Molecular Dissection of the Microtubule Depolymerizing Activity of Mitotic Centromere-associated Kinesin*

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Mitotic centromere-associated kinesin (MCAK) is a microtubule depolymerizer that is consistent with its role in promoting chromosome segregation during mitosis. Here we show that the conserved motor domain of MCAK is necessary but not sufficient for microtubule depolymerization in cells or in vitro. The addition of only 30 amino acids N-terminal to the motor restores depolymerization activity. Furthermore, dimerization studies revealed that the smallest functional MCAK deletion constructs are monomers. These results define a highly conserved domain within MCAK and related (Kin I) kinesins that is critical for depolymerization activity and show that this depolymerization is not dependent on MCAK dimerization.

Kinesins are molecules that convert chemical energy into physical work to perform tasks such as vesicle transport, chromosome segregation, and organization of the mitotic spindle. All kinesin-related motors share a conserved motor domain that contains a nucleotide and a microtubule-binding site (1). The position of the motor domain within the primary sequence of the protein predicts the direction that the kinesin travels along microtubules (2, 3). However, directionality is conferred by conformational interactions between the motor and other domains within the protein that impose a directional bias in the choice of the next tubulin-binding site (2–5). Structural variations such as these permit the conserved motor domain of kinesin to perform diverse functions in the cell.

Mitotic centromere-associated kinesin (MCAK)§ (6) belongs to a third subfamily of kinesin motors, the Kin I subfamily (1 for internal). This family of homodimeric kinesins has the conserved motor domain in the interior of the protein rather than at either end. Unlike most kinesins, which walk along microtubules, the Kin I kinesins depolymerize them (7, 8). Purified XKCM1 (the Xenopus homologue of MCAK) depolymerizes stabilized microtubules in the presence of ATP (7) and promotes microtubule depolymerization in Xenopus egg extracts (8, 9). In vivo, overexpression of MCAK protein in CHO cells results in a loss of mitotic spindle microtubules during mitosis, and the replacement of functional MCAK at the centromere with a motorless version delays the migration of chromosomes to the spindle poles at anaphase (10). This e+ is consistent with the in vitro results reported above because chromosomes are normally attached to depolymerizing microtubules during anaphase (11). Together, these results firmly establish MCAK and its homologues as microtubule depolymerizers whose activity is relevant to cellular functions.

The structural differences between kinesins that allow one to travel along a microtubule surface and another to induce its depolymerization are not understood. One possibility is that some feature of the protein other than the motor domain is responsible for inducing depolymerization. Another possibility is that a small change within the conserved motor itself produces the transformation to depolymerization. Finally, microtubule depolymerization may be dependent on the quaternary structure of MCAK. This report addresses these issues by describing the minimal amount of the MCAK structure necessary to induce microtubule depolymerization. Here we show that the conserved kinesin-like motor domain of MCAK is required but is insufficient to cause depolymerization. An additional 30 amino acids N-terminal to the motor region are required to restore this activity. In addition, it is demonstrated via three independent methods that the smallest functional MCAK constructs exist as monomers. This indicates that the microtubule depolymerization activity of MCAK is not dependent on its quaternary structure. These results improve our understanding of what makes a kinesin motor behave as a microtubule depolymerizer by elucidating the structural requirements responsible for depolymerization.

