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Polyploids require Bik1 for kinetochore–microtubule attachment

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The attachment of kinetochores to spindle microtubules (MTs) is essential for maintaining constant ploidy in eukaryotic cells. Here, biochemical and imaging data is presented demonstrating that the budding yeast CLIP-170 orthologue Bik1is a component of the kinetochore-MT binding interface. Strikingly, Bik1 is not required for viability in haploid cells, but becomes essential in polyploids. The ploidy-specific requirement for *BIK1* enabled us to characterize *BIK1* without eliminating nonhomologous genes, providing a new approach to circumventing the overlapping function that is a common feature of the cytoskeleton. In polyploid cells, Bik1 is required before anaphase to maintain kinetochore separation and therefore contributes to the force that opposes the elastic recoil of attached sister chromatids. The role of Bik1 in kinetochore separation appears to be independent of the role of Bik1 in regulating MT dynamics. The finding that a protein involved in kinetochore–MT attachment is required for the viability of polyploids has potential implications for cancer therapeutics.

Introduction

Eukaryotic cells preserve a constant DNA content or ploidy during most cell divisions. Ploidy is altered in some tissues during development, in certain congenital syndromes, and in many common cancers (Sen, 2000; Edgar and Orr-Weaver, 2001). Polyploid cells in normal tissues do not divide, possibly because increased ploidy limits the efficiency of mitosis. How the mechanism of mitosis is affected by ploidy changes is not known, but has potential implications for understanding the mechanism of antimitotic chemotherapeutic agents.

Constant ploidy is maintained by the mitotic spindle, which distributes replicated chromosomes to daughter cells. A crucial step in mitosis is the attachment of spindle microtubules (MTs)* to the kinetochores: sister kinetochores must form bivalent attachments to MTs emanating from opposite spindle poles before the initiation of anaphase (kinetochore MTs [kMTs]; Rieder and Salmon, 1998; Maney et al., 2000). Attachment occurs by a "search-and-capture" mechanism whereby the plus ends of kMTs insert into the kinetochore (Mitchison and Kirschner, 1984; Holy and Leibler, 1994; Desai and Mitchison, 1997).

Once attached, MTs exert force on the kinetochores (Koshland et al., 1988; Inoue and Salmon, 1995; Lombillo et al., 1995). Before anaphase, attached sister chromatids undergo oscillatory movements back and forth between the spindle poles (Skibbens et al., 1993; Waters et al., 1996; Straight et al., 1997). These oscillations involve depolymerization of kMTs attached to the leading kinetochore and polymerization of kMTs attached to the lagging kinetochore (Rieder and Salmon, 1998; Hunter and Wordeman, 2000). Before anaphase, tension exerted by kMTs pulls kinetochores apart (Shelby et al., 1996; Nabeshima et al., 1998; Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000; Pearson et al., 2001). The pulling force on kinetochores is resisted by the cohesion between sister chromatids (Tanaka et al., 2000). The tension experienced by attached kinetochores may enable the cell to monitor whether all chromosomes are successfully attached to the spindle (Nicklas,

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*Abbreviations used in this paper: +TIP, plus end–tracking protein; Chip, chromatin immunoprecipitation; kMT, kinetochore MT; MAP, MT-associated protein; MT, microtubule.

Key words: kinetechore; microtubule; ploidy; Bik1; plus end–tracking protein

1997). Once all of the kinetochores are attached, anaphase is initiated by the dissolution of sister chromatid cohesion. Recent experiments suggest that defects in kinetochore–MT attachment play an important role in generating the aberrant ploidy observed in common cancers (Cimini et al., 2001; Fodde et al., 2001; Kaplan et al., 2001).

Despite extensive study, the molecular composition of the kinetochore–MT interaction surface remains poorly defined. It is widely assumed that both MT motor proteins and nonmotor MT-associated proteins (MAPs) make important contributions to this binding surface (Hyman and Sorger, 1995; Hunter and Wordeman, 2000; Maney et al., 2000). In animal cells, motor proteins are necessary for kinetochore movement. Motor proteins may be involved in the initial capture of MTs at the kinetochore, or they may function later, to regulate kMT dynamics and generate force (Maney et al., 2000). Budding yeast has provided insight into the function of the kinetochore because of the many mutants that affect kinetochore structure and function. Despite the abundance of mutants, there is no evidence for a functional role for motor proteins at the yeast kinetochore (Hildebrandt and Hoyt, 2000). The failure to observe a defect in kinetochore behavior in motor mutants may be due to functional redundancy among the seven yeast motor proteins, none of which is essential for viability (Cottingham et al., 1999). Functional overlap, a common theme among cytoskeletal proteins, presents an obstacle to the use of mutants in defining the role of individual proteins.

The role of nonmotor MAPs in kinetochore–MT attachment is less well understood than that of motors. The primary approach to identifying such MT-binding proteins has been through the characterization of the protein components of the kinetochore (Hyman and Sorger, 1995; Maney et al., 2000). An alternative approach is to characterize the MAPs that associate with the plus ends of MTs. Plus end–associated proteins or protein complexes were recently shown to be required for the interaction of astral MTs and polarized membrane sites (Brunner and Nurse, 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000; Akhmanova et al., 2001; Schuyler and Pellman, 2001b). The fact that several of these proteins also localize to the kinetochore raises the possibility that MAPs at the MT plus end contribute to the kinetochore–MT binding surface (Dujardin et al., 1998; Fodde et al., 2001; Kaplan et al., 2001).

An important class of plus end–binding proteins is the plus end–tracking proteins (+TIPs, [Schuyler and Pellman, 2001a]). +TIPs bind the plus ends of MTs by a unique mechanism. For the prototype +TIP, CLIP-170, this mechanism involves treadmilling: addition of CLIP-170 molecules to the growing plus end accompanied by release of CLIP-170 molecules behind the region of polymerization (Perez et al., 1999). +TIPs clearly regulate MT assembly in vivo, and they may also serve as adapters that link MTs to target binding sites at the membrane or kinetochore (Schroer, 2001; Schuyler and Pellman, 2001a). What is not known is whether +TIPs participate in stable binding interactions or whether they are only transiently involved in binding to target sites. In particular, it is not known if these proteins contribute to force generation and motility.

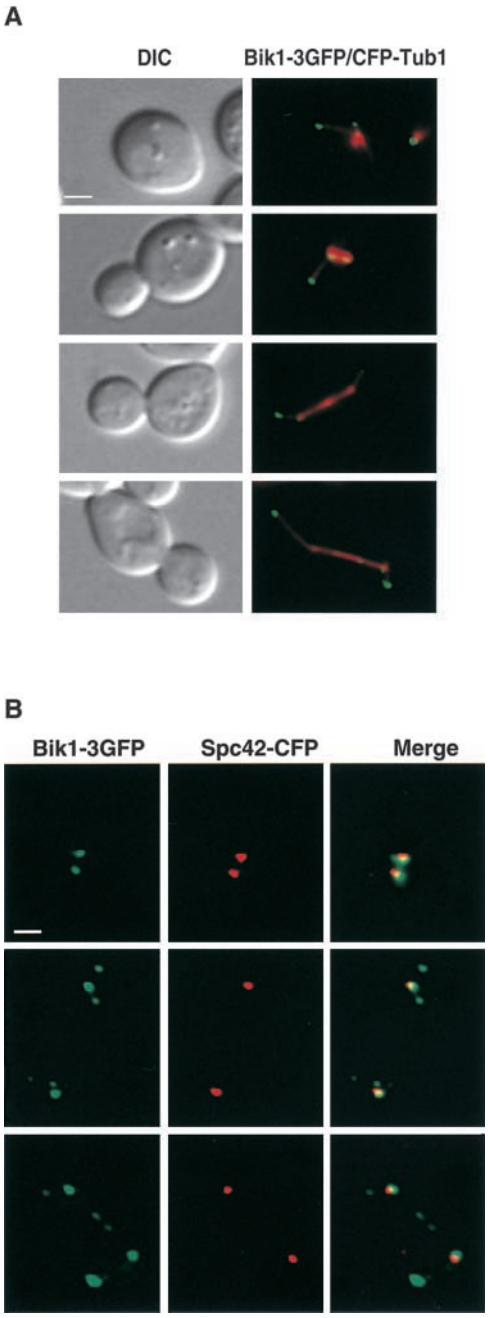

Figure 1. **Localization of Bik1 to MT plus ends and to the kinetochore.** (A) Colocalization of Bik1–3GFP (green) with CFP-Tub1 (red) at different stages of the cell cycle. Pairs of DIC (left) and merged fluorescence (right) images are shown. From top to bottom: G1 cell; preanaphase cell; anaphase cell; and telophase cell. Bar, 2 μm. (B) Bik1–3GFP localization at the kinetochore during mitosis in cells expressing Bik1–3GFP (green) and Spc42-CFP (red), a spindle pole body marker. Bar, 2 μm.

