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The molecular function of Ase1p: evidence for a MAP-dependent midzone-specific spindle matrix

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The midzone is the domain of the mitotic spindle that maintains spindle bipolarity during anaphase and generates forces required for spindle elongation (anaphase B). Although there is a clear role for microtubule (MT) motor proteins at the spindle midzone, less is known about how microtubule-associated proteins (MAPs) contribute to midzone organization and function. Here, we report that budding yeast Ase1p is a member of a conserved family of midzone-specific MAPs. By size exclusion chromatography and velocity sedimentation, both Ase1p in extracts and purified Ase1p behaved as a homodimer. Ase1p bound and bundled MTs in vitro. By live cell microscopy, loss of Ase1p resulted in a specific defect: premature spindle disassembly in mid-anaphase. Furthermore, when overexpressed, Ase1p was sufficient to trigger spindle elongation in S phase–arrested cells. FRAP revealed that Ase1p has both a very slow rate of turnover within the midzone and limited lateral diffusion along spindle MTs. We propose that Ase1p functions as an MT cross-bridge that imparts matrix-like characteristics to the midzone. MT-dependent networks of spindle midzone MAPs may be one molecular basis for the postulated spindle matrix.

Introduction

The equal distribution of chromosomes to daughter cells depends on the bipolar structure of the mitotic spindle. The spindle midzone is the region of overlap between antiparallel microtubules (MTs)* emanating from the spindle poles. The spindle midzone mediates the interaction between the two half-spindles, and is thought to be a site of force generation for spindle elongation (Sullivan and Huffaker, 1992; McIntosh, 1994; Hildebrandt and Hoyt, 2000; Mitchison and Salmon, 2001). In higher eukaryotes, the spindle midzone also establishes the site of cytokinesis (Straight and Field, 2000). Near the end of mitosis in plants, the spindle midzone is organized into the phragmoplast, a set of interdigitated antiparallel MTs that are required for the formation of the new cell plate (Staehelin and Hepler, 1996). Aside from kinesin motors, little is known about the molecules that determine spindle midzone organization (Rattner, 1992; Sharp et al., 2000).

A striking feature of spindle midzones from diverse organisms is the organization of antiparallel MTs into highly ordered geometrical arrays. From EM studies it has been inferred that this organization is established either by proteinacious cross-bridges or by a more diffuse electron-dense matrix substance (McIntosh et al., 1969; McDonald et al., 1977). However, there has been debate over whether or not the electron micrographs of cross-bridges or the matrix substance represent genuine in vivo structures. Recent studies using ultra-rapid freeze substitution methods have independently confirmed the presence of spindle cross-bridges (Ding et al., 1993; Mastronarde et al., 1993; Winey et al., 1995). Little is known about the molecular composition of these spindle cross-bridges, however there is compelling evidence that at least some contain kinesin motor proteins (Sharp et al., 1999).

The highly conserved BimC homotetrameric kinesin motors have been shown to form cross-bridges between MTs in vitro and to associate with cross-bridge structures in vivo (Kashina et al., 1996; Sharp et al., 1999). These motor proteins are thought to bind and bundle spindle MTs and to make a direct contribution to force generation during spindle elongation (Hoyt et al., 1992; Saunders and Hoyt, 1992; Sharp et al., 2000). In addition, the MKLP1 motor protein complex localizes to the spindle midzone in anaphase and is necessary for spindle integrity (Sharp et al., 2000; Adams et al., 2001). This complex may contain the microtubule-associated protein

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*Abbreviations used in this paper: APC, anaphase-promoting complex; CM, conserved motif; HU, hydroxyurea; MAP, microtubule-associated protein; MT, microtubule.

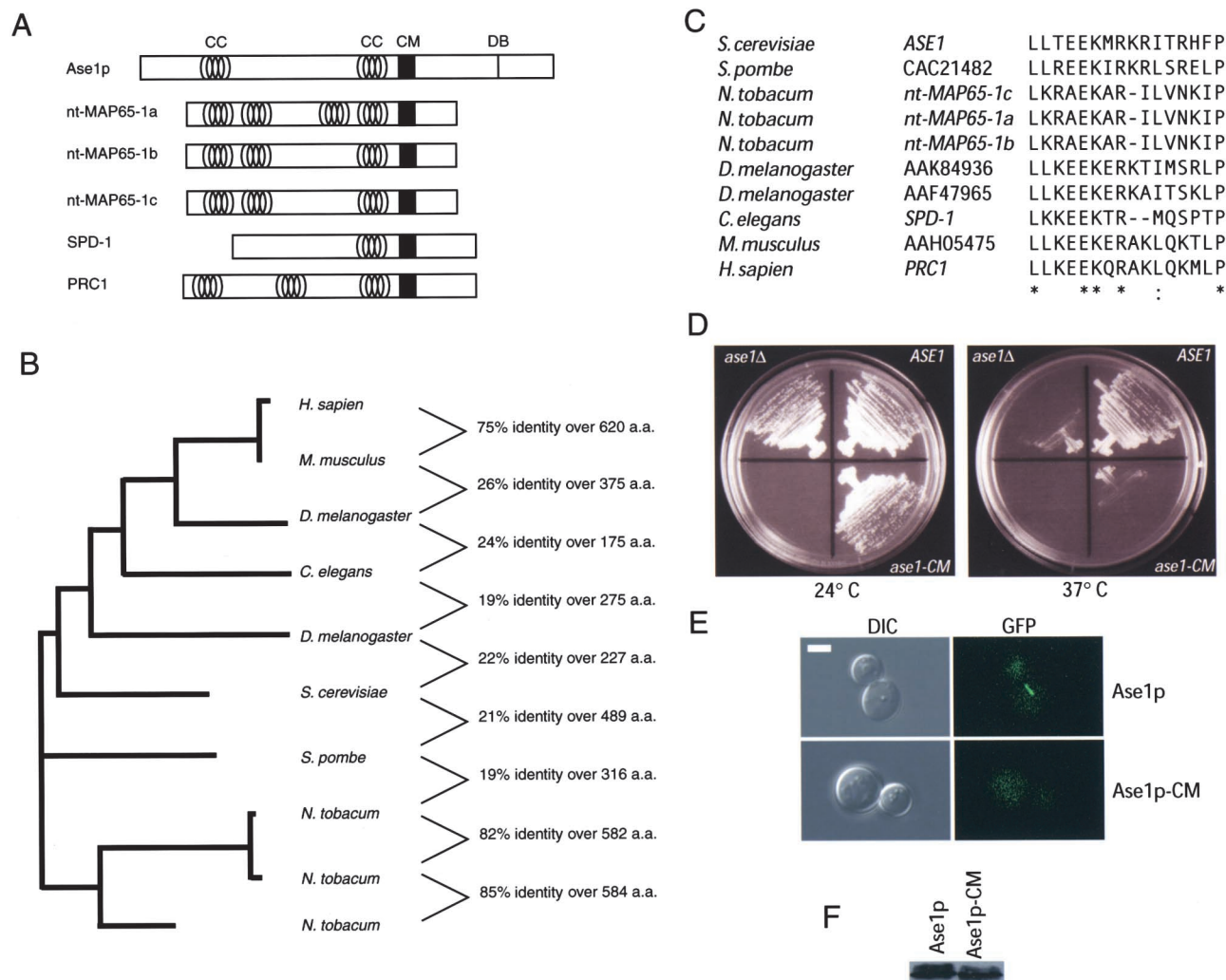Key words: mitosis; microtubule-associated protein; spindle midzone; anaphase; budding yeast

Figure 1. **Ase1p is a member of a conserved family of spindle midzone MAPs.** (A) Domain structure of Ase1p-related proteins; CC indicates predicted coiled-coil motifs, CM indicates the conserved motif, DB indicates the destruction box. (B) A neighbor-joining tree and the pair-wise identity between nearest neighbors of Ase1p-related proteins. There is a large set of proteins highly related to the tobacco MAP65 family predicted from ORFs in *Arabidopsis* that are not included in this diagram. (C) Amino acid sequence of the most highly conserved motif. Conserved (*) and similar (:) residues are marked. (D) GFP–Ase1p complements the temperature-sensitive synthetic lethality of an *ase1Δ bni1Δ* (Lee et al., 1999), whereas GFP–Ase1p-CM does not. (E) GFP–Ase1p-CM fails to localize properly to the spindle midzone. Shown are images of mitotic cells expressing GFP–Ase1p or GFP–Ase1p-CM. (F) Ase1p-CM is expressed at comparable levels to Ase1p.

