Protein Arms in the Kinetochore-Microtubule Interface of the Yeast DASH Complex

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Protein Arms in the Kinetochore-Microtubule Interface of the Yeast DASH Complex

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The yeast DASH complex is a heterodecameric component of the kinetochore necessary for accurate chromosome segregation. DASH forms closed rings around microtubules with a large gap between the DASH ring and the microtubule cylinder. We characterized the microtubule-binding properties of limited proteolysis products and subcomplexes of DASH, thus identifying candidate polypeptide extensions involved in establishing the DASH-microtubule interface. The acidic C-terminal extensions of tubulin subunits are not essential for DASH binding. We also measured the molecular mass of DASH rings on microtubules with scanning transmission electron microscopy and found that approximately 25 DASH heterodecamers assemble to form each ring. Dynamic association and relocation of multiple flexible appendages of DASH may allow the kinetochore to translate along the microtubule surface.

INTRODUCTION

Division of one parental cell into two genetically identical progeny requires accurate partitioning of newly replicated chromosomes. Of the many protein assemblies that monitor, regulate, and drive this process, only the kinetochore directly contacts centromeric DNA. In yeast kinetochores, over 60 proteins assemble into distinct subcomplexes on a ∼125-base pair centromere to bind a single microtubule (MT) of the mitotic spindle (McAinsh et al., 2003). Structural studies of these subcomplexes coupled with functional experiments have begun to add molecular detail to our picture of kinetochores. For example, the yeast Ndc80 complex forms a 570-Å coiled coil rod with two globular domains at each end (Wei et al., 2005), suggesting a role as a molecular adaptor. The globular domain of the human Ndc80 subunit, Hec1, folds into a MT-binding calponin homology domain (Wei et al., 2007), and the worm Ndc80 homolog directly binds MTs and facilitates association of additional kinetochore complexes (Cheeseman et al., 2006). The yeast DASH complex (also called the Dam1 complex) forms closed rings around MTs (Miranda et al., 2005). DASH can be induced to move along a MT and to translate with the depolymerizing end (Westermann et al., 2006). The interaction resists several picoNewtons of force (Asbury et al., 2006). DASH may therefore serve to help tether kinetochores to MTs during chromosome segregation.

The DASH complex is necessary for faithful segregation of chromosomes in mitosis. The Saccharomyces cerevisiae complex consists of 10 essential subunits: Dam1p, Duo1p, Dad1p, Dad2p, Spc19, Spc34p, Ask1p, Dad3p, Dad4p, and Hsk3p (Figure 1A) (Cheeseman et al., 2001, 2002; Janke et al., 2002, 2005). Loss of functional DASH results in sister chromatids attached to the same spindle pole body, an arrangement that leads to unequal segregation. The homologous complex in Schizosaccharomyces pombe contains similar subunits and localizes to kinetochores as well as to MT plus ends (Liu et al., 2005; Sanchez-Perez et al., 2005). Although not essential in fission yeast, loss of functional DASH also results in segregation defects. The ring structure observed in vitro may contribute to proper segregation by acting as a processivity factor for kinetochores, allowing chromosomes to remain attached to depolymerizing MT plus ends during anaphase. Rings are commonly found in assemblies that must remain attached to a linear substrate, for example, in the assemblies that carry out DNA replication (Hingorani and O’Donnell, 2000). The mechanism by which the DASH ring binds to and translates along MTs is therefore not only an integral part of how kinetochores work, but also an instance of a molecular solution to a ubiquitous structural challenge.

Negative stain electron microscopy (EM) of MTs decorated with DASH reveals a gap between the inner diameter of DASH and the outer diameter of the MT (Miranda et al., 2005; Westermann et al., 2005, 2006). No discrete mass of DASH can be seen abutting the MT, but polypeptide extensions not visibly contrasted by negative stain may position DASH rings around microtubules. On the MT side, the acidic C-termini of tubulin may project from the surface, but the 10–20 amino acids of these unstructured extensions (Lowe et al., 2001) are unlikely to bridge the 50–100-Å gap of the DASH-MT interface. Because rotary shadowed EM preparations show that DASH is relatively compact, but hydrodynamic measurements yield a Stokes’ radius much larger...
than expected for a globular particle (Miranda et al., 2005), we believe that extended projections are probably present. No obvious candidates for those bridging elements can be deduced from examination of amino acid sequences of DASH components. We have therefore carried out a series of experiments to determine the location and MT-binding properties of flexible, protease-sensitive polypeptides on DASH. This mapping adds detail to models of how DASH binds to and translates along MTs.

