The Regulation of Microtubule Dynamics in *Saccharomyces cerevisiae* by Three Interacting Plus-End Tracking Proteins^V

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Microtubule plus-end tracking proteins (+TIPs) are a diverse group of molecules that regulate microtubule dynamics and interactions of microtubules with other cellular structures. Many +TIPs have affinity for each other but the functional significance of these associations is unclear. Here we investigate the physical and functional interactions among three +TIPs in *S. cerevisiae*, Stu2, Bik1, and Bim1. Two-hybrid, coimmunoprecipitation, and in vitro binding assays demonstrate that they associate in all pairwise combinations, although the interaction between Stu2 and Bim1 may be indirect. Three-hybrid assays indicate that these proteins compete for binding to each other. Thus, Stu2, Bik1, and Bim1 interact physically but do not appear to be arranged in a single unique complex. We examined the functional interactions among pairs of proteins by comparing cytoplasmic and spindle microtubule dynamics in cells lacking either one or both proteins. On cytoplasmic microtubules, Stu2 and Bim1 act cooperatively to regulate dynamics in G1 but not in preanaphase, whereas Bik1 acts independently from Stu2 and Bim1. On kinetochore microtubules, Bik1 and Bim1 are redundant for regulating dynamics, whereas Stu2 acts independently from Bik1 and Bim1. These results indicate that interactions among +TIPs can play important roles in the regulation of microtubule dynamics.

INTRODUCTION

In living cells, individual microtubule ends alternate stochastically between phases of polymerization and depolymerization, a behavior known as dynamic instability. Polymerization dynamics are believed to be central to the biological function of microtubules, providing an effective mechanism to allow them to search three-dimensional space (Desai and Mitchison, 1997). The dynamics of microtubules in vivo are regulated by cellular proteins that interact directly with the distal ends of microtubules and termed plus-end tracking proteins (+TIPs; Schuyler and Pellman, 2001; Akhmanova and Hoogenraad, 2005). These include members of a variety of protein families such as those related to end-binding protein EB1, cytoplasmic linker protein CLIP-170, CLIP-associated protein CLASP, adenomatous polyposis coli (APC), XMAP215/Dis1, the dynactin subunit

p150^{Glued}, and the type 1 lissencephaly disease gene (Lis1). In addition, a variety of kinesin-related proteins have been shown to bind to microtubule ends and influence microtubule dynamics (Desai *et al.*, 1999; Cui *et al.*, 2005; Sproul *et al.*, 2005). The interplay between these factors is believed to play a key role in the regulation of microtubule dynamics and function.

Many +TIPs are known to physically interact with each other (Akhmanova and Hoogenraad, 2005) although the functional implications of these interactions are generally unclear. Nonetheless, evidence exists for both cooperation and competition between various +TIPs. The ability of EB1 to promote microtubule polymerization depends on APC (Nakamura *et al.*, 2001), and the microtubule-stabilizing activity of CLASPs depends on their interactions with EB proteins (Mimori-Kiyosue *et al.*, 2005). Recent work has also indicated that APC and EB1 act cooperatively to regulate mitotic spindle function (Green *et al.*, 2005). In contrast, the *Xenopus* protein XMAP215 stabilizes microtubules primarily by antagonizing the destabilizing activity of kinesin-13, XKCM1 (Tournibize *et al.*, 2000; Kinoshita *et al.*, 2001). Similarly, the human counterpart to XMAP215, chTOG, was found to have an antagonistic role with another kinesin-13 protein, MCAK, in establishing spindle bipolarity (Cassimeris and Morabito, 2004).

Several +TIPs have been identified in the yeast, *Saccharomyces cerevisiae*, and three of these, Stu2, Bik1, and Bim1, are the focus of this study. Stu2, a member of the XMAP215/Dis1 family, is an essential gene in yeast (Wang and Huffaker, 1997). In contrast, Bik1 and Bim1, members of the CLIP and EB1 families, respectively, are not essential (Berlin *et al.*, 1990; Schwartz *et al.*, 1997). Stu2, Bik1, and Bim1 each localize to the tips of cytoplasmic microtubules and on spindle microtubules (Tirnauer *et al.*, 1999; Kosco *et al.*, 2001; Lin

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Abbreviations used: FRAP, fluorescence redistribution after photobleaching; +TIP, microtubule plus-end tracking protein.