EXPERIMENTAL PROCEDURES

Cell Transfection and Immunofluorescence—Cell culture was performed using CHO cells as described in Wordeman and Mitchison (6). The cells were transfected for 4 h using LipofectAMINE (Life Technologies, Inc.) and fixed at either 18 or 42 h post-transfection depending on the experiment. For immunoprecipitations, the cells were transfected on 100-mm plates and lysed at 42 h post-transfection. Where paclitaxel was used, it was added at a concentration of 15 μM 2 h into the transfection until fixation. No GFP-expressing cells were ever observed at the 2-h time point using visual fluorescent inspection. The cells were fixed in −20 °C methanol with 1% paraformaldehyde for 1 min. To view microtubules, the coverslips were labeled with the rat YL anti-tubulin antibody. Myc-containing cells were labeled with an anti-Myc antibody from CLONTECH. Ten fields of −20–100 transfected cells were scored by eye for assembled microtubules per construct. The transfection efficiencies and GFP expression levels were consistent from coverslip to coverslip, allowing rapid assignment of MT polymer loss based on the fluorescence level of assembled microtubules for hundreds of cells. In non-paclitaxel-treated cells, the extent of polymer loss corresponded to the following formula (one focal plane): + + + + + , no MTs to less than 10/cell; + + + + , 10–30 MTs/cell; + + + , 30–70 MTs/cell; + + , greater than 70 MTs but fewer than control cells; −, MT polymer level equivalent to control cells. In paclitaxel-treated cells individual MTs were not dis-
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cernible because of bundling, but fluorescence levels of assembled MTs were normalized relative to control cells as compared with untreated transfectants. The samples were observed using a Nikon FX-A photomicroscope and photographed using either a SenSys Digital Camera or Kodak Technical Pan film. Images were processed using Adobe Photoshop 5.5.

**Immunoprecipitation**—Two plates of CHO cells were transfected with either GFP-MCAK and Myc-MCAK or GFP-A182 and Myc-A182. The cells were lysed for 30 min on ice with 1 ml of chilled lysis buffer (150 mM NaCl, 100 mM KCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100) and protease inhibitors (peptatin, leupeptin, aprotinin, antipain, phenylmethyl sulfonyl fluoride). The cells were scraped, DNA was homogenized, and then spun to remove debris from the lysate. Myc-antibody-agarose beads (CLONTECH) were added to the lysate and incubated for 3 h at 4 °C. The beads were collected and washed three times with lysis buffer. The protein was eluted in 100 mM glycine, pH 2.5, at room temperature for 1 h and neutralized by the addition of 1/5 volume of 1 M Tris, pH 8.0. The samples were run on a 4–18% gel (Novex, San Diego, CA) and blotted onto polyvinylidene fluoride membrane (Bio-Rad) and stained with either an anti-GFP antibody (CLONTECH) or an anti-Myc antibody (CLONTECH). Primary antibodies were visualized by incubating the blots in alkaline phosphatase-coupled secondary antibodies followed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium staining.

**MCAK DNA Constructs**—All GFP constructs were created using the TOPO/TA GFP cloning kit (Invitrogen). When expressed, these constructs contain the MCAK sequence listed in the name of the construct, linker residues from the TOPO vector, and the GFP protein at the N or C terminus, depending on the TOPO vector used. All of the paper are from constructs using an N-terminal GFP with the exception of Glu201–Ser263 which has a C-terminal GFP. Myc-tagged constructs were made using the CLONTECH c-Myc eukaryotic expression vector and polymerase chain reaction-generated MCAK fragments. Bacterial expression was achieved by cloning MCAK fragments into the pTrcHis TOPO vector (Invitrogen). Two-hybrid constructs were made using the CLONTECH pGBK7 vector and polymerase chain reaction-generated MCAK fragments. Motorless MCAK was made as described in Maney et al. (10) and inserted into the two-hybrid constructs listed above. Polymerase chain reaction was performed using Pfu Turbo polymerase (Stratagene) and oligonucleotides from Genset (La Jolla, CA). Construct sequences were verified by DNA sequencing. The specific amino acids in each construct are listed in the text. Amino acid numbers correspond to the MCAK peptide and not MCAK fusion peptides.