MATERIALS AND METHODS

Cloning

Constructs of S. cerevisiae DASH or subcomplexes were cloned into a poly-cistronic coexpression system (Miranda et al., 2005). The plasmid containing Asklp with a C-terminal Strep-tag was generated in an analogous manner. Constructs without HSK3 were obtained by omitting the subcloning step incorporating HSK3 from pET21aTr into the expression vector. This procedure was repeated to generate similar plasmids encoding Asklp, Dadlp, Dad2p, Dad3p, Dad4p, Scp3p, and Scp3p with C-terminal hexahistidine tags. A construct without DAD1 was obtained by omitting the subcloning step incorporating DAD1 from pET2aTr into the expression vector. This procedure generated a plasmid encoding Scp3p with a C-terminal hexahistidine tag except that the ion exchange step was repeated to generate similar plasmids encoding Dadlp and Dad3p with C-terminal hexahistidine tags.

Protein Expression and Purification

DASH complexes were purified with combinations of affinity, ion exchange, and size-exclusion chromatography (Miranda et al., 2005). DASH subcomplexes were purified in the same manner as heterodimeric DASH containing a protein with a C-terminal hexahistidine tag except that the ion exchange column and peptide treatments were omitted. DASH containing Asklp with a C-terminal Strep-tag was purified in a similar manner with modifications. Cell extracts made in 50 mM phosphate, 500 mM NaCl, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete Protease Inhibitors (Roche, Indianapolis, IN), pH 7.5. The supernatant was bound to a Strept-Tactin Sepharose (IBA, St. Louis, MO) column and washed in the same buffer without protease inhibitors but including 1 mM ATP and 0.1 mg/ml of the synthetic peptide NRRLLT. The complex was eluted in the wash buffer supplemented with 2.5 mM desthiobiotin. The eluant was concentrated, fresh ATP added to 1 mM, and additional NRRLLTT peptide added to 250-fold molar excess relative to the DASH. The complex was then purified using a 10/300 column (Amersham, Piscataway, NJ) equilibrated in 25 mM HEPES, 500 mM NaCl, 1 mM mercaptoethanol, and 1 mM EDTA, pH 7.4.

Microtubule Decoration

Bovine tubulin was polymerized in the presence of pactinactin (Miranda et al., 2005). MT cosedimentation assays were performed by mixing 10 µl of 1 mg/ml DASH or subcomplex in 25 mM HEPES, 500 mM NaCl, 1 mM mercaptoethanol, 1 mM EDTA, pH 7.4, with 90 µl of 0.055 mg/ml MTs in 25 mM HEPES, 100 mM NaCl, 1 mM GTP, 10 mM pactinactin, and 1% dimethyl sulfoxide (DMSO), pH 7.4. Control samples were treated with buffer instead of DASH or subcomplex. All samples were incubated for 20 min at room temperature and then spun at 16,000 × g for 10 min at room temperature.

Limited Proteolysis

DASH at 1 mg/ml and elastase (Sigma, St. Louis, MO) at 0.1 mg/ml, both in 25 mM HEPES, 500 mM NaCl, 1 mM mercaptoethanol, and 1 mM EDTA, pH 7.4, were mixed at a ratio of 9:1. Reactions were incubated at 4°C and quenched by the addition of SDS-PAGE buffer and boiling. The initial time-point sample was treated with buffer instead of elastase. For further purification, 100 µl of DASH protected for 30 min was fractionated on a Superose 6 10/300 column (Amersham) equilibrated in 25 mM HEPES, 500 mM NaCl, 1 mM mercaptoethanol, and 1 mM EDTA, pH 7.4. For experiments testing the ability of MTs to protect DASH against elastase digestion, 0.05 mg/ml MTs decorinated with 0.1 mM DASH was mixed with 0.01 mg/ml elastase in 25 mM HEPES, 150 mM NaCl, 1 mM GTP, 10 µM pactinactin, 1% DMSO, pH 7.4, at room temperature. The initial time-point sample was treated with buffer instead of elastase. Control samples were treated with buffer instead of MTs. For sublithium treatment of MTs, 9 vol of polymerized MTs at 5 mg/ml were mixed with 1 vol of 2 mg/ml sublithium (Sigma) in 80 mM PIPES, 1 mM MgCl₂, and 1 mM EGTA, pH 6.9. The reaction was incubated at 30°C for 60 min (Skinholt et al., 2004), quenched by the addition of 10 mM PMSF from a 100 mM stock in ethanol, spun at 16,000 × g for 10 min, and resuspended in 25 mM HEPES, 100 mM NaCl, 1 mM GTP, 10 µM paclitaxel, 1% DMSO, and 1 mM PMSF, pH 7.4. Control samples were treated with buffer instead of sublithium. All subsequently used buffers contained 1 mM PMSF. Western blots verifying cleavage of the C-terminus of β-tubulin were probed with the mAb JDR388 (Sigma), which is specific for that epitope (Banerjee et al., 1988). Similarly, cleavage of the C-terminus of α-tubulin was verified by probing with the mAb YL1/2 (AbD Serotec, Raleigh, NC), which is specific for the C-terminal tyrosine residue (Wehland et al., 1984).