Table 1. Yeast strains

Strain	Genotype
CUY25	<i>MATa ade2 his3-Δ200 leu2-3,112 ura3-52</i>
CUY1243	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 stu2Δ::URA3::P_{ANB1}UB-R-STU2 TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52</i>
CUY1244	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52::URA3</i>
CUY1247	<i>MATα STU2-GFP::HIS3MX6 his3-Δ200 leu2-3,112 ura3-52</i>
CUY1316	<i>MATa P_{GALI}-HA-BIM::HIS3 his3-Δ200 leu2-3,112 lys2-801 ura3-52</i>
CUY1459	<i>MATα STU2-3GFP::HIS3MX6 his3-Δ200 leu2-3,112 ura3-52</i>
CUY1713	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 stu2Δ::URA3::P_{ANB1}UB-R-STU2 bik1Δ::kanMX TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52</i>
CUY1714	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 bik1Δ::kanMX TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52::URA3</i>
CUY1715	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 bim1Δ::kanMX TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52::URA3</i>
CUY1716	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 stu2Δ::URA3::P_{ANB1}UB-R-STU2 bim1Δ::kanMX TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52</i>
CUY1718	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 bik1Δ::URA3::P_{ANB1}UB-R-BIK1 TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52</i>
CUY1721	<i>MATa P_{ACE1}-UBR1 P_{ACE1}-ROX1 bik1Δ::URA3::P_{ANB1}UB-R-BIK1 bim1Δ::kanMX TUB1::TRP1::GFP-TUB1 ade2-101 lys2-801 trp1-Δ1 ura3-52</i>
CUY1742	<i>MATa/MATa STU2/STU2-3GFP::HIS3MX6 ura3-52::TUB1-CFP::URA3/ura3-52 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-3,112 trp1-Δ63/TRP1</i>
CUY1763	<i>MATa ade2-101 his3-Δ200 leu2-3,112 ura3-52 (YCp BIK1-3HA LEU2)</i>
CUY1764	<i>MATa ade2-101 his3-Δ200 leu2-3,112 ura3-52 (YCp BIK1-3HA LEU2; YCp BIK1-3Myc URA3)</i>
CUY1765	<i>MATα STU2-GFP::HIS3MX6 his3-Δ200 leu2-3,112 ura3-52 (YCp STU2-3HA LEU2)</i>
CUY1766	<i>MATa P_{GALI}-HA-BIM::HIS3 his3-Δ200 leu2-3,112 lys2-801 ura3-52 (YCp BIM1-3Myc URA3)</i>
CUY1767	<i>MATα BIM1-3GFP::TRP1 his3-Δ200 leu2-3,112 trp1-1 ura3-52</i>
CUY1768	<i>MATa BIM1-3GFP::TRP1 URA3::CFP-TUB1::ura3-52 his3-Δ200 leu2 lys2-801 trp1</i>
CUY1804	<i>pKAK162 (P_{ADH1}-GAL4_{AD}-STU2⁶⁵⁷⁻⁸⁸⁸ LEU2) and pKAB6 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵ P_{MET25}-BIK1 TRP1) in Y190</i>
CUY1805	<i>pKAK162 (P_{ADH1}-GAL4_{AD}-STU2⁶⁵⁷⁻⁸⁸⁸ LEU2) and pKAB3 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵ P_{MET25}-STU2 TRP1) in Y190</i>
CUY1806	<i>pKAK162 (P_{ADH1}-GAL4_{AD}-STU2⁶⁵⁷⁻⁸⁸⁸ LEU2) and pKAK213 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵) in Y190</i>
CUY1807	<i>pXC233 (P_{ADH1}-GAL4_{AD}-BIK1¹⁹⁹⁻⁴⁴⁰ LEU2) and pKAB1 (P_{ADH1}-GAL4_{BD}-STU2⁶⁵⁷⁻⁸⁸⁸ P_{MET25}-BIM1 TRP1) in Y190</i>
CUY1808	<i>pXC233 (P_{ADH1}-GAL4_{AD}-BIK1¹⁹⁹⁻⁴⁴⁰ LEU2) and pKAB4 (P_{ADH1}-GAL4_{BD}-STU2⁶⁵⁷⁻⁸⁸⁸ P_{MET25}-BIK1 TRP1) in Y190</i>
CUY1809	<i>pXC233 (P_{ADH1}-GAL4_{AD}-BIK1¹⁹⁹⁻⁴⁴⁰ LEU2) and pKAK214 (P_{ADH1}-GAL4_{BD}-STU2⁶⁵⁷⁻⁸⁸⁸ TRP1) in Y190</i>
CUY1810	<i>pKAK100 (P_{ADH1}-GAL4_{AD}-BIK1 LEU2) and pKAB3 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵ P_{MET25}-STU2 TRP1) in Y190</i>
CUY1811	<i>pKAK100 (P_{ADH1}-GAL4_{AD}-BIK1 LEU2) and pKAB6 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵ P_{MET25}-BIK1 TRP1) in Y190</i>
CUY1812	<i>pKAK100 (P_{ADH1}-GAL4_{AD}-BIK1 LEU2) and pKAK213 (P_{ADH1}-GAL4_{BD}-BIM1¹⁴⁵⁻³⁴⁵) in Y190</i>
Y151	<i>MATa P_{GALI}-lacZ gal4 gal80 his3-Δ200 leu2-3,112 ura3-52 ade2-101 trp1-94</i>
Y190	<i>MATa URA3::P_{GALI}-lacZ LYS2::P_{GALI}-HIS3 gal4 gal80 his3 leu2-3,112 ura3-52 ade2-101 trp1-901 cylh^r</i>

et al., 2001); Stu2 and Bik1 have also been shown to be associated with kinetochores (He *et al.*, 2001; Lin *et al.*, 2001). Loss of Stu2 reduces the dynamics of cytoplasmic and kinetochore microtubules *in vivo* and leads to defects in spindle orientation and elongation (Kosco *et al.*, 2001). Cells lacking Bik1 contain shorter cytoplasmic microtubules and display defects in spindle orientation (Berlin *et al.*, 1990), but the effect of Bik1 on microtubule dynamics in yeast has not yet been measured. In cells lacking Bim1, cytoplasmic microtubules are less dynamic than those in wild-type cells (Tirnauer *et al.*, 1999). Bim1 also plays a role in spindle positioning by mediating the interaction between cytoplasmic microtubules and the cortical protein Kar9 (Korinek *et al.*, 2000; Lee *et al.*, 2000; Miller *et al.*, 2000; Hwang *et al.*, 2003; Liakopoulos *et al.*, 2003). In this article, we describe both physical and functional interactions among Stu2, Bik1, and Bim1.

MATERIALS AND METHODS

Yeast Strains

The yeast strains used in this study are listed in Table 1. The PCR-based gene deletion and modification method (Longtine *et al.*, 1998) was used for deletion and epitope-tagging of chromosomal yeast genes. Construction of the Stu2 conditional depletion strain (*stu2^{cu}*) has been described (Kosco *et al.*, 2001). A Bik1 conditional depletion strain (*bik1^{cu}*) was made in a similar manner. Details on strain constructions are available on request.

A *STU2-3GFP* fusion was created at the endogenous *STU2* locus by the PCR-based gene modification method (Longtine *et al.*, 1998) using as PCR template a plasmid, pLY373, that contains three tandem copies of GFP. A

BIM1-3GFP fusion was created at the endogenous *BIM1* locus. A portion of *BIM1*, encoding the C-terminal 178 amino acids, was cloned into PB1960 (Lin *et al.*, 2001), containing three tandem copies of GFP, to create pKAB41. pKAB41 was then linearized within *BIM1* with PstI and transformed into yeast. Correct integrations were identified by PCR of genomic DNA and Western blotting.

Two-Hybrid Assays

Two-hybrid assays were performed as described by Durfee (Durfee *et al.*, 1993). Yeast strains Y190 and Y151 and the plasmids pAS2 (YE: *P_{ADH1}-GAL4_{AD}-HA LEU2*) and pACTII (YE: *P_{ADH1}-GAL4_{BD}-HA TRP1 CYH2*) were obtained from Steve Elledge (Harvard Medical School, Boston, MA). Deletions of *BIK1* and *BIM1* were created in the strain Y151. *GAL4_{AD}* and *GAL4_{BD}* fusions to full-length or truncated *BIK1*, *BIM1*, or *STU2* genes were made in pAS2 and pACTII, respectively. These were then cotransformed into Y190 or Y151. β -galactosidase activity was determined by the filter assay and scored for blue color by visual inspection.

