CrKCBP is also extracted from isolated axonemes in buffers of more physiologic salt concentration but containing 1 mM ATP (Fig. 2). These results suggest that the CrKCBP associated with flagella is most probably bound to the axoneme by the ATP-sensitive motor domain. As further evidence that CrKCBP is associated with the axoneme, we localized CrKCBP to isolated axonemes both before and after ATP extraction using indirect immunofluorescence to detect CrKCBP. CrKCBP is present along the entire length of isolated

axonemes and this staining is greatly reduced following exposure of axonemes to ATP (Fig. 3). These results are consistent with the data we obtained from immunoblots and provide supporting evidence for the specificity of our antibodies in immunolocalization studies.

In vitro functional analysis of bacterially expressed AtKCBP demonstrated that AtKCBP binds microtubules in an ATP-dependent manner (Song et al., 1997). These and additional studies have also shown that both ATPase activity and microtubule interactions of expressed AtKCBP are inhibited by the presence of Ca²⁺-calmodulin in vitro (Kao et al., 2000; Narasimhulu et al., 1997; Narasimhulu and Reddy, 1998; Song et al., 1997). We used two experimental approaches to investigate the calmodulin-binding activity of bacterially expressed CrKCBP. First, we conducted in vitro binding experiments using the expressed head domain of CrKCBP (amino acids 879-1306) and expressed *Chlamydomonas* calmodulin, followed by immunoprecipitation of calmodulin. Regardless of whether Ca²⁺ is present, precipitation of calmodulin fails to precipitate CrKCBP (data not shown). In addition we have used the bacterially expressed head domain of CrKCBP in binding experiments using calmodulin-agarose. Again, regardless of whether Ca²⁺ was present or not, CrKCBP failed to bind to the calmodulin-agarose (data not shown).

It is possible that these negative results indicate that the bacterially expressed head domain of CrKCBP is non-

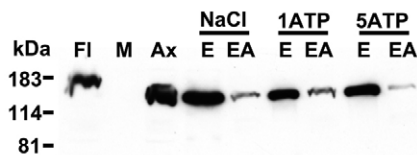


Fig. 2. Immunoblot. Flagella (Fl) were isolated from wild-type cells and demembrated as described in Materials and Methods. The resulting membrane and matrix fraction (M) and axonemes (Ax) were isolated by centrifugation. Axonemes were divided and extracted with either 0.6 M NaCl (NaCl), 1 mM ATP (1ATP), or 5 mM ATP (5ATP) to produce extracted axonemes (EA) and axonemal extracts (E). The resulting extracted axonemes and extracts were separated by centrifugation and extracted axonemes were resuspended in an equal volume of buffer. For immunoblot analysis, 25 μ l of each sample were loaded in each lane and anti-CrKCBP antibodies were used as a probe. These antibodies recognize a single band of approximately 140 kDa. This protein is extracted from axonemes under conditions of high salt and ATP. CrKCBP appears to be of higher molecular mass in flagella because of the large quantity of flagellar membrane in this sample, which interferes with protein migration.

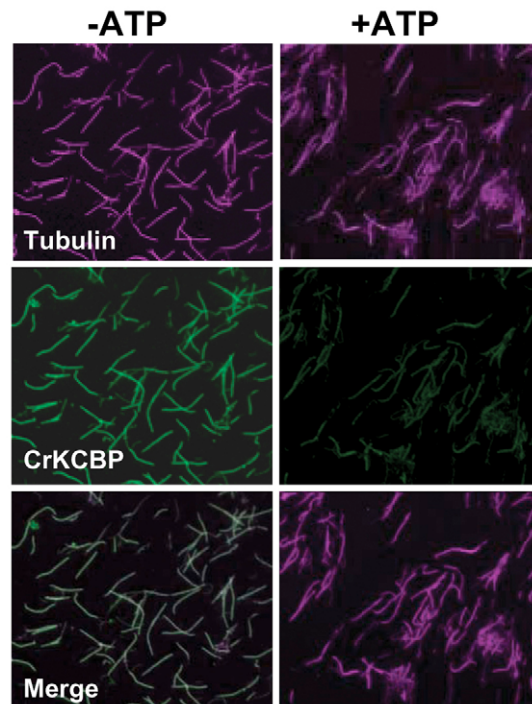


Fig. 3. Localization of CrKCBP by indirect immunofluorescence. Axonemes were isolated as described in the Materials and Methods. Samples in the right column were extracted with 5 mM ATP prior to processing for immunofluorescence. The axonemes were double labeled for tubulin (top row, Texas Red secondary antibody), and CrKCBP (middle row, Alexa Fluor 488 secondary antibody). The merged images are shown in the bottom row where white indicates co-localization of tubulin with CrKCBP. CrKCBP localizes along the length of the axoneme and is extracted upon exposure to 5 mM ATP.

functional. Therefore, to investigate the calmodulin and microtubule-binding properties of CrKCBP *in vitro*, we extracted CrKCBP from isolated wild-type axonemes using 1 mM ATP and treated the resulting extract with apyrase before binding to taxol-stabilized microtubules assembled from purified brain tubulin *in vitro*. Using a microtubule pelleting assay we found that approximately 60% of the ATP-extracted CrKCBP binds to microtubules *in vitro* (Fig. 4A). If the ATP extract was not treated with apyrase, no microtubule binding

was observed (not shown). Of the CrKCBP bound to microtubules, approximately 50% is released from the microtubules upon the addition of 1 mM ATP. These results indicate that about half of the CrKCBP that binds to microtubules *in vitro* is bound by the ATP-sensitive heads.

