Two Microtubule-associated Proteins Required for Anaphase Spindle Movement in Saccharomyces cerevisiae

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Abstract. In many eucaryotic cells, the midzone of the mitotic spindle forms a distinct structure containing a specific set of proteins. We have isolated ASE1, a gene encoding a component of the Saccharomyces cerevisiae spindle midzone. Strains lacking both ASE1 and BIK1, which encodes an S. cerevisiae microtubule-associated protein, are inviable. The analysis of the phenotype of a bik1 ase1 conditional double mutant suggests that BIK1 and ASE1 are not required for the assembly of a bipolar spindle, but are essential for anaphase spindle elongation. The steady-state levels of Ase1p are regulated in a manner that is consistent with a function during anaphase: they are low in G1, accumulate to maximal levels after S phase and then drop as cells exit mitosis. Components of the spindle midzone may therefore be required in vivo for anaphase spindle movement. Additionally, anaphase spindle movement may depend on a dedicated set of genes whose expression is induced at G2/M.

MITOSIS is achieved by sequential structural changes in the mitotic spindle. The centrosomes or microtubule organizing centers duplicate, a bipolar spindle is assembled, and then at anaphase, sister chromosomes are distributed to opposite poles so that each daughter cell receives a complete chromosome set. Anaphase is divided into two stages, anaphase A, when the chromosomes move to the spindle poles, and anaphase B, when the spindle is extended and the poles are segregated between mother and daughter cells. The typical mitotic spindle has three classes of microtubules: polar microtubules that extend from the centrosomes and interdigitate with polar microtubules from the opposite pole, kinetochore microtubules that connect chromosomes to the centrosome, and astral microtubules that radiate away from the spindle into the cytoplasm and to the cell cortex. The ordered changes in spindle structure are proposed to be determined primarily by proteins that bind along the length and at the ends of microtubules (23, 46, 50). These microtubule-associated proteins (MAPs), are thought to act either by regulating the polymerization of tubulin, or as “motors,” microtubule-based mechanochemical enzymes (30, 65).

Experimental evidence supports two contrasting mechanisms for anaphase pole separation—a pushing mechanism (the poles are pushed apart by forces generated from the central spindle), and a pulling mechanism (the poles are pulled apart by forces acting on astral microtubules) (5, 42). The idea of a pushing force is supported by three-dimensional electron microscopic reconstructions of mitotic spindles from a variety of organisms (17, 42, 47). During anaphase, polar microtubules increase in length and the region of overlap between polar microtubules decreases (in varying degrees in different organisms). This finding suggests that polar microtubules polymerize and slide past each other in the midzone of the spindle. Additional evidence for a pushing mechanism comes from experiments showing that the central spindle of diatoms and Schizosaccharomyces pombe can elongate in vitro in the absence of astral microtubules (14, 43). Evidence for a pulling force comes from the observation in Fusarium that the rate of separation of the poles is increased by breaking the central spindle (1), and in vertebrate epithelial cells, that the centrosomes continue to separate after their microtubule arrays no longer overlap (72).

The spindle midzone plays an important role in both the pushing and pulling models. In many organisms, the spindle midzone undergoes a dramatic morphological transformation during anaphase (17, 42, 48). Polar microtubules, which are initially distributed randomly throughout the central spindle, become organized into regular geometric arrays, with microtubules from one pole lying adjacent to microtubules from the opposite pole. This high degree of organization is likely to be imposed by the protein components of the spindle midzone. In animal cells, the spindle midzone is surrounded by an electron-dense material that has been termed the stem body matrix (18).

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number of animal cell proteins have now been identified that localize to the spindle midzone at specific stages of the cell cycle (18, 53). Although information about the in vivo function of these proteins is limited, several functions have been suggested. In models where the poles are pushed apart, the midzone is envisioned to have a role in generating the forces that drive anaphase B. In models where the poles are pulled apart, the midzone proteins are thought to serve as a guide to orient spindle movement and limit its rate (5, 42).

The ability to isolate mutations affecting mitosis in Saccharomyces cerevisiae has provided a key link to the in vivo function of spindle proteins in centrosome duplication, spindle assembly, and kinetochore function (54). Mutant analysis supports pushing forces exerted by polar microtubules as the primary mechanism for anaphase spindle elongation in S. cerevisiae (68). A cold-sensitive allele of TUB2, the gene encoding β-tubulin, polymerizes nuclear (containing both polar and kinetochore microtubules) but not cytoplasmic microtubules. Spindle elongation and nuclear division occur, but the spindle is misoriented, and nuclear division takes place entirely within the mother cell. This result suggests that in S. cerevisiae, cytoplasmic microtubules are required to orient the spindle with the plane of cell division, but that the nuclear microtubules generate the forces for anaphase spindle elongation.