Three-Hybrid Assays

For the three-hybrid experiments, pBridge (Clontech, Palo Alto, CA) was used in conjunction with pACTII constructs. pBridge allows for the expression of a *GAL4_{BD}* fusion from the *ADH1* promoter and another gene from *MET25* promoter. β -galactosidase activity was quantitated by a liquid assay (Clontech protocol PT3024-1). Cells were grown in SD medium lacking leucine and tryptophan and supplemented with the appropriate amount of methionine to an OD₆₀₀ of 0.5–0.9. Cells, 1.5 ml, were pelleted, washed with buffer 1 (100 mM HEPES, 154 mM sodium chloride, 4.5 mM L-aspartate, 1% BSA, 0.05% Tween 20), and concentrated by resuspending in 300 μ L of buffer 1. Cells, 100 μ L, were then frozen in liquid nitrogen and thawed at 37°C; this was repeated four times. Chlorophenol red- β -D-galactopyranoside (0.7 ml, 2.23 mM, CPRG; Roche Diagnostics, Indianapolis, IN) in buffer 1 was added to the cells. After development of red color, the reaction was stopped by adding 3 mM ZnCl₂. Cells were spun down, and the absorbance of the supernatant at 578 nm was read. β -galactosidase units equal $(1000 \times \text{OD}_{578}) / (T \times V \times \text{OD}_{600})$, where T

is elapsed time (in min) until development of red color and V is the volume of cells used (0.1 ml) times the concentration factor (5).

Immunoprecipitation Experiments

Immunoprecipitations were done as described previously (Chen *et al.*, 1998). For strains in which *BIM1-HA* is expressed from *GAL1* promoter, cells were grown in galactose-containing medium for 4 h before preparing extracts. Stu2 antibody was described previously (Kosco *et al.*, 2001). Bik1 antibody was made in rabbits using a Bik1 polypeptide containing amino acids 130–426. The rat monoclonal anti-yeast-tubulin antibody, YOL1/34 is from Accurate Chemical and Scientific (Westbury, NY). Mouse monoclonal antibodies HA11 and 9E10 that recognize the HA and myc epitopes, respectively, are from Berkeley Antibody Company (Berkeley, CA). HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse were purchased from Bio-Rad Laboratories (Hercules, CA).

In Vitro Binding Assays

BIM1 and *BIK1* were cloned into pET-GST-TEV (Moseley *et al.*, 2004) and transformed into BL21 DE3 *Escherichia coli* cells (Stratagene, La Jolla, CA). The cells were induced with IPTG and the GST fusion proteins were then purified on glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ). Soluble Bim1 was generated by treating the GST-Bim1 beads with TEV protease (Invitrogen, Carlsbad, CA), which cleaved between GST and Bim1. Bim1 was then purified from the His-tagged TEV protease by passage over Talon Resin (BD Biosciences Clontech, Mountain View, CA). A fragment of *STU2* encoding the C-terminal 385 amino acids (Stu2-ΔC) was previously cloned as a 6x-His fusion in pWP86 (Kosco *et al.*, 2001). pWP86 was transformed into BL21 DE3, induced with IPTG, purified onto Talon Resin (BD Biosciences Clontech), and eluted with imidazole. Binding assays were performed as described (Swaffield and Johnston, 1996). Rabbit polyclonal Bim1 antibody was a gift from Rita Miller (University of Rochester, Rochester, NY).

Fluorescence Microscopy and Image Analysis

All images were obtained with a 100× objective. Visualization of GFP- and CFP-conjugated proteins in live cells was done using a Zeiss Axioplan 2 Imaging microscope (Thornwood, NY) and Openlab software (Improvision, Lexington, MA). Time-lapse images of Stu2-3GFP and Bim1-3GFP were captured with a spinning disk confocal imaging system (UltraVIEW, Perkin-Elmer, Wellesley, MA). Analysis of cytoplasmic microtubule dynamics was done as described previously (Kosco *et al.*, 2001); images were taken at 20-s intervals.

FRAP experiments were performed on a Leica TCS SP2 confocal microscope system (Exton, PA). After photobleaching, a z-series stack consisting of five frames acquired at 0.5-μm intervals was acquired at each time point. For each z-series stack, a single image was constructed by maximum-brightness projection. Fluorescence intensities of bleached (F_b) and unbleached (F_u) half-spindles were measured. To calculate a rate constant k for redistribution, we assumed a first-order kinetic exchange of fluorescent molecules between the two half-spindles. The difference between fluorescence intensities in the two half-spindles ($F_u - F_b$) was calculated and divided by the total spindle fluorescence ($F_u + F_b$) to normalize for fluctuations in laser intensity, giving the ratio $r = (F_u - F_b)/(F_u + F_b)$. Then, $r(t)$ was modeled by $r(t)/r(0) = (1 - f)e^{-kt} + f$, where k is the rate constant and f is the asymptotic value of $r(t)/r(0)$ equivalent to the fraction of fluorescence that does not redistribute. k , f , and SEs were determined by nonlinear regression. Although f could in principle be determined from the asymptotic (extremely long time) difference between F_u and F_b , determining f and k together by nonlinear regression gives a more accurate estimate, particularly because the experiments do not extend into the fully asymptotic range. t tests were performed on the differences between k values. The $t_{1/2}$ of fluorescence redistribution was calculated by $t_{1/2} = \ln 2/k$. Note that the value of k computed here is the same as that computed by Maddox *et al.* (2000). The values of f we compute are based on the differences in fluorescence intensities in the bleached and unbleached half-spindles, whereas recovery (R) defined by Maddox *et al.* (2000) is calculated from the ratios of these intensities. These values are related by the following equation: $R = (1 - f)/(1 + f)$.

RESULTS

Stu2 and Bim1 Track on the Plus Ends of Growing and Shrinking Microtubules

Stu2, Bik1, and Bim1 are observed in the cytoplasm as one to several dots per cell often near the cell cortex (Tirnauer *et al.*, 1999; Kosco *et al.*, 2001; Lin *et al.*, 2001). This localization suggests that these proteins reside at the plus ends of cytoplasmic microtubules. In the case of Bik1, this has been demonstrated directly in cells double-labeled for microtubules and Bik1 (Carvalho *et al.*, 2004). To demonstrate the

Figure 1. Stu2 and Bim1 track on the plus ends of growing and shrinking cytoplasmic microtubules. (A and B) Images of live cells expressing CFP-Tub1 and Stu2-3GFP (CUY1742) or CFP-Tub1 and Bim1-3GFP (CUY1768). (C and D) Time-lapse images of Stu2-3GFP (CUY1459) and Bim1-3GFP (CUY1767). Times indicated are in seconds. White arrowheads indicate positions of the spindle pole bodies; each colored arrowhead indicates the position of a particular GFP dot over time. See Supplementary Videos 1 and 2, Stu2-3GFP; Supplementary Videos 3 and 4, Bim1-3GFP.

plus-end localization of Stu2 and Bim1 on cytoplasmic microtubules, we tagged both Stu2 and Bim1 with three tandem copies of GFP and expressed them in cells along with CFP-Tub1 (α -tubulin). The Stu2 and Bim1 dots consistently colocalized with the distal ends of cytoplasmic microtubules at all stages of the cell cycle (Figure 1, A and B). Time-lapse imaging of Stu2-3GFP and Bim1-3GFP demonstrated that these proteins bound the plus ends of both growing and shrinking microtubules (Figure 1, C and D; Supplementary Videos 1–4). Similar results have been reported for Bik1 previously (Carvalho *et al.*, 2004).