**The Yeast Two-hybrid Assay**—The yeast two-hybrid assay was performed by cloning MCAK deletion constructs in the pGBK7 vector (DNA-binding domain fusion) and cloning motorless MCAK into the pADT7 vector (activation domain fusion). The constructs were then transformed into yeast strain AH109 (purchased from CLONTECH) pretransformed with the motorless-activation domain fusion construct. The transformed cells were plated on plates lacking leucine and trypsinoprotein for presence of both plasmids. After 3 days of growth at 30 °C, the colonies were streaked onto plates lacking adenine, histidine, leucine, and tryptophan to select for protein interactions. Plates selecting for interaction were allowed to grow for 15 days. The positive results reported in the paper were seen no later than 5 days following plating on the restrictive media. All media were prepared using the Yeast Protocols Handbook from CLONTECH (PT3024–1). False positive tests were conducted with the MCAK motorless construct in each vector. Motorless MCAK was incapable of inducing growth when transfected alone, in conjunction with an empty complementing vector or with a complementing vector fused to a random protein (SV40 large T-antigen in pADT7 or lamin C in pGBKT7).

**Hydrodynamic Analysis**—Full-length MCAK was expressed and purified as described by Maney et al. (10). Sucrose density sedimentation and size exclusion chromatography of Ala182 and Asp246 were performed as described by Maney et al. (10). Molecular weights were calculated from the apparent Stoke’s radius \( r_s \) and sedimentation co-efficient \( S_{20, w} \) using the following relationship: 
\[
M_c = S_{20, w} N v/6 \pi \eta p(1-\psi)
\]
where \( \eta \) = solvent viscosity, \( v \) = calculated protein density from the amino acid composition, and \( p \) = solvent density. Gels were scanned on a UMAX scanner and quantified using NIH Image.

**Microtubule Depolymerization Assays**—The depolymerization of paclitaxel-stabilized microtubules was performed essentially as described in Desai et al. (7) except that 70 mM KCl was omitted in some assays. Motor concentration was determined from the proportion of active ATP-binding sites in the preparation (12). Equivalent amounts of either baculovirus expressed full-length MCAK (10) or bacterially expressed Ala182 or Asp246 was added to paclitaxel-stabilized microtubules. The reactions were incubated in the presence or absence of 1.5 mM ATP for 10 min at room temperature and then centrifuged in the top speed of an airfuge for 10 min. Supernatants and pellets were assayed for the presence of tubulin on Coomassie-stained SDS-polyacrylamide gels (Novex) and quantified using NIH Image.

**RESULTS**

**In Vivo Depolymerization Activity of MCAK and MCAK Deletion Constructs**—CHO cells were transfected with GFP-MCAK and a series of GFP-MCAK deletion constructs to determine what domains of MCAK were important for in vivo microtubule depolymerization. Transfection of full-length GFP-MCAK (A and B) results in a decrease of microtubule polymer. Overexpression of deletion constructs containing the entire MCAK motor domain in addition to 65 amino acids of the N terminus also produce loss of polymer (C and D). Transfection GFP alone (E and F) has no visible effect on microtubules. G–L, paclitaxel-treated CHO cells transfected with GFP-MCAK and various GFP-MCAK deletion constructs. Paclitaxel-stabilized microtubules are partially depolymerized by GFP-MCAK (G and H) or A182-S583 (I and J). These constructs bind microtubules in paclitaxel-treated cells (G–J, insets). GFP-stathmin is unable to depolymerize paclitaxel microtubules (K and L). The arrows (I and L) show untransfected cells with large amounts of tubulin polymer. Bars, 10 microns.

**FIG. 1.** Depolymerization of microtubules by MCAK and MCAK fragments in vivo. A, C, E, G, I, and K show GFP expression, and B, D, F, H, J, and L show tubulin staining in the same cells. A–F, CHO cells transfected with GFP-MCAK and GFP-MCAK deletion constructs. Overexpression of full-length GFP-MCAK (A and B) results in a decrease of microtubule polymer. Overexpression of deletion constructs containing the entire MCAK motor domain in addition to 65 amino acids of the N terminus also produce loss of polymer (C and D). Transfection GFP alone (E and F) has no visible effect on microtubules. G–L, paclitaxel-treated CHO cells transfected with GFP-MCAK and various GFP-MCAK deletion constructs. Paclitaxel-stabilized microtubules are partially depolymerized by GFP-MCAK (G and H) or A182-S583 (I and J). These constructs bind microtubules in paclitaxel-treated cells (G–J, insets). GFP-stathmin is unable to depolymerize paclitaxel microtubules (K and L). The arrows (I and L) show untransfected cells with large amounts of tubulin polymer. Bars, 10 microns.