Phosphorylation In Vitro

One vol of DASH at 1 mg/ml in 25 mM HEPES, 500 mM NaCl, 1 mM mercaptoethanol, 1 mM EDTA, pH 7.4, and 1 vol of human Cdk2-cyclin B (New England Biolabs, Beverly, MA) in 50 mM HEPES, 100 mM NaCl, 1 mM diithiothreitol, 100 µM EDTA, 50% glycerol, and 0.01% Brij 35, pH 7.5, 1 vol of 500 mM Tris, 100 mM MgCl₂, 20 mM diithiothreitol, 10 mM EDTA, 50% glycerol, and 0.1% Brij 35, pH 7.5, and 1 vol of 2 mM ATP were diluted into water to a total of 10 vol. Alternatively, 2 vol of human Cdk2-cyclin A (New England Biolabs) in HEPES and 2 mM ATP were incubated at 37°C overnight. Control samples were treated with water instead of ATP. For gel electrophoresis, samples were quenched by the addition of SDS-PAGE buffer and boiling. EM and mass spectrometry (MS) experiments were performed with reactions including human Cdk2-cyclin A and DASH containing Scp3p with a C-terminal His-tag.

Mass Spectrometry

Peptide maps of proteins in gel bands were obtained by in-gel trypsinolysis and peptide extraction using conventional methods. Mass mapping by matrix-assisted laser desorption ionization time-of-flight MS (Bucker et al., 2005). Intact proteins were desalted by polylysine-divinylbenzene microbeads reversed-phase liquid chromatography, and intact masses were measured by matrix-assisted laser desorption ionization time-of-flight MS (Bucker et al., 2005). Purification was assessed by matrix-assisted laser desorption ionization-fourier transform ion cyclotron resonance mass spectrometer (Bucker).

Electron Microscopy

Negatively stained specimens of decorated MTs were prepared with uranyl formate (Miranda et al., 2005). Samples of MTs decorated by DASH were fixed with 2% glutaraldehyde for 1 h in 0.1 mg/ml and 2.2 mg/ml DASH and H9251 or H9262. All subsequently used buffers contained 1 mM PMSF. Western blot analysis of samples containing Cdk2-cyclin A and DASH containing Scp3p with a C-terminal His-tag.

RESULTS

Limited Proteolysis of DASH

We used limited proteolysis to probe for protein “arms” that could bridge the gap between DASH rings and the MT. Mild digestion of the intact heterodecamer with elastase, which cleaves C-terminal to aliphatic side chains, yields only eight strong bands (Figure 1A). The proteolysis product resulting from 30 min of treatment represents the most easily isolated kinetic end point, which we therefore used for subsequent experiments. Asklp, Damlp, and Duo1p are cleaved. MS identified the new species appearing between Duo1p and Scp1p as the N-terminal portion of Duo1p. We detected 74% of the Duo1p sequence when mapping peptides in a molecular mass of 13.1 kDa for a tobacco mosaic virus, which is included as a control on all grids.