Despite the extensive characterization of yeast mitotic mutants, none encoding a spindle protein that is required primarily for anaphase spindle elongation has yet been described. A good candidate for a protein involved in anaphase pole movement is the nonmotor MAP encoded by the BIKI gene (8). The BIKI gene product colocalizes with the nuclear spindle (8). bikl null mutants are viable, but exhibit defects in several microtubule-dependent functions such as, chromosome segregation, nuclear migration, and karyogamy (8, 70). The structure of Biklp is similar to that of a human MAP, CLIP-170 (or restin) (9, 57). At the NH₂ terminus, both proteins contain copies of a 40-amino acid peptide motif found in a number of MAPs. The microtubule binding domain of CLIP-170 is at the NH₂ terminus and requires this peptide motif for microtubule binding (57). In both proteins the NH₂-terminal domain is followed by a region of heptad repeats, and then a COOH-terminal domain containing a zinc-binding motif termed the CCHC box.

To determine the role of BIKI in mitosis, mutations showing synthetic lethality with a bikl null mutation were isolated. One of the genes isolated in this screen, ASE1 (anaphase spindle elongation) encodes a new S. cerevisiae MAP that localizes to the midzone of the anaphase spindle. Like one human spindle midzone protein, Aselp levels are low in G1 but accumulate during mitosis (11). Analysis of strains deficient in BIKI and ASE1 function indicates that these genes are essential for anaphase spindle elongation.

**Materials and Methods**

**Strains and Microbial Techniques**

Media and genetic techniques were as described in Sherman et al. (66). Yeast plasmids and linear DNA fragments for gene replacement were transformed into yeast by lithium acetate transformation (34). The mating pheromone, α-factor was added to log phase cultures in synthetic complete media to a final concentration of 6 µg/ml for 3–4 h until >80% of the cells had arrested with an unbudded shmo polymorph. To release cells from α-factor arrest, cells were collected by filtration, washed, and resuspended in fresh media. Hydroxyurea was added directly to cultures in synthetic complete media to a final concentration of 0.1 M for ~4 h until >80% of cells had a large budded morphology. Lists of yeast strains and plasmids used in this study are provided in Tables I and II.

**Genetic Techniques**

The ADE3 sectoring assay and the strains for the synthetic lethality screen have been described (7, 36). MATa bikl::TRP1 ade2 ade3 strains were transformed with a BIKI URA3 ADE3 2µ plasmid. ade2 ade3 strains are white but ade2 ADE3 strains accumulate a red pigment. Because BIKI is not an essential gene, the BIKI ADE3 URA3 plasmid will be lost during growth on nonselective medium. This results in colonies that are red but contain white sectors that are the result of plasmid loss. Strains that con-

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Table II. Plasmids Used in This Study

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<td>PKK-1</td>
<td>A 177-bp fragment encoding three copies of the myc tag in pUC119</td>
<td>D. Kornitzer and S. Kron (Whitehead Institute, Cambridge, MA)</td>
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*PKK-1 A 177-bp fragment encoding three copies of the myc tag in pUC119 D. Kornitzer and S. Kron (Whitehead Institute, Cambridge, MA)*

*Table II. Plasmids Used in This Study*
the nonsectoring phenotype of asel strains. In addition, a different strain (W303) than the one used for the studies (7) described in this paper has a weak temperature-sensitive growth defect when the ASEL gene is deleted. The epitope-tagged ASEL complements the growth defect in this strain.

**Microscopic Analysis of Cells**

To examine cell morphology culture cells were stained with 4,6-diamidino-2-phenylindole (DAPI). Microtubule structures were observed after fixation in formaldehyde with the rat anti-tubulin antibody, YOLO/34 (58). Spindle pole bodies were observed after fixation for 5 min at 24°C in formaldehyde with a mixture of mAbs against a 90-kD SPB protein (61, 63). To visualize the intracellular location of ASEL marked with the epitope tag, cells were double labeled YOLO/34 and the 9E10 mAb directed against the myc epitope. Fluorochrome-conjugated secondary antibodies were from Jackson ImmunoResearch Labs., Inc. (West Grove, PA). For double labeling, control experiments demonstrated that cross-species reactivity did not contribute to the final images when species-specific secondary antibodies were used. These experiments also demonstrated that light channel spillover did not contribute to the final images. For quantitative analysis greater than 200 consecutive cells were counted.

Cells were prepared for thin-section electron microscopy by a modification of the procedure of Byers and Goetsch (13). Glutaraldehyde fixed cells were digested in 0.02 mg/ml Oxalyticase (Enzogenetics, Corvallis, OR) and Glusulase (Du Pont NEN, Boston, MA) at a dilution of 1/5 in 0.17 M KH2PO4, 30 mM sodium citrate, and 0.03 M 2-mercaptoethanol until the majority of cells had become spheroplasts as determined by phase microscopy. Spheroplasts were post-fixed with 2% osmium tetroxide in 0.05 M sodium acetate, pH 6.1, and en block stained with 1% aqueous uranyl acetate at 4°C overnight. Samples were dehydrated in graded ethanol and embedded in polyBed 812 (Polysciences, Inc., Warrington, PA). Thin sections were collected on 75 mesh pioloform (Ted Pella, Inc., Irvine, PA). Samples were examined with a Philips EM410 microscope at 60 kV. To determine the percentage of bikel asel cells that had splayed microtubules consecutive fields were scanned for cells with short spindles. These were then qualitatively assessed to have normal or splayed microtubules.