**RESULTS**

**In Vivo Depolymerization Activity of MCAK and MCAK Deletion Constructs**—CHO cells were transfected with GFP-MCAK and a series of GFP-MCAK deletion constructs to determine what domains of MCAK were important for in vivo microtubule depolymerization. Transfection of full-length GFP-MCAK results in significant microtubule loss after 18 h (Fig. 1, A and B). Deletion of the entire portion of MCAK C-terminal to the motor domain does not negatively affect this activity (not shown). Deleting amino acids 1–181 (construct Ala182–Ser583 hereafter called “A182”) results in similar microtubule polymer loss at the same time points as full-length MCAK (Fig. 1, C and D). Deleting the N terminus to Glu201 (construct Glu201–Ser583 hereafter called “E201”) also produces a construct capable of decreasing polymer levels in cells (Fig. 2), although this construct is not as effective as A182.

Deletion of the entire N terminus abolishes the microtubule depolymerizing ability of MCAK (Fig. 2). The construct Glu232–Ser263 (hereafter called “E232”), which represents only the conserved MCAK motor domain, was not able to depolymerize microtubules in CHO even though it can still bind them as judged by the microtubule binding/extraction assay (data not shown). Overexpression of GFP alone did not produce any visible microtubule depolymerization (Fig. 1, E and F).

To control for possible effects of GFP on the quaternary structure of the expressed protein, these experiments were repeated with Myc-tagged constructs of MCAK, A182 and E201, with identical results. The A182, E201, and E232 con-
structs were also tested with GFP fused to either end with no change in results (data not shown).

One concern with expressing GFP-MCAK constructs in living cells is that the observed microtubule loss may not be a result of direct MCAK-dependent depolymerization. The reduction in polymer may simply be a result of free tubulin sequestration by an association with the overexpressed MCAK. To address this issue, transfected CHO cells were incubated with 15 μM paclitaxel to stabilize microtubules. This concentration of paclitaxel renders the microtubules less sensitive to depolymerization triggered by a decrease in the concentration of free tubulin dimers (13). The cells were transfected with GFP-MCAK, GFP-MCAK deletion constructs, and the microtubule depolymerizing protein stathmin and expressed for 2 days in the presence of paclitaxel. GFP-MCAK is capable of depolymerizing paclitaxel-stabilized microtubules (Fig. 1, G–L). The amount of depolymerization is not as significant as without paclitaxel but is still dramatic compared with the microtubule density of untransfected cells (see untransfected cells in Fig. 1, J and L, arrows). In addition to depolymerizing microtubules, the GFP-MCAK construct became associated with the microtubules in the cells treated with paclitaxel (Fig. 1, G–J, insets). It is interesting that lattice binding of MCAK is promoted in the presence of paclitaxel (see "Discussion"). All of the deletion constructs that could depolymerize microtubules in the absence of paclitaxel could also do so in the presence of paclitaxel, but to varying degrees.

The A182 and the E201 mutants were both functional, but it is clear that some depolymerization activity is lost between them (Fig. 2). GFP-stathmin depolymerizes microtubules in the assay without paclitaxel (data not shown) but is unable to depolymerize microtubules stabilized with 15 μM paclitaxel when transfected at similar levels as the GFP-MCAK constructs (Fig. 1, K and L). This is consistent with previous reports indicating that stathmin cannot depolymerize taxol-stabilized microtubules. GFP alone had no effect on paclitaxel-stabilized microtubules (data not shown). Myc-tagged versions of these proteins were also able to depolymerize microtubules (data not shown).

