The Role of Self-association in Fin1 Function on the Mitotic Spindle*

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Stabilization of spindle microtubules during anaphase is essential for proper chromosome segregation. Fin1 is a budding yeast protein that localizes to the poles and microtubules of the spindle during anaphase and contributes to spindle stability. The N-terminal half of Fin1 is phosphorylated at multiple sites by the cyclin-dependent kinase Clb5-Cdk1, and dephosphorylation in anaphase triggers its localization to the spindle. The C-terminal half of Fin1 contains coiled-coil motifs that are required for its self-association. Here we investigated the functional importance of the two regions of Fin1. Fin1 mutants lacking the C-terminal coiled-coil domains localized to spindle pole bodies but not along spindle microtubules. These mutants failed to self-associate and displayed reduced binding to microtubules in vitro but were functional in vivo and stabilized anaphase spindles when dephosphorylated. Deletion of the Fin1 C terminus suppressed the lethal phenotypes of the phospho-mutant Fin1SA. Our findings suggest that the N-terminal region of Fin1 is sufficient for its regulated function as a spindle-stabilizing factor and that this function involves association with the spindle pole body. The ability of the C-terminal region to promote Fin1 self-association and microtubule binding may underlie the lethal effects of the deregulated Fin1SA mutant.

The faithful segregation of chromosomes during mitosis requires precise regulatory control of spindle microtubule dynamics. During metaphase, microtubules exhibit high dynamic instability, which facilitates the capture of chromosomes at their kinetochores. At the onset of anaphase, microtubule dynamics are silenced, and the spindle is thereby stabilized (1–3). The change in microtubule dynamics at the onset of anaphase depends on regulated changes in the activity of proteins that influence microtubule behavior (4).

The budding yeast protein Fin1 is a microtubule-stabilizing factor that associates with the spindle during anaphase. The activity of Fin1 is carefully regulated during the cell cycle (5). Phosphorylation in early mitosis by the cyclin-dependent kinase Clb5-Cdk1 inhibits Fin1 function and prevents its association with the spindle. Dephosphorylation in anaphase promotes its localization to the spindle, where it acts as a stability factor. A Fin1 phospho-mutant (Fin1SA) localizes prematurely to the spindle, alters metaphase spindle structure, and causes lethal errors in chromosome segregation (5).

Fin1 purified from yeast self-associates to form a 10-nm-diameter filament (6). Fin1–Fin1 interactions have also been demonstrated in vivo (7, 8). Oligomerization of Fin1 is mediated by sequences in the C-terminal portion of the protein, which are predicted to form coiled-coils (9). It has been suggested that Fin1 filaments provide structural support to the mitotic spindle, but the importance of Fin1 self-association for spindle stability is unknown (10, 11).

We tested the importance of Fin1 self-association by analyzing the properties of a series of Fin1 truncations. We found that deletion of the predicted coiled-coil regions in the Fin1 C terminus blocked self-association, eliminated Fin1 localization to spindle microtubules, and lowered the affinity of Fin1 for microtubules in vitro. Deletion of the predicted coiled-coil domains suppressed the lethality of the Fin1 phospho-mutant and prevented its effects on metaphase spindle structure. However, the N-terminal region of Fin1 was sufficient to target the protein to spindle poles, where it functioned to stabilize the spindle despite the absence of the coiled-coil domains.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—All strains were derivatives of W303 and grown at 30 °C unless otherwise noted. Plasmid p416GALS was used to express Fin1-GFP2 proteins under the control of the GAL promoter, an attenuated version of the GAL promoter (12). Plasmid p414ADH was used to express Fin1-GFP proteins under the control of the alcohol dehydrogenase (ADH) promoter (13).

Protein Analysis—Protein extracts for immunoblots were prepared as described (14). For co-immunoprecipitation, 50 ml of cells were grown to log-phase and lysed by bead-beating two times for 1 min in 20 mM Hepes, pH 8.0, 500 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 80 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin, leupeptin, and aprotinin. Protein G Dynabeads (Invitrogen) were preincubated with anti-GFP antibody for 30 min at 4 °C and then washed with lysis buffer. Protein extract (1 mg) was incubated with the bead mixture for 2 h at 4 °C. Beads were washed three times with lysis buffer and then resuspended in protein sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting. Fin1-3HA

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2 The abbreviations used are: GFP, green fluorescent protein; SPB, spindle pole body; PIPES, 1,4-piperazinediethanesulfonic acid; HA, hemagglutinin; 5-FOA, 5-fluoroorotic acid; TEV, tobacco etch virus.
was detected with the 16B12 antibody (Covance Laboratories Inc.) at 1:1000, and Fin1-GFP was detected with the anti-GFP antibody clones 7.1 and 13.1 (Roche Applied Science) at 1:1000.