**Flow Cytometry**

Yeast strains were grown to early log phase at 24°C, collected by filtration and resuspended at 36°C. The cells were fixed with 70% ethanol for 30 min at 24°C. Cells were stained with propidium iodide as described (33). Stained cells were analyzed on a FACSTAR (Becton Dickinson Immuno cytometry Sys., Mountain View, CA) using LYSIS II version 1.2. Cell sorting was performed based on the flow cytometry profile. Sorted cells were collected, restrained with propidium iodide, and nuclear morphology was examined by epifluorescence.

**Western and Northern Blot Analysis**

Cells were prepared for Western blotting by vortexing for 3 min with an equal volume of glass beads in buffer containing 50 mM TRIS, pH 7.5, 1 mM EDTA, 5% 2-mercaptoethanol and protease inhibitors. Extracts were then boiled in Laemmli sample buffer and the amount of Aselp and β-tubulin in 50 μg of total cell protein was determined by an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Epitope-tagged Aselp was detected with 9E10 mAb. β-tubulin was detected with a monoclonal antibody to 63). To visualize the intracellular location of ASE1 detected with 9E10 mAb. 13-tubulin was detected with a monoclonal antibody to 9E10 mAb. 13-tubulin was detected with a monoclonal antibody to

**Isolation and Sequence Analysis of ASE1**

The ASE1 gene was isolated by complementation of an asel strain using a plasmid library. Targeted integration was used to establish that the cloned fragment contains the ASE1 gene (see Materials and Methods). The chromosomal location of the ASE1 gene was determined by hybridization of an ASE1 probe to separated chromosomes and to a mapped yeast DNA library. ASE1 is on the right arm of chromosome 15~100 kb from the centromere (see Materials and Methods).

Sequence analysis of the ASE1 locus revealed that the ASE1 open reading frame is predicted to encode an 885-amino acid protein with an unmodified molecular mass of 102 kD and an estimated pI of 9.80 (GenBank accession number 420235). Aselp is predicted to have an α-helical secondary structure throughout most of the length of the protein (22, data not shown). Two short regions (amino acids 142-171 and amino acids 493-527) of Aselp are predicted to have a probability of greater than 0.8 of forming a coiled-coil structure by the method of Lupas et al. (40). Data base searches revealed low level homology to many proteins that have a coiled-coil α-helical structure. Although ASE1p does not have homology to known microtubule-associated proteins, the basic pI and regions predicted to form a coiled-coil structure are common features of MAPs from other organisms.

A Null Allele of ASE1 Is Viable

A deletion of ASE1 lacking the coding sequence for amino acids 1-858 was created by homologous recombination (see Materials and Methods). Strains carrying the asel deletion showed no difference from ASE1 strains when analyzed for the following phenotypes: growth rate, temperature sensitivity, and unilateral and bilateral karyogamy defects. However, like bikel strains, the asel strain is moderately supersensitive to the microtubule depolymerizing drug benomyl (the asel strain has a growth defect at 5 μM/ml as compared to 15 μM/ml for the isogenic control). To determine the phenotype of a strain containing deletions of both BIK1 and ASE1, a MATα asel null strain was crossed to a MATα bikel null strain. No double mutant progeny were recovered from this cross indicating that the asel deletion allele, like the asel alleles isolated in our
screen, shows synthetic lethality with a bik1 null mutant (see Materials and Methods).

*Ase1p Has a Novel Localization on the Mitotic Spindle*

A simple explanation for the lethality of bik1 asel double mutants would be that ASE1 encodes a MAP that performs a similar function to Bik1p. To test this hypothesis, the intracellular location of Ase1p was determined by immunolocalization of a fully functional epitope-tagged Ase1p (see Materials and Methods). Three tandem copies of a DNA sequence encoding a peptide epitope from the myc protein (21) were introduced into the COOH-terminal coding sequence of ASE1. A centromere-based plasmid containing epitope-tagged ASE1 was then transformed into an asel null strain. An asynchronous culture of this strain was fixed and stained with two mAbs, one directed against tubulin and the other against the myc epitope. The localization of Ase1p in cells in different stages of the cell cycle was visualized by indirect immunofluorescence (Fig. 1). In G1 cells that only contain astral microtubule structures, no specific staining of Ase1p is observed (Fig. 1, A–C). In cells that contain short bipolar spindles, Ase1p shows clear localization along the mitotic spindle (Fig. 1, D–F). Little or no staining is observed at the ends of the spindle corresponding to the region of the spindle pole bodies (the yeast centrosomes). In cells that have completed anaphase with segregated DNA and long spindles (telophase), Ase1p staining is restricted to an ~2-μm bar at the midzone of the spindle (Fig. 1, G–I). This pattern of localization was observed in almost all cells examined. 98% of cells with G1 astral microtubules (n = 200) had no

![Figure 1. Intracellular localization of epitope-tagged Ase1p. The photographs are from an asynchronous culture containing epitope-tagged Ase1p on a centromere-based plasmid. (A–C) Two G1 cells. (D–F) Three S/G2 cells with short bipolar spindles. (G–I) Three anaphase/telophase cells. (A, D, and G) Cells stained with 9E10, an α-myc antibody recognizing epitope-tagged Ase1p. The cells were stained with a CY3-conjugated goat anti-mouse secondary antibody and visualized with rhodamine fluorescence. (B, E, and H) Cells stained with Yol1/34 an anti-tubulin mAb. The cells were stained with a fluorescein isothiocyanate-conjugated secondary antibody and visualized with fluorescein isothiocyanate fluorescence. (C, F, and I) DAPI staining of DNA to visualize nuclei. Control experiments with an isogenic strain carrying ASE1 plasmid lacking the epitope tag showed no staining. Bar, 5 μm.](image-url)
specific staining of Ase1p. 92% of cells with short bipolar spindles (n = 231) showed staining of Ase1p on the nuclear microtubules, and 97% of cells with long spindles and segregated DNA (n = 206) showed Ase1p staining at the midzone of the spindle.