Stu2, Bik1, and Bim1 Interact in All Pairwise Combinations

Previously, our laboratory performed a two-hybrid screen using Stu2 as bait and identified Stu2, Bik1, and Bim1 (Chen *et al.*, 1998). We also found that Bik1 and Bim1 interact in the two-hybrid assay. To confirm these two-hybrid interactions,

Figure 2. Associations of Stu2, Bik1, and Bim1 in vivo and in vitro. (A) Stu2, Bik1, and Bim1 associate with each other in vivo. Protein extracts from a strain expressing Bim1-HA (CUY1316) or a control strain (CUY25) were immunoprecipitated with antibodies that recognize Stu2, Bik1, or HA (indicated as IP at bottom). Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with the antibodies indicated at left. WCE, whole cell extract. (B) Stu2, Bik1, and Bim1 associate with themselves in vivo. Strains expressing two tagged forms of Stu2, Bik1, or Bim1 (CUY1765, CUY1764, or CUY1766) were precipitated using antibody to one tag and blotted with antibody to the other tag. Control strains are CUY1247, CUY1763, and CUY1316. (C) Binding assays in vitro. GST-Bik1, GST-Bim1, or GST alone were immobilized on beads and incubated with Stu2- Δ C or Bim1 as indicated. Bound protein was detected by Western blotting after SDS-PAGE.

we performed coimmunoprecipitation assays using a yeast strain that expresses an epitope-tagged version of Bim1 (Bim1-HA) from the *GAL1* promoter. Antibodies specific for Stu2, Bik1, or Bim1-HA were used to immunoprecipitate these proteins from yeast cell extracts. In each case, the other two proteins coimmunoprecipitated with the target protein (Figure 2A). The Stu2-Bim1 interaction appears to be less prominent based on the weaker Stu2 signal in the Bim1-HA precipitation and the weaker Bim1-HA signal in the Stu2 precipitation. In addition, the Stu2-Bim1 interaction was not detected in cells expressing Bim1 from its endogenous promoter, although the Bik1-Bim1 interaction was clearly observed in these cells (unpublished data). There was no detectable tubulin in any of the immunoprecipitates indicating that these interactions are not mediated by microtubule subunits. Thus, we can detect interactions of Stu2, Bik1, and Bim1 in vivo in all pairwise combinations.

The identification of Stu2 itself in the two-hybrid screen suggested that Stu2 self-associates. Similarly, we have found that both Bik1 and Bim1 self-associate in the two-hybrid assay. We further investigated these self-interactions using coimmunoprecipitation. To assay the Stu2 self-interaction, we created a strain that expresses both Stu2-HA and Stu2-GFP. When anti-HA antibody was used to immunoprecipitate Stu2-HA, the Stu2-GFP was precipitated along with it (Figure 2B). In similar experiments, we assayed the Bik1 and Bim1 self-interactions by creating strains that express both Bik1-HA and Bik1-myc or Bim1-HA and Bim1-myc, respectively. In both cases, immunoprecipitations using anti-myc antibody pulled down the HA-tagged version of these proteins (Figure 2B). These results demonstrate that Stu2, Bik1, and Bim1 self-associate in vivo.

Having established that Stu2, Bik1, and Bim1 interact in vivo, we next investigated whether these proteins could associate directly in vitro. Full length Bik1 and Bim1 were fused to GST, expressed in *E. coli*, and immobilized on glutathione-Sepharose resin. Purified, soluble full-length Bim1 and a C-terminal fragment of Stu2 (Stu2- Δ C) that contains the Bik1- and Bim1-binding domain (see below) were tested for their association with these immobilized proteins (Figure 2C). GST-Bik1 precipitated both Bim1 (lane 6) and Stu2 (lane 9), whereas GST alone was unable to associate with either Bim1 (lane 5) or Stu2 (lane 7). However, we could not demonstrate an association between GST-Bim1 and Stu2 (lane 8). Full-length Stu2 purified from insect cells gave results similar to Stu2- Δ C (unpublished data).

Identification of Stu2, Bik1, and Bim1 Interaction Domains

To investigate the domains of Stu2, Bik1, and Bim1 that are sufficient for interactions with themselves and one another, we created a series of N- and C-terminal truncations of each of these proteins to be used in the two-hybrid assay (Figure 3). The region of Stu2 required to interact with itself is contained within a 190 amino acid segment (612-801), that also includes a portion of its microtubule-binding domain (amino acids 558-657; Wang and Huffaker, 1997). The C-terminal 34 amino acids of Stu2 (amino acids 855-888) are sufficient for interaction with both Bim1 and Bik1. The fact that this domain of Stu2 does not overlap its microtubule-binding domain provides further evidence that these interactions are not mediated by microtubules.

In a similar manner, we determined that the N-terminal 189 amino acids of Bik1, which contain its microtubule-binding domain (Lin *et al.*, 2001), are sufficient for interaction with Bim1. The C-terminal 242 amino acids of Bik1 (amino acids 199-440), which contain its coiled-coil and metal-binding motif, are sufficient for an interaction with Stu2. A somewhat longer C-terminal fragment of Bik1 (amino acids 123-440) is required for interaction with itself. Finally, the C-terminal 200 amino acids of Bim1 (amino acids 145-345) interact with Stu2, Bik1, and itself (Figure 3).

Stu2, Bik1, and Bim1 Compete for Binding to Each Other

Given that Stu2, Bik1, and Bim1 interact in all pairwise combinations, we wanted to investigate the nature of these interactions. There are three distinct possibilities: 1) The interactions could be independent. For example, if Stu2 binds Bik1 and Bim1 independently, the binding of Bik1 to Stu2 would not affect the binding of Bim1 to Stu2 and vice versa. 2) The interactions could be dependent. For example, Stu2 may interact directly only with Bik1 which in turn binds Bim1, thus bridging the interaction between Stu2 and Bim1. 3) The interactions could be competitive. For example, Bik1 and Bim1 may compete for binding to Stu2.

Figure 3. Mapping protein-protein interaction domains in Stu2, Bik1, and Bim1. Two-hybrid interactions were tested between the fragments of Stu2, Bik1, and Bim1 diagrammed in the drawings at the left and full-length Stu2, Bik1, and Bim1. Interactions were determined using a colony filter β -galactosidase color assay and scored for blue color. (+), positive interaction; (-), negative interaction. Protein structural features are indicated: CAP, CAP-Gly domain; CC, predicted coiled-coil region; MT, microtubule-binding domain; HEAT, HEAT repeats.