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tryptic digestion of the band; the identified peptides spanned residues 2–212 of Duo1p, which has 247 residues total. Edman degradation yielded no sequences, a result explained by observation of an acetylated N-terminal peptide. A ladder of unsequenced bands that appears between Duo1p and Dad1p/Dad3p suggests that Dam1p and Ask1p have been cleaved into a number of different polypeptides. The low abundance and heterogeneity of the putative Dam1p and Ask1p proteolysis products has precluded identification. When further purified with size-exclusion chromatography (Supplementary Figure 1A), all eight components and the unsequenced cleavage products remained associated (Supplementary Figure 1B). We could detect no other globular complex of coeluting, protease-resistant protein. We refer to the purified product as elastase-treated DASH (eDASH).

Recombinant DASH binds MTs as judged by an in vitro MT cosedimentation assay (Figure 1B; Miranda et al., 2005; Westermann et al., 2005), but eDASH does not (Figure 1C). Nonetheless, MTs do not protect DASH from elastase digestion (Figure 1D). The limited proteolysis experiments suggest Ask1p, Dam1p, and Duo1p as candidates for providing MT bridges. Because DASH lacks 3mer contains Ask1p and does not cosediment with MTs, we conclude that this subunit is not sufficient for establishing a functional DASH-MT interaction. DASHΔHsk3p 6mer contains both

**Figure 1.** Biochemical properties of eDASH. (A) Time-dependent limited proteolysis treatment of DASH with elastase. (B) Cosedimentation assay of MTs with DASH. (C) Cosedimentation assay of MTs with eDASH. S and P, supernatant and pellet fractions, respectively. (D) Time-dependent limited proteolysis treatment of DASH with elastase in the absence and presence of MTs.

Microtubule Binding of DASH Subcomplexes

Characterization of the MT-binding properties of DASH subcomplexes suggests which proteins are likely to contribute extended arms to the MT-DASH interface. We have purified a variety of distinct subcomplexes by deleting different subunits from our coexpression vector (Miranda, 2006). DASHΔHsk3p 3mer, which contains Ask1p, Dad2p, and Dad4p (Figure 2A), does not cosediment with MTs (Figure 3A), but DASHΔHsk3p 6mer, which contains Dam1p, Duo1p, Spc34p, Spc19p, Dad1p, and Dad3p (Figure 3B), does (Figure 3B). The limited proteolysis experiments suggest Ask1p, Dam1p, and Duo1p as candidates for providing MT bridges. Because DASHΔHsk3p 3mer contains Ask1p and does not cosediment with MTs, we conclude that this subunit is not sufficient for establishing a functional DASH-MT interaction. DASHΔHsk3p 6mer contains both
Dam1p and Duo1p, suggesting that the extensions cleaved by elastase on these two proteins are sufficient for association with MTs. Comparing EM images of undecorated (Figure 4A) and decorated MTs (Figure 4B) reveals an unorganized clustering of DASHΔHsk3p 6mer on the surface of the MT rather than organized rings. Extended elements of Dam1p, the C-terminus of Duo1p, or both are sufficient to form some interaction with MTs, but ring assembly and MT binding remain separable functions. Lack of MT binding by other subcomplexes corroborates the importance of Dam1p and Duo1p in MT binding. DASHΔDam1p contains Ask1p, Spc34p, Spc19, Dad2p,
Dad4p, and Hsk3p (Figure 2B). MS analysis of the species between Spc34p and Spc19p identified 41% of Spc34p; mapped tryptic peptides spanned residues 99–274 out of 295. Edman degradation yielded the sequence MKRNRR, indicating that the observed band corresponds to an N-terminal truncation beginning at residue 93. The limited proteolysis experiments and MT cosedimentation assays of DASH/H9004/Hsk3p subcomplexes predict that DASH/H9004/Dam1p 6mer will not bind MTs, and we have confirmed this prediction (Figure 3C). During coexpression experiments, we also observed frequent overexpression of a Dad1p/Dad3p heterodimer relative to DASH (data not shown). The Dad1p/Dad3p heterodimer can be expressed and purified independently (Figure 2C). This subcomplex does not interact with MTs (Figure 3D), again consistent with previous conclusions. The absence of Dam1p, Duo1p, Dad1p, and Dad3p from DASH/Dam1p 6mer is noteworthy. Because Dad1p and Dad3p form a separable structural unit, Dam1p and Duo1p may form another distinct structural unit, a suggestion supported by their joint role in providing flexible extensions from MT interaction. The sum of the cosedimentation experiments is consistent with the interpretation that Dam1p and Duo1p cooperate to form the principal connection between DASH and MTs.