We also repeated the microtubule-binding experiments in the presence of Ca^{2+} and exogenously added *Chlamydomonas* calmodulin expressed *in vitro* (Fig. 4A). For these experiments, axonemes and axonemal extracts were prepared in either the presence or absence of high Ca^{2+} ; in either case, the resulting extract was subjected to the microtubule-binding assay in either the presence or absence of high Ca^{2+} and calmodulin (see Materials and Methods for details). If the axonemal extracts were prepared in low Ca^{2+} conditions, we saw no difference in microtubule binding of CrKCBP, despite the presence of high Ca^{2+} and calmodulin in the microtubule-binding assay. If the axonemes were extracted with ATP under high Ca^{2+} conditions, we observed a modest decrease in the amount of CrKCBP that bound to microtubules *in vitro*. Of the CrKCBP that did bind to microtubules, approximately 50% was released upon the addition of ATP. These results suggest the microtubule-binding properties of CrKCBP may not be as sensitive to Ca^{2+} -calmodulin as those of AtKCBP.

Based on the ATP-sensitive binding of CrKCBP to isolated axonemes, we suspected that CrKCBP is associated with the membrane matrix fraction of flagella *in vivo* and that upon solubilization of the flagellar membrane in the absence of ATP, CrKCBP binds to the axonemal microtubules by its motor domain. Previous studies have shown that a significant amount of calmodulin associates with the membrane matrix fraction of flagella (Gitelman and Witman, 1980). To further explore the putative calmodulin-binding properties of CrKCBP, we demembrated isolated flagella in the presence of 1 mM ATP and high Ca^{2+} and used our anti-calmodulin antibodies in immunoprecipitation experiments. We have previously shown that antibodies we generated against *Chlamydomonas* calmodulin work extremely well for precipitating calmodulin and proteins associated with calmodulin (Wargo et al., 2005). Our anti-calmodulin antibodies precipitated virtually all of the calmodulin from the membrane-matrix extract, however all of the CrKCBP was found in the unbound fraction, regardless of whether the extract was treated with apyrase before precipitation (Fig. 4B). We obtained the same results when we used a dialyzed 0.6 M NaCl extract in immunoprecipitation experiments (not shown).

To further characterize the flagellar-associated CrKCBP we subjected the axonemal ATP extract to sucrose density gradient centrifugation. Immunoblots of the resulting gradient fractions reveal that CrKCBP sediments in a peak fraction at approximately 11S (Fig. 4C). We have been unable to definitively detect CrKCBP in silver-stained gels of sucrose gradient fractions or using microtubule-binding studies. Therefore, we are unable to determine if CrKCBP includes any associated light chains. We suspect that the antibodies we generated against CrKCBP are very high affinity and that CrKCBP is not a very abundant flagellar protein.

Cellular localization of CrKCBP during interphase

The localization of CrKCBP was investigated by indirect immunofluorescence of interphase cells to determine any association with cytoplasmic microtubule structures. We found

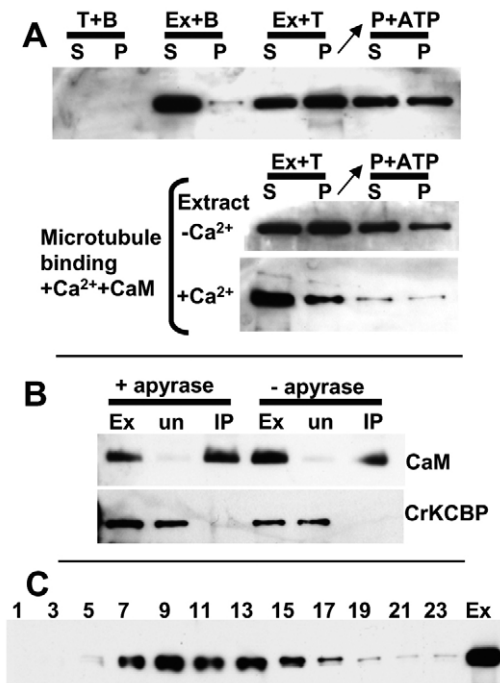


Fig. 4. (A–C) Immunoblots. (A) Blots from microtubule-binding studies probed with the anti-CrKCBP antibody. Extracts (Ex) were prepared by extracting axonemes with 1 mM ATP and treating with apyrase. The resulting extracts were then added to microtubules (T) assembled *in vitro* or to an equivalent volume of buffer (B). The microtubules were sedimented, the supernatant collected (S) and the pellet (P) resuspended in an equivalent volume of buffer. In some experiments ATP was added to the resulting pellet (P+ATP) to assess ATP-sensitive binding of CrKCBP to microtubules. (Top panel) Microtubule binding was conducted in the absence of calmodulin under low Ca^{2+} conditions; (second panel) binding was performed in the presence of high Ca^{2+} -calmodulin (+ Ca^{2+} +CaM) using an extract prepared in low Ca^{2+} conditions (– Ca^{2+}); (third panel) binding was performed in the presence of Ca^{2+} +CaM using an extract prepared in high Ca^{2+} conditions (+ Ca^{2+}). Microtubule binding of CrKCBP is only mildly sensitive to the presence of Ca^{2+} -CaM. (B) Blots from immunoprecipitation experiments using anti-calmodulin antibodies for precipitation and probed with either anti-calmodulin (CaM) or CrKCBP antibodies. Axonemes were extracted with 1 mM ATP in high Ca^{2+} conditions; half of the resulting extract (Ex) was treated with apyrase (+apyrase) and half was not (–apyrase). Precipitations were carried out as described in Materials and Methods and the resulting unbound (un) and precipitated (IP) proteins were processed for gel electrophoresis. The antibodies precipitate virtually all of the calmodulin in the extract but do not precipitate CrKCBP. (C) Blots of sucrose density gradient fractions probed with anti-CrKCBP antibodies (fractions 1–23=20–5% sucrose, respectively). CrKCBP sediments at approximately 11S, peaking in fraction 11.

