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Two yeast genes with similarity to TCP-1 are required for microtubule and actin function *in vivo*

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ABSTRACT **We have isolated cold-sensitive mutations in two genes of the yeast *Saccharomyces cerevisiae*, *BIN2* and *BIN3*, that cause aberrant chromosome segregation *in vivo*. *BIN2* and *BIN3* encode essential proteins that are similar to each other and to TCP-1. TCP-1 and TCP-1-like proteins are components of the eukaryotic cytoplasmic chaperonin that facilitates folding of tubulins and actin *in vitro*. Mutations in *BIN2* and *BIN3* cause defects in microtubule and actin assembly *in vivo* and confer supersensitivity to the microtubule-destabilizing drug benomyl. Overexpression of *TCP1*, *BIN2*, *BIN3*, or *ANC2*, a fourth member of the TCP-1 family in yeast, does not complement mutations in the other genes, indicating that the proteins have distinct functions. However, all double-mutant combinations are inviable; this synthetic lethality suggests that the proteins act in a common process. These results indicate that Bin2p and Bin3p are components of a yeast cytoplasmic chaperonin complex that is required for assembly of microtubules and actin *in vivo*.**

The correct folding and oligomeric assembly of newly synthesized proteins *in vivo* are greatly enhanced by interaction with accessory proteins known as molecular chaperones (1, 2). These molecules bind unfolded polypeptides and use the energy of ATP hydrolysis to generate correctly folded proteins. Two major classes of chaperones have been defined. Members of the first class are exemplified by the heat shock protein hsp70 and function as monomers or dimers. Members of the second class, exemplified by the heat shock protein hsp60 and called chaperonins, function as multisubunit toroidal complexes. Initially, chaperonins were found only in eubacteria, mitochondria, and plastids, all of which are thought to be related in their evolutionary origins. More recently, a chaperonin was identified in the cytosol of eukaryotic cells (3–5). Biochemical purification of the cytosolic chaperonin identified seven to nine polypeptides in the size range 52–65 kDa. One of these is the ubiquitous eukaryotic protein, t-complex polypeptide 1 (TCP-1). Mouse cDNAs corresponding to six of the other chaperonin polypeptides have been isolated (6). These encode proteins that are ≈30% similar to TCP-1 and ≈30% similar to each other in all pairwise combinations. Thus, the eukaryotic cytoplasmic chaperonin is a heteromeric complex composed of related proteins.

Several experiments indicate that the cytoplasmic chaperonin is involved in assembly of the cytoskeletal proteins tubulin and actin. It has been shown that chicken α- and β-tubulins made in rabbit reticulocyte extracts become transiently bound to the cytoplasmic chaperonin (7). The addition of ATP releases the bound chains to produce αβ-tubulin heterodimers that are able to assemble into microtubules. In independent studies, the same chaperonin was purified from reticulocyte lysates and shown to refold denatured α-, β-, and γ-tubulin and actin (4, 8, 9). The yeast *TCP1* gene encodes a protein that is ≈60% identical to the mouse protein (10, 11). *TCP1* is essential for the growth of yeast and a cold-sensitive mutation in this gene affects microtubule-mediated processes (11).

We have identified two yeast genes, *BIN2** and *BIN3*, that encode *TCP1*-related proteins. The phenotypes of cold-sensitive *bin2* and *bin3* mutants indicate that these genes are essential for microtubule and actin function *in vivo*. In addition, genetic evidence suggests that Tcp1p, Bin2p, Bin3p, and Anc2p, a fourth member of this family (12), play functionally distinct roles in a common cellular process.

MATERIALS AND METHODS

Isolation of *bin* Mutants. A collection of ≈700 heat-sensitive and cold-sensitive yeast strains was obtained from D. Botstein (Stanford University). All are derived from the S288C strain DBY473 (*MATα his4-619*) after mutagenesis with ethyl methanesulfonate. Cells were grown in yeast extract/peptone/dextrose medium (13) at 26°C to early logarithmic phase, shifted to the restrictive temperature for two generation times (4 h at 37°C for heat-sensitive strains, 16 h at 14°C for cold-sensitive strains), and stained with 4′,6′-diamidino-2-phenylindole to visualize DNA (14). Each *bin* mutant was backcrossed three times to a wild-type strain; in all cases cold sensitivity and benomyl sensitivity cosegregated as a single gene defect.