Limited Proteolysis of Microtubules

We have previously reported preliminary EM experiments suggesting that removal of the acidic C-termini of both α and β tubulin by subtilisin does not abolish DASH binding (Miranda et al., 2005). The opposite conclusion has been drawn by others on the basis of EM and fluorescence binding assays (Westermann et al., 2005). Our treatment of MTs with subtilisin alters electrophoretic mobility of both tubulin subunits on a gel (Figure 5A). Although this mobility shift is often a sufficient indicator of removal of both the α and β tubulin termini, we have also verified cleavage with epitope-specific monoclonal antibodies. The JDR.3B8 antibody detects the C-terminus of β tubulin on mock-treated MTs, but not on subtilisin-treated MTs (Figure 5B). Similarly, the YL1/2 antibody detects the intact α subunit C-terminus, but almost all of the epitope is removed by subtilisin treatment (Figure 5C). DASH binds to subtilisin-digested MTs with similar affinity as it does to mock-digested MTs (Figure 5D). Moreover, we observe DASH rings with the same frequency on mock-treated (Figure 5E) and subtilisin-treated MTs (Figure 5F). The acidic C-termini of α and β tubulin are therefore not essential for proper formation of the DASH-MT interface, and we suggest that the flexible extensions of DASH dock against the cylindrical wall of the microtubule.

Phosphorylation of DASH

Ask1p is modified by a yeast cyclin-dependent kinase, Cdc28, in a cell cycle-dependent manner (Li and Elledge, 2005). Our treatment of DASH with Cdc28 also results in the observed band. The JDR.3B8 antibody detects the C-terminus of β tubulin on mock-treated MTs, but not on subtilisin-treated MTs (Figure 5B). Similarly, the YL1/2 antibody detects the intact α subunit C-terminus, but almost all of the epitope is removed by subtilisin treatment (Figure 5C). DASH binds to subtilisin-digested MTs with similar affinity as it does to mock-digested MTs (Figure 5D). Moreover, we observe DASH rings with the same frequency on mock-treated (Figure 5E) and subtilisin-treated MTs (Figure 5F). The acidic C-termini of α and β tubulin are therefore not essential for proper formation of the DASH-MT interface, and we suggest that the flexible extensions of DASH dock against the cylindrical wall of the microtubule.

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Figure 4. Negative stain EM micrographs of MT decoration by DASH/Hsk3p 6mer. Gallery of images showing a (A) MT and (B) MT with DASH/Hsk3p 6mer. Scale bars, 250 Å.

Figure 5. DASH binding to subtilisin-treated MTs. (A) Coomassie-stained gel of MT preparations, probed with JDR.3B8, an antibody specific for the C-terminus of β tubulin. (C) Western blot of MT preparations probed with YL1/2, an antibody specific for the C-terminus of α tubulin. (D) Cosedimentation assay of subtilisin-treated MTs with DASH. S and P, supernatant and pellet fractions, respectively. (E) Negative stain EM micrograph of a mock-treated MT decorated by DASH. (F) Negative stain EM micrograph of a subtilisin-treated MT decorated by DASH. Scale bars, 250 Å.
allow mass measurement of each particle. Unstained, lyoph-
single rings spaced sufficiently apart from each other to
data (Figure 6B) and double rings (Figure 6C) yielded molecular
masses of 8 MDa (Table 1), close to the 7.8 MDa expected for
one and only one site is modified; no masses corresponding to
unphosphorylated or diphosphorylated species are observed.
Peptide mass mapping of modified Ask1p found the phos-
phorylated state with no detection of a mass corresponding to
the phosphorylated form. Phosphorylated DASH cosediments
with MTs as efficiently as unphosphorylated DASH (data not
shown). EM experiments also show that DASH phosphory-
lated by Cdk still forms rings around MTs with a similar
mass of C/1011
frequency as does unphosphorylated DASH (data not shown).
Cdkp therefore modify DASH in vitro only on S250 of Ask1p,
and this phosphorylation event does not affect ring assembly.
We conclude that the anaphase defects seen when S250 is
mutated must involve regulation of some other aspect of
DASH activity.