To determine whether the Stu2-Bik1, or Stu2-Bim1 interaction is bridged through Bim1 or Bik1, respectively, we performed two-hybrid assays in *bim1* Δ and *bik1* Δ strains. *STU2* is an essential gene so we were not able to test the Bik1-Bim1 interaction in a *stu2* Δ strain (Wang and Huffaker, 1997). We found that deletion of *BIK1* does not diminish the Stu2-Bim1 interaction, and deletion of *BIM1* does not diminish the Bik1-Stu2 interaction. Because Kar9 is known to interact with both Stu2 (Miller *et al.*, 2000) and Bim1 (Korinek *et al.*, 2000; Lee *et al.*, 2000), we also tested whether deletion of Kar9 affected the Stu2-Bim1, Stu2-Bik1, or Bik1-Bim1 interaction; it did not in any of these cases. Thus, we found no evidence for interdependence among the Stu2/Bik1/Bim1 interactions.

To test for competition in binding, we used a three-hybrid system. In this system, in addition to the expression of the bait and prey constructs, a third gene is placed under the control of the conditional *MET25* promoter and fused to a nuclear localization sequence. If overexpression of the third protein competes with the two-hybrid interaction, then an inhibitory effect should be observed on reporter gene expression. In these experiments, the overexpression of Bim1 and Bik1 in the absence of methionine was lethal, so a concentration of 5 or 7.5 μ g/ml methionine was used, respectively. In the presence of 150 μ g/ml methionine only low levels of Stu2, Bim1, or Bik1 are expressed, whereas in cells grown in 0, 5 or 7.5 μ g/ml methionine, the levels of Stu2, Bim1, or Bik1, respectively, are much greater (Figure 4A). Strikingly, overexpression of Bim1 reduced the Stu2-Bik1 interaction to 14% of that obtained with low levels of Bim1 (Figure 4B). This is comparable to the reduction observed in control cells overexpressing Bik1. The Bik1 fragment expressed in these cells (Bik1¹⁹⁹⁻⁴⁴⁰) does not bind Bim1 so this result indicates that Bik1 and Bim1 compete for binding to Stu2.

Figure 4. Stu2, Bik1, and Bim1 compete for binding to each other. Strains were grown on 150 μ g/ml methionine to repress expression from *P_{MET25}* and 0 μ g/ml methionine to induce expression of *P_{MET25}-STU2*, 5 μ g/ml methionine to induce expression of *P_{MET25}-BIM1*, or 7.5 μ g/ml methionine to induce expression of *P_{MET25}-BIK1*. (A) Yeast extracts were subjected to SDS-PAGE followed by Western blotting using anti-HA antibody to detect Bim1, Stu2, or Bik1 (each is fused to an NLS and the HA tag). (B) Each strain contains a protein fused to the Gal4-binding domain (BD), a protein fused to the Gal4 activation domain (AD), and a protein that is overexpressed (OE) from the *MET25* promoter (except for controls indicated as none). Values shown for β -galactosidase activity are averages from three experiments. The percent activity for each strain equals 100 $\times$ activity in low methionine (induction)/activity in high methionine (repression).

Similarly, overexpression of Bik1 reduced the Stu2-Bim1 interaction to 7%. In addition, overexpression of Stu2 reduced the Bim1-Bik1 interaction by about half, an effect similar to that observed in control cells overexpressing Bik1.

Combined Effects of Stu2, Bik1, and Bim1 on Cytoplasmic Microtubule Dynamics

We wanted to know how the combined activities of Stu2, Bim1 and Bik1 influence microtubule dynamics. To determine this, we constructed single and double mutant strains that lack one or two of these proteins, respectively. We anticipated three simple models that might describe their interplay. 1) The two proteins act independently. In this case, loss of either protein would have an effect and these effects would be additive upon loss of both proteins. 2) The two proteins act cooperatively. For example, the proteins could form a complex such that loss of either protein would destroy its activity. In this case, loss of either protein alone or loss of both proteins would have a similar effect. 3) The two proteins are redundant. In this case, loss of either protein alone would have little effect but loss of both would be significant.

To perform these experiments, we created strains that lacked Stu2, Bik1, and Bim1. *BIM1* and *BIK1* are not essential genes so we used *bim1* Δ and *bik1* Δ strains. *STU2* is an essential gene so we used the copper-inducible Stu2 depletion strain that we described previously and refer to as *stu2^{cu}* (Kosco *et al.*, 2001). In this strain, Stu2 levels are reduced by >90% within 2 h after addition of copper to the medium. A *bim1* Δ *bik1* Δ double mutant is also inviable so we created a copper-inducible Bik1 depletion strain (referred to as *bik1^{cu}*) that can be used in combination with *bim1* Δ . In the *bik1^{cu}* strain, the levels of Bik1 are reduced by >90% after 6 h of exposure to copper (unpublished data). Therefore, to de-

Table 2. Cytoplasmic microtubule dynamics

	Growth rate ($\mu\text{m}/\text{min}$)	Shrinkage rate ($\mu\text{m}/\text{min}$)	Rescue frequency (events/s)	Catastrophe frequency (events/s)	Pause time (%)	Dynamicity (dimers/s)
G1						
WT	0.85 ± 0.47	1.04 ± 0.76	0.0130	0.0104	8	20.5
<i>stu2^{cu}</i>	0.96 ± 0.56	0.90 ± 0.66	0.0034	0.0040	55	8.6
<i>bim1Δ</i>	0.70 ± 0.46	0.81 ± 0.56	0.0039	0.0040	43	9.2
<i>bik1Δ</i>	0.38 ± 0.18	0.51 ± 0.26	0.0025	0.0021	54	4.9
<i>bik1^{cu}</i>	0.54 ± 0.34	0.55 ± 0.32	0.0042	0.0044	33	8.0
<i>stu2^{cu} bim1Δ</i>	0.82 ± 0.97	0.76 ± 0.41	0.0041	0.0038	40	9.8
<i>stu2^{cu} bik1Δ</i>	0.29 ± 0.24	0.40 ± 0.39	0.0009	0.0011	74	2.0
<i>bik1^{cu} bim1Δ</i>	0.42 ± 0.21	0.53 ± 0.28	0.0008	0.0016	73	3.2
Peanaphase						
WT	0.79 ± 0.32	0.86 ± 0.37	0.0089	0.0109	14	17.2
<i>stu2^{cu}</i>	0.88 ± 0.58	1.06 ± 0.48	0.0040	0.0047	52	10.7
<i>bim1Δ</i>	0.91 ± 0.57	0.93 ± 0.48	0.0086	0.0097	15	17.5
<i>bik1Δ</i>	0.48 ± 0.27	0.52 ± 0.26	0.0042	0.0028	37	7.0
<i>bik1^{cu}</i>	0.45 ± 0.23	0.49 ± 0.22	0.0037	0.0038	39	7.2
<i>stu2^{cu} bim1Δ</i>	0.65 ± 0.58	0.62 ± 0.33	0.0047	0.0021	45	8.4
<i>stu2^{cu} bik1Δ</i>	0.32 ± 0.33	0.52 ± 0.65	0.0008	0.0014	59	2.6
<i>bik1^{cu} bim1Δ</i>	0.45 ± 0.22	0.47 ± 0.19	0.0020	0.0036	38	6.8