Cloning and Disruption of the *BIN2* and *BIN3* Genes. The *BIN2* and *BIN3* loci were cloned from a YCp50-based yeast genomic library (15) by complementation of the cold sensitivity of *bin2-1* and *bin3-1* strains, respectively. Each DNA fragment directed integration of a plasmid to a chromosomal location that is genetically linked to the original mutation. The N-terminal coding region of *BIN3* lies ≈260 bp upstream of the N-terminal coding region of *SSL2* (16). A *bin2::HIS3* deletion mutation was made by replacing an 1157-bp *Bam*HI internal fragment of *BIN2* with a 1.8-kb *HIS3*-containing fragment. This construction deletes *BIN2* sequences coding for amino acids 50–436. A *bin3::HIS3* deletion mutation was made by replacing a 644-bp *Spe* I/*Sph* I internal fragment of *BIN3* with the 1.8-kb *HIS3*-containing fragment. This construction deletes *BIN3* sequences coding for amino acids 94–308. Linear DNA fragments containing these deletions were transformed separately into a wild-type diploid strain homozygous for the *his3-Δ200* deletion. Southern hybridization analysis of genomic DNA from the transformants indicated that the constructions had integrated at the *BIN2* and *BIN3* loci, respectively (data not shown). Tetrads resulting from sporulation of these diploids contained two viable and two inviable spores; all viable spores were His⁻. Thus, both deletions create recessive-lethal mutations.

Fluorescence Staining of Cells. Diploid cells, wild-type or homozygous for the *bin* allele, were grown at 30°C and shifted to 14°C for 16 h. Microtubules and actin were visualized by

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09480).

immunofluorescence as described (14). Anti-tubulin antibody YOL1/34 was a gift from J. Kilmartin (Medical Research Council, Cambridge, U.K.); anti-actin antibody was a gift from D. Drubin (University of California, Berkeley). Fluorescein-conjugated secondary antibodies were obtained from Cappel.

RESULTS

Identification of *bin* Mutants. In the yeast *Saccharomyces cerevisiae*, cytoplasmic microtubules extend from both poles of an intranuclear spindle. These microtubules are required to orient the yeast spindle in the bud neck prior to anaphase. We have previously described a β-tubulin mutation that causes the specific loss of cytoplasmic microtubules (14). In these cells, spindles fail to be properly oriented and nuclear division takes place entirely within the mother cell. Subsequent cytokinesis produces one cell with two nuclei (binucleate) and one without a nucleus (anucleate). Binucleate cells are also produced by mutations in actin, suggesting that the actin cytoskeleton is involved in spindle orientation, perhaps through interactions with cytoplasmic microtubules (17, 18). To identify cellular components that are necessary for the assembly and function of these cytoskeletal elements, we screened a random collection of cold-sensitive and heat-sensitive yeast mutants for defects in nuclear segregation. After a shift to the restrictive temperature, each strain was stained to visualize DNA. Seven cold-sensitive mutants produce a substantial fraction of cells containing two nuclei in one cell body. We refer to these as *bin* mutants for binucleate. Complementation and linkage tests showed that these mutations identify four genes; we obtained one allele of *BIN1*, *BIN2*, and *BIN4* and four alleles of *BIN3*. Mutant alleles of *BIN2* and *BIN3* also confer supersensitivity to the microtubule-destabilizing drug benomyl. All of the *bin2* and *bin3* strains fail to grow on benomyl at 10 μg/ml, less than half the amount required to inhibit growth of the wild-type strain. We describe here characterization of the *bin2* and *bin3* mutants.

After incubation at 14°C for 16 h (≈2 generation times), *bin2* and *bin3* cells display a normal population distribution of unbudded, small-budded, and large-budded cells (Fig. 1). However, in each case, ≈10% of the cells contain two nuclei in one cell body. In addition, an unusually large fraction of the *bin2-1* and *bin3-1* large-budded cells (>40%) contain an undivided nucleus. In contrast, most wild-type large-budded cells have completed nuclear division; <10% contain an undivided nucleus. Thus, the *bin2-1* and *bin3-1* mutations inhibit nuclear division as well as spindle orientation.