Multiple Centrosomes in Strains Lacking BIK1 and ASE1 Function

The lethality of bik1 asel double mutants and the viability of the single null mutants indicates that either gene can perform a function that is essential for viability. To determine the essential function missing in the double mutant, we created a conditional (temperature sensitive) bik1 asel strain whose defects could be analyzed at the nonpermissive temperature. The desired double mutant was identified by combining several in vitro constructed alleles of BIK1 with the five alleles of ASE1 isolated in the screen for synthetic lethality with bik1. One double mutant, bik1-S419 asel-1, has a strong recessive temperature-sensitive growth defect. The bik1-S419 asel-1 strain has a modest growth defect at 24 and 30°C but does not grow at 36°C. The bik1-S419 allele encodes a point mutation in the COOH-terminal zinc binding motif of Bik1p. The bik1-S419 mutation is a partial loss of function allele because bik1-S419 tub1-1 double mutants have a growth defect (but are viable) and because strains carrying bik1-S419 have a partial karyogamy defect (data not shown). The bik1-S419 asel-1 strain will hereafter be referred to as bik1 asel.

The bik1 asel strain exhibits dramatic defects in spindle morphology at the nonpermissive temperature. bik1 asel and BIK1 ASE1 strains were grown at 24°C, shifted to 36°C, fixed, and then prepared for indirect immunofluorescence. BIK1 ASE1 strains show characteristic microtubule structures representing all stages of the yeast cell cycle: G1 asters, S/G2 short bipolar spindles, and long anaphase/telophase spindles (Fig. 2, A and B). By contrast, the bik1 asel strain displays three classes of abnormal microtubule structures (Fig. 2, C and D): large budded cells with very short (less than 1 μm) bipolar spindles, budded cells with single spindle poles, and budded or unbudded cells with multiple short spindles and multiple spindle poles. These three classes of spindle structures are observed in approximately equal numbers in the bik1 asel strain (Table III). The finding that the bik1 asel strain accumulates cells with multiple spindle pole bodies (SPB) was confirmed by staining with a mixture of monoclonal antibodies against the 90-kD spindle pole body component (61), (Fig. 2, G and H). Although it was not possible to photograph more than three spindle pole bodies in a single focal plane, most cells with more than two SPBs appeared to have four SPBs when the entire cell was examined by changing focal planes. A small percentage of cells appeared to have more than four SPBs, but the exact number of poles in these cells was difficult to determine because of the overlap of adjacent poles. Perhaps the most notable feature of the bik1 asel strain at 36°C is the absence of post-anaphase cells with long spindles and segregated DNA (two foci of DAPI staining material).

Microtubules in bik1-S419 and asel-1 single mutant

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Cultures of BIK1 ASE1, bik1-S419 ASE1, BIK1 asel-1, and bik1 asel strains were shifted to 37°C for 6 h and examined by anti-tubulin immunofluorescence and DAPI staining. The percentage of cells with the morphologies shown on the left were determined by counting greater than 200 cells. From top to bottom, the following classes of cells were seen: unbudded cells with a single spindle pole, budded cells with single spindle poles, budded cells with short bipolar spindles, post-anaphase cells with long spindles and segregated DNA, post-anaphase cells with broken spindles, either unbudded or budded cells with multiple spindle poles in a single nucleus, and anucleate cells.

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strains were examined by indirect immunofluorescence (Table III). The spindle structures in the bik1-S419 strain are indistinguishable from the BIK1 ASE1 strain. The microtubule structures in the asel-1 strain had abnormalities similar to the bik1 asel strain but were less dramatic and less frequent. Many large budded cells had short bipolar spindles. Additionally, there were large budded cells with single poles, and rare cells with multiple SPBs.

The bik1 asel strain was examined by transmission electron microscopy (Fig. 3). In wild-type yeast cells, the SPB is composed of a trilaminar structure and the spindle is formed from arrays of microtubules emanating from the opposite SPBs (12; Fig. 3 A). In the bik1 asel strain at 36°C the SPBs had a normal morphology, but microtubule structures were abnormal. In bik1 asel cells with short bipolar spindles, the microtubules from opposite poles appeared splayed (Fig. 3 B), suggesting that BIK1 and ASE1 may be required to maintain the organization of anti-parallel microtubules. The frequency of the splayed spindle structure was 75% when consecutive cells (n = 24) with short spindles were examined. In the cells with single spindle poles, duplicated SPBs were found adjacent to each other (Fig. 3 C).