(MAP) INCENP and the signaling molecule aurora B kinase and, in *Caenorhabditis elegans*, appears to be part of the "central spindlin" complex (Mishima et al., 2002). The purified MKLP1 motor protein alone binds to and bundles MTs and slides antiparallel MTs past each other in vitro (Nislow et al., 1992). However, the precise mechanism by which the BimC and MKLP1 motor proteins contribute to spindle midzone organization remains unknown. Specifically, it is not known if these motor proteins are sufficient to create proper spindle midzone organization or whether other nonmotor elements make a contribution.

The major class of nonmotor elements identified in the spindle midzone is MAPs. This class includes the highly conserved "chromosomal passenger" INCENP (Kim et al., 1999; Adams et al., 2001; Morishita et al., 2001; Petersen et al., 2001; Rajagopalan and Balasubramanian, 2002). In budding yeast, the best candidates for midzone MAPs are Ase1p and Stu1p (Pellman et al., 1995; Yin et al., 2002).

Ase1p was originally identified as a protein that is essential in cells lacking the MT plus end tracking protein Bik1p (Pellman et al., 1995). Because *ase1-1 bik1-S419* cells fail to elongate anaphase spindles, but do not undergo cell cycle arrest, these cells accumulate multiple spindles and spindle pole bodies within a single nucleus. Ase1p is a substrate for the anaphase-promoting complex (APC), and its degradation appears to be required for the normal timing of spindle disassembly (Juang et al., 1997; Huang et al., 2001). It was also shown that telophase spindles in *cdc15-2* arrested cells are unstable in the absence of Ase1p (Juang et al., 1997). The analysis of specific double mutants (*ase1-1 bik1-S419* and *ase1Δ cdc15-2*) previously suggested that Ase1p is important for anaphase spindle stability. However, this hypothesis has not yet been tested directly by live cell microscopy of *ase1Δ* cells. Furthermore, how Ase1p contributes at the molecular level to spindle bipolarity and elongation is unknown.

To probe the role of a nonmotor protein in midzone function, we have studied Ase1p function in vitro and in vivo. First, we note that Ase1p is a member of a conserved family of spindle midzone proteins. Next, we found that Ase1p acts as a homodimer that binds to and bundles MTs. Ase1p is required for anaphase spindle elongation, and overexpression of Ase1p is sufficient to induce spindle elongation in S phase–arrested cells. In addition, FRAP analysis has revealed that Ase1p is relatively immobile within the midzone during spindle elongation. We propose that Ase1p functions as a spindle cross-bridge that imparts matrix-like characteristics to the spindle midzone, maintaining anaphase spindle integrity.

Results

Ase1p is a member of a conserved family of spindle midzone proteins

Ase1p is an 885–amino acid polypeptide with a predicted molecular weight of 102 kD (Fig. 1 A). Homology between Ase1p and human PRC1 has been noted previously, with an identity of 23% over a stretch of 333 amino acids (Jiang et al., 1998; Mollinari et al., 2002). Although the similarity in midzone localization is intriguing, the low degree of sequence identity made it difficult to conclude that Ase1p and PRC1 are members of the same conserved family. More recently, a large number of Ase1p-related proteins have been identified in many organisms. All possess putative coiled-coil sequences and all of the family members tested so far localize to the spindle midzone. The Ase1p-related proteins include *Daucus carota* MAP65, the nt-MAP65 family from *Nicotiana tobacum*, SPD-1 from *C. elegans*, and human PRC1 (Fig. 1, A and B; Chan et al., 1999; Smertenko et al., 2000; Mollinari et al., 2002; K. Verbrugghe and J. White, personal communication).

The highest sequence identity among the Ase1p-related proteins resides in a 16–amino acid conserved motif (CM) located in the COOH terminus (Fig. 1, A and C). It was recently shown that a 213–amino acid fragment of PRC1 containing this sequence binds to MTs in vitro (Mollinari et al., 2002). To determine if this same region was required for Ase1p function, an *ASE1* allele was constructed that lacks the coding sequence for this CM (*ase1-CM*). This mutant fails to complement the temperature-sensitive synthetic lethality of an *ase1Δ bni1Δ* double mutant (Fig. 1 D; Lee et al., 1999). Furthermore, Ase1p-CM fails to localize properly in mitotic cells (Fig. 1 E), even though it is expressed at comparable steady-state levels to Ase1p (Fig. 1 F). These results suggest that the CM is required for Ase1p function in vivo.

Ase1p is a homodimer with an extended rod shape

The molecular function of Ase1p is not known. The cellular localization of Ase1p and the phenotypes of cells lacking Ase1p are consistent with it being a structural component of the spindle. However, it could also be a component of a regulatory complex, for example, like the INCENP subunit of the aurora B kinase complex (Adams et al., 2001). If Ase1p is a structural component of the spindle, it could either be a homomeric MAP or a component of a macromolecular complex, for example, a motor protein complex. As a first step to distinguish among these possibilities, the size and
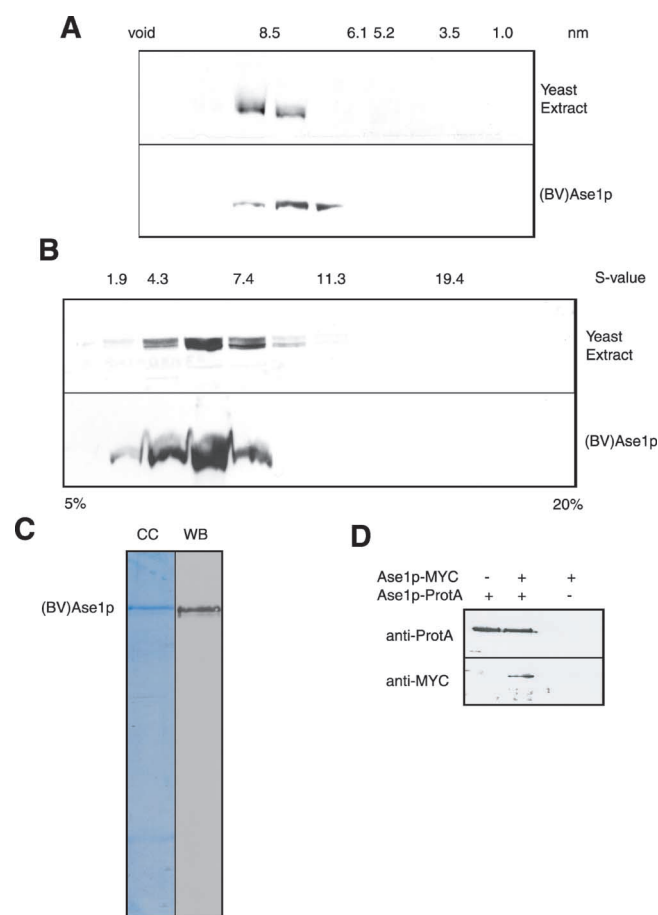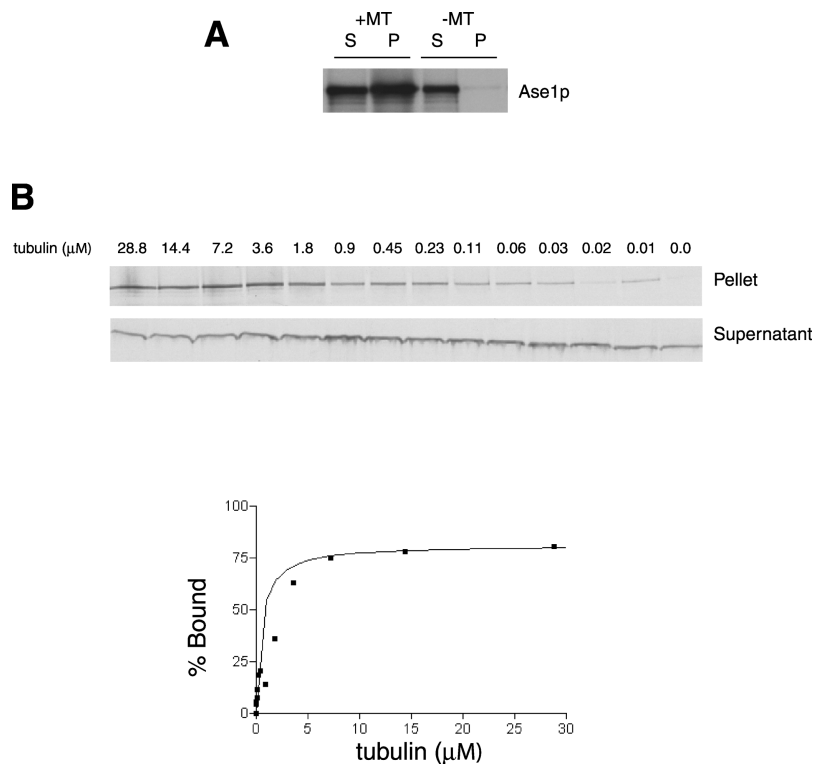