WT (CUY1244), G1 (n = 10, t = 5480 s), PA (n = 10, t = 5240 s); *stu2^{cu}* (CUY1243), G1 (n = 17, t = 9600 s), PA (n = 8, t = 4420 s); *bik1 Δ* (CUY1714), G1 (n = 10, t = 5660 s), PA (n = 10, t = 5340 s); *bik1^{cu}* (CUY1718), G1 (n = 16, t = 8560 s), PA (n = 16, t = 8960 s); *bim1 Δ* (CUY1715), G1 (n = 10, t = 5480 s), PA (n = 10, t = 5440 s); *stu2^{cu} bik1 Δ* (CUY1713), G1 (n = 17, t = 9680 s), PA (n = 8, t = 4520 s); *stu2^{cu} bim1 Δ* (CUY1716), G1 (n = 10, t = 5520 s), PA (n = 11, t = 5980 s); *bik1^{cu} bim1 Δ* (CUY1721), G1 (n = 10, t = 5600 s), PA (n = 10, t = 5380 s). n, number of time-lapse sequences; t, total length of time-lapse sequences obtained. Event rates are average values $\pm$ SD.

plete Stu2 or Bik1, strains were grown in copper-containing medium for 2 or 6 h, respectively.

We analyzed cytoplasmic microtubule dynamics in live cells expressing Tub1-GFP. Lengths of individual microtubules were measured at time intervals and these measurements were used to determine the rates of growth and shrinkage, the frequencies of catastrophes (transitions to shrinkage after a growth or a pause) and rescues (transitions to growth after a shrinkage or a pause), and the fraction of time spent paused (no significant growth or shrinkage). We analyzed microtubules in both G1 cells that lacked spindles and preanaphase cells that contained short bipolar spindles. Because loss of Stu2 inhibits spindle elongation, we did not measure microtubule dynamics in anaphase cells. Microtubule dynamics have been examined previously in both *stu2^{cu}* and *bim1 Δ* cells (Tirnauer *et al.*, 1999; Kosco *et al.*, 2001), and the results we obtained here are similar to these reports. Both *bik1 Δ* and *bik1^{cu}* cells were used to examine microtubule dynamics in cells lacking Bik1.

The results of these experiments are shown in Table 2. Loss of either Stu2 or Bim1 did not significantly alter the rates of microtubule growth and shrinkage. However, loss of Stu2 in G1 and preanaphase cells increased several-fold the percentage of time that microtubules spent in the paused state. Although microtubules in wild-type cells paused $\sim 10\%$ of the time, microtubules in *stu2^{cu}* cells spent $\sim 50\%$ of the time in a paused state. This increase in pausing is the primary reason for a greater than twofold reduction in catastrophe and rescue frequencies. Loss of Bim1 had an effect on pausing similar to the loss of Stu2 in G1 cells, but no apparent effect in preanaphase cells. Loss of Bik1, either by deletion (*bik1 Δ*) or depletion (*bik1^{cu}*), resulted in increases in pausing and decreases in transition frequencies similar to cells lacking Stu2. In addition, cells lacking Bik1 showed a nearly twofold decrease in growth and shrinkage rates in both G1 and preanaphase cells ($p < 0.005$ in all cases).

A convenient parameter for indicating the level of microtubule dynamics is termed dynamicity (Toso *et al.*, 1993). Dynamicity is a measure of the mean rate of tubulin exchange on microtubules and is equivalent to the number of tubulin dimers gained or lost per second. This value reflects growth and shrinkage rates as well as the fraction of time microtubules spend growing and shrinking. Dynamicity is about twofold lower in G1 and preanaphase cells lacking Stu2 or Bik1. In cells lacking Bim1, dynamicity is reduced about twofold in G1 cells but not in preanaphase cells. Thus, all three proteins stimulate cytoplasmic microtubule dynamics, but Bim1 does so only in G1 cells.

Figure 5. Comparison of cytoplasmic microtubule dynamics in single and double mutants. (A) Dynamicity in single mutants. (B) Dynamicity in *stu2^{cu} bim1 Δ* double mutant versus *stu2^{cu}* and *bim1 Δ* single mutants. (C) Dynamicity in *stu2^{cu} bik1 Δ* double mutant versus *stu2^{cu}* and *bik1 Δ* single mutants. (D) Dynamicity in *bik1^{cu} bim1 Δ* double mutant versus *bik1^{cu}* and *bim1 Δ* single mutants. Values are taken from Table 2.

Figure 6. Spindle microtubule dynamics in single and double mutants. Quantitation of fluorescence redistribution of photo-bleached half-spindles. Each point is an average value $\pm$ SEM of the number (n) of spindles examined; WT, CUY1244; *stu2^{cu}*, CUY1243; *bik1 Δ* , CUY1714; *bik1^{cu}*, CUY1718; *bim1 Δ* , CUY1715; *stu2^{cu} bik1 Δ* , CUY1713; *stu2^{cu} bim1 Δ* , CUY1716; and *bik1^{cu} bim1 Δ* , CUY1721. Curves were modeled from data as described in *Materials and Methods*. Values for n , $t_{1/2}$, and redistribution are in Table 3.

Next we analyzed cytoplasmic microtubule dynamics in the double mutants (Table 2 and Figure 5). In G1 and preanaphase *stu2^{cu} bik1 Δ* cells, dynamicity decreased more than four- and two fold compared with that in *stu2^{cu}* and *bik1 Δ* cells, respectively. Thus, the effects of losing Stu2 and Bik1 appear to be additive, suggesting that Stu2 and Bik1 act independently in both of these phases of the cell cycle. In G1 *bik1^{cu} bim1 Δ* cells, dynamicity is also reduced $\sim$ 3-fold and 2.5-fold compared with that in *bim1 Δ* and *bik1^{cu}* cells, respectively, indicating that Bik1 and Bim1 act independently in G1. In G1 *stu2^{cu} bim1 Δ* cells, dynamicity is similar to that in *stu2^{cu}* and *bim1 Δ* cells, indicating that Stu2 and Bim1 act cooperatively in G1. Dynamicity in preanaphase *bik1^{cu} bim1 Δ* and *stu2^{cu} bim1 Δ* cells is similar to that in *bik1^{cu}* and *stu2^{cu}* cells, respectively, consistent with the idea that Bim1 does not influence cytoplasmic microtubule dynamics in preanaphase.