***BIN2* and *BIN3* Encode Proteins Similar to Tcp1p.** The wild-type *BIN2* and *BIN3* genes were cloned and sequenced.

	Cell Morphology			Nuclear Morphology: No bud			Small bud		Large bud			
	○	○○	○○○	⊙	◔	○	⊙⊙	⊙◔	⊙⊙⊙	⊙⊙◔	⊙◔◔	◔◔◔
WT	42	35	23	100	0	0	100	0	91	9	0	0
bin2-1	39	36	25	89	9	2	84	16	38	14	30	18
bin3-1	44	30	26	91	7	2	90	10	36	16	28	20
bin3-2	56	28	16	89	6	5	80	20	80	8	3	9
bin3-3	42	42	16	100	0	0	95	5	60	15	3	22
bin3-4	39	41	20	97	3	0	88	12	69	12	6	13

FIG. 1. *bin* mutants produce binucleate cells. Cells were grown at 30°C, shifted to 14°C for 16 h, and stained with 4',6-diamidino-2-phenylindole. Cell morphology: percentages of cells that were unbudded, small budded, or large budded are indicated. A large-budded cell possesses a bud whose diameter is at least three-fourths the diameter of the mother cell. Nuclear morphology: percentages of unbudded, small-budded, and large-budded cells that possessed a particular nuclear morphology are indicated. WT, wild type.

BIN2 and *BIN3* encode proteins of 58.7 and 57.2 kDa, respectively. These proteins are ≈30% identical to each other and 30–35% identical to the *S. cerevisiae* protein Tcp1p (Fig. 2). *BIN3* is identical to a gene on chromosome IX recently identified through the efforts of the Yeast Genome Project (19). A fourth member of this family, Anc2p, is described in the accompanying manuscript (12); these four proteins are 27–35% identical in all pairwise combinations (Table 1). Gene disruptions show that *BIN2* and *BIN3*, like *TCP1* (11) and *ANC2* (12), are essential for viability in yeast (see *Materials and Methods*).

Yeast Tcp1p is ≈60% identical to its mouse homolog TCP-1 (11), which has recently been renamed CCTα (6). Similarly, Bin2p and Bin3p are ≈60% identical to the mouse chaperonin subunits CCTγ and CCTβ, respectively (Table 1). Thus, Bin2p and Bin3p likely represent the yeast equivalents of CCTγ and CCTβ.

Cytoskeletal Assembly in *bin* Mutants. Experiments from several laboratories demonstrate that the cytoplasmic chaperonin complex from animal cells is capable of folding tubulins and actin *in vitro* (3, 4, 7, 8). These results and the knowledge that defects in microtubule and actin function can produce binucleate cells prompted us to examine the assembly of cytoskeletal structures in the *bin2* and *bin3* mutants. In wild-type cells microtubules form a brightly staining intranuclear spindle; cytoplasmic microtubules extend from the spindle poles and stain less brightly (20). All of the *bin* mutants display defects in microtubule assembly (Fig. 3). *bin3-1* cells show the most severe microtubule assembly defect. After a shift to 14°C, about half of the *bin3-1* cells lack detectable microtubules and half possess only a single faint dot of staining. Fewer than 5% contain a bipolar spindle; these are usually found in binucleate cells and often fail to be properly aligned through the bud neck. The phenotype of *bin2-1* and the other *bin3* mutants is slightly less severe. Most cells possess short microtubule fibers that appear to originate from a single point. As was the case for *bin3-1* cells, bipolar spindles are infrequently observed in these other *bin* mutants.

Actin assembly is also aberrant in the *bin* mutants. Wild-type cells contain a network of cytoplasmic actin cables in the mother cell and brightly staining cortical patches of actin concentrated in the bud (20). In the *bin* mutants, cortical patches are abnormally small and actin cables are less prominent (Fig. 3). In addition, the polarized distribution of actin cables and patches is missing in the *bin* mutants. Both patches and faint cables are evenly located throughout the mother cell and bud. Intensely staining actin bars are also observed in about half of the *bin3-1* cells and in ≈10% of the *bin3-2* cells. Similar actin bars are produced by some mutations in the yeast actin gene and presumably represent aberrant actin polymerization (17, 21). Thus, each of the *bin* mutations interferes with the proper assembly of microtubules and actin *in vivo*.

Genetic Interactions of Tcp1-Like Proteins. Two types of genetic criteria suggest that Bin2p, Bin3p, Tcp1p, and Anc2p play distinct roles in a common cellular process. We tested whether multiple copies of one of these genes could compensate for a mutation in any of the other genes. A wild-type copy of each gene on a high-copy-number 2-μm plasmid was transformed into *bin2-1*, *bin3-1*, *tcp1-1*, and *anc2-1* strains. In each case, the plasmid-borne gene could complement the conditional lethality of the mutation only in the same gene. Thus, each gene product appears to be functionally distinct.