In this paper, we provide evidence that the budding yeast orthologue of CLIP-170, Bik1, is required for the viability of polyploid cells, and in polyploids, Bik1 contributes to the pulling force that maintains sister kinetochore separation before anaphase. The creation of polyploids thus provides a new experimental approach for studying the overlapping function of cytoskeletal proteins. Our finding that a protein involved in kinetochore–MT attach-

ment is selectively required for the viability of polyploid cells also may have implications for the development of antimitotic chemotherapeutic drugs.

Results

Bik1, like CLIP-170, is a plus end–tracking protein

Bik1 has sequence homology to the MT plus end–associated protein CLIP-170 (Pierre et al., 1992), but previous localization studies on cells overexpressing Bik1 indicated that, unlike CLIP-170, the protein was present along the length of all MT structures (Berlin et al., 1990). To determine whether this distribution was a consequence of overexpression, we reexamined Bik1 localization in cells expressing the protein at native levels. Bik1 was fused to three tandem copies of GFP (Bik1–3GFP). This chimera was functional as assayed by complementation of *bik1Δ* cells (see Materials and methods). Bik1–3GFP gave bright and strikingly discontinuous labeling of MTs, with discrete dots located at the plus ends of MTs (Fig. 1 A). During mitosis, Bik1–3GFP was also present in a bilobed distribution on spindle MTs where the kinetochores are known to localize. This bilobed distribution of Bik1–3GFP near the spindle poles persisted throughout all stages of mitosis (Fig. 1 B). This contrasts with a previous study that found that CLIP-170 is only transiently associated with kinetochores at prometaphase in tissue culture cells (Dujardin et al., 1998). Overall, these data show that the localization of Bik1 is similar to that of human CLIP-170 and to that of the other budding yeast MT plus end–binding protein Bim1 (Pierre et al., 1992; Perez et al., 1999; Tirnauer et al., 1999).

Human CLIP-170 has the unusual property of associating with the growing plus ends of MTs (Perez et al., 1999). Recently, human EB1 (a Bim1 orthologue), the adenomatous polyposis coli tumor suppressor protein, and components of the dynein motor complex (Xiang et al., 2000; Han et al., 2001) were found to have a similar pattern of association with MT plus ends in vivo (Brunner and Nurse, 2000; Mimori-Kiyosue et al., 2000a,b). We term this behavior "plus end-tracking" (Schuyler and Pellman, 2001a). By time-lapse fluorescence microscopy we found that Bik1–3GFP at the ends of astral MTs moved toward the periphery of the cell as was previously described for CLIP-170 and EB1 (Fig. 2 A). The Bik1–3GFP spots moved at a rate of 3.6 ± 1.5 μm/min in G1 cells ($n = 10$) and at a rate of 2.9 ± 1.1 μm/min in mitotic cells ($n = 20$). Although there is some variation in the reported growth rate of astral MTs in budding yeast (measured with different GFP reporters expressed from different promoters), the velocity of the Bik1–3GFP dots was within the range of these values (for review see Adames and Cooper, 2000). Furthermore, we found that in cells where tubulin was labeled with CFP-Tub1, the Bik1–3GFP dots were found at the distal ends of astral MTs, not along the body of the MTs, 96% of the time ($n = 170$). Together, these data show that Bik1 has the characteristics of a plus end–tracking protein.

We tested whether the MT-binding domain of CLIP-170 could functionally substitute for the corresponding region of Bik1 using a chimera that contains the CLIP-170 MT-binding domain fused to the coiled-coil and "cargo-binding" domain of Bik1. The dynamic binding of human CLIP170 to MT plus ends is mediated by its NH_2-terminal MT-binding domain (known as the CAP-Gly domain) and occurs by a

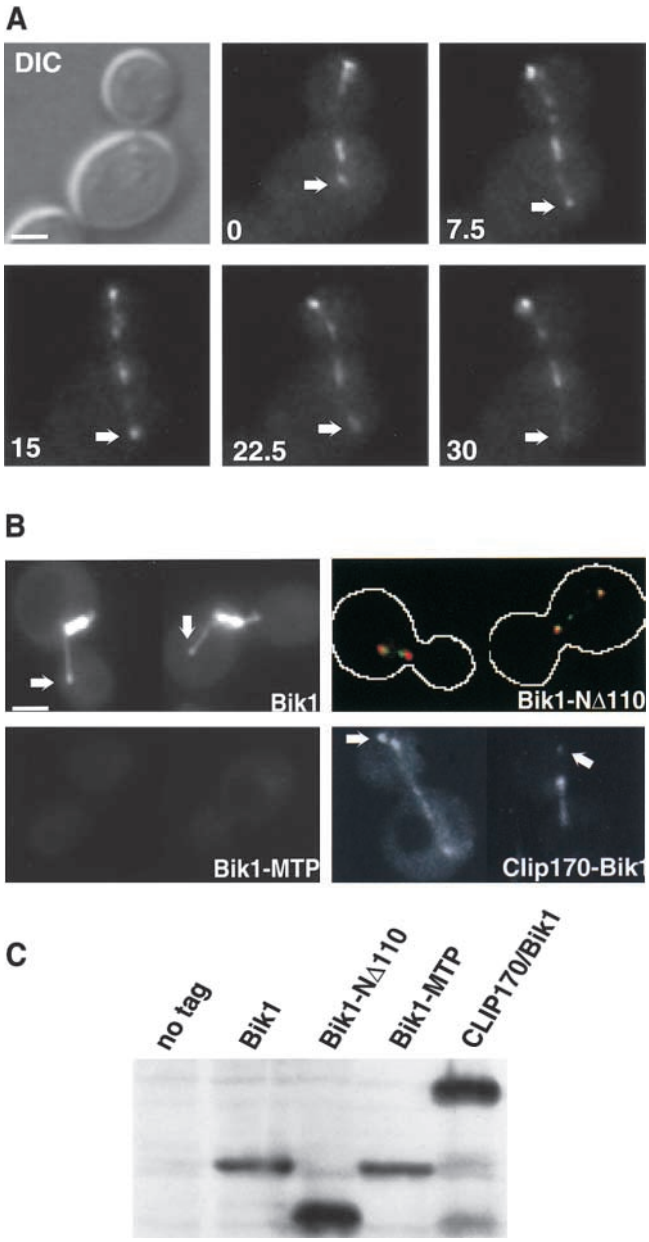

Figure 2. **Bik1 is a plus end–tracking protein and the yeast orthologue of human CLIP-170.** (A) Time-lapse series of Bik1–3GFP. Time is in s. The images are two-dimensional projections of a 0.5-μm Z-focal plane image stack. Arrows indicate Bik1–3GFP spots moving toward the cell periphery. (B) The localization of Bik1, Bik1-NΔ110, Bik1-MTP (which contains four amino acid substitutions in the CAP-Gly MT-binding domain identical to that previously done for CLIP-170; Pierre et al., 1992), and CLIP-170-Bik1. For Bik1, Bik1-MTP, and CLIP-170-Bik1 black-and-white images of the GFP fluorescence are shown. Arrows indicate localization of Bik1 and CLIP-170-Bik1 to the plus ends of astral MTs. For Bik1-NΔ110, a color image is shown of the localization of Bik1-NΔ110-GFP (green) adjacent to Spc42-CFP (red, a SPB marker). All Bik1 constructs are expressed as COOH-terminal fusions to one copy of GFP. (C) Western blot showing the steady-state protein levels of the indicated Bik1 derivatives detected with a polyclonal anti-GFP antibody (50 μg of cell extract were loaded in each lane). Bars, 2 μm.