Combined Effects of Stu2, Bik1, and Bim1 on Kinetochore Microtubule Dynamics

At metaphase, the haploid *S. cerevisiae* spindle contains 16 kinetochore microtubules and a few polar microtubules originating from each spindle pole (Winey *et al.*, 1995). Kinetochore microtubules are dynamic with their growth and shrinkage coupled to oscillations of kinetochores along the length of the spindle. However, their close association precludes the observation of individual microtubules in the spindle. Therefore, we used fluorescence redistribution after photobleaching (FRAP) as a measure of kinetochore microtubule dynamics (Maddox *et al.*, 2000). Half of a metaphase spindle labeled with GFP-Tub1 was selectively photobleached. Then, the fluorescence intensities of both the bleached and unbleached half-spindles were measured over time. From these values, we calculated the extent of redistribution and a time to half-maximal redistribution ($t_{1/2}$), assuming that redistribution was due to a first-order kinetic exchange of fluorescent molecules between the two half-

Table 3. Measured parameters of FRAP

Strain	n	$t_{1/2}$ (s)	f
WT	11	61.1 ± 10.8	0.11 ± 0.06
<i>stu2^{cu}</i>	11	nd	nd
<i>bim1Δ</i>	8	60.8 ± 12.6	0.08 ± 0.07
<i>bik1Δ</i>	10	53.2 ± 9.3	0.21 ± 0.04
<i>bik1^{cu}</i>	6	64.4 ± 10.3	0.26 ± 0.04
<i>stu2^{cu} bim1Δ</i>	12	nd	nd
<i>stu2^{cu} bik1Δ</i>	10	nd	nd
<i>bik1^{cu} bim1Δ</i>	9	128.4 ± 31.5	0.32 ± 0.07

n , number of spindles examined; f , fraction that does not redistribute; nd, not determined because the rates of redistribution were too low.

spindles (see *Materials and Methods*). This assumption is based on the data of Maddox *et al.* (2000), who showed that the rate of redistribution of fluorescence in the bleached half-spindle matches the rate of decay of fluorescence in the unbleached half-spindle.

Fluorescence redistribution in wild-type cells occurred with a $t_{1/2}$ of 61 s (Figure 6 and Table 3). Loss of Bik1 (*bik1 Δ* and *bik1^{cu}*) or Bim1 (*bim1 Δ*) had no significant effects on $t_{1/2}$ ($p > 0.3$). However, loss of Bik1 (*bik1 Δ* and *bik1^{cu}*), but not Bim1 (*bim1 Δ*), slightly increased the fraction that does not redistribute (f). As reported previously (Kosco *et al.*, 2001), depletion of Stu2 has a dramatic effect on spindle microtubule dynamics, with *stu2^{cu}* cells failing to show any measurable redistribution over this time period. Because of the low amount of redistribution, a $t_{1/2}$ could not be accurately calculated. Redistribution rates in cells lacking Bik1, or Bim1, in addition to Stu2, were similar to that in cells lacking Stu2 alone. Interestingly, cells lacking both Bik1 and Bim1 showed an intermediate level of redistribution with a $t_{1/2}$ of 128 s, approximately half the wild-type rate. This rate is significantly different from that measured in wild-type cells or in cells lacking either Bik1, or Bim1 alone ($p < 0.015$ in all cases). Given that loss of Bik1, or Bim1 alone had little effect on redistribution rates, this result indicates that these proteins play redundant roles in maintaining spindle microtubule dynamics.

DISCUSSION

Stu2, Bik1, and Bim1 Are Plus-End Tracking Proteins

Bik1 has been shown to track along the plus ends of both growing and shrinking microtubules (Carvalho *et al.*, 2004). Here we show that Stu2 and Bim1 also track along growing and shrinking microtubules. These abilities of Bik1 and Bim1 are distinct from those of their mammalian counterparts, CLIP170 and EB1, that track along the ends of only growing microtubules (Perez *et al.*, 1999; Tirnauer *et al.*, 2002). The plus end localization of Bik1 requires the activity of the kinesin-like protein, Kip2 (van Breugel *et al.*, 2003; Carvalho *et al.*, 2004), suggesting that active transport is the mechanism by which Bik1 remains associated with microtubule ends. Kinesin-like proteins are not required for plus-end localization of Stu2 (unpublished data), but Stu2 has the intrinsic ability to bind plus-ends of stabilized microtubules in vitro (van Breugel *et al.*, 2003). Although the exact mechanisms underlying the localization of Stu2, Bik1, and Bim1 remain to be elucidated, it appears likely that these will be varied (Carvalho *et al.*, 2003).

Physical Interactions among Stu2, Bik1, and Bim1

Like many +TIPs (Akhmanova and Hoogenraad, 2005), Stu2, Bik1, and Bim1 display a mutual affinity. Two-hybrid, coimmunoprecipitation, and *in vitro* binding assays indicate that Bik1 interacts directly with both Stu2 and Bim1. The evidence for a Stu2-Bim1 interaction is not as strong. Stu2 and Bim1 do interact in the two-hybrid assay and by coimmunoprecipitation when Bim1 is expressed from the *GAL1* promoter. However, Bim1 is overexpressed in both of these assays, and we do not observe coimmunoprecipitation of Stu2 and Bim1 when Bim1 is expressed at endogenous levels. These results indicate that Stu2 and Bim1 have the ability to interact but this interaction is limited in the cell. One possibility is that these proteins interact only at the plus-ends of cytoplasmic microtubules where a small fraction of the total Stu2 and Bim1 is located. In addition, we do not observe binding of Stu2 and Bim1 *in vitro*, which might suggest that Stu2 and Bim1 are linked by another protein in the cell. Bik1 and Kar9 are known to bind both Stu2 and Bim1, but neither of these is essential for the Stu2-Bim1 two-hybrid interaction. Alternatively, Stu2 and Bim1 may interact directly in the cell, but this interaction requires posttranslational modifications that do not occur in *E. coli* or insect cells. The fact that Stu2 and Bim1 functionally interact only in G1 cells suggests that this interaction is regulated by the cell cycle, a process that could involve post-translational modifications of either protein.