Cells lacking BIK1 and ASE1 Assemble Bipolar Spindles, but Fail to Elongate the Spindle during Anaphase

A culture of the bik1 asel strain was synchronized in G1 at the permissive temperature and then shifted to the non-permissive temperature to determine when the first observable defects in cell division occur. A MATa bik1 asel strain and wild-type control were synchronized in G1 with α-factor at 24°C. Cell cultures were then released from this block into fresh media at 36°C and samples were removed at times after release and examined by phase microscopy and fluorescence microscopy for tubulin distribution. Progression into the cell cycle was measured by scoring the percentage of cells with large buds and the percentage of cells containing both segregated DNA and long spindles spanning the length of the mother and daughter cells (telophase cells). In the BIK1 ASE1 strain, cells progress normally into the cell cycle after release from an α-factor

![Figure 3. Electron microscopic analysis of BIK1 ASE1 and bik1 asel cells. Cells were prepared for electron microscopy after a 4-h shift to 36°C. (A) Two BIK1 ASE1 cells. (B) A bik1 asel cell with a short spindle exhibiting splayed microtubules. (C) A bik1 asel cell with a single pole having two side by side SPBs. (D) A bik1 asel cell with three SPBs in the plane of the section. Bar, 1 μm.](image-url)
block. Dividing cells form large buds around the time of bipolar spindle assembly and remained with large buds until cytokinesis (Fig. 4 A).

In the bikl ase1 strain, mitosis is uncoupled from bud growth and cytokinesis (Fig. 4 A). Although the bikl ase1 strain initiates and completes budding with similar kinetics as the BIK1 ASE1 control, bikl ase1 cells assembled bipolar spindles, but fail to complete anaphase normally (Fig. 4 B). After release from α-factor, when mothers and daughters are roughly the same size, wild-type cells have completed anaphase, but the bikl ase1 cells (85%) have short spindles and an undivided nucleus. After longer periods at the nonpermissive temperature, a percentage (15%) of bikl ase1 cells show highly defective anaphase spindle structures, with very short anaphase spindles and nuclear division occurring entirely within the mother cell (Fig. 4 B, 90 min).

α-Factor block and release experiments performed with a single mutant bikl-5419 strain gave similar results to those performed with the BIK1 ASE1 strain. After release from α-factor, the majority of single mutant ase1-1 cells complete anaphase but do so more slowly than the control strain. Some cells showed spindles defects reminiscent of those seen in bikl ase1 cells (data not shown).

Loss of BIK1 and ASE1 Function During S Phase Does Not Destroy the Bipolar Spindle or Alter the Subsequent Mitosis

Experiments were performed to test whether BIK1 and ASE1 are required during S phase, when the bipolar spindle is assembled (12). Yeast cells treated with the DNA synthesis inhibitor, hydroxyurea (HU), arrest in S phase as large budded cells with an undivided nucleus and a short spindle. If BIK1 and ASE1 were required to maintain spindle pole separation in cells arrested with hydroxyurea, then the spindle would be predicted to collapse after a shift to the nonpermissive temperature. bikl ase1 and control strains were arrested with HU at the permissive temperature (24°C), and then shifted for 1.5 h to the nonpermissive temperature (36°C) in the presence of HU. At 24°C both strains arrest with short spindles (96% of BIK1 ASE1 cells; 84% of bikl ase1 cells). After 1.5 h at 36°C both strains remained arrested with intact short spindles (98% of BIK1 ASE1 cells; 83% of bikl ase1 cells). Although we do not know precisely how rapidly BIK1 and ASE1 function is lost in the bikl ase1 strain at 36°C, marked defects in spindle structure were observed at 36°C 70 min after release from a G1 block (Fig. 4 B). Therefore, incubation at 36°C for 1.5 h should have been sufficiently long to observe an effect. Under similar conditions, the spindle collapses in strains such as cin8-3 kip1Δ (63) that have a defect in spindle assembly.

Loss of BIK1 and ASE1 function might result in a defect in S phase, but this defect might not be observable until later in the cell cycle. This hypothesis was tested by the following experiment. bikl ase1 and control cells were arrested in HU at 24°C. The cells were then split into three samples. One sample was shifted to 36°C for 1.5 h and then released from the HU block at 24°C. Another sample was
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were arrested in S phase with 0.1 M hydroxyurea for 4 h. Each percentage of cells that successfully completed anaphase were identified through the experiment. Open squares indicate cells that were maintained at 24°C for 1.5 h and then released from the HU block at 36°C. The final sample was maintained in HU at 36°C. (A) A diploid BIK1/ASE1 strain after a 6 h shift to 36°C. (B) A homozygous bikl asel diploid strain after 6 h at 36°C. Fixed cells were stained with propidium iodide and analyzed by flow cytometry. Cell cycle position was assessed by cell morphology and by the level of histone (H2B) mRNA. H2B transcripts reach maximal levels at the G1/S boundary and then decline at the end of S phase (28). Ase1p is not detectable in cells arrested with α-factor but then increases to maximal levels after the decline of H2B mRNA.