that CrKCBP primarily localizes between the bases of the two flagella with less prominent staining in the cytoplasm including some co-localization with the cytoplasmic microtubules (Fig. 5A). Because the intensity of staining in this region is very high, the CrKCBP signal in the flagella in Fig. 5A is below the detection limit of the camera at the exposure shown (compare with Fig. 3). To quantify the amount of CrKCBP localized near the base of the flagella relative to that associated with the flagella, we isolated both flagellar basal body complexes and flagella from wild-type cells. The samples were prepared from equal numbers of cells and subjected to immunoblotting using antibodies against CrKCBP as well as a known flagellar protein, PF20 (Smith and Lefebvre, 1997). We predicted that if CrKCBP is primarily localized near the base of the flagella, then we would observe significantly less CrKCBP protein in an equivalent load of isolated flagella; flagella prepared by the dibucaine method lack this region. CrKCBP was observed in both flagellar basal body complexes and isolated flagella, however, significantly more CrKCBP was found in flagellar basal body complexes (Fig. 5B). By contrast, when the same blot was probed using antibodies against PF20, virtually equal amounts of PF20 protein were observed in equivalent loads of flagella and flagellar basal body complexes. These results are consistent with our localization studies.

The basal apparatus of *Chlamydomonas* is an elaborate assemblage of structures that connect the basal bodies that nucleate the doublet microtubules of the flagella to each other as well as to both the rootlet microtubules that protrude into the cytoplasm and the nucleus-basal body connectors that associate with the nucleus. The most prominent and well-studied constituent of these structures is the Ca^{2+} -binding protein centrin. Geimer and Melkonian (Geimer and Melkonian, 2005) have recently published a detailed study of centrin localization relative to these structures in *Chlamydomonas* and propose that centrin forms a continuous filament system that includes the nucleus-basal body connectors, distal connecting fiber and transition region fibers (Geimer and Melkonian, 2005).

To further characterize the localization of CrKCBP near the base of the flagella, we used antibodies against CrKCBP as

well as against centrin in immunofluorescence localization studies. As shown in Fig. 6, only a small region of CrKCBP staining co-localizes with centrin and most likely represents staining of the distal connecting fiber. The majority of CrKCBP present in this region of the cell appears to localize more anteriorly and laterally to the centrin fiber system. These results indicate that CrKCBP may be associated with the plasma membrane and/or the minus ends of the cytoplasmic microtubules immediately adjacent to the plasma membrane.

Localization of CrKCBP during mitosis and cytokinesis

Antibody injection experiments in plants have led to a proposed role for KCBP in mitosis and cell division (Vos et al., 2000). To investigate a possible role for CrKCBP in mitosis and cell division, we first synchronized *Chlamydomonas* cultures using the methods of Umen and Goodenough (Umen and Goodenough, 2001). We then fixed and triple labeled cells using antibodies against CrKCBP, centrin and tubulin at time points that we empirically determined to provide the greatest chance of capturing cells in mitosis. Representative images from these studies are shown in Fig. 7 and reveal that the localization of CrKCBP changes dramatically as the cells proceed through M-phase and cytokinesis. During prophase, much of the CrKCBP is associated with each of the duplicated microtubule organizing centers (MTOCs), however, CrKCBP is also diffusively distributed throughout the cytoplasm. As the cells progress into metaphase, the more diffuse cytoplasmic CrKCBP staining seen in interphase and prophase is absent; virtually all of the CrKCBP is concentrated at the MTOCs. From anaphase and into cytokinesis, a fraction of CrKCBP remains localized near the MTOCs, however, CrKCBP is also more diffusively distributed in the cytoplasm as well as co-localized with the microtubules making up the phycoplast. This redistribution of CrKCBP suggests a role for CrKCBP during cell division.

Discussion

KCBP is a minus-end-directed kinesin that was first discovered in flowering plants. This unique motor protein was shown to be negatively regulated by Ca^{2+} -calmodulin in vitro and is thought to play a role in trichome morphogenesis and cell division in vivo. Based on sequence alignment analysis true KCBP homologues have only been found in the genomes of plants and green algae (Abdel-Ghany et al., 2005). Given the differences in microtubule organization in plants compared with that in green algae, we predicted that KCBP might be involved in additional microtubule-based processes in *Chlamydomonas* that are not observed in flowering plants. Using a combination of biochemical and immunolocalization approaches, we have obtained evidence supporting a role for CrKCBP in flagellar assembly/function as well as in cell division.

