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

JJ L. Miranda,† David S. King,‡ and Stephen C. Harrison§

*Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138; †Department of Molecular and Cell Biology, and Howard Hughes Medical Institute, University of California, Berkeley, CA 94720; and §Department of Biological Chemistry and Molecular Pharmacology, and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

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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., 2001, 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.

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; Li 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...
of the C-terminal His-tag. MT cosedimentation assays were performed by mixing 10 μl of polymerized MTs at 5 mg/ml DASH or subcomplexes with 1 vol of 2 mM ATP and 1 M paclitaxel, 1% DMSO, and pH 7.4. Control samples were treated with buffer instead of DASH. Samples of MTs decorated by DASH containing Spc34p with the mAb YL1/2 (AbD Serotec, Raleigh, NC) were analyzed for visual criteria for background and proximity to other particles. Molecular masses of proteins in gel bands were obtained by in-gel trypsinolysis. Peptide maps of proteins in gel bands were obtained by in-gel trypsinolysis and electrophoresis, samples were quenched by the addition of SDS-PAGE buffer and boiling. Mass spectrometry (MS) experiments were performed with reactions including human Cdk2-cyclin A and DASH containing Spc34p with a C-terminal His-tag, and Phosphorylation In Vitro. Denatured and reduced 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 Ask1p with a C-terminal hexahistidine tag and Ask1p with a C-terminal Strep-tag was generated in an analogous manner. Cloning Constructs of S. cerevisiae DASH or subcomplexes were cloned into a poly- cysticin coexpression system (Miranda et al., 2005). The plasmid containing Ask1p with a C-terminal Streep-tag was generated in an analogous manner. Constructs without HSK3 were obtained by omitting the subcloning step incorporating HSK3 from pET2aTr into the expression vector. This procedure was repeated to generate similar plasmids encoding Ask1p, Dad1p, Dad2p, Dad3p, Dad4p, Spc19p, and Spc34p with C-terminal hexahistidine tags. A construct without DAD1 was obtained by omitting the subcloning step incorporating DAD1 from pET3aTr into the expression vector. This procedure generated a plasmid encoding Spc34p with a C-terminal hexahistidine tag and boiling. Vectors with only the procedure generated a plasmid encoding Spc34p with a C-terminal hexahistidine tag and Ask1p with a C-terminal Strep-tag. Vectors with only the procedure generated a plasmid encoding Spc34p with a C-terminal hexahistidine tag and Ask1p with a C-terminal Strep-tag was generated in an analogous manner. Vectors with only DAD1 and DAD3 were generated by sequentially subcloning DAD1 and DAD3 from pET3aTr into the compatible restriction sites of the first and third cassettes in pET39 as Nhel/BamHI and SacI/KpnI fragments, respectively. This procedure was repeated to generate similar plasmids encoding Dad1p and Dad3p with C-terminal hexahistidine tags. 

**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. Ask1p, Dad1p, and Duo1p are cleaved. MS identified the new species appearing between Duo1p and Spc19p as the N-terminal portion of Duo1p. We detected 74% of the Duo1p sequence when mapping peptides in 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 subtilisin. All subtilisin used buffers contained 1 mM PMSF. Western blots verifying cleavage of the C-terminus of β-tubulin were probed with the mAb JDR3.88 (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).

**Mass Spectrometry**

Peptide maps of proteins in gel bands were obtained by in-gel trypsinolysis and peptide extraction using conventional methods to identify matrix-assisted laser desorption ionization time-of-flight (MS) analysis (Matsubara et al., 2004). Protein complexes were desalted by polyisocyanide-divinylbenzene microbeads reversed-phase liquid chromatography, and intact masses were measured by matrix-assisted laser desorption ionization time-of-flight MS (Bruker, Billerica, MA). Intact proteins were desalted by polyisocyanide-divinylbenzene microbeads reversed-phase liquid chromatography, and intact masses were measured by matrix-assisted laser desorption ionization time-of-flight MS (Bruker, Billerica, MA). Phosphorylation was assessed by microwave trypsinolysis (CEN, Matthews, NC) of the proteins of interest for 7 min with 25 W at 50°C followed by desalting on a ZipTip (Millipore, Bedford, MA) and flow-injection analysis of the entire tryptic digest on a 9.4-T electrospay ionization-Fourier transform ion cyclotron resonance mass spectrometer (Bruker).
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 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 size-exclusion chromatography (data not shown), a property observed with DASH (Miranda et al., 2005). This oligomerization suggests that the interface between eDASH heterodecamers within a ring may still be intact. Lack of protection of MT-bound DASH from proteolysis also suggests that DASH provides a strong interface with MTs, and that the on and off rates of binding to MTs are high enough to free a reasonable fraction of the contacting peptides at any one moment. The width of the gap between the major mass of a DASH ring and the MT surface is 50–100 Å, so a polypeptide extension from a ring subunit might be easily accessible to elastase, an enzyme ~40–50 Å in diameter (Shotton and Watson, 1970), even when the tip of the extension is docked against the MT wall. Thus, the cleavage data suggest that exposed, extended parts of DASH bridge the ring and the MT lattice.