Figure 2. **Ase1p is a homodimer.** (A) Analytical gel filtration of Ase1p in a native yeast extract or purified (BV)Ase1p. Stokes' radii of protein standards are indicated above the blots. (B) Sucrose gradients of Ase1p in a native yeast extract or purified (BV)Ase1p. Svedberg coefficients for the proteins used as globular standards are shown above the blots. (C) Purified (BV)Ase1p from Sf9 cells. Purified (BV)Ase1p was run on an SDS-PAGE gel and visualized by colloidal Coomassie (CC) staining or Western blotting (WB). (D) An anti-ProtA coimmunoprecipitation shows that Ase1p–ProtA coimmunoprecipitates Ase1p–MYC. Anti-IgG (upper) and anti-MYC (lower) Western blots are shown.

shape of an epitope-tagged Ase1p (Ase1p–MYC) in native yeast extracts was measured by velocity sedimentation and size exclusion chromatography. For these experiments, the lysis buffer used for extraction contained 150 mM salt and no detergent. The Stokes' radius of the native yeast molecule was determined to be 8.8 nm (Fig. 2 A), and the Svedberg coefficient was 5.9S (Fig. 2 B). From these values, we calculated that the native form of Ase1p has a molecular weight of ∼225 ± 45 kD (Siegel and Monty, 1966; Schuyler and Pellman, 2002). The predicted molecular weight of Ase1p–MYC is 107 kD. The ratio between the measured and calculated value is 2:1, suggesting that Ase1p may form a homodimer in vivo. The axial ratio, if a prolate ellipsoid is assumed, is 17:1 (Schuyler and Pellman, 2002). This suggests that Ase1p in native extracts has an extended rod shape, which is consistent with the high content of coiled-coil structure predicted from its primary sequence.

To ask if other proteins are present in the Ase1p complex, we purified recombinant Ase1p–MYC–6xHis ([BV]Ase1p)

Figure 3. **Ase1p is an MT-binding protein.** (A) In vitro–translated Ase1p copellets with 28 µM Taxol-stabilized MTs (s, supernatant; p, pellet). The maximum percent bound in this assay is 75%. (B) Copelleting of in vitro–transcribed/translated Ase1p with a serial dilution of Taxol-stabilized MTs to determine the binding constant.

from Baculovirus-infected insect cells (Fig. 2 C). Purified (BV)Ase1p was homogeneous, migrating as a single peak by velocity sedimentation and size exclusion chromatography (Fig. 2, A and B). The Stokes' radius of (BV)Ase1p was 8.4 nm and the sedimentation coefficient was 5.9S. The calculated molecular weight from these hydrodynamic values is 213 kD, closely matching the calculated molecular weight of native Ase1p from yeast extracts. The comparison of the hydrodynamic properties of purified Ase1p with Ase1p from yeast extracts at native levels strongly suggests that Ase1p is a homodimer. To directly test whether Ase1p self-associates, two forms of the protein tagged with different epitopes were coexpressed in cells at native levels. Cell extracts were immunoprecipitated with antibodies against one epitope (protein A), and the immunoprecipitates were probed with antibodies against the other epitope (MYC; Fig. 2 D). This experiment demonstrated that the two differently tagged forms of the protein are associated with each other and supports the conclusion that Ase1p is a homodimer with an extended rod shape.

Ase1p is an MT binding and bundling protein

Next, we determined if Ase1p interacts with MTs. Ase1p expressed in a rabbit reticulocyte lysate copelleted with bovine Taxol-stabilized MTs (Fig. 3 A). Thus, Ase1p can interact with MTs in the absence of other yeast proteins. Furthermore, Ase1p bound to MTs with an apparent K_d of 0.3 ± 0.13 µM ($n = 2$; Fig. 3 B), which is similar to the binding constants of other known MAPs.

The purified (BV)Ase1p also bound Taxol-stabilized MTs in the copelleting assay, demonstrating that Ase1p bound MTs directly and that the purified (BV)Ase1p was functional (Fig. 4 A). Next, we mixed the purified (BV)Ase1p with Oregon green–labeled MTs for 15 min and determined if it bundled MTs by fluorescence microscopy (Fig. 4 B). Obvious

MT bundling was observed in Ase1p-containing sample after a 15-min incubation, and a more extensive MT bundling was apparent after a 30-min incubation (Fig. 4 B). We confirmed the formation of Ase1p-dependent MT bundles by electron microscopy of negative stained samples (Fig. 4 C).