Although Stu2, Bik1, and Bim1 can interact in all pairwise combinations, our results argue against the notion that these proteins associate in a single unique complex. First, three-hybrid experiments indicate that Stu2, Bik1, and Bim1 compete for binding to each other. Specifically, the C-terminal 34 amino acids of Stu2 are sufficient to bind both Bik1 and Bim1, but cannot bind both simultaneously. Second, deletion of *BIK1* or *BIM1* does not disrupt the Stu2-Bim1 or Stu2-Bik1 interaction, respectively, as might be expected for a multi-protein complex. These findings suggest that cells contain distinct Stu2-Bik1, Stu2-Bim1, and Bik1-Bim1 complexes that may play unique roles in regulating microtubule function.

Stu2, Bik1, and Bim1 can associate with microtubules directly, but three lines of evidence indicate that their interactions do not require microtubule binding. 1) Tubulin does not coimmunoprecipitate with any of these proteins. 2) The regions of Stu2, Bik1, and Bim1 that mediate all but one of these interactions do not contain microtubule-binding domains. The microtubule-binding domain of Stu2 resides in amino acids 558-657 (Wang, 1998), that of Bik1 lies within the CAP-Gly domain (Lin *et al.*, 2001), and that of Bim1 likely resides in the N-terminal domain defined in the EB1 homologue RP1 (Juwana *et al.*, 1999). Thus, the only interaction region containing a microtubule-binding domain is Bik1¹⁻¹⁸⁹, which interacts with Bim1. 3) *In vitro* binding studies, which confirmed the Stu2-Bik1 and Bik1-Bim1 interactions, were done in the absence of tubulin.

Self-interactions are likely mediated by each protein's coiled-coil domain. In CLIP-170, a mammalian homolog of Bik1, the coiled-coil region has been shown to mediate its dimerization (Scheel *et al.*, 1999). We find that a C-terminal fragment of Bik1 containing the entire predicted coiled-coil region is required for the Bik1-Bik1 interaction. Interestingly, a smaller C-terminal fragment Bik1¹⁹⁹⁻⁴⁴⁰ did not interact with Bik1, perhaps because it is lacking the first 10 amino acids of this coiled-coil region. Similarly, the fragments of Stu2 and Bim1 that are required for self-interactions both contain a predicted coiled-coil domain (see Figure 3).

Functional Interactions on Cytoplasmic Microtubules

Cytoplasmic microtubules are much less dynamic in cells lacking Stu2, Bik1, or Bim1. Although Stu2 and Bik1 stimulate cytoplasmic microtubule dynamics in both G1 and preanaphase cells, Bim1 does so only in G1 cells (this study and Tirnauer *et al.*, 1999). This latter result is curious given that Bim1 localizes to the plus ends of cytoplasmic microtubules in both G1 and preanaphase cells (Tirnauer *et al.*, 1999). Evidently, plus-end localization of Bim1 *per se* is not sufficient to stimulate dynamics. The primary effect of Stu2 and Bim1 is to reduce the amount of time cytoplasmic microtubules spend in the paused state. Bik1 has this effect as well, but, in addition, increases the rates of growth and shrinkage about twofold. Thus, Bik1 appears to stimulate cytoplasmic microtubule dynamics by a somewhat different mechanism than Stu2 or Bim1.

Bik1 acts independently of Stu2 and Bim1 in stimulating cytoplasmic microtubule dynamics, indicating that the physical interactions of Bik1 with Stu2 or Bim1 are not required for these activities. In contrast, the Stu2-Bim1 interaction does play a role in regulating cytoplasmic microtubule dynamics. Stu2 and Bim1 act cooperatively in G1; loss of both proteins produces an effect that is very similar to loss of either protein alone for all parameters measured. These results suggest that Stu2 and Bim1 may be essential components of a complex that regulates cytoplasmic microtubule dynamics. Interestingly, Stu2 continues to play this role in preanaphase cells but Bim1 apparently does not. This raises a couple of questions for future investigation: 1) why is Bim1 required for normal cytoplasmic microtubule dynamics in G1 but not preanaphase and 2) does the functional independence of Stu2 and Bim1 at preanaphase coincide with a physical separation, and, if so, how is this regulated?

Functional Interactions on Kinetochores Microtubules

As reported previously, loss of Stu2 has a dramatic effect on FRAP, indicating that this protein plays a major role in stimulating kinetochore microtubule dynamics (Kosco *et al.*, 2001). On the other hand, loss of either Bik1 or Bim1 has no significant effect on the half-time of redistribution. Loss of Bik1 does increase the fraction of microtubules that do not redistribute from ~10% in wild-type cells to ~20% in *bik1Δ* cells. The fraction that does not redistribute has been attributed to the presence of polar microtubules that are believed to turnover at a much lower rate than kinetochore microtubules (Maddox *et al.*, 2000). Thus, these results indicate that Bik1 may influence the ratio of kinetochore to polar microtubules in the spindle.

Interestingly, analysis of the *bik1^{cu} bim1Δ* double mutant shows that Bik1 and Bim1 do play roles in stimulating kinetochore microtubule dynamics but that their activities are redundant. The primary role of Stu2 and the redundancy of Bik1 and Bim1 suggest that both a Stu2-Bik1, and a Stu2-Bim1 complex may play roles in stimulating kinetochore microtubule dynamics. However, the loss of Stu2 produces a much larger effect than loss of Bik1 and Bim1 together, indicating that at least part of Stu2's activity is independent of both Bik1 and Bim1.

CONCLUSIONS

This study has shown that Stu2, Bik1, and Bim1 interact physically in all pairwise combinations *in vivo*. In spite of these interactions, however, in most cases, these proteins appear to act independently in regulating microtubule dynamics (Table 4). The only compelling example of coopera-

Table 4. Functional interactions affecting microtubule dynamics

	G1 Cytoplasmic MTs	Preanaphase	
		Cytoplasmic MTs	Kinetochore MTs
Stu2-Bik1	Independent	Independent	Independent
Stu2-Bim1	Cooperative	Independent	Independent
Bik1-Bim1	Independent	Independent	Redundant

tive function is between Stu2 and Bim1 in controlling cytoplasmic microtubule dynamics in G1 cells. Bik1 and Bim1 act redundantly in regulating spindle microtubule dynamics but redundancy does not imply a physical interaction. Why do pairwise interactions among these +TIPs often appear to be unimportant to their roles in regulating microtubule dynamics? One possible explanation is redundancy. Like most +TIPs, Stu2, Bik1, and Bim1 interact with a variety of other proteins at the microtubule plus end. Although the sum total of these interactions may be important to the activity of any particular +TIP, it is possible that no one interaction is critical. It should also be noted that this study has focused exclusively on microtubule dynamics. It is possible that Stu2, Bik1, and Bim1 interactions are important for other aspects of microtubule function such as mediating the interactions between microtubule ends and other cellular structures. Also, a fraction of these proteins is located at the spindle poles, suggesting that functionally important interactions among Stu2, Bik1, and Bim1 may be occurring somewhere other than the plus-ends of microtubules.

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