The results of these in vivo depolymerization experiments are summarized in Fig. 2. From these experiments, it is clear that the conserved kinesin-related motor domain of MCAK is not sufficient for microtubule depolymerization in cells. An additional 31 amino acids are necessary to achieve significant depolymerization using this visual assay. An additional 19 more are necessary to reach levels equal to or better than full-length MCAK. These results also demonstrate that the N- and C-terminal regions of MCAK important for centromere binding are unnecessary for its depolymerizing activity.

Analysis of MCAK Deletion Construct Dimerization—Native MCAK appears to be a homodimer (10). Therefore, it is essential to test whether the depolymerization activity exhibited by some of the constructs is dependent on the quaternary structure of the native expressed protein. One possible explanation for the return of depolymerization activity in the A182 and E201 mutants is that multimerization is a prerequisite for depolymerization and these 30–50 amino acids added to the N terminus of the MCAK motor domain promote dimer formation. To determine whether functional motor constructs are multimers, the yeast two-hybrid assay, immunoprecipitation, and hydrodynamic assays were performed.

The constructs used in the yeast two-hybrid assay consisted of the same amino acids used in the depolymerization assay with the exception of the motor domain (Fig. 3A). The motor domain was left off of these constructs because the motor is toxic to the yeast cells. All of the N-terminal MCAK fragments to be tested were cloned into the "bait" vector and transformed into yeast containing motorless MCAK cloned into the "prey" vector. Motorless MCAK was also cloned into the "bait" vector as a positive control. Interaction between an MCAK N-terminal fragment and motorless MCAK should result in colony growth. As shown in Fig. 3, the yeast cells expressing motorless MCAK in each vector exhibited a large amount of growth, indicating strong dimerization between these constructs. This demonstrates that the motor domain is not important for multimer formation. The fragment containing the complete N terminus

![Fig. 2. Microtubule depolymerization by GFP-MCAK deletion constructs in the presence and absence of paclitaxel. Fields of transfected cells were scored immunofluoresecently for MT polymer loss (see "Experimental Procedures"). Complete microtubule loss was scored as ++++++, and MT polymer levels indistinguishable from control cells were scored as −. DP, depolymerizing activity; DP+T, depolymerizing activity in paclitaxel-treated cells.](image)

![Fig. 3. Dimerization and analysis of MCAK deletions. A, yeast two-hybrid analysis of MCAK fragments. The Ala<sup>182</sup>–Lys<sup>215</sup> and Glu<sup>203</sup>–Lys<sup>231</sup> MCAK fragments produced no growth of transformed yeast, suggesting that there is no interaction between these protein fragments and motorless MCAK. B, co-immunoprecipitation analysis of MCAK and A182-S583, CHO cell lysates containing GFP-MCAK and Myc-MCAK (MCAK column) or GFP-Ala<sup>182</sup>–Ser<sup>215</sup> and Myc-Ala<sup>182</sup>–Ser<sup>231</sup> (A182 column) were immunoprecipitated with anti-Myc antibody-coupled agarose beads. Post-immune lysate (L) and eluate from the pelletted beads (P) are shown probed with anti-Myc (Myc) or Anti-GFP (GFP) antibodies. All four proteins were successfully expressed (lanes 1, 2, 5, and 6) and Myc-MCAK and Myc-A182 were present in large amounts in the bead pellets (lanes 3 and 4). GFP-MCAK was also present in the Myc antibody-agarose bead pellet (lane 7), indicating an interaction between Myc-MCAK and GFP-MCAK. In contrast, GFP-Ala<sup>182</sup>–Ser<sup>215</sup> was not seen in the pellet with Myc-Ala<sup>182</sup>–Ser<sup>231</sup> (lane 8), suggesting that these proteins do not interact. The bands in all four lanes of the Myc panel represent proteins in the lysate that cross-react with the Myc antibody that were then concentrated in the pellet fraction.](image)
of MCAK (M1-K231) also promoted growth, although an extra 2 days of incubation was required. Deleting a significant portion of the N terminus (the S150 construct) severely reduced growth to where only a few colonies were present after 5 days of incubation. This amount of growth is possibly spurious but may represent a weak interaction. The next constructs tested (Ala182–Lys231 and Glu201–Lys231), both of which exhibit microtubule depolymerization activity when fused to the MCAK motor, did not exhibit any growth even after 15 days of incubation. These results suggest that the smallest functional deletion constructs used in the depolymerization experiments are not dimers. Finally, the C terminal of the motorless MCAK construct was tested. This construct produced a very low, but consistent, amount of growth after 5 days incubation, suggesting a role for the C terminus in MCAK dimerization in addition to the N terminus.