A fraction of CrKCBP localizes to flagella

The conclusion that CrKCBP is associated with flagella is supported by: northern blot analysis demonstrating that the CrKCBP transcript increases in abundance following deflagellation, immunoblots of isolated flagella and flagellar basal body

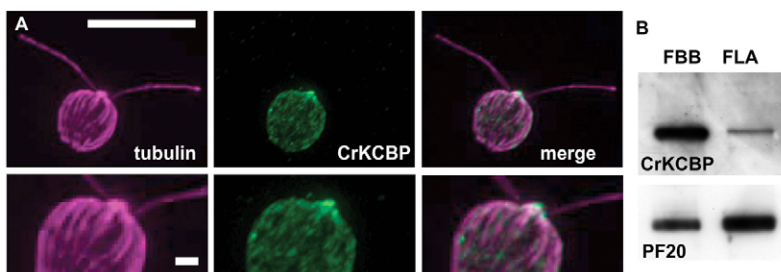


Fig. 5. Indirect immunofluorescence localization of CrKCBP during interphase. (A) Localization of tubulin (Texas Red secondary antibody) and CrKCBP (Alexa Fluor 488 secondary antibody) in *Chlamydomonas* interphase cells. Areas of co-localization appear white in the merged image. Lower panels are enlargements of the region with the greatest CrKCBP staining. The significant fraction of CrKCBP localizes near the base of the flagellar microtubules. Bars, top row, 10 μm ; bottom row, 1 μm . (B) Immunoblot. Flagellar basal body complexes (FBB) and flagella (FL) were prepared from equal numbers of cells and equal amounts were loaded onto the gel. The resulting blot was probed with antibodies against CrKCBP as well as PF20, a known axonemal component.

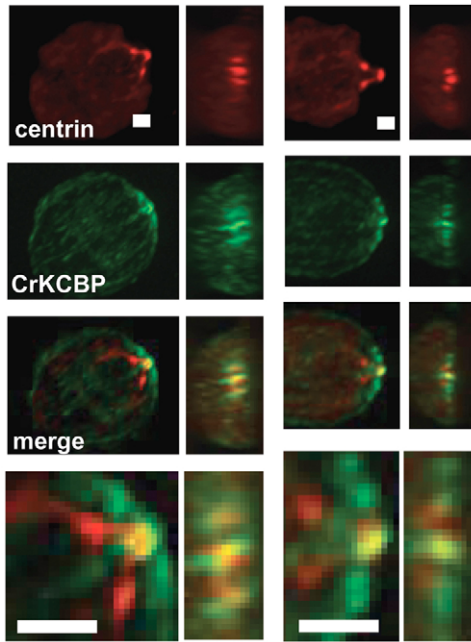


Fig. 6. Cellular localization of CrKCBP and centrin by indirect immunofluorescence. Interphase cells were double labeled with anti-centrin (Texas Red secondary antibodies) and CrKCBP (Alexa Fluor 488 secondary antibodies) antibodies. Regions of co-localization appear yellow in the merged images. Panels to the right of each main image show an end-on view of protein localization. Lower panels are enlargements of the regions with greatest CrKCBP staining. CrKCBP co-localizes with centrin in a very small region, most probably representing the distal connecting fiber. Bars, 1 μm .

complexes, and immunolocalization studies. CrKCBP is the fourth kinesin found to be associated with flagella in *Chlamydomonas*. Two of these kinesins are associated with the central apparatus microtubules and one is associated with the flagellar membrane and involved in intraflagellar transport (IFT) (Bernstein et al., 1994; Fox et al., 1994; Kozminski et al., 1995; Mitchell and Sale, 1999; Walther et al., 1994; Yokoyama et al., 2004). Based on our observations that mutant flagella lacking subsets of axonemal structures retain CrKCBP and that CrKCBP is released from axonemes upon exposure to 1 mM ATP, we suspect that CrKCBP is not a structural component of the axoneme. The simplest interpretation of these results is that CrKCBP is associated with the flagellar membrane and that upon solubilization of the flagellar membrane, CrKCBP binds to the axonemal microtubules by the ATP-sensitive heads.

What function might a flagellar membrane-associated CrKCBP have? One function might include a role in intraflagellar transport (IFT). IFT involves a plus-end-directed heterotrimeric kinesin as well as a minus-end-directed cytoplasmic dynein and is required for the assembly, disassembly and structural maintenance of cilia and flagella (for a review, see Cole, 2003). Presumably, a minus-end-directed kinesin (CrKCBP) and cytoplasmic dynein would have redundant functions in IFT. However, it is possible that two minus-end-directed motors are required during rapid flagellar resorption. In *Chlamydomonas*, flagellar resorption

can be induced by removal of Ca^{2+} from the medium and is reversed by re-addition of Ca^{2+} to the medium (Lefebvre et al., 1978). If CrKCBP is negatively regulated by Ca^{2+} -calmodulin in the same way as AtKCBP (see Song et al., 1997), removal of Ca^{2+} from the medium would activate KCBP, consistent with a role for CrKCBP during flagellar resorption.

Several key observations provide arguments against a direct role for CrKCBP during flagellar resorption or IFT. First, as shown in this study, transcript levels of CrKCBP decrease in abundance during resorption. Secondly, flagellar resorption is negatively regulated by Ca^{2+} and yet, we have only been able to detect a very modest change in microtubule-binding properties of CrKCBP in the presence of Ca^{2+} -calmodulin; we suspect that this motor is not regulated in the same way as AtKCBP. Third, we have used our antibodies in immunolocalization studies of CrKCBP during flagellar resorption and do not detect any increase in CrKCBP in flagella or near the base of flagella (data not shown). And finally, we determined the cellular localization of CrKCBP in *fla10^{ts}* cells shifted to the restrictive temperature (FLA10 encodes the IFT kinesin) (Kozminski et al., 1995; Walther et al., 1994) as well as in the *dhc1b* mutant which is defective in the IFT dynein (Pazour et al., 1999). In either mutant, the localization of CrKCBP is similar to that in wild-type cells.