Table I. **Efficiency of karyogamy in *bik1* strains**

	% of zygotes with fused nuclei	
	× *bik1Δ*	× *BIK1*
BIK1	67	86
bik1Δ	8	74
bik1-NΔ110	12	76
bik1-MTP	9	76
CLIP-170-BIK1	38	78

Karyogamy efficiency was measured by counting fused and unfused nuclei by DAPI staining of zygotes from the indicated crosses as described (Kurihara et al., 1994). Plasmids bearing the different *BIK1* constructs were integrated into the *LEU2* locus. Zygotes containing a single-fused nucleus underwent successful karyogamy. ≥50 zygotes were scored for each cross. *CLIP-170-BIK1* also complements the benomyl sensitivity of *bik1Δ* strains (unpublished data).

treadmilling mechanism (Perez et al., 1999). The function of the cargo-binding domain of CLIP-170 family proteins is not well characterized, but it contains a unique zinc-binding motif and is proposed to mediate interactions with various targets (Pierre et al., 1992, 1994; Schuyler and Pellman, 2001a). *BIK1* mutants containing point mutations (*bik1-MTP*) or a deletion (*bik1-NΔ110*) in the coding sequence for the CAP-Gly domain were not functional, as assayed by complementation of the karyogamy defect and benomyl sensitivity (a MT-depolymerizing drug) of a *bik1Δ* strain (Table I). By contrast, *CLIP-170-BIK1* complemented the karyogamy defect and benomyl sensitivity of *bik1Δ* strains ~50% as efficiently as *BIK1*. Thus, the MT-binding domains of Bik1 and CLIP-170 are functionally exchangeable.

Next, the *CLIP-170-BIK1* chimera and a series of control constructs were fused to *GFP* and, to facilitate detection of the chimera, introduced into cells on 2-μm plasmids (20–50 copies/cell). Overexpressed Bik1-GFP had a similar labeling pattern as native level Bik1–3GFP; however, as expected, it labeled the length of MTs more frequently (Fig. 2 B). The association of Bik1 with MTs was abolished either by the introduction of point mutations in the CAP-Gly domain of Bik1 (Bik1-MTP) or deletion of this domain (Bik1-NΔ110). The loss of in vivo MT association was not due to decreased steady state levels of the mutant proteins (Fig. 2 C). Exchange of the MT-binding domain from CLIP-170 for the corresponding Bik1 sequence restored MT association, and strikingly, the chimera was targeted to the MT plus end (Fig. 2 B). This suggests that the plus end tracking of Bik1 is mediated by a treadmilling mechanism that is similar or identical to the treadmilling mechanism documented for CLIP-170 (Perez et al., 1999).

MT-independent binding of Bik1 to the kinetochore

Because of the prominent Bik1 signal at kinetochores, chromatin immunoprecipitation (Chip) was used to determine whether Bik1 bound to the kinetochore (Meluh and Koshland, 1997). Epitope-tagged Bik1 was specifically cross-linked to centromeric DNA but not to flanking chromosome arm sequences (Fig. 3 A). Further, the interaction of Bik1 with centromeric DNA was completely dependent upon Ndc10, a core component of the CDEIII DNA–binding subcomplex of the kinetochore (Fig. 3 B). Finally, we found that Bik1 specifically coimmunoprecipitated with another candidate kinetochore protein, Stu2 (Fig. 3 C). Stu2 is the budding yeast member of the XMAP215 family of MT-binding proteins (Gard and Kirschner, 1987; Wang and Huffaker, 1997; Chen et al., 1998; He et al., 2001; Nakaseko et al., 2001). Stu2 was previously shown to interact with Bik1 by two-hybrid analysis (Chen et al., 1998). Both Stu2 and its *S. pombe* orthologue dis1 were recently shown to bind to the kinetochore by Chip (He et al., 2001; Nakaseko et al., 2001). These data strongly suggest that Bik1 is a component of the yeast kinetochore.

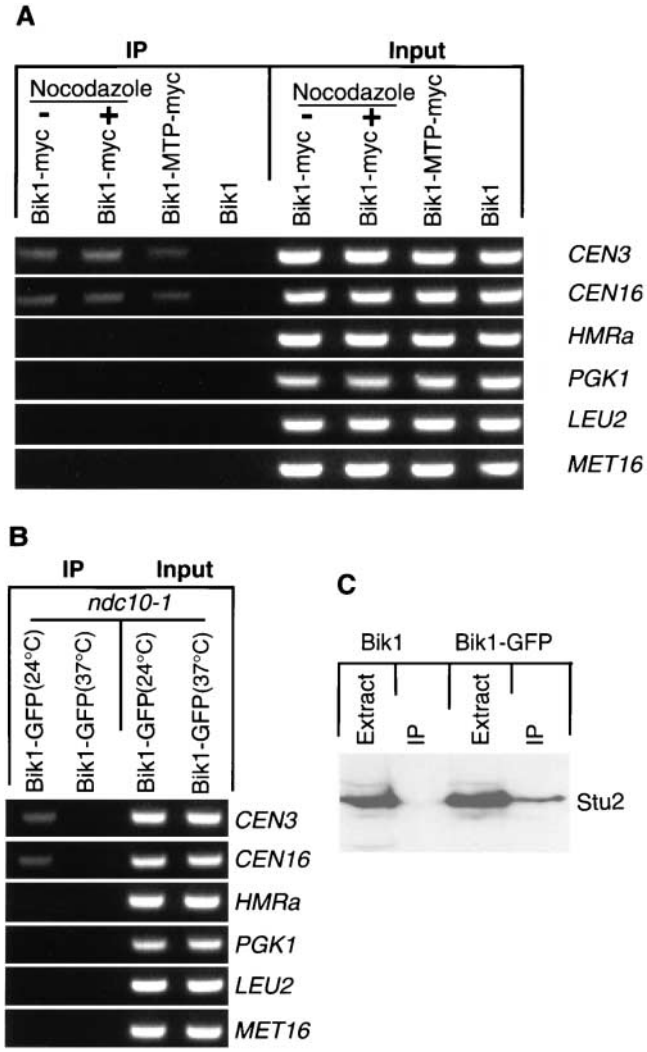

Figure. 3. **MT-independent binding of Bik1 to the kinetochore.** (A) Bik1 can be cross-linked to *CEN* DNA in the absence of MTs. Chip with strains bearing the indicated Bik1 derivatives. Each construct is expressed from a *CEN* plasmid in a *bik1Δ* strain and contains the coding sequence for 3 tandem copies of the myc epitope at the 3′ end of the *BIK1* sequence. *CEN3* and *CEN16* are centromere DNA sequences. *HMRa, PGK1, LEU2, and MET16* are control flanking sequences. The nocodazole-treated cells were incubated in medium containing 15 μg/ml nocodazole for 1 h. Complete MT depolymerization was confirmed in a parallel culture expressing GFP-Tub1. (B) Cross-linking of Bik1 to *CEN* DNA requires a functional kinetochore. Chip assay of Bik1–3GFP in an *ndc10–1* strain after incubation at the indicated temperature for 3 h. (C) Coimmunoprecipitation of Bik1 with Stu2. Cell extracts from strains expressing either Bik1–3GFP or untagged Bik1 were immunoprecipitated with a polyclonal anti-GFP antibody. Stu2-HA was detected by Western blotting with an anti-HA monoclonal antibody.