The multimerization question was further analyzed in vivo by performing co-immunoprecipitation assays with differentially tagged MCAK constructs. Because the focus of this paper is whether or not the minimal functional MCAK fragments can operate as monomers, we chose to analyze only the A182 mutant rather than perform co-immunoprecipitations with each construct. The A182 construct was chosen over the E201 construct because its paclitaxel-stabilized microtubule depolymerization activity rivaled that of wild-type MCAK. CHO cells were transfected with Myc and GFP-tagged versions of MCAK and GFP-tagged versions of the A182 deletion mutant. Because the focus of this paper was to determine whether or not the minimal functional MCAK fragments can operate as monomers, we chose to analyze only the A182 mutant rather than perform co-immunoprecipitations with each construct. The A182 construct was chosen over the E201 construct because its paclitaxel-stabilized microtubule depolymerization activity rivaled that of wild-type MCAK. CHO cells were transfected with Myc and GFP-tagged versions of MCAK and GFP-tagged versions of the A182 deletion mutant. After expressing these constructs, the cells were lysed, and the Myc-tagged proteins were immunoprecipitated (Fig. 3B, lanes 1–4). These pellets were then tested to see whether they contained any GFP-tagged MCAK, indicating dimerization (Fig. 3B, lanes 5–8). Full-length GFP-tagged MCAK was pulled down with the Myc-tagged MCAK, indicating dimerization between these proteins (Fig. 3B, lane 7). The GFP-A182 construct, however, did not co-immunoprecipitate with Myc-tagged A182, suggesting that these proteins are not dimerizing (Fig. 3B, lane 8). This result is consistent with the two-hybrid data described above.

Size exclusion chromatography and sucrose gradient centrifugation was performed on bacterially expressed A182, which exhibits strong microtubule depolymerization activity in vitro. The same analysis was performed on Asp246–Glu581 (hereafter called “D246”) as a nondepolymerizing control. This control construct consists of the minimal catalytic core motor domain for kinesin-related as defined by the crystal structure (14). The results of the size exclusion chromatography are shown in Fig. 4. The measured Stoke’s radius and sedimentation co-efficient for both A182 and D246 are consistent with both of these proteins existing as monomers in solution (Table I). The molecular weight for A182 can be calculated from the apparent Stoke’s radius (3.1 nm) and the sedimentation co-efficient ($S_{20, w} = 3.6$) as 43,800 (see “Experimental Procedures”). Interestingly, both the Stoke’s radius and the sedimentation co-efficient for D246 were slightly larger than those measured for A182. This suggests that D246 may have a slightly different shape or hydration state than A182. D246 is known from previous studies on similar sized constructs of kinesin heavy chain to be a monomer in solution (15). Our hydrodynamic measurements for D246 are also consistent with a monomeric quaternary structure. The predicted molecular weights for A182 and D246 including the His tag and leader sequences are 49,500 and 41,800, respectively (Table I).