A second possible function of a flagellar membrane-associated kinesin would be to support flagellar surface motility. Surface motility in *Chlamydomonas* cells includes gliding along substrates using flagellar membrane adherence, flagella-flagella contact between two cells during mating, and surface motility observed using polystyrene beads adhered to the flagellar membrane (for a review, see Bloodgood, 1992b). Flagellar surface motility is predicted to involve a microtubule-based motor protein associated with the flagellar membrane and linked to flagellar glycoproteins that adhere to a surface. Studies of flagellar glycoprotein movement in *Chlamydomonas* indicate that an influx of intraflagellar Ca^{2+} is required to activate surface motility (Bloodgood, 1992a; Bloodgood and Salomonsky, 1990). If CrKCBP is regulated in the same way as AtKCBP, this influx of Ca^{2+} would inhibit surface motility. However, as elaborated below, we have no evidence to indicate that CrKCBP is negatively regulated by Ca^{2+} .

Using several assays we were unable to detect any association of Ca^{2+} -calmodulin with CrKCBP. Using anti-calmodulin antibodies that we previously demonstrated to be highly effective in precipitating calmodulin-containing protein complexes (Wargo et al., 2005), we have shown here that CrKCBP is not precipitated from flagellar membrane matrix extracts. We have also used calmodulin-Sepharose affinity column chromatography as well as calmodulin overlay assays [for methods see Wargo et al. (Wargo et al., 2005)] to detect calmodulin binding. However, in both cases CrKCBP does not bind to calmodulin in either high or low Ca^{2+} conditions (not shown). In addition, we have detected only very modest inhibition of microtubule-binding activity upon the addition of exogenous calmodulin to the sedimentation assay.

One possibility for the difference in calmodulin binding and kinesin regulation in our study compared with those of others could be the difference in source of the kinesin. In our studies we used native CrKCBP that we isolated from the flagellar axoneme. In studies with AtKCBP, the head domain of AtKCBP was bacterially expressed and used in microtubule-binding and

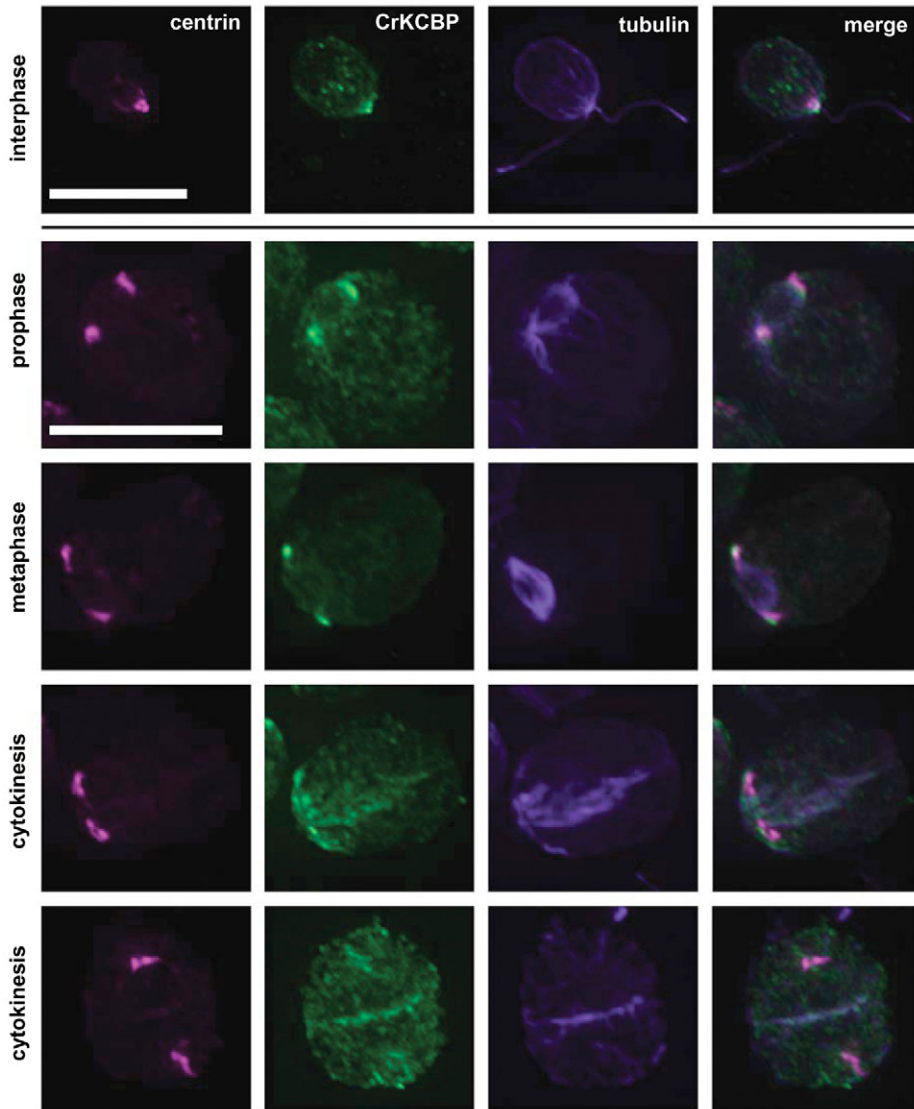


Fig. 7. Cellular localization of CrKCBP during mitosis as shown by indirect immunofluorescence. Cells were triple labeled with anti-centrin (Alexa Fluor 594 secondary antibodies), anti-CrKCBP (Alexa Fluor 488 secondary antibodies) and anti-tubulin (Alexa Fluor 350 secondary antibodies) antibodies. As the cells progress through mitosis the CrKCBP staining appears concentrated at the duplicated microtubule organizing centers. However, as the cells progress to cytokinesis CrKCBP appears to localize to the phycoplast. Bar, top row, 10 μm ; bottom row, 1 μm .