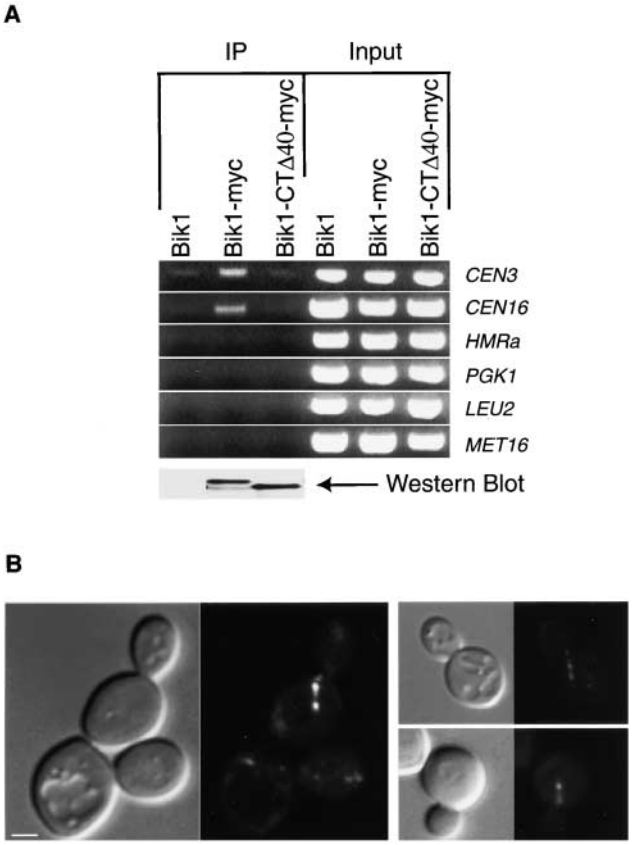

Figure 4. **Bik1-CTΔ40 interacts with MTs but not kinetochores.** (A) Cross-linking of Bik1 to *CEN* DNA requires the cargo-binding domain. The Chip experiment was performed as described in the legend to Figure 3 except that Bik1 and Bik1-CTΔ40 are tagged at the COOH terminus with 13 tandem copies of the myc epitope (Longtine et al., 1998). Bik1 and Bik1-CTΔ40 are expressed at the same levels. Western blot with an anti-myc monoclonal antibody is shown at the bottom (100 μg of cell extract was loaded in each lane). (B) The localization of Bik1-CTΔ40-GFP. Pairs of DIC and fluorescence images are shown. Bar, 2 μm.

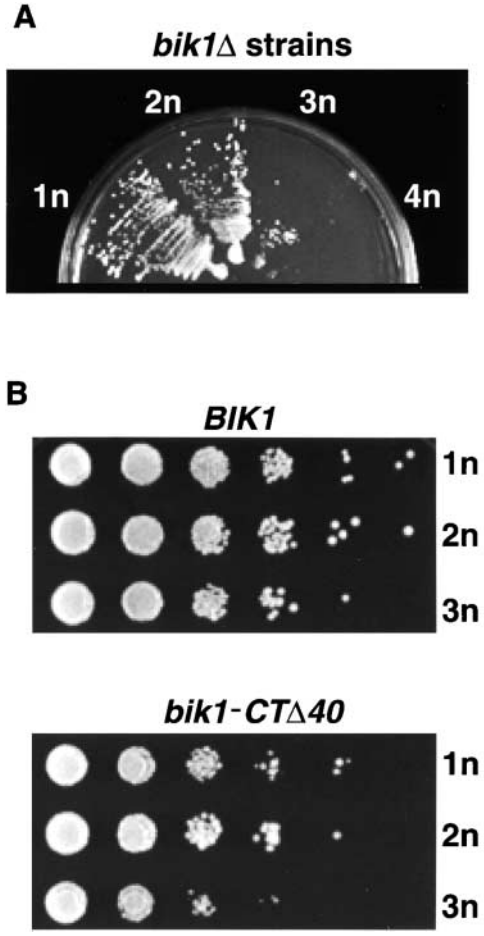

Figure 5. ***BIK1* is essential for viability of polyploids.** (A) A series of *bik1Δ* strains of the indicated ploidy was transformed with a *2μ BIK1 URA3* plasmid and plated on medium containing 5-fluoroorotic acid (5-FOA). 5-fluoroorotic acid selects for the loss of the *BIK1 URA3* plasmid revealing the growth defect of triploid and tetraploid *bik1Δ* strains at 24°C. Control *BIK1* strains of the same ploidy do not have a detectable growth defect (Galitski et al., 1999; unpublished data). (B) Growth of *BIK1*- and *bik1-CTΔ40*–containing triploids. Serial fivefold dilutions from cultures of the indicated strains at a density of 5×10^7 cells/ml were plated. The indicated strains were spotted onto YPD medium.

Importantly, three experiments suggested that the binding of Bik1 to kinetochores is not dependent on the binding of Bik1 to MTs. First, Bik1-MTP, which contains point mutations in the CAP-Gly domain that abolished its association with MTs (Fig. 2 B), was still cross-linked to *CEN* DNA by the Chip assay (Fig. 3 A). Second, wild-type Bik1 was still cross-linked to *CEN* DNA when MTs were depolymerized by treatment of cells with nocodazole (Fig. 3 A). Finally, Bik1-NΔ110, a deletion lacking the CAP-Gly MT-binding domain, localized near the SPB at the expected position of the kinetochore (Fig. 2 B). The kinetochore localization of Bik1-NΔ110 was probably evident because it was present at higher steady state levels than Bik1-MTP (Fig. 2 C). The fact that Bik1 could bind the kinetochore independently of MTs strongly suggests that Bik1 physically links the ends of MTs to the kinetochore.

The COOH-terminal cargo-binding domain of CLIP-170 family proteins is proposed to link MT plus ends to various targets (Pierre et al., 1992). However, the cargo-binding hypothesis has not been directly tested. To test this idea, a *bik1* mutant lacking the coding sequence for the cargo-binding domain (*bik1-CTΔ40*) was generated by homologous recombination at the *BIK1* locus (see Materials and methods). Bik1-CTΔ40 is expressed at similar levels to Bik1 (Fig. 4 A) and, when expressed as a COOH-terminal GFP fusion, has a similar pattern of localization as Bik1 (Fig. 4 B). However, in contrast to Bik1, Bik1-CTΔ40 failed to bind to the kinetochore by Chip (Fig. 4 A). These data validate the idea that the COOH-terminal Bik1 domain binds cargo and suggest that Bik1 is indeed a bifunctional linker protein.

BIK1 is essential in polyploid cells

Although *BIK1* is not required for normal growth of haploid or diploid cells, surprisingly, we found that it is required for the maximal growth rate of triploids and becomes essential in tetraploid cells (Fig. 5 A, Table IV). The requirement for *BIK1* in polyploids does not reflect a global defect in MT sta-