In Vitro Depolymerization Activity of MCAK and MCAK Deletion Constructs—The bacterially expressed proteins used for these hydrodynamic analyses were tested in vitro for depolymerizing activity. Purified motor was added to paclitaxel-stabilized microtubules in the presence or absence of ATP (Fig. 5A). The ratio of motor heads to tubulin dimers was at least 1:16. This would be expected to more than saturate microtubule ends (average microtubule length equal to 8 microns) if the ends were preferentially bound by motor (7). The reaction progressed at room temperature for 10 min. After 10 min the microtubules were centrifuged, and the pellets and supernatants were assayed for the presence of tubulin. The reactions were not allowed to go to completion (complete depolymerization of all microtubule polymer), but they were capable of complete depolymerization if the reaction progressed for 30 min at room temperature (data not shown). It can be seen that tubulin was liberated into the supernatant in the presence of MCAK plus ATP and in the presence of the monomeric A182 construct plus ATP (Fig. 5A, seventh lane). However, little or no tubulin was liberated into the supernatant by D246 in the presence of ATP (Fig. 5A, eleventh lane). These results demonstrate that a monomeric form of MCAK motor is capable of depolymerizing microtubules both in vitro and in vivo.

To directly compare the depolymerization efficiency of A182 to full-length MCAK it was necessary to measure the proportion of motor heads that can bind ATP (active sites) in our preparations and then compare the number of active motor

![Table I](image-url)

| Truncation Proteins | MCAK$^a$ | A182 | D246 |
|---------------------|---------|------|------|
| Stokes radius (nm)  | 6.95    | 5.3  | 3.3  |
| Sedimentation Co-efficient (S) | 5.3 | 3.6  | 3.9  |
| Predicted molecular weight | 80,580 | 49,500 | 41,800 |
| Calculated native molecular weight | 153,000 | 43,600 | 49,700 |

$^a$ Values from Maney et al. (10) for baculovirus expressed and purified MCAK.

$^a$ Molecular weights predicted from amino acid sequence.
heads required to release an equivalent concentration of tubulin dimer. The results of this experiment are shown in Fig. 5B. In BRB80, a standard buffer for assaying kinesin motility, MCAK and A182 show equivalent ATP-dependent microtubule depolymerizing activity over the course of 10 min at room temperature. In BRB80 plus an additional 70 mM KCl (7), the activities of both motors suffer, but A182 appears more sensitive to added salt than MCAK.

We have prepared four DNA constructs consisting of the “neck” region of MCAK fused to the motor domain of KIF5B and successively increasing portions of the MCAK motor domain. Each of these constructs displayed no microtubule depolymerizing activity when expressed in cultured cells (data not shown). This suggests that regions within the core MCAK motor domain are required for full depolymerizing activity in addition to the neck region of MCAK.

**DISCUSSION**

In this report, we have shown that the conserved catalytic motor domain of MCAK is necessary but not sufficient for microtubule depolymerization in cells and in vitro. Depolymerization activity can be partially restored with the addition of 30 amino acids of the N-terminal neck domain and fully restored with the addition of 20 more. These results define a minimal domain required for microtubule depolymerization and dissociate centromere localization and depolymerization activities of MCAK. We have constructed four MCAK-KIF5B chimeric motors that do not depolymerize microtubules in cells, suggesting that properties intrinsic to the MCAK motor must also be important for depolymerization. We also demonstrate, using three different procedures, that these functional deletion constructs are monomers and that the monomer can be as effective at depolymerizing microtubules as the dimer.