Figure 5. Loss of BIK1 and ASE1 Function during S Phase does not block subsequent anaphase. Early exponential cultures of control BIK1/ASE1 (A) and bikl asel double mutant (B) strains were arrested in S phase with 0.1 M hydroxyurea for 4 h. Each culture was then split. Closed circles indicate cells that were shifted to 36°C for 1.5 h in HU, washed and then released at 24°C. Sectioned squares indicate cells that were maintained at 24°C throughout the experiment. Open squares indicate cells that were shifted to 36°C for 1.5 h in HU, washed and then released at 36°C. Cells were collected at intervals for DAPI staining and the percentage of cells that successfully completed anaphase were identified as large-budded cells with two separate masses of DAPI-staining material.

shifted to 36°C for 1.5 h and then released from the HU block at 36°C. The final sample was maintained in HU at 24°C for 1.5 h and then released from the HU block at 24°C. The percentage of cells that completed anaphase, as reflected by successful nuclear division, was then scored by DAPI staining. Fig. 5 B shows that a 1.5-h temperature shift during S phase does not affect the subsequent mitosis when bikl asel cells are released at 24°C. These experiments suggest that the bikl asel double mutant does not have an S phase defect.

Loss of BIK1 and ASE1 Function Uncouples Mitosis from Cytokinesis and DNA Replication

Despite the defect in mitosis, the bikl asel strain at 36°C does not undergo arrest with a uniform terminal phenotype (Table III). After a 6-h shift to 36°C, both budded and unbudded cells are present in roughly equal proportions. A significant proportion of the unbudded cells are anucleate. The proportion of anucleate cells that were observed in this experiment is comparable to the proportion of cells with multiple spindle pole bodies (Table III), consistent with the idea that these two cell types result from division of the same parent cell. The finding that the number of anucleate cells is slightly greater than the number of cells with multiple SPBs is likely to be due to multiple rounds of division by cells with multiple SPBs. The fact that the strain fails to undergo cell cycle arrest is also confirmed by the fact that in synchronized cells at the nonpermissive temperature the percentage of large budded cells decreases with time (Fig. 4 A).

To test the hypothesis that the bikl asel strain continues DNA replication despite the block in nuclear division, the DNA content of a homozygous bikl asel temperature-sensitive diploid strain was measured at 36°C by flow cytometry. By contrast with a wild-type diploid strain (Fig. 6 A), the double mutant accumulates two abnormal populations of cells, a hypoploid population and a hyperploid population (Fig. 6 B). Microscopic examination of cells isolated from the hypoploid peak by fluorescence-activated cell sorting showed that these cells were unbudded anucleate cells. Flow cytometry of the double mutant at 24°C also identified hypoploid and hyperploid peaks that were smaller than the peaks observed from cultures at 36°C. This finding is consistent with the growth defect of the double mutant at the permissive temperature. The homozygous double mutant, therefore, accumulates anucleate cells and cells with increased DNA content.

Cell Cycle-specific Accumulation of Ase1p

One explanation for the absence of immunofluorescence staining for Ase1p in G1 cells is that there is less Ase1p in these cells (Fig. 1). The steady state levels of Ase1p were therefore examined in synchronized cells after release from a G1 block with α-factor. Samples were collected at intervals for phase microscopy, Western blot, and Northern blot analysis. Cell cycle position was assessed by cell morphology and by the level of histone (H2B) mRNA. H2B transcripts reach maximal levels at the G1/S boundary and then decline at the end of S phase (28). Ase1p is not detectable in cells arrested with α-factor but then increases to maximal levels after the decline of H2B mRNA.

Figure 6. DNA content of bikl asel and control cells at 36°C. (A) A diploid BIK1/ASE1 strain after a 6 h shift to 36°C. (B) A homozygous bikl asel diploid strain after 6 h at 36°C. Fixed cells were stained with propidium iodide and analyzed by flow cytometry. Fluorescence intensity is indicated on the X axis. Cell number is indicated on the Y axis. After the 6-h shift the bikl asel strain accumulates anucleate cells (peak A) and a hyperploid population of cells (peak H).
Figure 7. Ase1p accumulates after S phase. (A) Cell cycle changes in the steady state levels of Ase1p. A strain carrying epitope-tagged ASE1 on a centromere plasmid was arrested in G1 with α-factor. Cells were released from the α-factor block at 30°C and aliquots were removed at the indicated times for analysis of cell morphology, Ase1p and β-tubulin levels, and H2B RNA levels. (B) Comparison of Ase1p levels in exponentially growing cells and in cells arrested in S phase with hydroxyurea. An isogenic control strain containing an ASE1 plasmid lacking the epitope is included to show the specificity of the α-myc antibody for epitope-tagged Ase1p. (C) Comparison of Ase1p localization in exponentially growing cells and in cells arrested in S phase with hydroxyurea. The top panels show Ase1p localization detected with α-myc antibody. The bottom panels show tubulin staining detected with YOL1/34. Bar, 5 μm.

When cells have developed large buds, Ase1p levels then decline as cells exit mitosis and undergo cytokinesis (Fig. 7 A). Because the S. cerevisiae bipolar spindle is assembled during S phase (12), Ase1p probably does not reach maximal levels until after spindle assembly. An independent test of this conclusion was performed by examining Ase1p levels and localization in cells arrested with HU. Cells arrested with HU have significantly reduced levels of Ase1p relative to cells from an asynchronous culture (Fig. 7 B). Furthermore, indirect immunofluorescence shows that Ase1p is not associated with the spindle in HU-arrested cells (Fig. 7 C). These experiments suggest that Ase1p becomes a significant component of the bipolar spindle only after S phase.