We also examined the effect of placing two of the mutations in a single haploid cell. A cross of *bin2-1* to *bin3-1* produced tetrads with four, three, and two viable spores. In four viable-spore tetrads, all four segregants were cold sensitive; in three viable-spore tetrads, two were cold sensitive; in two viable-spore tetrads, neither was cold sensitive. This pattern of segregation indicates that the double-mutant haploids are

FIG. 2. Comparison of the yeast Bin2p, Bin3p, and Tcp1p amino acid sequences. Sequences were aligned by the Clustal method. Identical amino acids present in at least two of the three aligned sequences are boxed.

inviable. Thus, the *bin2-1 bin3-1* double-mutant haploid fails to grow at 30°C, a temperature that is permissive for each single mutant. Analogous crosses demonstrated that both *bin2-1* and *bin3-1* are lethal in combination with *tcp1-1* and *anc2-1* as well.

DISCUSSION

We have identified two genes encoding proteins with similarity to *TCP1*. One of these genes, *BIN3*, has been recently described in an independent study and is referred to as *TCP1β* (19). However, this latter designation is not in keeping with the universally accepted nomenclature for *S. cerevisiae* genes (13), so we have continued to refer to it as *BIN3*. Tcp1p, Bin2p, Bin3p, and Anc2p form a family of yeast proteins that are ≈30% identical in all pairwise combinations. Seven mouse proteins, including TCP-1 (CCTα), have been identified as part of a cytoplasmic chaperonin complex (6). These proteins are also ≈30% identical in all pairwise combinations. Yeast Tcp1p is ≈60% identical to mouse CCTα and presumably represents the yeast homolog of this chaperonin subunit (10, 11). Bin2p and Bin3p are ≈60% identical to CCTγ and CCTβ, respectively, and are likely to represent yeast homologs of these mammalian subunits as well. The relatively high degree of sequence conservation across species, as compared to the level of sequence conservation within species, suggests that the chaperonin subunits have evolved to perform separate functions. In agreement with this idea, we found that none of these yeast genes supplied in extra copies could rescue a mutation in any of the other genes.

Table 1. Sequence similarity among TCP-1-like proteins

Yeast proteins	Yeast proteins			Mouse proteins		
	Bin2p	Bin3p	Anc2p	CCTα	CCTβ	CCTγ
Tcp1p	32	35	31	62	34	32
Bin2p		29	27	30	30	59
Bin3p			31	35	65	29

Percentages of identical amino acids for pairwise sequence comparisons determined using the GAP computer program (Genetics Computer Group software) are shown.