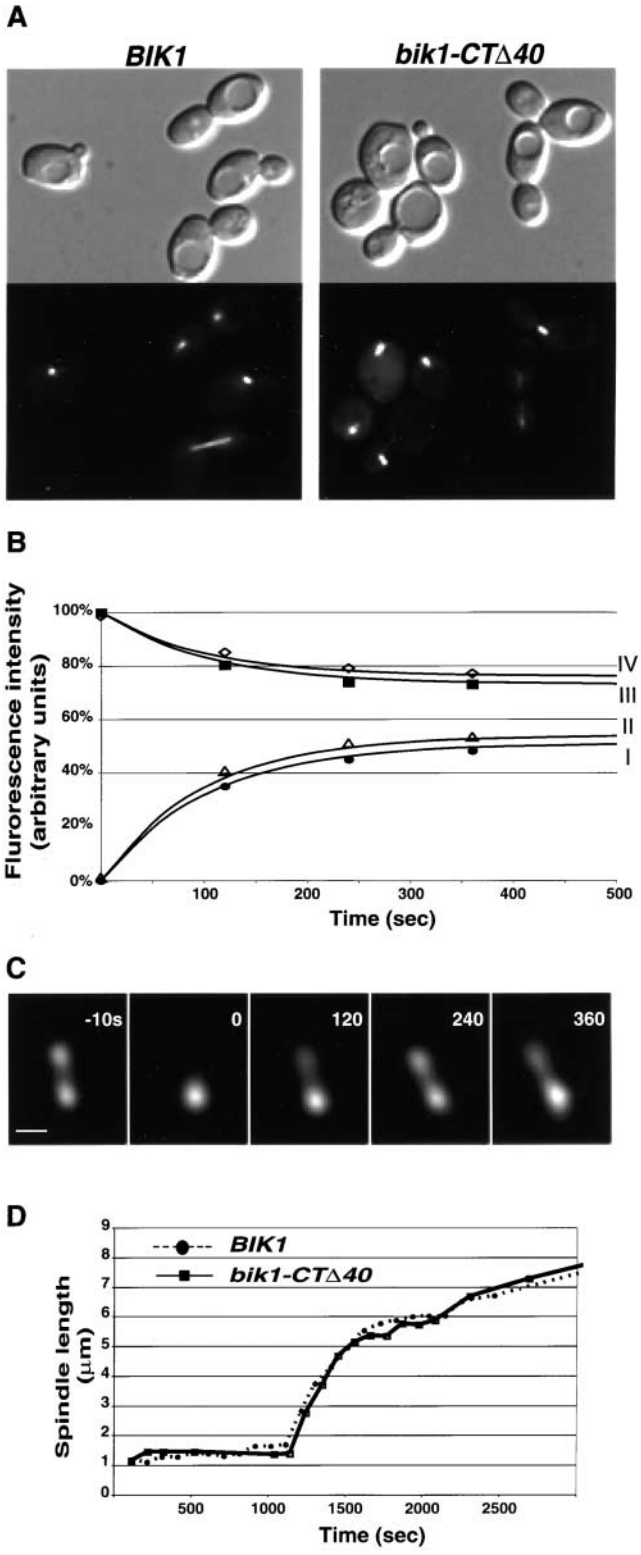

Figure 6. **Characterization of the mitotic spindle in triploids bearing *bik1-CTΔ40*.** (A) Triploid cells containing *bik1-CTΔ40* have normal spindle morphology. The left panel shows spindles in triploid cells containing *BIK1*. The right panel shows spindles in triploid cells containing *bik1-CTΔ40*. MTs are labeled in both strains by GFP-Tub-1. (B) FRAP in *BIK1*- and *bik1-CTΔ40*–containing triploid cells. MTs are labeled by GFP-Tub1. The graph shows intensity measurements of a 5 × 5 pixel area from preanahase spindles in triploid cells bearing either *BIK1* (Δ, bleached; ◇, unbleached) or *bik1-CTΔ40* (●, bleached; ■, unbleached). The predicted exponential curves for FRAP are shown as the plotted lines. Curves I and III represent the bleached and unbleached regions of triploid cell bearing *bik1-CTΔ40*, respectively. Curves II and IV represent the bleached and unbleached regions of cells bearing *BIK1*, respectively. These theoretical curves were derived from the first-order rate constant, calculated as described in the Materials and methods (also see Table III). The intensity of the unbleached region at the 0 time point was assumed to be 100% and the intensity of bleached region at the 0 time point was assumed to be 0%. (C) Example of a FRAP experiment with a preanaphase triploid cell bearing *bik1-CTΔ40*. Time is in seconds. Bar, 1 μm. (D) The kinetics of anaphase spindle elongation are not altered in *bik1-CTΔ40*–containing triploids relative to *BIK1*-containing triploids. Time-lapse images were acquired from GFP-Tub1–containing strains at ~90-s intervals.

Table II. **FRAP parameters in triploids bearing *BIK1* or *bik1-CTΔ40***

	k	$t_{1/2}$	*R*
BIK1	0.0102 ± 0.0025	71.0 ± 16.1	71 ± 15%
bik1-CTΔ40	0.0098 ± 0.0025	75.0 ± 17.8	70 ± 13%

The first-order rate constant (*k*) and the half-time ($t_{1/2}$) of fluorescence recovery were calculated as described in the Materials and methods. The fluorescence recovery (*R*) of the bleached region was the ratio of the bleached to the unbleached region after recovery. The table shows the mean ± SD for six independent cells. Values of *k*, $t_{1/2}$, and *R* were not significantly different between triploid cells bearing *BIK1* and triploid cells bearing *bik1-CTΔ40* (p = 0.7, 0.7, and 0.5, respectively).

bility in polyploids, because triploids and tetraploids are only mildly sensitive to the MT depolymerizing drug benomyl relative to diploids (see Materials and methods). However, tetraploids may have a defect in kinetochore function because they are reported to have a 1,000-fold increase in the rate of chromosome loss relative to haploids (Mayer and Aguilera, 1990). In light of these findings, we considered the possibility that the mitotic defect in polyploids could be a consequence of a defect in kinetochore–MT attachment.

Bik1 is required for MT stability, perhaps because all CLIP-170 family proteins inhibit MT catastrophes (Berlin et al., 1990; Brunner and Nurse, 2000). Like *bik1Δ* haploids, polyploid cells lacking *BIK1* have short astral and spindle MTs and also highly abnormal spindles. To create a strain that potentially retained normal spindle structure, but would enable the requirement for Bik1 in kinetochore dynamics to be tested, the *bik1-CTΔ40* allele was introduced into triploid cells.

Triploids bearing *bik1-CTΔ40* had a modest growth defect relative to *BIK1*-containing isogenic controls (Fig. 5 B). By contrast tetraploids bearing *bik1-CTΔ40* had a severe growth defect and exhibited striking variability in colony size (unpublished data). The variable growth of *bik1-CTΔ40*–containing tetraploids prevented the characterization of kinetochore dynamics in this strain. However, because the triploid strains bearing *bik1-CTΔ40* grew fairly robustly, but had readily observable defects in kinetochore function (see below), subsequent analysis was focused on this strain.

Spindle morphology, spindle MT dynamics, and anaphase spindle elongation are not altered in triploids bearing *bik1-CTΔ40*

Triploid cells bearing *bik1-CTΔ40* had morphologically normal mitotic spindles (Fig. 6 A). In addition, the average

Table III. **Kinetochore separation in *BIK1* or *bik1-CTΔ40*–containing triploids**

	Total time	Time separated/ total time	Time separated <30 s/ time separated
	s		
BIK1	3347	53.5%	14.3%
bik1-CTΔ40	2784	21.9%	38.4%

Kinetochores in preanaphase triploid cells bearing *BIK1* (14 cells) or *bik1-CTΔ40* (14 cells) were followed by time-lapse microscopy. Images were acquired every ~2 s. The average spindle length in *BIK1*- and *bik1-CTΔ40*–containing triploids was 1.27 ± 0.20 μm and 1.24 ± 0.16 μm, respectively, throughout the time of imaging.

length of preanaphase spindles in triploids bearing *bik1-CTΔ40* was not significantly different from *BIK1*-containing triploids (1.14 ± 0.3 μm [n = 197] and 1.10 ± 0.3 μm [n = 157], respectively, $p > 0.05$). We calculated the approximate volume of preanaphase spindles by measuring the width and length of the GFP-Tub1–labeled preanaphase spindles (Tub1 is the major α-tubulin isoform), assuming that they are cylinders. These values were also not significantly different between *bik1-CTΔ40*–containing triploids and the control (0.9 ± 0.3 μm^3 and 1.0 ± 0.4 μm^3, respectively, $p > 0.05$). By contrast, the spindle volume was smaller in isogenic wild-type haploids (0.4 ± 0.2 μm^3, $p < 0.001$). Thus, *bik1-CTΔ40* does not alter the size or shape of preanaphase spindles in triploid cells. The preanaphase spindles in triploids are similar in length to the preanaphase spindles in haploids, but they are somewhat thicker. The relatively short length of preanaphase spindles in triploids might reflect increased tension on the spindle poles from the additional bioriented kinetochores (Goshima et al., 1999).

We next determined if *bik1-CTΔ40* affected the turnover rate of MTs in preanaphase spindles of triploid cells. In budding yeast, bundles of antiparallel MTs are the major component of the preanaphase spindles (Winey and O'Toole, 2001). There is one kMT for each chromosome as well as several polar MTs that maintain spindle bipolarity. Although the lack of spatial resolution precludes the analysis of individual kMT dynamics, bulk preanaphase spindle MT dynamics can be measured by FRAP of preanaphase spindles (Salmon and Wadsworth, 1986; Maddox et al., 2000). GFP-Tub1 was expressed in triploid cells containing *bik1-CTΔ40* or *BIK1*. A targeted laser was used to bleach selectively one half of a 1.5–2-μm preanaphase spindle, the cells were imaged before and after bleaching, and the rate and percentage of fluorescence recovery were measured (Fig. 6, B and C; Table II). Recovery rates were fit to an exponential function in order to calculate the turnover rate. We found that the half-time to recovery was 71 s in the *BIK1*-containing cells and 75 s in the *bik1-CTΔ40*–containing cells. The exchangeable fraction, which is the percentage of the fluorescence intensity that recovers after bleaching, was 71% for the *BIK1*-containing cells and 70% for the *bik1-CTΔ40*–containing cells. From previous studies in haploids, where the ratio of kinetochore to polar MTs is known (Winey et al., 1995), it was inferred that the exchangeable fraction reflects the turnover of kMTs, whereas the nonexchangeable fraction reflects the signal from polar MTs (Maddox et al., 2000). Although in triploids the ratio of kinetochore to polar MTs is not known, our data are consistent with the notion that this ratio is similar in triploids and in haploids. This suggests that the fluorescence recovery rates we measured reflect the turnover of kMTs. Importantly, this experiment did not reveal a difference in the turnover of spindle MTs between triploid cells bearing *BIK1* and *bik1-CTΔ40*.