translocation assays. Similarly, Rogers et al. (Rogers et al., 1999) detected Ca^{2+} -sensitive binding to a calmodulin-affinity column using the bacterially expressed motor domain of SpKinC. Although the expressed head domains of these kinesins are functional in terms of microtubule translocation capabilities, they may not be regulated in the same manner as the holoenzyme. It is also possible that the buffers used to prepare the axonemes disrupt calmodulin binding of CrKCBP or that our preparation of CrKCBP contains flagellar proteins that inhibit calmodulin binding. And finally, these differences in calmodulin binding may be due to the variability of the calmodulin-binding domain of CrKCBP compared with that of AtKCBP or SpKinC. CrKCBP varies in 10 out of the 23 amino acids defined by Abdel-Ghany et al. (Abdel-Ghany et al., 2005) as the AtKCBP calmodulin-binding domain. These changes may result in a weaker affinity for calmodulin, which prevents detection of calmodulin binding using the assays we currently have available.

CrKCBP primarily localizes near the base of the flagella in interphase

Our studies of the localization of CrKCBP demonstrate that the

majority of CrKCBP localizes near the base of the flagella during interphase but does not co-localize with centrin. This general region in the cell represents the meeting place for the minus ends of flagellar microtubules as well as the rootlet and accessory cytoplasmic microtubules. This location is also predicted to be a staging area for the pre-assembly of axonemal components awaiting transport by IFT (for a review, see Rosenbaum and Witman, 2002). Given that KCBP is reported to be a minus-end-directed motor, its concentration in a locale with a high number of microtubule minus ends suggests a role for CrKCBP in the translocation and localization of cellular components to this region of the cell, including the possible translocation of flagellar precursor proteins translated in the cytosol towards the base of the flagella.

Is it possible that KCBP plays a role in flagellar assembly or ciliogenesis in other organisms? Whereas true KCBP homologues have only been found in plants and green algae, very closely related kinesins with predicted calmodulin-binding domains are found not only sea urchin, but also in *Ciona intestinalis* and *Tetrahymena thermophila* (Abdel-Ghany, 2005). In addition, a search of the *Paramecium*

tetrauralia genome database (<http://paramecium.cgm.cnrs-gif.fr/>) also reveals a closely related kinesin with a calmodulin-binding domain. Rogers et al. (Rogers et al., 1999) reported that SpKinC is present in sea urchin embryos and suggested that this kinesin plays a role in early development. It is interesting to note that the life cycle of marine invertebrates such as sea urchins and sea squirts (*Ciona*) includes a ciliated larval phase. And like *Chlamydomonas*, the unicellular organisms *Tetrahymena* and *Paramecium* are ciliated. Determination of what role these closely related kinesins play in other organisms will require both localization studies as well as mutant analysis.

CrKCBP associates with the phycoplast during cytokinesis

We have demonstrated that CrKCBP becomes concentrated at the duplicated centrosomes during the early phases of mitosis but then redistributes to the phycoplast during cytokinesis. Unlike localization studies in plants (Bowser and Reddy, 1997), we found very little if any co-localization of CrKCBP with the spindle microtubules during mitosis. The diffuse cytoplasmic staining seen in interphase gradually diminishes and CrKCBP becomes concentrated at the spindle poles along with centrin; upon assembly of the phycoplast, CrKCBP dramatically redistributes to these microtubules. This localization supports a role for CrKCBP in cytokinesis.

In green algae such as *Chlamydomonas*, the phycoplast presumably serves much the same function as the phragmoplast in dividing plant cells, in that these microtubules play a role in the assembly and positioning of the cleavage furrow (Ehler and Dutcher, 1998). The key difference between these two structures is that the phycoplast microtubules assemble parallel to the plane of cleavage and perpendicular to the spindle axis (Johnson and Porter, 1968), whereas the phragmoplast microtubules form perpendicular to the plane of cleavage. In either case, these microtubules are believed to play a role in the recruitment and transport of cleavage furrow components for cytokinesis (reviewed by Jurgens, 2005). In the plant phragmoplast, two sets of microtubules are oriented in an antiparallel fashion with respect to their structural polarity; the plus ends are oriented towards the phragmoplast midline whereas the minus ends are oriented away from the cleavage plane. Based on this orientation and polarity of phragmoplast microtubules, it has been suggested that AtKCBP does not play a role in vesicle transport but rather in phragmoplast stability (Vos et al., 2000) (reviewed by Hepler et al., 2002). In *Chlamydomonas*, the orientation of the phycoplast microtubules with respect to microtubule structural polarity is not known.

Our combined results indicate that CrKCBP may play a role in flagellar assembly or function as well as cell division. To definitively resolve the function of KCBP in *Chlamydomonas* will require knockout or knockdown of CrKCBP expression. Towards this end, we have screened all available mutants at the *Chlamydomonas* Genetics Center that are defective in surface motility for the loss of CrKCBP, and all of these strains have wild-type levels of CrKCBP. We have also screened an entire mutant collection, generated by insertional mutagenesis and generously provided by George Witman (University of Massachusetts, Worcester, MA), using PCR and have not identified any mutant that lacks the gene encoding CrKCBP.