**Scanning Transmission Electron Microscopy**

We have determined the molecular masses of MTs deco-
rated with DASH rings by direct mass measurement with
STEM. Substoichiometric amounts of DASH relative to tu-
bulin were bound to MTs in order to favor the formation of
single rings spaced sufficiently apart from each other to
allow mass measurement of each particle. Unstained, lyoph-
ilized preparations were examined in the STEM. Images of
particles from dark field micrographs were boxed into 400 ×
800-Å arrays for analysis; high background as a result of
binding conditions and specimen damage from mechanical
manipulations resulted in only a small number of particles
suitable for analysis. MTs appear as cylinders without the
characteristic tubulin heterodimer repeat pattern probably
because of radiation damage (Figure 6A). Our measure-
ments of a 400-Å-long MT segment yielded a molecular
mass of ~8 MDa (Table 1), close to the 7.8 MDa expected for
a 14 protofilament MT. We performed similar measurements
for MT segments decorated with DASH. MTs with single
(Figure 6B) and double rings (Figure 6C) yielded molecular
masses of ~13 and 18 MDa, respectively. The SD of our
measurements ranged from 1 to 2 MDa, ~4–13% depending
on the data set. The precision and accuracy of our measure-
ments are similar to those obtained with kinesin-MT com-
plexes (Hoenger et al., 2000). We calculate the molecular
mass of a ring as ~5 MDa, with propagated errors ranging
from 0.5 to 2.5 MDa, ~10–60%, depending on the data set
(Table 1). The molecular mass of one DASH heterodecamer
is 0.2 MDa; each DASH ring therefore contains 25 ± 5
heterodecamers.

**DISCUSSION**

Chromosomes move along MTs during various stages of
mitosis. In anaphase, progressive MT depolymerization at
the plus end drives this translation so that net movement is
toward the spindle pole body. We have proposed that the
DASH rings are processivity factors that allow kineto-
chores to translate along a MT without dissociating
(Miranda et al., 2005), and the rings can indeed translate
quite freely (Asbury et al., 2006). The processivity factors
that keep a DNA replication complex from dissociating
slide loosely along the double helix (Hingorani and O’Donnell, 2000). A similar electrostatic sliding has been
suggested for DASH (Westermann et al., 2006), but the
observations reported here lead us to seek an alternative
mechanism. In particular, two results favor direct con-
acts. First, we find that the negatively charged tubulin
C-termini on the MT surface do not appear to be essential
for ring formation. Second, the DASHΔHsk3p 6mer sub-
complex, which contains Dam1p and Duo1p, binds without
forming rings. Were encirclement the only property
holding DASH components onto a MT, we would expect
ring formation to be essential for binding. We also note
that sliding clamps in DNA replication need an energy-
dependent clamp loader, whereas DASH rings assemble
spontaneously around MTs.

Systematic analysis of the MT-binding properties of lim-
ited proteolysis products and various subcomplexes defines
the nature of the DASH-MT interface. Polypeptide arms
from both DASH and MTs could contribute to a molecular
bridge across the gap observed between a DASH ring and
the MT in EM images, but we determined that DASH alone
makes functionally significant contacts across this space.
Specifically, Dam1p, Duo1p, or both are probably responsi-
ble for MT binding (Figure 7A). Cleavage of these proteins

![Figure 6](image-url)

**Table 1. Molecular mass of DASH rings determined by STEM**

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<th>Particle</th>
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<td>MT</td>
<td>54 (32)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.4 ± 1.0 (7.9 ± 0.7)</td>
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<tr>
<td>MT and one DASH ring</td>
<td>24 (12)</td>
<td>13.6 ± 1.8 (12.4 ± 0.6)</td>
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<tr>
<td>MT and two DASH rings</td>
<td>23 (6)</td>
<td>18.2 ± 1.9 (18.0 ± 0.7)</td>
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<tr>
<td>(MT and one DASH ring)</td>
<td>n/a (n/a)</td>
<td>5.2 ± 2.0 (4.5 ± 0.9)</td>
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<tr>
<td>(MT)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n/a (n/a)</td>
<td>4.5 ± 2.5 (4.6 ± 0.9)</td>
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<tr>
<td>(MT and two DASH rings)</td>
<td>n/a (n/a)</td>
<td>4.9 ± 1.1 (5.1 ± 0.5)</td>
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<sup>a</sup> Results in parentheses obtained from a second data set with more stringent criteria used in picking particles for analysis.

<sup>b</sup> The difference between the measurements of MTs with one DASH ring and MTs.

<sup>c</sup> The difference between the measurements of MTs with two DASH rings and MTs with one DASH ring.