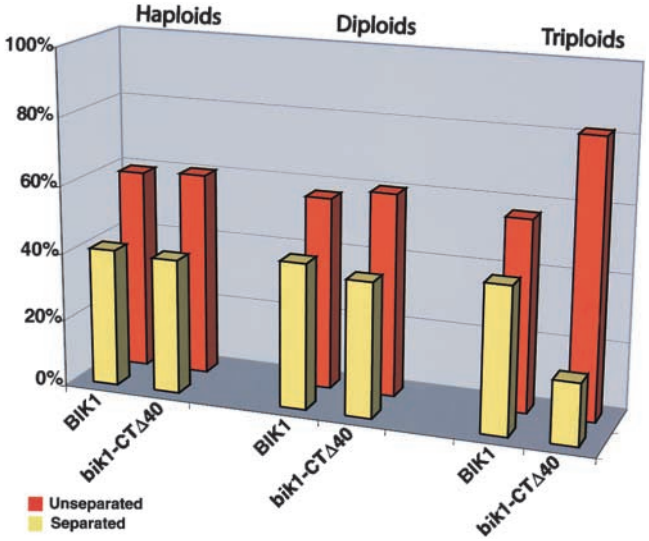

Figure 7. ***bik1-CTΔ40*–containing triploids have a defect in preanaphase kinetochore separation.** Kinetochore separation was scored in the indicated strains by coimmunostaining for tubulin (monoclonal antibody YOL1/34) and GFP-marked *CEN5* (polyclonal anti-GFP). Unseparated kinetochores are seen as one fluorescent dot, whereas separated kinetochores are seen as two dots. The fraction of cells with separated kinetochores and the average spindle lengths were as follows. *BIK1*-containing cells: 35/86 and 1.2 μm (haploids); 68/158 and 1.2 μm (diploids); and 68/157 and 1.1 μm (triploids). *bik1-CTΔ40*–containing cells: 35/88 and 1.3 μm (haploids); 63/158 and 1.2 μm (diploids); and 36/197 and 1.2 μm (triploids).

As a final test of spindle integrity in *bik1-CTΔ40*–containing triploids, we measured the rate of anaphase spindle elongation in these and control cells. There was no significant difference between the rates of spindle elongation between *BIK1*-containing triploids and the *bik1-CTΔ40*–containing triploids: for the initial fast phase of spindle elongation the values were 0.43 ± 0.07 μm/min (n = 8) and 0.35 ± 0.11 μm/min (n = 6), respectively, $p > 0.05$; for the subsequent slow phase, the values were 0.13 ± 0.05 μm/min (n = 8) and 0.11 ± 0.03 μm/min, respectively, (n = 6, $p > 0.05$). Thus, in triploids, *bik1-CTΔ40* did not affect spindle size or morphology, nor did it affect preanaphase spindle MT dynamics or anaphase kinetics. Therefore, the cargo-binding domain of Bik1 does not appear to be required for the normal assembly dynamics of spindle MTs.

BIK1 is required for the pulling force that maintains preanaphase kinetochore separation

We next determined if the strain containing *bik1-CTΔ40* had a defect in preanaphase kinetochore separation. A GFP-based system was used to visualize kinetochores in *bik1-CTΔ40* and *BIK1* cells (Straight et al., 1996). Tandem arrays of the Tet operator sequence (224 copies) were introduced 1.9 Kb from

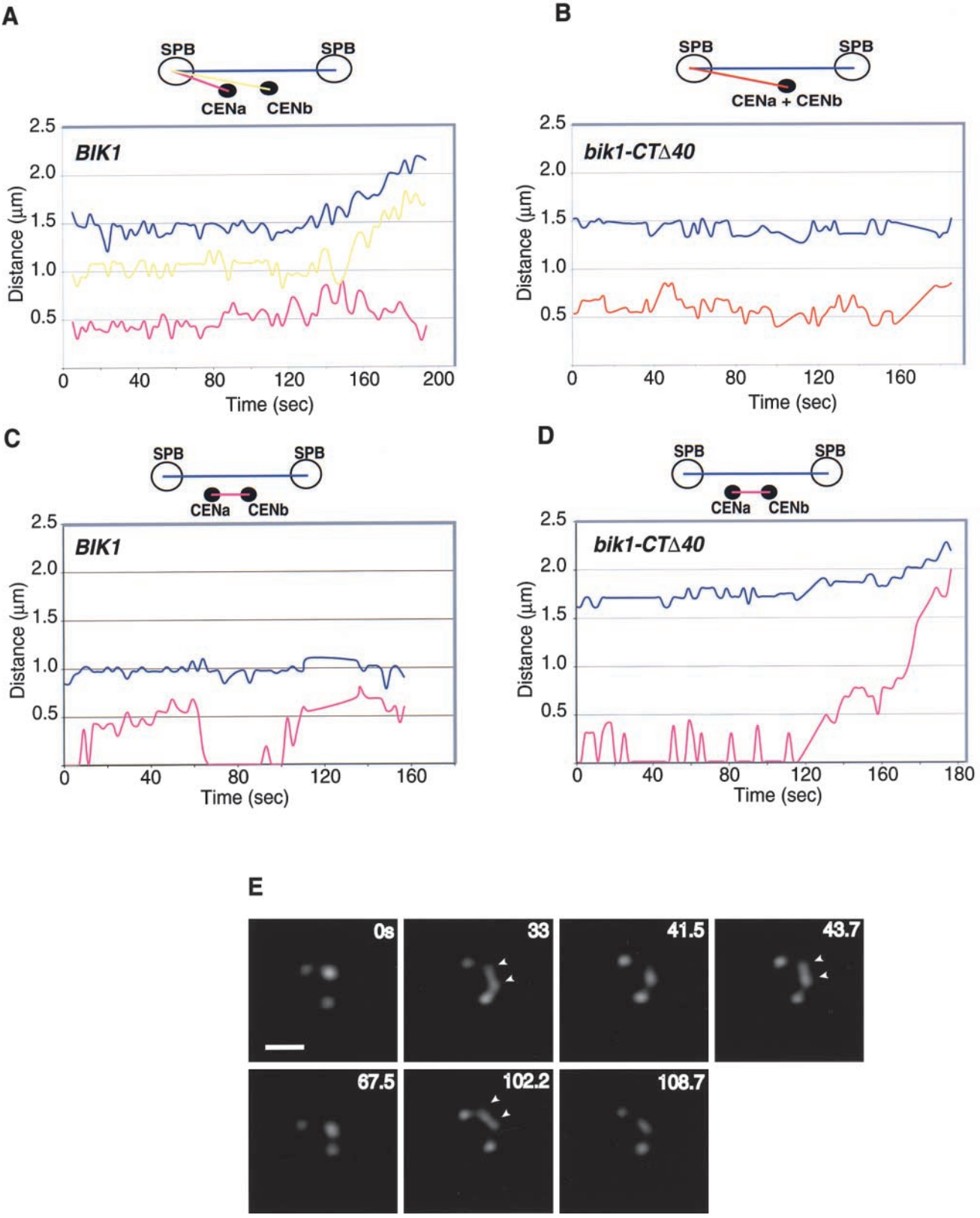

Figure 8. **Defective kinetochore dynamics in *bik1-CTΔ40*–containing triploid cells.** Rapid single-focal plane imaging of GFP-tagged kinetochores relative to GFP-tagged SPBs in *BIK1*- and *bik1-CTΔ40*–containing triploid cells. Images were acquired approximately every 2 s. >1,300 data points were analyzed for each strain. (A) An example of a *BIK1*-containing triploid cell where kinetochores remain separated throughout most of the time of imaging. (B) An example of a *bik1-CTΔ40*–containing triploid cell where kinetochores do not separate. (C) An example of a *BIK1*-containing triploid cell where kinetochores separate on average for an interval >30 s. (D) An example of a *bik1-CTΔ40*–containing triploid cell where 9 very short kinetochore separations occur within a 2-min period. (E) Time-lapse images illustrating very short kinetochore separations in a *bik1-CTΔ40*–containing triploid cell. Arrow indicates separated kinetochores. Bar, 1 μm.

