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Saccharomyces cerevisiae Genes Required in the Absence of the *CIN8*-encoded Spindle Motor Act in Functionally Diverse Mitotic Pathways

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Kinesin-related Cin8p is the most important spindle-pole–separating motor in *Saccharomyces cerevisiae* but is not essential for cell viability. We identified 20 genes whose products are specifically required by cells deficient for Cin8p. All are associated with mitotic roles and represent at least four different functional pathways. These include genes whose products act in two spindle motor pathways that overlap in function with Cin8p, the kinesin-related Kip1p pathway and the cytoplasmic dynein pathway. In addition, genes required for mitotic spindle checkpoint function and for normal microtubule stability were recovered. Mutant alleles of eight genes caused phenotypes similar to *dyn1* (encodes the dynein heavy chain), including a spindle-positioning defect. We provide evidence that the products of these genes function in concert with dynein. Among the dynein pathway gene products, we found homologues of the cytoplasmic dynein intermediate chain, the p150^{Glued} subunit of the dynactin complex, and human LIS-1, required for normal brain development. These findings illustrate the complex cellular interactions exhibited by Cin8p, a member of a conserved spindle motor family.

INTRODUCTION

The mitotic spindle achieves the segregation of replicated chromosomes through a series of motility events. Microscopic observations of this process clearly indicate the extent of its complexity. Spindle structural transitions and several distinct motility events must be carefully coordinated with each other and other cell cycle events. Biochemical and genetic studies have revealed that proper spindle function requires the actions of a large number of proteins. These include the components of microtubules, kinetochores, spindle poles, microtubule-based motor proteins, and various regulatory enzymes. In such a system, one might expect that a single component would display numerous and complex interactions with other components of the system. These types of cellular interactions can often be revealed by genetic interactions. Combinations of mutant alleles of genes encoding interacting proteins may display new phenotypes reflecting the nature of their cellular interaction.

In recent years, many of the microtubule-based motor proteins that drive mitotic movements have been identified and characterized (Hoyt, 1994; Barton and Goldstein, 1996; Hoyt and Geiser, 1996). In studies using genetically tractable organisms, combinations of mutant motor gene alleles often were observed to result in phenotypic enhancement or suppression (reviewed in Hoyt and Geiser, 1996). It has been proposed that these observations reflect cooperative or antagonistic physical interactions occurring between the motor proteins.

Most of the characterized spindle motor proteins belong to families that are conserved in primary sequence throughout the eukaryotes. Members of two kinesin-related families, BimC and Kar3, have been observed to be associated with the spindle microtubules located between the poles. BimC-related motors participate in spindle assembly and pole separation

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(Enos and Morris, 1990; Hagan and Yanagida, 1990; Sawin *et al.*, 1992; Heck *et al.*, 1993; Blangy *et al.*, 1995). In the budding yeast *Saccharomyces cerevisiae*, two BimC-related motors, Cin8p and Kip1p, overlap in their abilities to perform these functions (Hoyt *et al.*, 1992; Roof *et al.*, 1992). Extra copies of *KIP1* were found to suppress *cin8* loss-of-function alleles. In addition, elimination of the function of either was tolerated, but elimination of both caused lethality. Studies in which the activities of both were eliminated at various stages of mitosis revealed that these motor proteins act to assemble the bipolar spindle and are required to produce an outwardly directed force acting upon the poles (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders and Hoyt, 1992; Saunders *et al.*, 1995). Prior to the onset of anaphase, Cin8p and Kip1p are antagonized by the actions of Kar3p (Saunders and Hoyt, 1992; Hoyt *et al.*, 1993). Kar3p apparently contributes an inwardly directed force that can drive the poles back together when Cin8/Kip1p function is eliminated.

During anaphase spindle pole separation, Cin8p and Kip1p are assisted by the actions of another motor protein, cytoplasmic dynein. After the onset of anaphase, pole separation could only be halted by the elimination of the functions of Cin8p, Kip1p, and Dyn1p [the dynein heavy chain gene product, also known as Dhc1p (Saunders *et al.*, 1995)]. Functional overlap between Cin8p and Dyn1p was also suggested by the observation that elimination of both of these nonessential gene products caused lethality. Dynein also participates in nuclear migration (Eshel *et al.*, 1993; Li *et al.*, 1993). This motility process positions the dividing nucleus and associated spindle in the neck between mother and bud cell bodies such that anaphase results in an equal distribution of the nuclear contents. Dynein is not essential for nuclear migration at normal growth temperatures (i.e., 26°C) presumably due to the presence of an auxiliary system. In contrast to Cin8p and Kip1p, dynein is believed to act upon the cytoplasmic microtubules that extend from the outer edges of the spindle poles toward the cell cortex (Yeh *et al.*, 1995).