Discussion

An S. cerevisiae Component of the Midzone of the Anaphase Spindle

Using a screen for mutations that show synthetic lethality with BIK1, we have identified ASE1, which encodes a component of the midzone of the S. cerevisiae anaphase spindle. The association of Ase1p with microtubules varies during the cell cycle. In G1 cells there is no Ase1p staining on astral microtubules, in S/G2 cells, Ase1p staining is seen along the nuclear microtubules of short spindles, and in late anaphase/telophase cells the Ase1p staining is restricted to an ~2-μm bar in the midzone of the spindle. The extent of the midzone staining corresponds to the extent of overlap between polar microtubules during anaphase in S. cerevisiae (73).

The absence of Ase1p staining in G1 cells can be attributed to cell cycle changes in steady state levels of Ase1p. Ase1p is not detectable in G1 cells arrested with α-factor, but accumulates to maximal levels after the decline of H2B mRNA at the end of S phase. Since the bipolar spindle in S. cerevisiae is assembled late in S phase (12), it is likely that Ase1p accumulates after spindle assembly. This interpretation is further supported by the fact that Ase1p levels are low, and do not show detectable association with the bipolar spindle of cells arrested with the DNA synthesis inhibitor, HU.

The variation in the levels of Ase1p is due, at least in part, to changes in the steady state level of the ASE1 mRNA. The level of the ASE1 transcript oscillates in a similar manner to the messages for B-type cyclins and other G2/M-specific transcripts (D. Pellman, unpublished results). Furthermore, it is likely that ASE1 is coordinately regulated with these other G2/M-specific transcripts. The ASE1 promoter (−287 to −251: tcccaagttaaaatataaa) contains an element that binds MCM1 and a factor termed SFF that is found in the promoters of several G2/M-regulated transcripts (35, 41).

One other fungal protein, S. pombe cut7, which encodes a protein related to the microtubule-based motor, kinesin, is associated with the spindle midzone (26, 27). Unlike Ase1p, cut7 is also strongly associated with the spindle poles. Temperature-sensitive mutants of S. pombe cut7 are defective in spindle assembly. Because the cut7 gene product localizes to the spindle poles in addition to the central spindle, the defect in spindle assembly could reflect cut7 function at either or both sites.

In view of the functional overlap between BIK1 and ASE1, one might expect colocalization of Ase1p and Bik1p. The interpretation of the experiments on Bik1p localization is complicated by the fact that we have been able to detect Bik1p by immunofluorescence only when it is overexpressed approximately 10-fold from a 2μ plasmid. When overexpressed, Bik1p is not associated with microtubules in G1 cells but is observed along the entire length of the spindle (nuclear microtubules) during mitosis (reference 8 and D. Pellman, unpublished results). Failure to observe restricted localization of Bik1p to the spindle mid-
The bik1 ase1 Mutant Has a Defect in Anaphase B

The simplest hypothesis to explain the phenotype of the bik1 ase1 strain at 36°C is that the growth limiting defect in this strain is in spindle elongation. The mutant phenotypes can then be explained by the following model. The bik1 ase1 strain can duplicate the SPB normally and establish a short (metaphase-like) bipolar spindle. However, this bipolar spindle is defective and fails to elongate, resulting in failure of nuclear division. If there is no cell cycle arrest, subsequent cytokinesis will produce an anucleate cell and an unbounded cell with two SPBs. This “G1” cell with two SPBs will reinitiate the cell cycle resulting in a cell with four SPBs and increased DNA content (diagrammed in Fig. 8).

The hypothesis that bik1 ase1 is defective in spindle elongation is supported by the analysis of the spindle morphology of cells at the nonpermissive temperature. After release from a G1 block, the earliest point in mitosis that the bik1 ase1 mutant exhibits a spindle defect is after the assembly of a bipolar spindle. Although bik1 ase1 cells form bipolar spindles, these structures are abnormally short and the microtubules appear splayed. The double mutant therefore manifests a functional defect in anaphase, where spindle elongation either does not occur or is markedly abnormal. The fact that the bik1 ase1 strain does not undergo cell cycle arrest and undergoes abnormal spindle elongation supports the idea that spindle elongation itself is defective and not simply entry into anaphase.

The conclusion that bik1 ase1 cells are able to form bipolar spindles is supported by several observations. First, 85% of bik1 ase1 cells form bipolar spindles after release at the nonpermissive temperature from a G1 block. Second, bik1 ase1 cells arrested in HU maintain bipolar spindles even after prolonged incubation at the nonpermissive temperature. Finally, loss of bik1 ase1 function in HU-arrested cells does not prevent subsequent anaphase when cells are released from the S phase block at the permissive temperature. Because bipolar spindle structure is abnormal in bik1 ase1 cells (Fig. 3 B), we suggest that BIK1 and ASE1 are required after the bipolar spindle is assembled to establish structures required for anaphase.