Ase1p is necessary for spindle elongation in anaphase B

Taken together with the in vivo localization, the biochemical properties of Ase1p suggested that it might bundle antiparallel MTs during anaphase. We therefore characterized mitosis in cells lacking Ase1p by live cell fluorescence microscopy. GFP–Tub1p (α-tubulin) was expressed in *ase1Δ* and control wild-type cells to label MTs. Anaphase B in yeast and other eukaryotes consists of a fast phase of spindle elongation followed by a slow phase of spindle elongation (Oppenheim et al., 1973; McIntosh, 1994; Kahana et al., 1995; Yeh et al., 1995; Straight et al., 1997, 1998). The mitotic fast phase is thought to result from the sliding of antiparallel MTs and has a rate of 0.54 µm/min in budding yeast (McIntosh 1994; Kahana et al., 1995; Yeh et al., 1995; Straight et al., 1997, 1998; Maddox et al., 2000). The mitotic slow phase is thought to result from midzone MT polymerization together with antiparallel MTs sliding and occurs at a rate of 0.21 µm/min in budding yeast (McIntosh, 1994; Kahana et al., 1995; Yeh et al., 1995; Straight et al., 1997, 1998; Maddox et al., 2000). In contrast to the control cells, the spindle MTs in *ase1Δ* cells collapse abruptly in mid-anaphase at the transition between the fast and slow phase (Fig. 5, A and B). All wild-type cells ($n = 26$) underwent both the mitotic fast and slow phases of spindle elongation. However, 41 out of 43 *ase1Δ* cells completed the fast phase, but underwent premature spindle disassembly at the beginning of the slow phase (Fig. 5 C). Thus, the anaphase spindle collapse of *ase1Δ* mutant cells is highly penetrant. In the 2 out of 43 *ase1Δ* cells that failed to com-
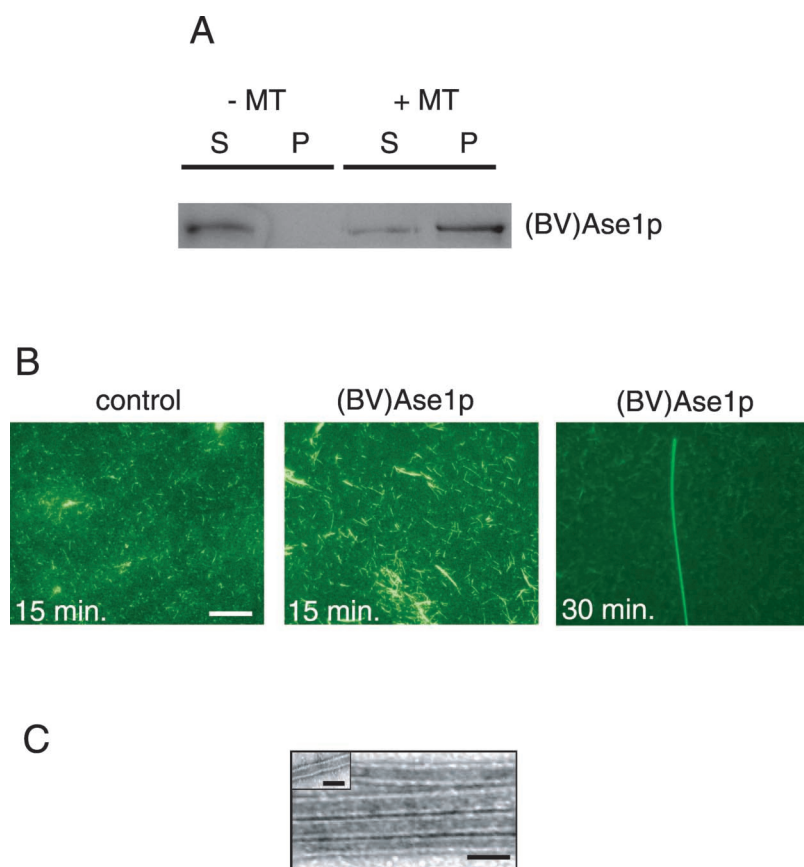
A

Figure 4.   **Ase1p is an MT bundling protein.**
(A) Purified (BV)Ase1p binds MTs directly. 2.5 μg of (BV)Ase1p was bound to Taxol-stabilized MTs as in Fig. 3 A (s, supernatant; p, pellet). (B) Ase1p bundles MTs. Pure recombinant protein was mixed with Oregon green–labeled MTs (Bar, 5 μm). (C) Electron micrograph of MT bundles prepared by negative staining of samples incubated for 15 min with (BV)Ase1p or buffer only control (inset) (Bars, 50 nm).

B

C

plete the fast phase, the mitotic spindle collapsed during the fast phase. In wild-type cells, the mean rate for the mitotic fast phase was 0.56 ± 0.19 μm/min and the slow phase was 0.18 ± 0.06 μm/min, which is consistent with values previously reported (Kahana et al., 1995; Yeh et al., 1995; Straight et al., 1997, 1998). In the *ase1Δ* mutant cells, the fast phase rate of 0.42 ± 0.17 μm/min was slightly slower then wild type, but not significantly different by an unpaired *t* test. Thus, Ase1p is essential for the mitotic slow phase.

The conclusion that Ase1p is required for the slow phase of anaphase is supported by genetic analyses. Previous studies have suggested that neither the mitotic fast phase nor slow phase is essential, but cells impaired in both functions are inviable (Hoyt et al., 1992; Straight et al., 1998). Cells lacking Cin8p do not execute a mitotic fast phase, whereas cells lacking Kip1p have an impaired slow phase (Straight et al., 1998). We have found that *ase1Δ* is synthetically lethal with *cin8Δ*, but not with *kip1Δ*. From 19 tetrads, we obtained 5 viable *ase1Δ cin8Δ* double mutant spores out of the 19 expected, where all 5 of the double mutants displayed a marked growth defect, and from 14 tetrads, 14 viable *ase1Δ kip1Δ* double mutant spores out of the 14 expected. These genetic data are consistent with Ase1p being essential for the mitotic slow phase.

Finally, we determined whether in the absence of Ase1p spindle midzone MTs depolymerize, in addition to losing bipolar interactions. Using rapid single focal plane imaging (streaming) of *ase1Δ* cells, we found that MTs abruptly depolymerize during spindle collapse (Fig. 5 D). The rate of MT depolymerization was similar to the rate observed for
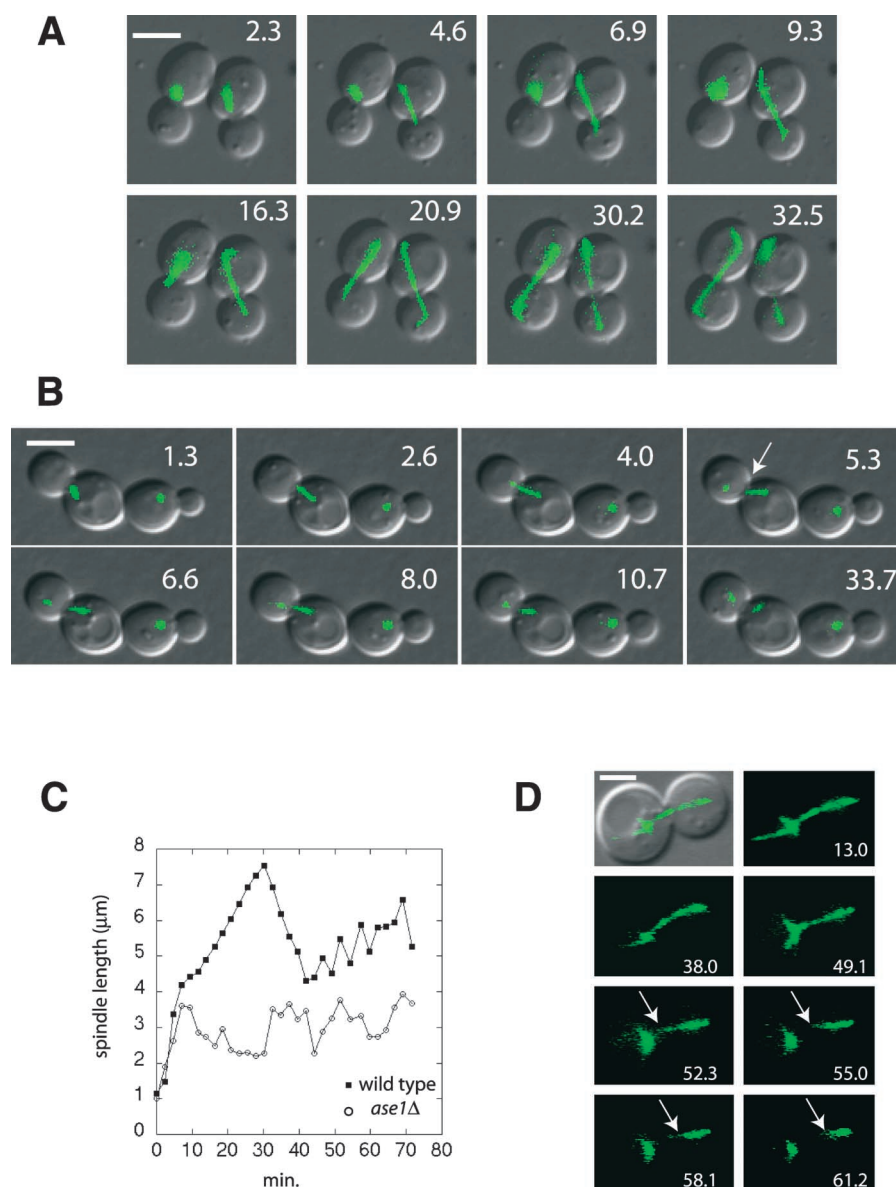
MT depolymerization upon spindle disassembly in wild-type cells at the end of mitosis ($\sim$0.18 μm/sec; Maddox et al., 2000). Taken together, our data suggest that loss of Ase1p results in little or no defect in the mitotic fast phase of spindle elongation, but rather a highly penetrant spindle collapse at the beginning of the slow phase.