Immunofluorescence Microscopy—Cells were fixed overnight at 4 °C in 100 mM potassium phosphate, pH 6.4, 0.5 mM MgCl2, 3.7% formaldehyde. After fixation, immunofluorescence was performed as described (15). The anti-tubulin antibody YOL134 (AbCam Ltd.) was used at 1:500, and the anti-Tub4 antibody was used at 1:500. Anti-rat Cy3 and anti-rabbit Cy3 (Jackson Immunoresearch) were used at 1:1000. Images were captured with a Hamamatsu Orca-AG camera on a Zeiss Axiosvert 2000MAT microscope with a ×63 objective lens, using Image Pro Plus 5.1 software.

Microtubule Binding Assays—Microtubule binding was analyzed as described previously (5). That is, purified Fin11–152-His6 was incubated with serially diluted taxol-stabilized microtubules in BRB80 buffer (80 mM PIPES, pH 6.8, 2 mM MgCl2, 1 mM EDTA) for 20 min at room temperature. The reaction was centrifuged at 90,000 rpm for 10 min at 22 °C. Supernatant and pellet were separated and analyzed by SDS-PAGE.

RESULTS

Subcellular Localization of Fin1 Mutants—Fin1 is a 291-amino-acid protein that contains two putative coiled-coil domains near its C terminus. The N-terminal half of Fin1 is a regulatory region that contains five consensus Cdk1 phosphorylation sites and a destruction box (D-box), which is required for anaphase-promoting complex-mediated proteolysis of Fin1 (Fig. 1A) (5).

To determine which regions of Fin1 are required for its spindle association, we expressed a series of truncations fused to green fluorescent protein (GFP) under the control of the inducible GALS promoter in fin1Δ cells. During anaphase, wild-type Fin1-GFP localizes along spindle microtubules and as two foci at the spindle poles. Fin11–197-GFP and Fin11–152-GFP, which lack the C-terminal coiled-coil domains but contain all five Cdk1 phosphorylation sites, appeared diffuse in the nucleus and as foci near spindle poles but were not detected on spindle microtubules (Fig. 1B). Further truncation of the C terminus (Fin11–132-GFP) caused an increase in the cytoplasmic GFP signal and a reduction of the signal near the spindle pole body (SPB). Fin140–152-GFP localization was similar to that of Fin11–132-GFP. Fin1132–291-GFP, which contains the predicted coiled-coil domains but not the regulatory domain, localized diffusely in the nucleus and never appeared at the spindle poles or along the length of the spindle (Fig. 1B). Expression of each mutant in wild-type cells yielded similar results, indicating that the presence of endogenous Fin1 has no effect on the localization of the mutants (data not shown). These results indicate that both the C-terminal and the N-terminal regions of Fin1 are required for its association with spindle microtubules but that amino acids 1–132 are sufficient to target Fin1 to spindle poles.

Kinetochore sites, the sites of microtubule attachment to chromosomes, cluster near the spindle poles during anaphase (16). Thus, the Fin1 foci we saw could represent Fin1 at the SPB and/or the kinetochore. To determine whether the appearance of Fin1 dots depends on kinetochore function, we compared the localization of Fin15A in wild-type cells with that in cells expressing a temperature-sensitive version of Ndc10 (ndc10–1), an essential kinetochore component (17). Kinetochore structure is extensively disrupted at the restrictive temperature in this mutant. Expression of Fin15A in these cells allowed us to
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We also investigated the localization of Fin1 truncations in ndc10-1 cells. Unlike Fin15A, Fin1 truncations had no effect on spindle structure in ndc10-1 cells. Fin11–197-5A-GFP formed foci that co-localized with SPBs in wild-type and ndc10-1 cells (Fig. 2B). Fin11–152-GFP displayed a similar localization pattern (data not shown).

Fin1-Fin1 Interactions Require the C-terminal Coiled-Coil Domain—The C terminus of Fin1 has been characterized as a self-association domain (7, 8). To confirm that Fin1 interacts with itself, we performed co-immunoprecipitations from a strain carrying two different epitope-tagged versions of Fin1. Fin1 was tagged at the C terminus with triple hemagglutinin epitopes (3HA) or with GFP. Tagged Fin1 proteins were co-expressed in fin1Δ cells, and protein extracts were immunoprecipitated with anti-GFP antibody. Anti-GFP immunoprecipitates contained Fin1-3HA, confirming that Fin1 self-associates in vivo (Fig. 3). Truncation of the predicted coiled-coil domains eliminated the interaction of Fin1 mutants with full-length Fin1. A mutant containing the coiled-coil domains (Fin1122–291) maintained the ability to associate with full-length Fin1. Together with the localization data, these results suggest that Fin1 self-association is required for Fin1 localization to spindle microtubules but not to SPBs.