Table IV. **Isogenic series of yeast strains of varying ploidy**

Strain	Genotype
PY 4000	MAT α; BIK1::3HA::KAN^r ade2 his3Δ200 leu2-3,112 lys2-201 trp1Δ1 ura3-52 can1 cyh2
PY 4001	MAT α; bik1CTΔ40::3HA::KAN^r ade2 his3Δ200 leu2-3,112 lys2-201 trp1Δ1 ura3-52 can1 cyh2
PY 4002	MAT**a**/MATα; bik1-1::TRP1/BIK1::3HA::KAN^r ura3-52/ura3-52 leu2-3,112/leu2-3,112 lys2-201/lys2-201 his3Δ200/his3Δ200 ade2/ade2 cyh2/cyh2 can1/can1
PY 4003	MAT**a**/MATα; bik1-1::TRP1/bik1CTΔ40::3HA::KAN^r ura3-52/ura3-52 leu2-3,112/leu2-3,112 lys2-201/lys2-201 his3Δ200/his3Δ200 ade2/ade2 cyh2/cyh2 can1/can1
PY 4004	MAT**a**/MAT**a**/MATα; bik1-1::TRP1/bik1-1::TRP1/BIK1::3HA::KAN^r ura3-52/ura3-52/ura3-52 leu2-3,112/leu2-3,112/ leu2-3,112 lys2-201/lys2-201/lys2-201 his3Δ200/his3Δ200/his3Δ200 ade2/ade2/ade2 cyh2/cyh2/cyh2 can1/can1/can1
PY 4005	MAT**a**/MAT**a**/MATα; bik1-1::TRP1/bik1-1::TRP1/bik1CTΔ40::3HA::KAN^r ura3-52/ura3-52/ura3-52 leu2-3,112/leu2-3,112/ leu2-3,112 lys2-201/lys2-201/lys2-201 his3Δ200/his3Δ200/his3Δ200 ade2/ade2/ade2 cyh2/cyh2/cyh2 can1/can1/can1

the chromosome V centromere (*CEN5;* He et al., 2000), allowing the position of *CEN5* to be detected by a Tet repressor–GFP fusion. In fixed populations of wild-type haploids kinetochores were separated in the majority of preanaphase cells (Goshima and Yanagida, 2000). Preanaphase kinetochore separation was also observed in our wild-type strains. In haploid and diploid cells bearing the *bik1-CTΔ40* allele, there was no defect in kinetochore separation relative to control strain ($p > 0.05$, Fig. 7). By contrast, kinetochore separation was reduced from 43% in the control triploid strain to 18% in the *bik1-CTΔ40* triploid strain ($p < 0.001$). Thus, Bik1, and specifically its cargo-binding domain, was required for preanaphase kinetochore separation in triploid cells.

Using the Tet operator system, we also determined if there was visually detectable chromosome missegregation in our strains (Cheeseman et al., 2001). Visual inspection of anaphase cells did not reveal a difference in the fraction of cells with incorrectly segregated chromosomes in *BIK1-* and *bik1-CTΔ40*–containing triploids: 5/113 *BIK1*-containing triploids and 5/124 *bik1-CTΔ40*–containing triploids were missing a kinetochore signal from one nucleus. The absence of very high level chromosome missegregation in the *bik1-CTΔ40*–containing triploids is expected from the mild growth defect of these mutants (Fig. 5 B). Presumably, error correction mechanisms, such as the spindle checkpoint, compensate for the defect in kinetochore separation.

The defect in kinetochore separation observed in triploid *bik1-CTΔ40* cells could be due to a defect in the ability of MTs to attach to kinetochores, a defect in pulling kinetochores apart after attachment occurs, and/or a defect in maintaining kinetochore separation. High time resolution live cell imaging was used to distinguish among these possibilities. Consistent with the data from fixed preanaphase cells, the live cell imaging revealed a striking defect in sister kinetochore separation in *bik1-CTΔ40*–containing triploids (Fig. 8). Relative to the control cells, sister kinetochores were separated a substantially smaller fraction of the time (Table III). In addition, when separation of kinetochores was initiated in *bik1-CTΔ40*–containing triploids, it often lasted for very short intervals (lasting <30 s). By contrast, only a minority of kinetochore separation events in the *BIK1*-containing cells lasted less than 30 s (Fig. 8 E, Table III). These very short separations indicate that *bik1-CTΔ40*–containing triploids fail to maintain normal kinetochore separation. The inability of *bik1-CTΔ40*–containing triploids to maintain kinetochore separation strongly suggests that these cells have a defect in maintaining kinetochore–MT attachment.

Discussion

How the mitotic apparatus adapts to changes in ploidy is not known, but it may have implications for cancer therapeutics. In polyploid yeast, large numbers of chromosomes must be attached to the mitotic spindle in a similar period of time as is allotted in haploid cells (Galitski et al., 1999). Also, the presence of large numbers of chromosomes in the small region around the spindle poles might interfere with the poleward movement of additional chromosomes. These restrictions raise the possibility that kinetochore–MT attachment might be compromised in polyploid yeasts and possibly in other cells with increased ploidy such as cancer cells. Here, we report biochemical, genetic, and imaging experiments demonstrating that Bik1is a component of the kinetochore–MT binding interface. Furthermore, *BIK1* is an essential gene only in polyploids, and *BIK1* is required for preanaphase kinetochore separation only in polyploid cells. These findings thus provide the first experimental support for the hypothesis that kinetochore–MT attachment is a limiting factor for the viability of polyploid cells. Moreover, the ability to construct isogenic polyploid yeast strains has enabled us to characterize ploidy-specific effects on the mechanism of mitosis.

Bik1 and kinetochore–MT attachment

CLIP-170 family proteins are conserved MT regulators that bind to MT plus ends. They are also proposed to provide a connection to other cellular components through their COOH-terminal "cargo-binding" domains (Pierre et al., 1992, 1994; Dujardin et al., 1998; Schroer, 2001; Schuyler and Pellman, 2001a). Considerable evidence supports the notion that CLIP-170 family proteins regulate MT dynamics (Schuyler and Pellman, 2001a). However, the proposed linker function of these proteins is less well characterized.

Here, we demonstrate that the budding yeast CLIP-170 orthologue Bik1, through its cargo-binding domain interacts with the kinetochore and contributes to the connection between the plus ends of MTs and the kinetochore. Our data suggest that Bik1 bound to the plus ends of MTs by a similar, if not identical, mechanism as CLIP-170 (Perez et al., 1999). Bik1 also bound to the kinetochore as assayed by Chip. Importantly, the binding of Bik1 to kinetochores was independent of its binding to MTs suggesting that Bik1 is a bona fide component of the kinetochore. Further, deletion

of the cargo-binding domain abolished kinetochore binding, providing direct evidence that this domain is required to attach Bik1, and presumably kMT plus ends, to the kinetochore. Very recently, another study reported localization and Chip results for full-length Bik1; however, the functional consequences of these findings for kinetochore–MT attachment were not characterized (He et al., 2001). Here, a functional role for Bik1 at the kinetochore was revealed by our analysis of polyploid cells bearing a *BIK1* allele lacking the coding sequence for the cargo-binding domain. These cells had a striking defect in initiating and maintaining preanaphase kinetochore separation. Together, these data demonstrate that Bik1 is important for generating the pulling force on kinetochores that occurs before anaphase.

Bik1 might contribute to the pulling force on kinetochores by several mechanisms: regulation of kMT assembly dynamics, recruitment of motor proteins or other MAPs, and/or direct binding to kinetochore proteins. The growth phenotype and short MTs in polyploid *bik1Δ* mutants suggested that Bik1 contributes to MT stability in polyploids like it does in haploids (Berlin et al., 1990). However, our analysis of triploids bearing *bik1-CTΔ40* suggests that the Bik1 cargo-binding domain has an additional role in kinetochore–MT attachment that is more direct. Triploids bearing *bik1-CTΔ40* had spindles of normal size and shape relative to the control strain; they had normal preanaphase spindle MT dynamics as measured by FRAP, and finally, they had normal rates of anaphase spindle elongation. Thus, by all of these measures, the cargo-binding domain of Bik1 is not required for the integrity or dynamics of spindle MTs. We therefore propose that the cargo-binding domain of CLIP-170 family proteins either binds to kinetochore proteins or recruits proteins to the kinetochore–MT binding interface.