However, Ase1p may play additional roles at other stages in mitosis. For example, in time-lapse movies of *ase1Δ* cells, we noticed a delay in the initiation of anaphase in many cells. This led us to suspect that Ase1p may play a role within the metaphase spindle. One possible explanation for the observed delay is that loss of Ase1p function leads to the activation of the spindle checkpoint (Hoyt, 2001). We have observed that *ase1Δ* shows synthetic lethality with a *mad1Δ* mutant, and that in *ase1Δ* mutant cells released from α-factor, there is about a 20-min delay in the metaphase to anaphase transition (unpublished data). The simplest explanation for this observation is that loss of Ase1p decreases the stability of the polar MTs in preanaphase spindles and therefore indirectly affects the function of kinetochore MTs. However, we cannot exclude the possibility that during preanaphase, Ase1p has a more direct role in regulating kinetochore MTs.

Ase1p overexpression is sufficient to induce premature spindle elongation

Having found that Ase1p was necessary for anaphase spindle stability, we next determined if Ase1p overexpression might be sufficient to trigger spindle elongation. Cells carrying a *GAL::ASE1* centromeric plasmid were arrested by hydroxyurea (HU) treatment, which arrests cells in late S phase with

Figure 5. **Ase1p is required for anaphase spindle stability.** (A) Anaphase in a wild-type cell. MTs are labeled with GFP–Tub1p. Numbers indicate the time in minutes (Bar, 5 μm). (B) Anaphase in *ase1Δ* cells. Arrow indicates spindle collapse. (C) Spindle length in wild-type and *ase1Δ* cells. The examples shown are for the wild-type cell on the right in A and the *ase1Δ* cell on the left in B. (D) Single focal plane imaging of an *ase1Δ* cell during mid-anaphase. Arrow indicates the end of the depolymerizing spindle MTs. Numbers indicate the time in seconds (Bar, 2.5 μm).

short (1.5–2 μm) preanaphase mitotic spindles. High levels of Ase1p expression were induced by addition of galactose to the medium. Images of cells were acquired at 1-h intervals after induction, and spindle lengths were measured. Ase1p overexpression was sufficient to induce spindle elongation. Within the first hour after induction, the spindles grew to twice the normal length and then displayed a slow increase to approximately three times the normal length by 6 h (Fig. 6 A). At the later time points, we also observed that this spindle elongation was sufficient to deform the shape of the nucleus and, in rare cases, appeared to actually separate DNA masses (Fig. 6 B, bottom panel). The spindle elongation induced by Ase1p overexpression could be due to Ase1p-promoted MT polymerization or to Ase1p-induced advancement of the cell cycle. To distinguish between these two possibilities, the steady-state levels of Pds1p and Clb2p were monitored during the course of the experiment. The levels of both Pds1p and Clb2p remained constant throughout the time course of Ase1p induction, suggesting that the cells neither initiate anaphase (i.e., degrade Pds1p) nor exit mitosis (i.e., degrade

Clb2p; Fig. 6 C). Additionally, we have found that Ase1p overexpression also induced spindle elongation in cells lacking *CDC23* function, demonstrating that the ability of Ase1p to promote spindle elongation was not restricted to S phase (unpublished data). This demonstrates that Ase1p is sufficient to promote premature spindle elongation, probably by cross-linking and stabilizing spindle MTs.

Ase1p is immobile within the spindle midzone during spindle elongation

Because Ase1p is a MAP and is required for the integrity of the midzone, we considered the possibility that Ase1p might be part of a static or semi-static spindle matrix. We therefore characterized the turnover of Ase1p on anaphase spindles. First, we monitored the behavior of GFP–Ase1p expressed at native levels in living cells. We monitored GFP–Ase1p localization during anaphase relative to the localization of the kinetochore protein Nuf2p–GFP (Kahana et al., 1995). During anaphase, the kinetochores are closely associated with the spindle poles. As expected, GFP–Ase1p (*n* = 3) localizes
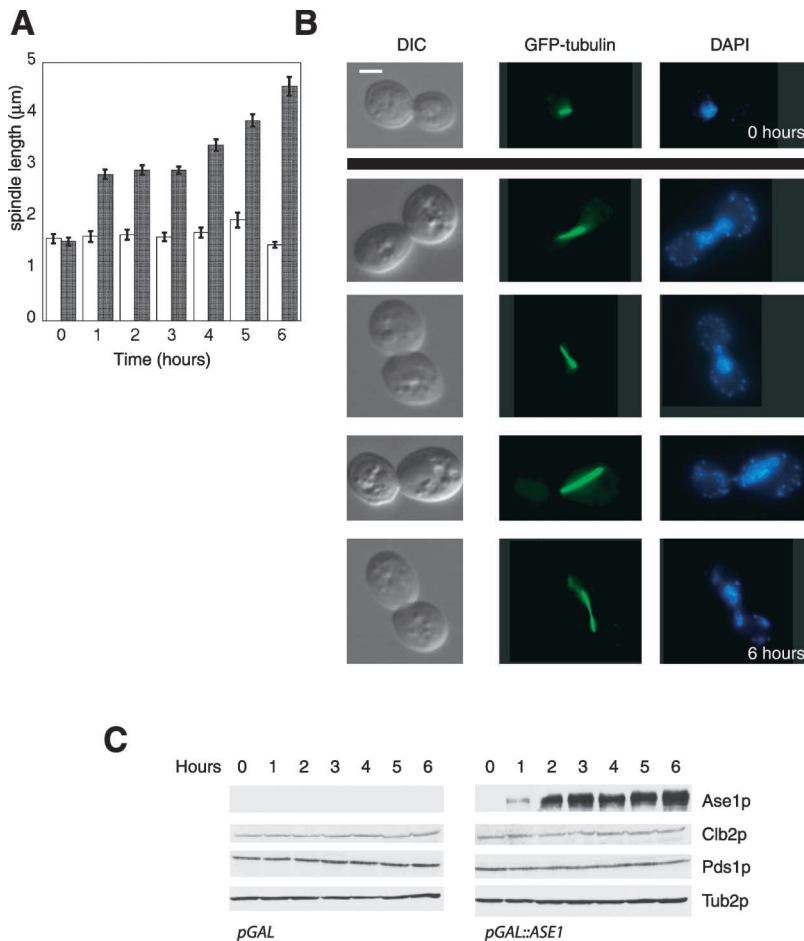
Figure 6. **Ase1p overexpression induces spindle elongation in HU-arrested cells.** (A) Measurements of spindle length upon the overproduction of Ase1p in HU-arrested cells. The average spindle lengths for vector only control are shown in open columns, and the average spindle lengths for Ase1p-overexpressing cells are in dark gray columns. More then 60 cells were counted at each time point. Error bars show standard deviations of the means. (B) Images of Ase1p-overexpressing cells at the indicated time points. HU was used at a final concentration of 200 mM (Bar, 2 μm). (C) Ase1p overexpression does not induce cell cycle progression. Shown are Western blots of extracts from the indicated time points probed with rabbit polyclonal antibodies against the indicated proteins.
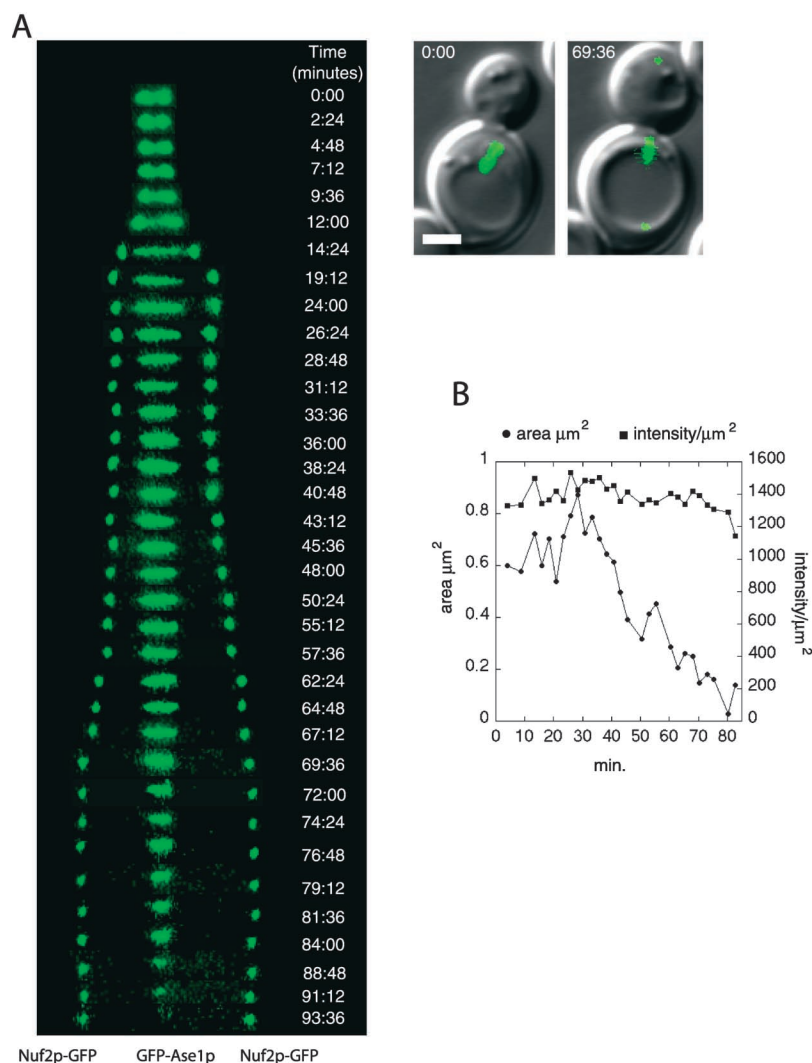
to the midzone. Strikingly, the length of the GFP–Ase1p midzone bar decreases as the spindles complete anaphase (Fig. 7, A and B; Pellman et al., 1995). The decrease in the length of the GFP–Ase1p midzone bar closely parallels what is known to be the decrease in antiparallel MT overlap as the spindle elongates (McIntosh, 1994; Winey et al., 1995). Similar observations were made with a GFP–Ase1p-DB ($n = 3$), which contains a mutation in the "destruction box" required for ubiquitin-dependent proteolysis of Ase1p at the end of mitosis (Juang et al., 1997). This suggests that Ase1p proteolysis is not necessary for the observed decrease in GFP–Ase1p midzone localization. Quantitative measurements (the average grayscale value per pixel) of GFP–Ase1p fluorescence (Fig. 7 B) revealed that the fluorescence per unit area does not change throughout anaphase. This provides support for the idea that Ase1p is not accumulating within the spindle midzone.