**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 Dam4p (Figure 2A), does not cosediment with MTs (Figure 3A), but DASHΔHsk3p 6mer, which contains Dam1p, Duo1p, Spc34p, Spc19p, Dad1p, and Dad3p (Figure 2A), 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 DASH/H9004/Hsk3p 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. DASH\Delta Dam1p 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 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,
particles from dark field micrographs were boxed into 400 Å and 800-Å arrays for analysis; high background as a result of radiation damage (Figure 6A). Our measurements of a 400-Å-long MT segment yielded a molecular mass of 74 (expected 82) Da suggests that 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 phosphorylated peptide QAHEEHINNGDNGDENSNNIESS-FLK, which contains S250. The unphosphorylated form of the peptide was not found. A peptide containing S216, I5LLQQQYGSMSMVPSPIVPNK, is observed in the unphosphorylated state with no detection of a mass corresponding to the phosphorylated form. Phosphorylated DASH coexist with MTs as efficiently as unphosphorylated DASH (data not shown). EM experiments also show that DASH phosphorylated by Cdk still forms rings around MTs with a similar frequency as does unphosphorylated DASH (data not shown). Cdk5 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 decorated with DASH rings by direct mass measurement with STEM. Substoichiometric amounts of DASH relative to tubulin 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, lyophilized preparations were examined in the STEM. Time-of-flight mass spectrometry of unmodified and modified Ask1p yields a molecular weight of 32058 (expected 32071) and 32132 (expected 32151) Da, respectively. The shift of 74 (expected 82) Da suggests that 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 phosphorylated peptide QAHEEHINNGDNGDENSNNIESS-FLK, which contains S250. The unphosphorylated form of the peptide was not found. A peptide containing S216, I5LLQQQYGSMSMVPSPIVPNK, is observed in the unphosphorylated state with no detection of a mass corresponding to the phosphorylated form. Phosphorylated DASH coexist with MTs as efficiently as unphosphorylated DASH (data not shown). EM experiments also show that DASH phosphorylated by Cdk still forms rings around MTs with a similar frequency as does unphosphorylated DASH (data not shown). Cdk5 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.

**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 kinetochore fibers to translate along a MT without dissociating 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 responsible for MT binding (Figure 7A). Cleavage of these proteins (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 contacts. 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 subcomplex, which contains Dam1p and Duo1p, binds without forming rings. Were encirclement the only property suggested for DASH (Westermann 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 heterodimer is 0.2 MDa; each DASH ring therefore contains 25 ± 5 heterodimers.

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

<table>
<thead>
<tr>
<th>Particle</th>
<th>N</th>
<th>Molecular mass (MDa)</th>
</tr>
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<tbody>
<tr>
<td>MT</td>
<td>54 (32)</td>
<td>8.4 ± 1.0 (7.9 ± 0.7)</td>
</tr>
<tr>
<td>MT and one DASH ring</td>
<td>24 (12)</td>
<td>13.6 ± 1.8 (12.4 ± 0.6)</td>
</tr>
<tr>
<td>MT and two DASH rings</td>
<td>18 (6)</td>
<td>18.2 ± 1.9 (18.0 ± 0.7)</td>
</tr>
<tr>
<td>(MT and two DASH rings) – (MT)</td>
<td>n/a (n/a)</td>
<td>5.2 ± 2.0 (4.5 ± 0.9)</td>
</tr>
<tr>
<td>(MT and two DASH rings) – (MT)/2</td>
<td>n/a (n/a)</td>
<td>4.5 ± 2.5 (4.6 ± 0.9)</td>
</tr>
<tr>
<td>(MT and two DASH rings) – (MT)/2</td>
<td>n/a (n/a)</td>
<td>4.9 ± 1.1 (5.1 ± 0.5)</td>
</tr>
</tbody>
</table>

- Results in parentheses obtained from a second data set with more stringent criteria used in picking particles for analysis.
- The difference between the measurements of MTs with two DASH rings and MTs.
- The difference between the measurements of MTs with two DASH rings and MTs with one DASH ring.
- Half the difference between the measurements of MTs with two DASH rings and MTs.

Figure 6. Dark field STEM micrographs of MT decoration by DASH. Gallery of images showing (A) MTs, (B) MTs with one DASH ring, and (C) MTs with two DASH rings. Scale bars, 250 Å.
upon removal of the acidic C-termini of both H9251 (Westermann slightly lowers the affinity of recombinant DASH for MTs and a 138-amino acid truncation of the Dam1p C-terminus vitro–translated Dam1p binds MTs (Hofmann reported observations from cosedimentation assays. In both criteria. Our conclusion is consistent with previously subcomplex allows MT-binding. No other subunits meet the DASH-MT interaction; presence of these proteins in a subcomplex contains more sets of MT-binding extensions than the DASH ring contains. 

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 (Joglekar et al., 2006). STEM is a direct measurement of the molecular mass of an isolated particle and depends on 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 available on the 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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