The Fin1 C Terminus Contributes to Fin1 Affinity for Microtubules—The localization of Fin1 truncations to SPBs but not spindle microtubules suggested that deletion of the C terminus might lower the affinity of Fin1 for microtubules. To test this hypothesis, we examined the ability of recombinant Fin11–152-His6 to bind purified, taxol-stabilized microtubules in vitro. In our previous work, we showed that full-length Fin1 binding to microtubules was half-maximal between 0.13 and 0.25 μM tubulin (Fin11–152-His6) retained some ability to bind microtubules, but affinity was reduced significantly, resulting in half-maximal binding between 0.5 and 1.0 μM tubulin. These results, along with the self-association data, suggest that amino acids 1–152 contain a weak microtubule-binding domain but that full microtubule binding requires the

analyze Fin1 foci in both metaphase and anaphase. Wild-type and ndc10-1 cells were synchronized in G1 at 23 °C and released at 37 °C in the presence of galactose to induce expression of Fin11–152-GFP. Cells were harvested 60 and 90 min after release for Fin1-GFP visualization and immunofluorescence of Tub4 (to visualize spindle poles). Images in the right column represent a composite of Fin1 (green) and Tub4 (red).

**FIGURE 2.** *Fin1 localizes to the SPB independent of kinetochore function.* Wild-type (WT) or ndc10-1 cells carrying Fin15A-GFP (A) or Fin11–152-5A-GFP (B) under the GALS promoter were grown to log-phase at 23 °C and then arrested in G1 with α-factor (12.5 μg/ml) for 3.5 h. Cultures were shifted to 37 °C for 2 h, washed twice to remove the α-factor, and then released at 37 °C into galactose-containing medium to induce Fin1 expression. Cells were harvested 60 and 90 min after release for Fin1-GFP visualization and immunofluorescence of Tub4 (to visualize spindle poles). Images in the right column represent a composite of Fin1 (green) and Tub4 (red).
C terminus, perhaps because Fin1 oligomerization enhances microtubule binding.

Functional Analysis of Fin1 Truncations—Deletion of FIN1 is synthetic lethal with deletion of ASE1 (18), which encodes a protein that contributes to anaphase spindle stability by cross-linking microtubules at the spindle midzone (19). To assess the functionality of Fin1 truncations, we tested whether they could support growth as the only copy of Fin1 in ace1Δ cells. We constructed a fin1Δase1Δ strain that is viable due to the presence of a URA3-marked plasmid that expresses FIN1 from its own promoter. We expressed Fin1 truncations from the constitutive ADH promoter in these cells and then assessed cell viability on medium containing 5-fluoro-orotic acid (5-FOA), which selects against the FIN1-URA3 plasmid. Fin11−197 and Fin11−152 supported robust colony growth on 5-FOA, indicating that these mutants retain Fin1 function (Fig. 5A). A small number of colonies survived on 5-FOA when Fin11−132 was the only copy of Fin1, suggesting that this mutant is partially functional. Cells expressing Fin140−152 or Fin1132−291 could not survive in the absence of full-length Fin1.

To exclude the possibility that differences in the functionality of the mutants were caused by variations in expression, we analyzed the levels of the mutant proteins by immunoblotting. Mutant proteins were expressed at levels higher than those of full-length Fin1 but similar to each other (Fig. 5B).

We next assessed the ability of Fin1 truncations to stabilize spindles in an artificial anaphase system (4, 20). In this assay, cells are arrested in metaphase by depletion of Cdc20, an essential activator of the anaphase-promoting complex. Sister chromatid separation is induced by expression of TEV protease, which cleaves an engineered site in Scc1, a protein required to maintain sister chromatid cohesion. Anaphase ensues, but chromosome movements and spindle elongation occur abnormally, and spindles in these cells eventually break. These defects are thought to result because Cdk phosphorylation inhibits the activity of important anaphase spindle-stabilizing factors in this arrest (4, 21). Expression of non-phosphorylated Fin1 (Fin15A) reduces the frequency of spindle breakage in these cells, whereas expression of Fin1, which remains phosphorylated, has no effect (5).

We found that Fin11−197-5A and Fin11−152-5A promoted spindle stability in this system, although to a lesser extent than full-length Fin1 (Fig. 6). Fin11−152 also partially stabilized spindles, indicating that this mutant is not properly regulated by phosphorylation. This mutant lacks a KXL motif that is important for interaction with Clb5-Cdk1 (22) and might therefore be incompletely phosphorylated. Spindle-stabilizing activity was lost in mutants with further deletion of either the N terminus or the C terminus.