Next, we wished to determine if Ase1p behaves in a manner similar to other MAPs, which usually show a very dynamic and rapid rate of association and dissociation with MTs. As the turnover rates for nontubulin spindle components have not been measured in budding yeast, we first measured the FRAP of Cin8p–GFP. Based on the behavior of its BimC homologue, Eg5, this kinesin motor protein is predicted to have a very dynamic association with the mitotic spindle (Kapoor and Mitchison, 2001). In wild-type cells, we observed that Cin8p–GFP in anaphase spindles has a half-time of recovery of 28 ± 7 s ($n = 5$; Fig. 8 A).

This rate of recovery is similar to that of Eg5 (Kapoor and Mitchison, 2001). Next, we wished to test if Ase1p function had an effect on the overall recovery rate of Cin8p–GFP. A similar rate of recovery in anaphase cells was observed in $ase1\Delta$ mutant cells, with a half-time of 26 ± 10 s ($n = 6$, Fig. 8 B). Thus, Cin8p–GFP has a highly dynamic association with the mitotic spindle in budding yeast, and it does not appear that the presence of Ase1p affects the overall population turnover rate of Cin8p. However, whether Ase1p affects the lateral mobility of GFP–Cin8p along spindle MTs could not be determined because of limitations in spatial resolution.

The slow turnover rate of Ase1p contrasts sharply with the turnover rate of Cin8p. In initial experiments, we targeted a small portion of the central GFP–Ase1p midzone signal and observed that there was no recovery within the first 2 min after photobleaching (unpublished data). We therefore repeated the FRAP protocol acquiring images at 5-min intervals. We photobleached a small central part of the GFP–Ase1p signal to ensure that there would be a spindle-associated pool left to allow recovery (Fig. 8 C). The average half-time for recovery was ∼7.5 min ($n = 5$), where the percent recovery was at the level of 70–80% (Fig. 8 D). It is important to note that because of the need to preserve a robust GFP–Ase1p signal from the unbleached region, we were unable to acquire a large number of post-bleach images. Due to this restriction, imposed by the relatively low signal of GFP–Ase1p, our measurements may underestimate the half-time for recovery of

Figure 7. **Ase1p localization during spindle elongation.** (A) A kymograph of GFP–Ase1p and Nuf2p–GFP localization during anaphase B. As the spindle elongates, the Ase1p bar decreases in length. Numbers indicate the time in minutes (Bar, 2 μm). (B) The area occupied by Ase1p in the spindle midzone decreases during anaphase. The average area of GFP–Ase1p signal and the average fluorescence intensity (grayscale values per pixel) of Ase1p are plotted versus time, for the cell shown in A. The area occupied by one pixel in these images is 0.004 μm².

GFP–Ase1p. Together our data demonstrate that Ase1p is relatively immobile within the midzone during anaphase.

Discussion

Ase1p is the founding member of a recently recognized family of midzone-specific MAPs. Although the overall homology between the family members is modest, they do share one CM and a conserved organization (Mollinari et al., 2002; this work). The family members contain coiled-coil motifs followed by a CM, which in turn is followed by a divergent COOH terminus. We have shown here that in Ase1p, the most highly conserved 16–amino acid motif is necessary for in vivo function. This CM may contribute to MT binding and/or MT stabilization, an idea that is supported by the observation that in PRC1, a 213–amino acid region, encompassing the CM, has MT binding activity in vitro (Mollinari et al., 2002). Ase1p, carrot MAP65, and PRC1 appear to be rod-shaped, and Ase1p, carrot MAP65, nt-MAP65, and PRC1 all bind and bundle MTs in vitro (Chan et al., 1999; Smertenko et al., 2000; Mollinari et al., 2002). Furthermore, nt-MAP65 has been shown to stabilize MTs directly in vitro (Smertenko et al., 2000). Finally, Ase1p, carrot MAP65, PRC1, and nt-MAP65 all localize to

the spindle midzone in mitosis (Pellman et al., 1995; Jiang et al., 1998; Chan et al., 1999; Smertenko et al., 2000). These similarities in molecular design, biochemical function, and in vivo localization strongly suggest that the Ase1p-related proteins constitute a distinct class of midzone MAPs.

Our experiments suggest that Ase1p is not a component of a large macromolecular complex and likely functions as a homodimer. Importantly, our study excludes the possibility that Ase1p is a kinesin motor light chain or a stoichiometric subunit of a mitotic regulator (for example, INCENP in the aurora B complex). Whether or not other family members exist as dimers remains to be determined, but dimerization might be expected because of coiled-coil motifs found in all family members. Although our findings demonstrate that Ase1p is not in a stable complex with other proteins, Ase1p may participate in important transient or low-affinity interactions.