<sup>d</sup> Half the difference between the measurements of MTs with two DASH rings and MTs.
with elastase in limited proteolysis experiments abrogates the DASH-MT interaction; presence of these proteins in a subcomplex allows MT-binding. No other subunits meet both criteria. Our conclusion is consistent with previously reported observations from cosedimentation assays. In vitro–translated Dam1p binds MTs (Hofmann et al., 1998), and a 138-amino acid truncation of the Dam1p C-terminus slightly lowers the affinity of recombinant DASH for MTs (Westermann et al., 2005). Our experiments also show that upon removal of the acidic C-termini of both α and β tubulin, DASH still binds to and forms rings around MTs. A contrary conclusion was reached with different MT proteolysis protocols, cleavage verification methods, and binding assays (Westermann et al., 2005); we cannot rationalize the difference in results. Our data suggest a DASH-MT interface in which extensions from DASH rings reach across a gap between the ring and MT and dock on the MT wall (Figure 7, B and C).

Our mass measurements indicate that each DASH ring contains ~20–30 heterodecamers, thereby defining the multiplicity of potential MT-binding contacts. This number is somewhat larger than other estimates. An apparent 16-fold symmetry of rare EM images of DASH bound to MTs led to the conclusion that rings contain 16 heterodecamers (Westermann et al., 2006); the possibility of 32 copies was not considered. Fluorescence measurements of Ask1p copy number in vivo suggest 16–20 heterodecamers (Westermann et al., 2006); the possibility of 32 copies was not considered. Fluorescence measurements of Ask1p copy number in vivo suggest 16–20 heterodecamers (Joglekar et al., 2006). STEM is a direct measurement of the molecular mass of an isolated particle and depends on no assumptions other than appropriate calibration of the instrument. In any case, all the various measurements lead to the conclusion that the polyvalent DASH ring contains more sets of MT-binding extensions than the number of MT protofilaments.

We note that polyvalent attachment through extended arms or loops is fully compatible with diffusive motion, provided that the individual interactions are weak and that an individual arm may reach multiple tubulin docking sites. A range of potential attachment directions is consistent with the variable geometry with which DASH can decorate MTs in vitro, as rings and helices of various pitch. The lack of protection from proteolysis of Dam1p and Duo1p suggests that the cleavable extensions on these subunits are in dynamic equilibrium between the on and off positions. Indeed, all of the arms are unlikely to be engaged at once as the resulting avidity would probably yield too strong an attachment for translation along a MT. Thus, we imagine that at any moment only a fraction of the arms contact the MT. As the population of attached arms shifts, the ring will undergo diffusional translation along the MT, moving in one direction or the other at each step depending on whether a preponderance of the newly attaching arms lie toward one end or the other.

Molecular motors have been thought to move the kinetochore actively toward the poles during anaphase (Hyman et al., 1992), but various lines of evidence suggest that motors are not essential in mitosis. Chromosome movement in S. cerevisiae, as measured by transient sister separation during metaphase, is unaffected by individual deletion of any of the nuclear kinesin-like motors (Tytell and Sorger, 2006). Poleward kinetochore movement in S. pombe during anaphase is unaffected by deletion all three known minus end–directed motors (Grishchuk and McIntosh, 2006). Experiments with chromosomes and MTs in vitro demonstrate that MT depolymerization alone is sufficient to drive chromosome segregation toward the MT minus end (Koshland et al., 1988).

At least two models have been proposed to explain how MT depolymerization drives chromosome motion in anaphase. The conformational wave model (Koshland et al., 1988) suggests that the curling of protofilaments at the depolymerizing end of a MT exerts force directly on the kinetochore, a process that propagates poleward. The diameter of the DASH ring would prevent it from falling off during depolymerization, making DASH a suitable force transducer (Miranda et al., 2005; Westermann et al., 2005). The biased-diffusion model (Hill, 1985) presumes that multiple attachment sites are present and that the energy required to move from one site on the MT lattice to another is sufficiently small to allow rapid diffusion of the kinetochore. At the depolymerizing plus end, new attachment sites are selectively available on one side of the ring and not the other, biasing diffusion toward the minus end. Curling could also contribute, especially if the docking sites were at the protofilament interface. Our picture of associating and dissociating bridges between the body of the DASH ring and the MT surface is compatible with either model.
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