Deletion of the Fin1 C Terminus Suppresses Fin15A Phenotypes—Proper regulation of Fin1 phosphorylation state is required for cellular viability; expression of Fin15A from the GAL5 promoter is lethal. However, expression of the truncated Fin1 mutants from the GAL5 promoter had no effect on cell viability (Fig. 7A). The Fin15A C terminus is therefore required for its lethal effects.

Overexpression of Fin15A in metaphase-arrested cells causes striking defects in metaphase spindle structure (5). These spindle defects likely contribute to the lethality of Fin15A. Since truncation of Fin15A suppressed its lethality, we hypothesized that Fin1 truncations would have no effect on metaphase spindle structure. We arrested cells in metaphase by depletion of Cdc20 and then induced Fin15A or truncated Fin1 phosphomutants from the GAL5 promoter. After 3.5 h, expression of full-length Fin15A caused spindles to collapse and astral microtubules to elongate (Fig. 7B), as in our previous work (5). A small percentage of cells expressing Fin11−197-5A or Fin11−152-5A contained these abnormal microtubule structures, but the majority of metaphase spindles appeared normal. Metaphase spindle structure was unaffected in cells expressing any of the other Fin1 mutants.
DISCUSSION

We conclude that the N-terminal half of Fin1 (amino acids 1–152) is sufficient for Fin1 localization to spindle poles and for its normal function as a stabilizer of the anaphase spindle. Phosphorylation sites within this region ensure that the spindle-stabilizing effects of the Fin1 N terminus are limited to anaphase.

The Fin1 C terminus promotes self-association but is not essential for the spindle-stabilizing function of Fin1. This region is required for high affinity binding of Fin1 to microtubules in vitro and for the localization of Fin1 to spindle microtubules in vivo. The C-terminal region is also required for the deleterious effects of the Fin1 phospho-mutant. We speculate that coiled-coil-dependent assembly of Fin1 into dimers or higher order multimers generates multivalent Fin1-microtubule interactions that enhance microtubule binding and bundling. This behavior is not required for the normal function of Fin1 in anaphase but may result in lethal spindle defects when the Fin15A phospho-mutant interacts with the spindle before anaphase.

Regulated changes in the behavior of spindle microtubules occur as cells progress through the cell cycle. Microtubules are highly dynamic in metaphase, but this behavior is silenced at anaphase onset. Our work suggests that Fin1 activity at the spindle pole contributes to the stabilization of microtubules during anaphase. The minus ends of microtubules are organized at spindle poles, perhaps suggesting that Fin1 affects spindle stability by changing microtubule dynamics at the minus end.

The dynamic behavior of spindle microtubules in animal cells depends in part on microtubule flux, which involves microtubule depolymerization at the minus end and polymerization at the plus end. In *Drosophila* embryos, the poleward flux of microtubules before anaphase is thought to result from the sliding activity of a kinesin-5 motor (KLP61F) at the plus end and the microtubule-depolymerizing activity of a kinesin-13 (KLP10A) at the minus end (23, 24). Elongation of the spindle in anaphase B appears to result from inhibition of minus end depolymerization by kinesin-13 (24). The inhibition of flux depends on the degradation of cyclin B (24, 25). Likewise, in human mitotic cells, inhibition of a kinesin-13 (Kif2A) causes a significant decrease in flux (26). Thus, a change in microtubule dynamics at the minus end, which likely depends on the dephosphorylation of Cdk substrates, contributes to the elongation and stabilization of animal spindles in anaphase B.

Fluorescent speckle microscopy of budding yeast spindles has revealed that microtubule assembly and disassembly occurs primarily at the plus end and that microtubule minus ends are stable (2). These studies were based on analyses of cytoplasmic...
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astral microtubules and anaphase interpolar spindle microtu-
bules, and the dynamics of metaphase spindle microtubules
were not determined. It therefore remains possible that micro-
tubule flux contributes to the dynamic behavior of microtu-
bules during metaphase in yeast and that the recruitment of
Fin1 to spindle poles accounts for the stabilization of minus
ends in anaphase. Alternatively, Fin1 associated with the spin-
dle poles might affect microtubule stability at the plus end
either by traveling to the plus end itself or by recruiting plus end
regulatory proteins to the spindle. Several plus end-interacting
proteins are loaded onto the spindle at the poles and subse-
quently transported to the plus end (27–29). Detailed analysis
of anaphase spindle dynamics in the absence of Fin1 function
would help distinguish between these possibilities and provide
new insights into spindle behavior and regulation in budding
yeast.

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