The central role of Ase1p in anaphase spindle elongation

As a homodimeric spindle midzone MT-bundling protein, Ase1p is ideally situated to regulate spindle elongation. We found that Ase1p was essential for the mitotic slow phase, a mitotic phase that is thought to be the result of midzone MT polymerization coupled with antiparallel MT sliding (Oppenheim et al., 1973; McIntosh, 1994). This role
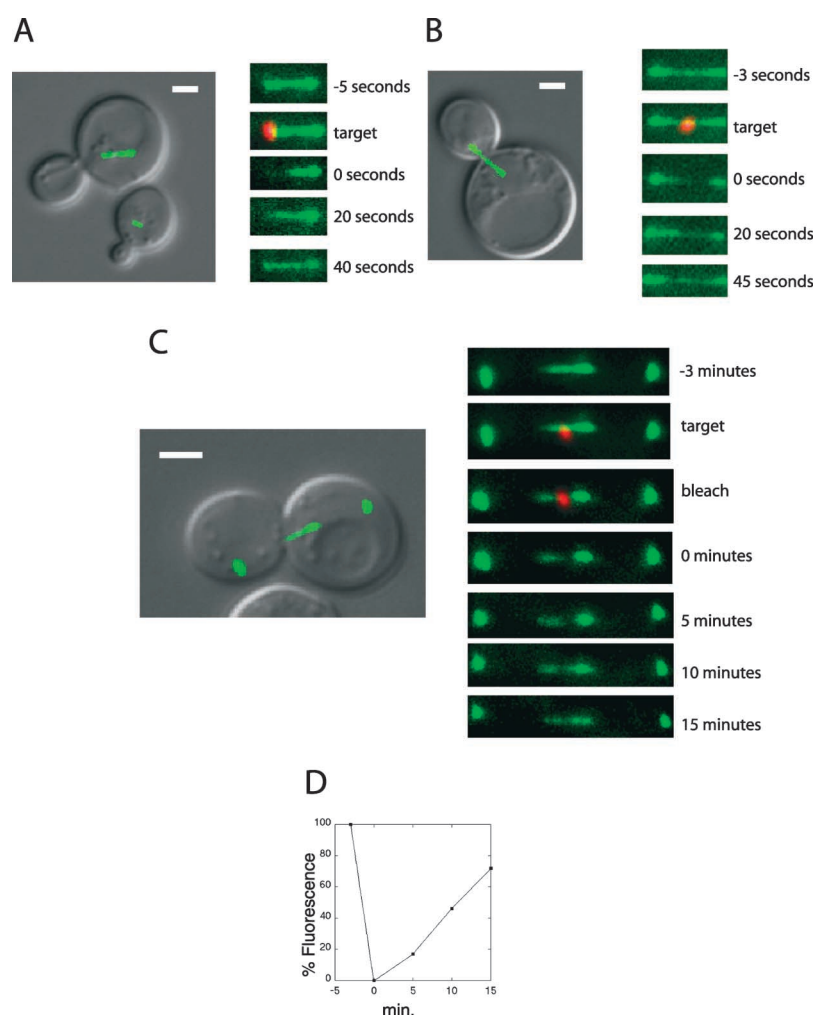
Figure 8. **Slow turnover of Ase1p at the spindle midzone.** (A) FRAP of Cin8–GFP in an anaphase cell. Red dot indicates the area targeted by the laser. Time points are indicated in seconds (Bar, 2 μm). (B) FRAP of Cin8–GFP in an *ase1Δ* anaphase cell. (C) FRAP of GFP–Ase1p in an anaphase cell. Time points are in minutes relative to the time of bleaching. Red dot indicates the area targeted by the laser (Bar, 2 μm). (D) Fluorescence intensity of a 0.35 μm² area at intervals after photobleaching. The values were normalized to the initial fluorescence intensity.

within the spindle midzone and mitotic slow phase appears to be conserved. In *C. elegans*, a mutant in *SPD-1*, the homologue of Ase1p, was found to have defects in spindle integrity in anaphase that ultimately gave rise to defects in cytokinesis (O'Connell et al., 1998; Verbrugghe, K., and J. White, personal communication). In cultured cells, RNAi treatments against PRC1 also led to defects of premature spindle collapse in mitosis (Mollinari et al., 2002). Thus, the Ase1p-related family of MAPs appears to be essential to maintain anaphase spindle bipolarity in evolutionarily distant organisms.

Strikingly, we found that overexpression of Ase1p is sufficient to trigger premature spindle elongation. This effect is not due to advancement of the cell cycle and is therefore most likely due to promoting polymerization of interdigitated MTs. We propose that the Ase1p-related family of MAPs plays a central and conserved role in spindle elongation, likely by bundling antiparallel MTs and by promoting their polymerization and/or stabilization. This hypothesis is supported by the previous observation that nt-MAP65 stabilizes MTs directly in vitro (Smertenko et al., 2000).

Finally, the cell cycle control of Ase1p is consistent with its molecular function described here. Ase1p expression is restricted to mitosis in a pattern similar to that of the mitotic cyclin Clb2p (Pellman et al., 1995). Like Clb2p, Ase1p is a substrate of the APC. Proteolysis of Ase1p destabilizes telo-

phase spindles and contributes to the timely disassembly of the mitotic spindle. Nondegradable Ase1p delayed, but did not block, spindle disassembly, leading to the supposition that the APC might have other important substrates that control anaphase spindle stability (Juang et al., 1997). More recent work has indeed identified a set of spindle proteins regulated by APC-dependent proteolysis, such as two budding yeast BimC motors, Cin8p and Kip1p (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Furthermore, the APC-mediated activation of separase and destruction of Pds1p are also required for normal anaphase spindle function (Uhlmann et al., 2000; Severin et al., 2001; Sullivan et al., 2001). The mechanisms underlying these regulatory events are just starting to be understood. However, defining the function of the spindle-associated targets is an important first step.

Ase1p is a static element of the spindle midzone

Ase1p shows a discrete localization to the spindle midzone that appears to mirror the extent of overlap of antiparallel MTs during anaphase. We have observed that the length of the GFP–Ase1p bar shrinks during late anaphase. This suggests that the GFP–Ase1p signal corresponds to the region of overlap between antiparallel MTs. This supports the idea that Ase1p, and perhaps other Ase1p-related proteins, preferentially binds antiparallel MTs.

In vivo, we observed that Ase1p is immobile relative to the kinesin motor protein Cin8p or in comparison to previous experiments on α-tubulin (Maddox et al., 2000). By FRAP analysis, photobleached GFP–Ase1p shows some recovery, albeit at a very slow rate. This recovery could be due to the very slow lateral diffusion of Ase1p within the spindle midzone MT lattice. Alternatively, it may be that Ase1p is completely immobile within the midzone and the appearance of recovery is the result of the addition of newly synthesized GFP–Ase1p to the spindle. Either way, the length of time it takes for GFP–Ase1p to recover is several orders of magnitude greater than would be expected from the diffusion constant that is predicted from our hydrodynamic data (2.5 × 10^{-7} cm²/s). It is important to note that our experimental design left a large pool of unbleached GFP–Ase1p adjacent to the bleached region that could diffuse into the bleached region if it were mobile.

There are several possible explanations for the limited diffusion of Ase1p along midzone MTs. First, the organization of the MT lattice in the spindle midzone is itself predicted to form a geometrical barrier to diffusion (Jacobson and Wojcieszyn, 1984; Blum et al., 1989). Second, the affinity of Ase1p for MTs, and perhaps the even higher affinity of Ase1p for antiparallel MTs, would further limit lateral mobility (Gershon et al., 1985; Blum et al., 1989). Combined, these effects could lower the effective diffusion constant for Ase1p by several orders of magnitude, explaining our FRAP results (Blum et al., 1989). One predicted consequence of the presence of an immobile midzone MT cross-bridge is that the midzone may act to decrease the rate of spindle elongation. Immobile spindle cross-linking MAPs could act to resist the work of kinesin motor proteins sliding apart antiparallel MTs during elongation. Indeed, laser ablation experiments in several organisms suggest that an intact spindle midzone slows the rate of spindle elongation (Aist and Berns, 1981; Aist et al., 1991, 1993).