These experiments suggest that the bik1 ase1 double mutant is defective in anaphase. However, we cannot exclude the possibility that either Bik1p or Ase1p has an additional role at an earlier stage of mitosis. For example, there could be a defect in the rate of spindle assembly that is not obvious in our experiments analyzing timepoints from fixed samples of cells. A delay in spindle assembly could be inferred from the fact that a proportion of bik1 ase1 cells at the nonpermissive temperature have monopolar spindles. However, note that synchronized cells at the nonpermissive temperature accumulate monopolar spindles after the majority of cells have formed bipolar spindles (see Fig. 4 legend). At least a proportion of the bik1 ase1 cells with monopolar spindles may therefore arise by spindle collapse after spindle assembly. A role in some other aspect of mitosis is perhaps more likely for BIK1 because BIK1 is expressed throughout the cell cycle (D. Pellman, unpublished results). The expression of ASE1 only after the spindle is assembled makes it unlikely that Ase1p is required earlier in the cell cycle.

One other S. cerevisiae mutant, esp1-1 has a multiple spindle pole body phenotype similar to that of the bik1 ase1 mutant (6, 45). esp1 cells fail to elongate short spindles and progress through the cell cycle with similar kinetics to wild-type cells (45, 69). Mutations in related genes from other fungi result in similar phenotypes (44, 71).

Based on our analysis of bik1 ase1 cells and on the analysis of esp1 cells by McGrew et al. (45), we suggest that the accumulation of multiple spindle bodies is a signature phenotype for a defect in anaphase B in S. cerevisiae.

Anaphase Spindle Elongation in Saccharomyces

Genetic experiments in S. cerevisiae have suggested a role for both pushing and pulling forces on the spindle poles during anaphase. Our experiments support the view that a pushing force generated by nuclear microtubules is of primary importance. Yeast strains with deletions of the genes encoding the cytoplasmic dynein heavy chain (20, 39), or candidate light chains (15, 49, 51), are viable and undergo anaphase B. However, these mutants are defective in orienting the spindle with the plane of cell division and, therefore, produce a significant number of cells with a binucleate phenotype. These results have been interpreted to suggest that pulling forces generated from cytoplasmic microtubules are important for spindle orientation, but make only a minor contribution to anaphase B.

Although the binucleate phenotype has been taken to indicate a defect in the function of cytoplasmic microtu-
It is also possible that this phenotype could be produced by a defect in nuclear microtubules. Because nuclear migration is dynamic, with extensive movement of the nucleus and rotation of the spindle before anaphase (reference 55 and J. Kahana and P. Silver, personal communication), a delay in spindle elongation relative to the timing of cytokinesis could produce binucleate cells. In fact, one of the most striking mitotic defects in \textit{bik1} strains is the accumulation of binucleate and anucleate cells (8). The colocalization of \textit{Bik1p} with nuclear microtubules supports the hypothesis that the binucleate phenotype of \textit{bik1} strains results from a defect in nuclear microtubule function.

Proteins associated with nuclear microtubules are the obvious candidates for generating a pushing force on the poles. Aside from \textit{Bik1p} and \textit{Ase1p}, several \textit{S. cerevisiae} proteins have been isolated that localize to nuclear microtubules, two kinesin-like proteins, Cin8p and Kip1p (32, 60), and one non-motor MAP, Stu1p (56). The phenotype of the \textit{bik1} \textit{asel} strain is strikingly different from strains containing mutations in these genes. Strains lacking \textit{STU1} or \textit{CIN8} and \textit{KIP1} display marked defects in spindle assembly. \textit{CIN8} and \textit{KIP1} are also required to maintain the structure of short bipolar spindles in cells arrested in S phase with HU (63). In addition to their function early in mitosis, recent experiments suggest that Cin8p and Kip1p also contribute to anaphase spindle movement. A triple mutant, lacking the function of \textit{CIN8}, \textit{KIP1} and \textit{DYN1}, is defective in anaphase B (64). Coordinated spindle movement may therefore require both proteins whose function is required throughout mitosis, such as Cin8p and Kip1p, as well as proteins whose function is restricted to specific stages of mitosis.

### The Spindle Midzone During Anaphase

Evidence from animal cells suggests that the spindle midzone may be a distinct sub-organelle composed of a unique set of proteins (18). The phenotype of the \textit{bik1 asel} double mutant suggests that in vivo the spindle midzone plays a critical role in anaphase B. We have considered two possible functions for \textit{Bik1p} and \textit{Ase1p}. First, these proteins may be components of a filamentous matrix associated with the spindle (4, 18, 52). This matrix could regulate microtubule polymerization, organize microtubules from opposite poles, or provide a scaffold for microtubule-based motors to generate movement of microtubules during spindle elongation. Alternatively, either \textit{Bik1p} or \textit{Ase1p} could be a subunit of a microtubule-based motor.

Our finding that \textit{Bik1p} and \textit{Ase1p} are required after the time at which the spindle is formed supports the view that mitosis is driven by the ordered assembly and functioning of spindle-associated proteins. Furthermore, the regulated pattern of \textit{Ase1p} expression suggests that anaphase is not simply a continuation of the process of spindle assembly, but requires the function of dedicated genes. The identification of \textit{ASE1} from \textit{S. cerevisiae} should facilitate the isolation of other regulated components of the anaphase spindle and help to reveal the mechanism by which the spindle midzone functions during anaphase.

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