Our results contrast with recent findings on the kinetochore-associated MAP Stu2 (He et al., 2001). Stu2 is a member of the XMAP-215 family of MT-binding proteins (Gard and Kirschner, 1987; Wang and Huffaker, 1997). Mutations in *STU2* block preanaphase kinetochore separation (He et al., 2001); however, this may be due to effects of Stu2 on kMT dynamics because loss of Stu2 was recently found to abolish spindle MT turnover by FRAP (Kosco et al., 2001).

Although our photobleaching experiments did not reveal a role for the cargo-binding domain of Bik1 in spindle MT dynamics, it is possible that this domain has a role in the regulation of astral MT dynamics. Based in part on the phenotype and genetic interactions of the *bik1-CTΔ40* mutation, we have evidence that Bik1 is required for the dynein-dependent step in nuclear migration (unpublished data). These results are consistent with a previous genetic study in budding yeast (Geiser et al., 1997). They are also consistent with colocalization and overexpression studies in animal cells that suggest a relationship between CLIP-170 and dynein components (Dujardin et al., 1998; Valetti et al., 1999; Vaughan et al., 1999). Although dynein regulates astral MT behavior in budding yeast, it is not required for normal kinetochore movement (Bloom, K., personal communication). Because Bik1 is present on the plus ends of the different populations of MTs (Fig. 1 A), it is not surprising that it might have distinct roles at these different locations.

It has recently become apparent that the yeast kinetochore has many more components than previously appreciated (Ortiz et al., 1999; Goshima and Yanagida, 2000; Cheeseman et al., 2001; He et al., 2001; Janke et al., 2001; Nakaseko et al., 2001; Wigge and Kilmartin, 2001). Furthermore, the yeast kinetochore may have a complex folded structure (Ortiz et al., 1999). Here, we found that Bik1 coimmunoprecipitated with the XMAP-215 orthologue Stu2. This raises the possibility that CLIP-170 and XMAP-215 family proteins in other organisms may interact. Although Stu2 is present at several locations in the cell like Bik1 (Wang and Huffaker, 1997; Kosco et al., 2001), these proteins may cooperate to form part of the complex kinetochore–MT binding surface.

Kinetochore–MT attachment and ploidy

We have uncovered an unexpected link between *BIK1* and the physiology of polyploid cells: *BIK1* is not an essential gene in haploids but becomes essential when ploidy is increased. Tetraploid yeasts are reported to have a 1,000-fold increase in the frequency of chromosome loss (Mayer and Aguilera, 1990). Our work, taken together with previous studies, supports the idea that chromosome loss may be due, at least in part, to a defect in kinetochore function. First, it is known that in haploids excess kinetochores are toxic and promote chromosome missegregation (Futcher and Carbon, 1986). Second, although chromosome loss rates are high in polyploid cells, they are only mildly sensitive to MT depolymerizing drugs such as benomyl (our results); this suggests that polyploid cells do not have a global defect in spindle stability. Third, the mitotic defect in tetraploid cells is not due to altered transcriptional regulation, because the transcription of only 17 genes is altered in tetraploids, and none of these genes is known to be involved in mitosis (Galitski et al., 1999). Finally, we found that Bik1 is important for kinetochore–MT attachment and becomes an essential gene in polyploid cells. Thus, kinetochore–MT attachment may be a key factor in limiting the ploidy that yeast, and perhaps other eukaryotic cells, can attain.

Unlike polyploid cells in normal tissues, many common cancers divide with increased numbers of chromosomes (Lengauer et al., 1998; Sen, 2000; Pellman, 2001). A notable but poorly understood feature of cancer cells is their sensitivity to antimitotic drugs. In a handful of cancers, mutations in mitotic checkpoint genes have been identified (Cahill et al., 1998). Recently, mutations in the adenomatous polyposis coli tumor suppressor have been suggested to cause defects in kinetochore–MT attachment (Fodde et al., 2001; Kaplan et al., 2001). Thus, it is appealing to speculate that cancer cells have an intrinsic mitotic defect that accounts for their sensitivity to antimitotic drugs. However, the genetic changes in cancer cells are complex. Aneuploidy involves complex chromosomal rearrangements in addition to missegregation of whole chromosomes (for review see Artandi et al., 2000). The lack of isogenic controls has made it difficult to distinguish ploidy-specific effects from other genetic changes in cancer cells. Using the approach we have developed in yeast, it should be possible to systematically characterize the genetic requirements for increased ploidy. Finally, our finding that the budding yeast CLIP-170 orthologue is required

for the viability of polyploid cells may therefore have implications for cancer therapeutics: proteins that are preferentially required in cells of increased ploidy may be useful targets for the development of new antimitotic drugs.

Materials and methods

Media and genetic techniques

Construction of polyploid strains was as previously described (Berlin et al., 1990; Galitski et al., 1999). The benomyl sensitivity of the isogenic series of polyploid strains was assayed by growth on medium containing 5, 7.5, 10, or 15 μg/ml benomyl (Du Pont). At 5 μg/ml of benomyl, there was no difference in growth for any of the strains. At higher concentrations of benomyl, the most significant difference in growth was between the haploids and the diploids. At 10 or 15 μg/ml of benomyl, there was a slight growth defect of triploids and tetraploids relative to diploids.

Plasmid constructs and epitope tagging

Details of the plasmid constructs and the sequences of oligonucleotides used are available upon request. PB1587 is a plasmid for integrating three tandem copies of GFP at the 3′ end of the *BIK1* coding sequence (Figs. 1, 2 A, and 3, B and C). *BIK1–3GFP* is fully functional as assayed by complementation of the benomyl sensitivity and bilateral mating defect of *bik1Δ* strains, and by genetic crosses to *bim1Δ* and to *kar9Δ*. Fig. 2 B: PB532 expresses Bik1-GFP from the native *BIK1* promoter; PB629 expresses a Bik1-GFP mutant lacking the first 110 amino acids of Bik1; PB645 expresses a Bik1-GFP mutant (termed MTP) containing mutations (N43A, G45A, K46E, and G49A) in the CAP-Gly domain (as in Pierre et al., 1992); PB631 expresses a chimera containing the first 271 amino acids of CLIP-170, amino acids 111–440 of Bik1, followed by GFP. Fig. 3 A: PB65 (*CEN*) expresses Bik1 from the *BIK1* promoter; PB457 is derived from PB65 and expresses Bik1–3myc; PB458 is derived from PB457 but contains the MTP mutations. The STU2::HA plasmid (pWP70) was a gift from T. Huffaker (Cornell University, Ithaca, NY). pAFS125, for the expression of GFP-Tub1 was a gift from A. Straight (Harvard Medical School, Boston, MA) and A. Murray (Harvard University, Cambridge, MA). Construction of strains bearing *BIK1::3HA, BIK1::13myc, bik1-CTΔ40::3HA, bik1-CTΔ40::13myc, or bik1-CTΔ40::GFP* was done by one-step gene replacement (Longtine et al., 1998).

Fluorescence imaging and FRAP

The system for fluorescence live cell microscopy has been previously described (Tirnauer et al., 1999). All image manipulations and measurements were performed using Openlab software from Improvision.

For FRAP, a 377-nm nitrogen pulse laser (Photonics Instruments, Inc.) was used. Incoherent light was synchronized and amplified using a Coumerin blue chemical chamber, which emits at a wavelength of 440 nm. The average target size had a diameter of 667 nm at the half-maximal diameter of a Gaussian profile.

Measurements of FRAP were made as previously described (Salmon and Wadsworth, 1986; Maddox et al., 2000). The fluorescence intensity of 5 pixel × 5 pixel squared (0.018 μm^2) was measured at each time point for both the bleached and unbleached half-spindles. For each intensity measurement, the value used for each pixel was taken from the point of maximal brightness within the z-focal plane series. The intensity measurements were corrected for both background fluorescence and loss of fluorescence that occurred during imaging. The correction for loss of signal due to imaging was derived from an average value of unbleached cells (3–6 for each sample). The first-order rate constant k was calculated as described (Salmon and Wadsworth, 1986) using Statview (SAS Institute Inc.).

Biochemical methods

Immunoprecipitation and Western blotting were performed as described (Lee et al., 2000). Chip was performed as described (Kuras and Struhl, 1999).

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