Finally, in mitotic spindles assembled in frog egg extracts, although Eg5 is dynamic, it exhibits unexpectedly low lateral mobility (Kapoor and Mitchison, 2001). This restricted lateral mobility of Eg5 provided evidence for an immobile spindle matrix. As expected, we found that loss of Ase1p does not affect the turnover of Cin8p on early anaphase spindles, the rate of which is determined by the ability of spindle-associated Cin8p to exchange with a soluble pool. However, the small compact size of early anaphase yeast spindles prevented us from measuring the lateral mobility of Cin8p.

Ase1p provides the molecular functions proposed for a spindle matrix to the spindle midzone

In general, there are three functional characteristics that have been proposed for a spindle matrix: promote the organization of midzone MTs into highly ordered arrays, participate in the execution of spindle elongation in anaphase B, and provide a static or relatively immobile structure that limits the lateral mobility of kinesin motor proteins (Pickett-Heaps et al., 1997; Scholey et al., 2001; Bloom, 2002; Kapoor and Compton, 2002). One of the most remarkable features of the spindle midzone in fungi and animal cells is its highly ordered geometrical arrays of antiparallel MTs (Ding et al., 1993; Mastronarde et al., 1993; Winey et al., 1995). We

found that Ase1p binds and bundles MTs, is required for spindle elongation, and is immobile within the spindle midzone. Thus, Ase1p appears to have many of the characteristics for a proposed spindle matrix.

One view of the spindle matrix envisions a static and MT-independent structure: essentially another cytoskeleton to organize the MT-based spindle (Pickett-Heaps et al., 1997; Walker et al., 2000; Scholey et al., 2001; Bloom, 2002; Kapoor and Compton, 2002). At present, there is little functional evidence to support the presence of such a structure in budding yeast. One spindle-associated protein, Fin1p, was recently found to assemble into MT-independent filaments in vitro. However, deletion of *FIN1* had no discernible mitotic defect (Bloom, 2002; van Hemert et al., 2002). More recently, the spindle matrix model has been expanded to encompass the idea that the spindle matrix might in fact be a dynamic assemblage of mitotic motor proteins, such as the bipolar tetramers related to BimC (Scholey et al., 2001). However, the very high dynamicity and low processivity of BimC motors are at variance with the notion that these proteins are the spindle matrix (Crevel et al., 1997; Gheber et al., 1999; Kapoor and Mitchison, 2001; Kapoor and Compton, 2002). Further, the unexpectedly low lateral mobility of Eg5 on spindle MTs suggested the presence of another immobile spindle component, hypothesized to be the genuine spindle matrix.

Whatever the molecular composition of this proposed spindle matrix, our work demonstrates that in the absence of Ase1p, the hypothetical matrix cannot maintain the stability of the anaphase spindle. An essential role for Ase1p-related proteins in anaphase spindle stability has also been observed in *C. elegans* and vertebrate tissue culture cells (O'Connell et al., 1998; Verbrugghe, K., and J. White, personal communication; Mollinari et al., 2002). Thus, in widely varied cell types, a MAP-independent matrix, if present, cannot maintain spindle bipolarity in the absence of an Ase1p-related protein.

Based upon our experiments, we propose a model for spindle midzone organization. We suggest that networks of immobile MAPs, particularly the Ase1p-related proteins, form the molecular basis of an MT-dependent spindle matrix in the spindle midzone in most cell types. Immobile cross-linking MAPs are ideally suited to couple MT organization with MT polymerization. This network of static cross-bridges within a highly organized MT lattice might work in concert with highly dynamic motor proteins to maintain spindle bipolarity and promote spindle elongation in anaphase B.

Materials and methods

Yeast genetics
Media and genetic techniques were as previously described (Rose et al., 1990). All strains used are in the W303 background. Details of plasmid and strain construction will be provided upon request.

Protein sequence analysis and multiple sequence alignments
Coiled-coil domains were identified using "MacStripe 2.0" (Lupas et al., 1991). Protein homology was determined using the standard settings of BLASTp (Altschul and Gish, 1996). Multiple sequence alignments were performed using T-COFFEE (Notredame et al., 2000). Values for pair-wise identities were determined using BLAST2 (Tatusova and Madden, 1999). The tree diagram was generated using the BLOCKS Multiple Sequence Alignment Processor (Henikoff et al., 2000).

Protein biochemistry

Native yeast extracts prepared by liquid nitrogen lysis, coimmunoprecipitations, and hydrodynamic measurements and calculations were performed as previously described (Schuyler and Pellman, 2002). The lysis buffer was 50 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 1 mM PMSF, and Complete Mini-EDTA Free protease inhibitor mix (Roche). Large-scale production of recombinant protein in insect cells was performed as previously described (Zaloudik et al., 1997). A frozen pellet of 1.23×10^9 Ase1p-expressing Sf9 cells from 1 liter of culture was resuspended in 10 ml of lysis buffer (as above, with 1.0% Triton X-100). The cells were extracted in detergent by incubation on ice for 10 min. The suspension was homogenized for 2 min using a tight-fitting Pyrex pestle (Fisher Scientific). Cell lysates were spun at 13,000 g at 4°C. The supernatants were collected and filtered using a 0.8-μm sterile syringe filter (Millipore). A dot-blot assay was used to follow the recombinant protein during purification.

The supernatant was loaded onto a 6-ml MONO S column (Bio-Rad Laboratories) preequilibrated in lysis buffer lacking detergent. Protein was eluted from the column with a linear NaCl gradient. Ase1p–MYC–6xHis elutes at 500 mM NaCl. Ase1p-containing fractions were pooled and run through a 2-ml Ni-NTA column (QIAGEN) preequilibrated with 25 mM Hepes-NaOH (pH 7.4), 75 mM NaCl, and 25 mM imidazole. Protein was eluted with an imidazole gradient. Fractions were pooled and loaded onto a 1-ml MONO Q (Bio-Rad Laboratories) preequilibrated in 25 mM Hepes-NaOH (pH 7.4) and 75 mM NaCl. Ase1p–MYC–6xHis was eluted with a NaCl gradient, at ∼200 mM NaCl. Fractions were pooled and diluted with an equal volume of ddH$_2$O and then loaded onto a 250-μl MONO S polishing column (Bio-Rad Laboratories) to concentrate the protein. The purified Ase1p–MYC–6xHis was eluted with a step gradient of NaCl.

MT binding and bundling was performed as previously described (Butner and Kirschner, 1991; Goode and Feinstein, 1994; Desai et al., 1999). Negative staining of MTs was performed as previously described (Desai et al., 1999).

Time-lapse video microscopy and FRAP

Fluorescence time-lapse imaging was performed at room temperature as previously described (Tirnauer et al., 1999). For photobleaching, a 377-nm Nitrogen pulse laser was used (Photonics Instruments, Inc.). Incoherent light was synchronized and amplified using a coumerin blue 440 chemical chamber, which emits at a wavelength of ∼440 nm. The emitted beam was focused and projected onto the back of a dichroic mirror in the filter house and reflected through the objective lens onto the sample. For photobleaching GFP–Ase1p, cells were typically exposed to 10 pulses at 2–6 ns per pulse during an 800-ms window. Laser targeting and calibration were performed on a mirror. The laser was attenuated with a graded neutral density filter. After setting the filter to maximum attenuation, the attenuation was decreased until a hole was spotted in the mirror after a single laser pulse. The average target size had a diameter of 667 nm at the 1/2-max of a Gaussian profile, as determined by using the "profiler" option in Openlabs (Improvision). This method of targeting gives an average target area of $0.35 \pm 0.2\ \mu m^2$. The intensities of the prebleached target site and a region adjacent to it were measured. The intensity values of these sites were set as 100% fluorescence. The adjacent region was measured at each time point to measure the photobleaching caused by imaging. The intensity of the post-bleach target area was measured, and this value was set as 0% fluorescence.

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