And finally, we have generated constructs for knocking down expression levels of CrKCBP by RNA-mediated interference using several different vectors (Koblenz and Lehtreck, 2005; Li et al., 2004; Rohr et al., 2004; Sineshchekov et al., 2002; Yokoyama et al., 2004) and have not recovered any transformed strains with stably reduced expression of CrKCBP. Although we have recovered some strains in which CrKCBP expression levels appear reduced by two-thirds, these strains resume production of wild-type levels of CrKCBP before their phenotypes can be adequately assessed. Based on our difficulty in stably knocking down CrKCBP expression, the lack of any available CrKCBP mutants, and the results we present here, we suspect that CrKCBP plays an essential role in normal cellular functions.

Materials and Methods

Strains and cell culture

Chlamydomonas reinhardtii strain A54-e18 (*nit1-1, ac17, srl, mt+*) is wild-type for motility and was obtained from Paul Lefebvre (University of Minnesota, St Paul, MN); the *pf30pf28* strain was obtained from Winfield Sale (Emory University, Atlanta GA); the strains *fla10, cw15, pf14, and pf18* were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The *dhc1b* mutant was obtained from George Witman (University of Massachusetts, Worcester, MA). For experiments involving asynchronous cultures, cells were grown in constant light in TAP (Tris-acetate phosphate) medium (Gorman and Levine, 1965). To establish growth curves for assessing culture synchronization, we followed the protocols of Umen and Goodenough (Umen and Goodenough, 2001). A54-e18 cells were grown in a flask containing TAP medium and continuously bubbled with air in constant light for 24 hours. The flasks were then placed in the dark for 36 hours. After re-exposure to light, aliquots of cells were removed at the indicated times and fixed with 0.1% glutaraldehyde. Cell concentration was determined using a hemocytometer.

RT-PCR and sequence analysis

Reverse transcription was performed using poly(A)⁺ RNA isolated as described below and Superscript III in the 5'RACE System kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA). The polymerase chain reaction (PCR) was performed using Taq DNA polymerase (Invitrogen); the resulting PCR products were ligated into the PCR2.1 vector using the TOPO-TA cloning kit (Invitrogen) and sequenced using the ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). The resulting sequences were assembled and compared with the KCBP peptide sequence predicted from the *Chlamydomonas* genome sequence version 3.0 (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>). Searches of the GenBank databases for sequence homologies were performed using the BLAST programs at NCBI (<http://www.ncbi.nlm.nih.gov/>).

RNA isolation and northern blots

For all experiments cells were grown in TAP medium, to mid to late log phase, and divided. For deflagellation experiments, 250 ml of cells were sedimented, resuspended in 100 ml of TAP medium, and deflagellated by pH shock. For flagellar resorption experiments 250 ml of wild-type cells were pelleted and resuspended in 100 ml low Ca²⁺-high sodium medium according to the methods of Lefebvre et al. (Lefebvre et al., 1978). Total RNA was prepared from non-deflagellated cells, cells 45 minutes after deflagellation, and cells 45 minutes after the induction of flagellum resorption (Wilkerson et al., 1994). Poly(A)⁺ RNA was prepared using the Oligotex mRNA Midi kit (Qiagen, Valencia, CA). Approximately 10 µg of poly(A)⁺ RNA was fractionated on formaldehyde agarose gels (Sambrook, 1982), then transferred to Hybond N+ membranes (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA), and hybridized with ³²P-labeled probes according to the manufacturer's instructions.

Flagellar basal body complex, axoneme and protein extract preparation

Flagellar basal body complexes were prepared according to the method of Wright et al. (Wright et al., 1985). 8×10⁸ cells were treated with autolysin to remove cell walls. The cells were subsequently lysed and the flagellar basal body complexes were purified on Percoll gradients and the final pellet resuspended in 50 µl of HMDEdNa (10 mM HEPES, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, 30 mM NaCl, pH 7.4) and loaded onto 8% acrylamide gels for SDS-PAGE. For axoneme preparations, flagella were severed from cell bodies using dibucaine (King et al., 1986; Witman, 1986), isolated by differential centrifugation, and resuspended in HMDEdNa. Axonemes were isolated using 0.5% NP-40 in HMDEdNa to remove flagellar membranes. For some experiments, axonemes were extracted using either

0.6 M NaCl, 1 mM ATP, or 5 mM ATP in HMDEdNa buffer for 20 minutes on ice. The extracts and extracted axonemes were isolated by differential centrifugation and the pellet of extracted axonemes was resuspended in an equal volume of HMDEdNa buffer. For immunoblot analysis, 25 μ l of each sample were loaded in each lane and anti-CrKCBP antibodies were used as a probe. For some experiments extracts were loaded onto 5–20% sucrose gradients and subjected to ultracentrifugation at 35,000 rpm for 16 hours in an SW41Ti rotor. Fractions (0.5 ml) were collected from the bottom of the tube and prepared for SDS-PAGE. For calmodulin immunoprecipitation experiments, see methods in Wargo et al. (Wargo et al., 2005).