One obvious question posed by these results is the nature of the domain that restores depolymerization activity to the motor. This segment can be divided into two regions: the Gly201–Lys231 region (segment II) that restores partial activity and the Ala182–Glu260 region (segment I) that restores full activity when added with segment II (Fig. 6). This area of MCAK has been called the neck region of the protein as it connects the motor to the rest of the molecule (1). Both of these regions are predicted to be helical in nature but are not predicted to be coiled-coils, either alone or in series (16). These predictions are in line with our current data because no dimerization is seen with these domains. Both segments are highly conserved between other Kin I kinesins but not as conserved with the dimerization-conferring coiled-coil neck of the minus end-directed kinesin NCD (Ref. 17 and Fig. 6). If these segments are not involved in dimerization, then how do they participate in motor activity? One possibility is that the neck interacts with microtubules. The positively charged neck region may interact with negatively charged microtubule polymer to either tether MCAK to the microtubule or wedge apart protofilaments or promote tubulin dimer curving. Another possibility is that the neck directly interacts with the motor domain and affects its conformation. Examples of neck/motor interactions exist for both conventional kinesin and NCD and were shown to be responsible for motor directionality and speed of translocation along the microtubule (3, 5, 18, 19). Regions of the NCD neck that interact with the motor map spatially to the area of MCAK represented by segment II (5). Interestingly, few of the NCD amino acid pairs responsible for the neck/motor interaction are conserved in MCAK. Perhaps these differences in NCD and MCAK neck/motor interactions assist in the transformation of MCAK to a depolymerizer.

Our data demonstrate that MCAK in monomer form can depolymerize microtubules. Under standard motility assay conditions the monomeric form of MCAK is as effective as the dimeric form. This indicates that depolymerization does not occur via coupled coordination between the heads of a dimeric motor. Kin I kinesins accumulate at both ends of microtubules under certain conditions (7). In theory there are 13 potential MCAK-binding sites at each end of the microtubule. It is possible that independent heads may cooperate at the end of a microtubule to depolymerize a paclitaxel-stabilized microtubule. We hypothesize that if this is the case, it would occur not necessarily though a physical coupling between the heads that...
introduces strain in the protofilament structure but through the combined action of several heads, each contributing a sub-critical perturbation at the microtubule end.

The monomeric minimal depolymerizing motor (A182) is as effective or even more effective at depolymerizing microtubules in standard motility buffer and also in cells (Fig. 2, DP-T). However, the addition of 70 mM KCl to the depolymerization assay causes the monomer to lose depolymerizing activity faster than the dimer. There are two possible explanations for this observation. Either the second head may bind an adjacent tubulin dimer, thus increasing the overall affinity of the MCAK molecule for the microtubule in higher salt, or alternatively regions within the N- or C-terminal tail of MCAK are useful in promoting depolymerization or increasing microtubule affinity under these conditions irrespective of the quaternary structure, perhaps by adding more microtubule-binding sites. Our KIF5B-MCAK chimera studies suggest that, in addition to the neck, differences between the kinesin and MCAK motor domains may contribute to the different functions of the proteins. Interestingly, recent studies have revealed microtubule-depolymerizing activity in the HIV-1 REV protein. A search for regions of homology with other microtubule depolymerizers revealed significant homology with the microtubule-binding domain at the C terminus of the motor domain of XKCM1 (20).

We are devising further experiments to test these possibilities.

Elegant studies by Desai et al. (7) have shown that depolymerization by the Kin I family of kinesins induces protofilament curling similar to that seen during microtubule depolymerization but without the coincident hydrolysis of GTP. They also demonstrated that Kin I kinesins will bind specifically to microtubule ends and trigger initial protofilament peeling in the presence of AMP-PNP. These data suggest that a high affinity binding step occurs when the motor is in the ATP-bound state and that this binding site can be accessed at either microtubule end but not in the lattice. Because the Kin I kinesins can depolymerize microtubules from both ends (7), this suggests that the high affinity binding site may be between protofilaments rather than at the end of the microtubule. Such a site would tend to be exposed preferentially at the ends where the lateral interactions between the protofilaments are easiest to break. We have produced an MCAK mutant that cannot hydrolyze ATP and binds uniformly along the lattice of the microtubules (21). This mutant has no effect on microtubules in cells. The MCAK motor domain may cycle between high affinity binding, which breaks the lateral bonds between protofilaments at the end of the microtubule, and benign association with the lattice depending on the ATP hydrolysis state of the motor head. Specifically, our data demonstrate that microtubule depolymerizing activity coupled to ATP hydrolysis is intrinsic to a monomeric motor domain.

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