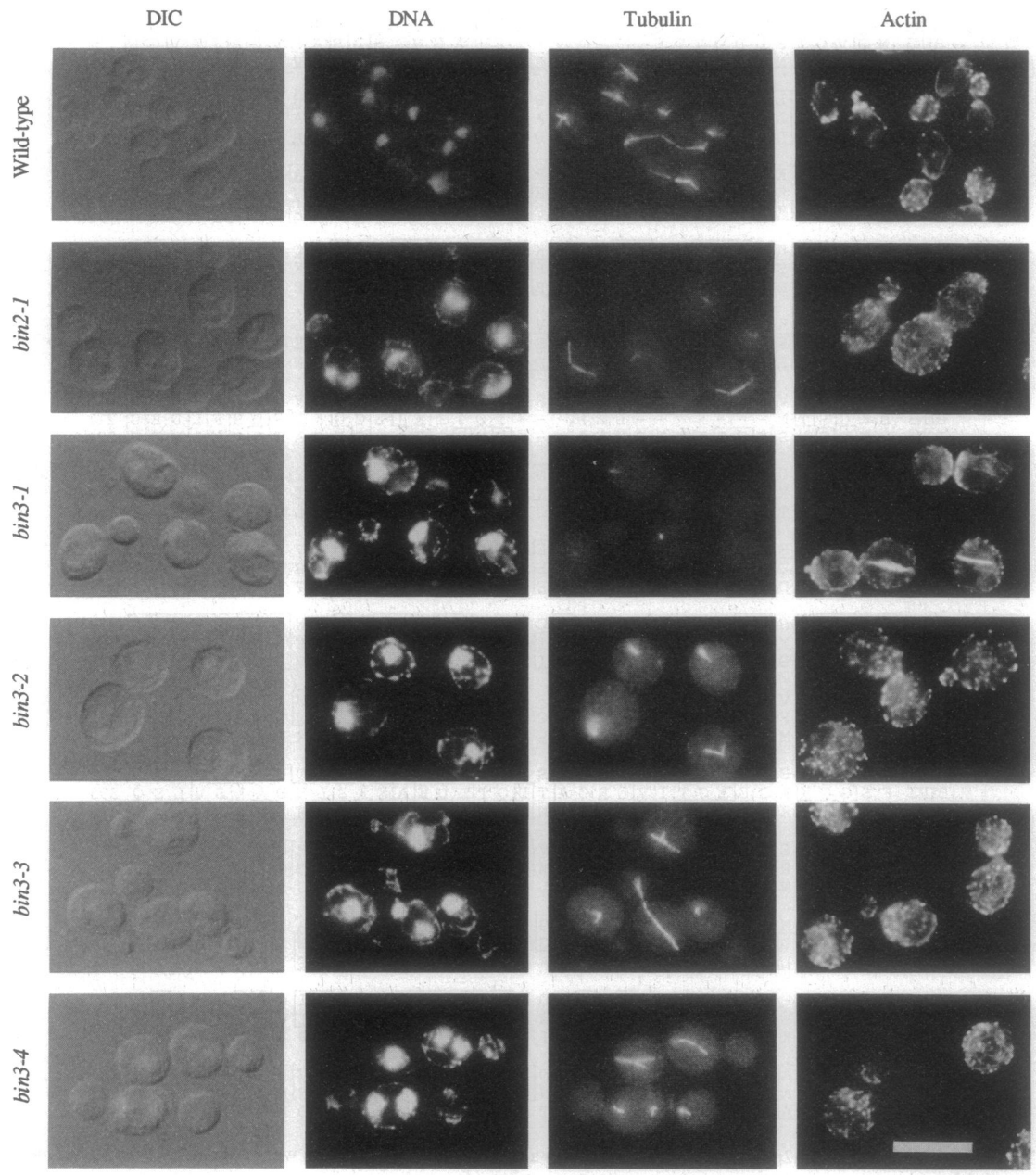

FIG. 3. *bin* mutations interfere with proper microtubule and actin assembly *in vivo*. Diploid cells, wild-type or homozygous for the indicated *bin* allele, were grown at 30°C and shifted to 14°C for 16 h. DNA and tubulin staining were performed on the same cells; actin staining was performed on a separate aliquot of cells. To illustrate the binucleate phenotype, binucleate cells and, hence, cells containing bipolar spindles are overrepresented in some fields. DIC, differential interference contrast microscopy. (Bar = 10 μm.)

Although the four yeast proteins appear to be functionally distinct, all double-mutant combinations between *bin2-1* or *bin3-1* and mutations in the other three genes are lethal. This phenomenon, termed synthetic lethality, often indicates that the gene products participate in a common process (22). These genetic data are consistent with the proposal that these proteins associate to form the cytoplasmic chaperonin complex, a suggestion that is supported by the finding that Tcp1p and Bin3p reside in a single complex (19). However, we do not know whether yeast contain one complex or a variety of complexes with different subunit compositions.

Experiments from several laboratories have shown that the mammalian cytoplasmic chaperonin is capable of refolding denatured tubulins and actin *in vitro* (3, 7, 8). The phenotypes of the *bin* mutants indicate that Bin2p and Bin3p are required for tubulin and actin assembly *in vivo*. All mutant alleles fail to assemble normal populations of microtubules and actin structures at the restrictive temperature. In addition, the *bin* mutants display defects in two microtubule-mediated processes, spindle orientation and nuclear division. The *bin* mutant phenotypes described here are quite similar to the phenotype of the cold-sensitive *tcp1-1* mutation. *tcp1-1* cells are supersensitive to benomyl and display similar defects in nuclear division, nuclear segregation, and microtubule assembly (11). A heat-sensitive allele of *BIN3* has also been described recently (19). This allele produces a different phenotype; cells arrest uniformly with large buds and a single undivided nucleus. While this latter mutation interferes with mitotic spindle formation, it has no apparent effect on actin biogenesis. Biochemical analysis of mutant chaperonin complexes should establish whether these phenotypic differences represent quantitative or qualitative changes in chaperonin activity.

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