Peptide and antibody production, antibody purification and immunoblots

Polyclonal antibodies were generated in rabbits against a peptide corresponding to the putative calmodulin-binding domain (CNKEMLRMKKQVEYWKEQAGL) of CrKCBP. The peptide was conjugated to keyhole limpet hemocyanin (KLH) and injected into rabbits for polyclonal antibody production. Peptide synthesis, peptide conjugation and antibody production were performed at Spring Valley Laboratories (Woodbine, MD, USA). The resulting antibodies were affinity purified using the peptide conjugated to a Sulfolink column according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

For immunoblots, equivalent loads of flagella (8 mg/ml), axonemes, extracts and/or extracted axonemes were subjected to SDS-PAGE using 7% polyacrylamide gels. Gels were transferred to PVDF (Immobilon P, Millipore, Billerica, MA, USA). Membranes were blocked for 1 hour in 5% milk in T-TBS (0.1% Tween, Tris-buffered saline, pH 7.5) and then incubated overnight at 4°C with primary antibody. The affinity purified KCBP antibodies were diluted 1:500 in block; the rabbit PF20 antibodies (Smith and Lefebvre, 1997) were diluted 1:5000; and the anti-calmodulin antibodies (Wargo et al., 2005) were diluted 1:7000. Following three 5-minute washes in T-TBS, the membrane was incubated for 1 hour with secondary antibody diluted in T-TBS. The anti-mouse-HRP secondary (Pierce) antibodies were diluted 1:10,000; the anti-rabbit-HRP secondary antibodies were diluted 1:20,000 (Amersham Pharmacia Biotech). The ECL Plus Western blotting detection system was used for chemiluminescent detection (Amersham Pharmacia Biotech).

Microtubule-binding experiments

Axonemes (isolated from *pf30pf28* with or without 1 mM CaCl₂ present) were extracted with 2 mM ATP for 10 minutes on ice. Extracts were treated for 10 minutes with apyrase (0.1 mU/ μ l) and dialyzed into HMDEdNa. Microtubules were polymerized and stabilized using a microtubule polymerization kit (Cytoskeleton, Inc. Denver, CO). Extracts were incubated with either buffer or microtubules for 20 minutes with gentle agitation. For some samples, the extract and microtubules included a final concentration of 1 mM CaCl₂ and 10 μ M *Chlamydomonas* calmodulin, which was expressed and purified as described by Wargo et al. (Wargo et al., 2005). After incubation, the microtubules were sedimented and resuspended in an equal volume of buffer with 2 mM ATP present. Samples were incubated for 10 minutes with ATP present, the microtubules sedimented, and then resuspended in an equal volume of buffer. For immunoblot analyses, 30 μ l of sample were loaded in each lane and anti-CrKCBP antibodies were used as a probe.

Immunolocalization using indirect immunofluorescence

For experiments using isolated axonemes, axonemes were prepared as described above and divided into two samples, one of which was extracted with 5 mM ATP. Following extraction, axonemes were pelleted by centrifugation and resuspended in an equivalent volume of HMDEdNa. Both extracted and unextracted samples were adhered to number 1.5 coverslips that had been washed with 0.2 N HCl and treated with 0.01% poly-L-lysine solution (Sigma, St Louis, MO) for 30 minutes. Axonemes were fixed with cold (–20°C) methanol for 20 minutes and rehydrated with three, 5-minute washes in phosphate-buffered saline (PBS). Coverslips were then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour. After blocking, coverslips were incubated with a 1% BSA in PBS solution containing mouse monoclonal anti- α -tubulin antibodies (clone B512, diluted 1:1000, Sigma) and affinity purified anti-CrKCBP antibodies (diluted 1:30) at 4°C overnight. Secondary antibody incubations are described below.

For whole cell experiments, *cw15* cells were placed in the dark for 48 hours to produce synchronous growth when reintroduced light. Aliquots of cells were taken from 8 hours to 9.5 hours after introduction to light in 15-minute increments and adhered to number 1.5 coverslips that had been washed with 0.2 N HCl and treated with 0.01% poly-L-lysine solution for 30 minutes. Cells were fixed with cold (–20°C) methanol for 15 minutes and rehydrated with three 5-minute washes in PBS. Coverslips were then blocked with 5% BSA in PBS for 1 hour. After blocking, cells were treated with a 1% BSA in PBS solution containing mouse monoclonal anti- α -tubulin (clone B512, diluted 1:1000), mouse monoclonal anti-centrin (clone 20h5, generously provided by J. Salisbury, Rochester, MN; diluted 1:400), and/or affinity purified anti-CrKCBP (diluted 1:30) antibodies, and incubated at 4°C overnight.

For all experiments using immunofluorescence, following incubation in primary antibody, coverslips were rinsed with three 5-minute washes in PBS and treated with

a 1% BSA in PBS solution containing various combinations of the following secondary antibodies: Alexa Fluor 350-labeled goat anti-mouse IgG₁ (diluted 1:200, for anti- α -tubulin), Texas Red-labeled anti-mouse IgG (diluted 1:500, for anti-tubulin or anti-centrin), Alexa Fluor 594-labeled goat anti-mouse IgG_{2a} (diluted 1:200, for anti-centrin), and Alexa Fluor 488-labeled goat anti-rabbit IgG₁ (diluted 1:285, for anti-CrKCBP). All Alexa secondary antibodies were purchased from Invitrogen-Molecular Probes; Texas Red secondary antibodies were purchased from Vector Labs (Burlingame, CA). After a 1-hour secondary incubation, coverslips were rinsed with three 5-minute washes in PBS and mounted on slides with ProLong Antifade (Invitrogen-Molecular Probes).

Fluorescent images were acquired on a Nikon Eclipse 80i microscope with a 60 \times oil-immersion objective lens utilizing Nikon UV-2E/C (DAPI), B-2E/C (FITC) and G-2E/C (TRITC) filters, connected to a Photometrics CoolSNAP ES camera (Tucson, AZ). Stacks of images, with a slice thickness of 0.2 μ m, were collected with the MetaVue Imaging System (Molecular Devices, Sunnyvale, CA). Image stacks were deconvolved, colored and merged in AutoDeblur 9.3 (AutoQuant Imaging, Troy, NY).

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