Due to functional overlap, none of the three motor proteins that lead to spindle pole separation (Cin8p, Kip1p, or Dyn1p) is essential for viability. Indeed, cells deficient for both Kip1p and Dyn1p grow well. Of the three, the Cin8p motor phenotypically appears to make the most important contributions to spindle function. Loss of the Cin8p motor causes the most severe effects on spindle pole separation (Saunders *et al.*, 1995). In addition, mutational elimination of the function of *CIN8* causes both *KIP1* and *DYN1* to become essential for viability. Inviability caused by the combination of two nonlethal mutations is often referred to as "synthetic lethality" (Huffaker *et al.*, 1987; Guarente, 1993). If the function of Kip1p or Dyn1p requires the actions of associated gene products, mutant forms of these should also display lethality when combined with *cin8*. To screen for gene products that act in the Kip1p or Dyn1p pathways and to identify other functional pathways that are essential in the absence of Cin8p, we collected mutations that cause lethality only when combined with a *cin8* deletion. New mutant alleles of *KIP1* and *DYN1* were identified. In addition, 18 other genes were identified that could mutate to a perish in the absence of *CIN8* (or *pac*) phenotype. Mutant alleles of eight of these caused phenotypes similar to *dyn1*, suggesting that their products participate in a common pathway with dynein. Genes required for microtubule stability and cell-cycle feedback ("checkpoint") regulation during mitosis were also identified.

Table 1. Yeast strains and plasmids

	Genotype
Strain	
MAY589	*MAT***a** *ade2 his3 leu2 ura3*
MAY591	*MATα lys2 his3 leu2 ura3*
MAY2301	*MAT***a** *ade2 his3 leu2 ura3 cyh2 cin8::URA3* (pMA1208)
MAY2830	*MAT***a** *ade2 his3 leu2 ura3 trp1 cyh2 cin8::HIS3* (pMA1208)
MAY2831	*MATα lys2 his3 leu2 ura3 trp1 cyh2 cin8::HIS3* (pMA1208)
Plasmid	
pMA1125	*CIN8 URA3 CEN*
pMA1208	*CIN8 CYH2 LEU2 CEN*
pMA1257	*CIN8 HIS3 CEN*

MATERIALS AND METHODS

Yeast Strains and Media

The yeast strains used in these experiments were derivatives of the strains listed in Table 1. For experiments in which we wished to convert cells from *CIN8* to *cin8*, we used strains related to MAY2301, MAY2830, and MAY2831. These strains are deleted for the chromosomal copy of *CIN8* but carry intact *CIN8* on a plasmid whose presence can be selected against (see below). For other experiments, we used our laboratory standard wild-type background (MAY589 and MAY591). The motor-gene deletion-insertion alleles were described previously [*cin8::URA3* and *kip1::HIS3* (Hoyt *et al.*, 1992), *cin8::HIS3* (Saunders *et al.*, 1995), and *dyn1::URA3* (Eshel *et al.*, 1993)] except for *dyn1::HIS3*, which was constructed by insertion of a *HIS3*-containing fragment between the *Eco*RV site (bp 3271) and the *Sac*I site (bp 4972) of *DYN1*. (Note that in our descriptions of deletion alleles, nucleotides are numbered with the first A of the predicted open reading frame assigned number 1.) The *KAR3-898* allele was described by Hoyt *et al.* (1993). Rich (YPD), minimal (SD), and sporulation media were as described (Sherman *et al.*, 1983). Standard yeast manipulations were carried out as described (Guthrie and Fink, 1991).

pac Mutant Screen

Mutant strains that require an intact *CIN8* gene for viability (perish in the absence of *CIN8* or *pac*) were derived from three strains,

MAY2301, MAY2830, and MAY2831. These strains have in common a chromosomal deletion of *CIN8*, a cycloheximide-resistance–conferring allele of *CYH2*, and a defective allele of *LEU2*. They also harbor a plasmid carrying the wild-type alleles of these three genes (pMA1208). These strains were mutagenized with methanesulfonic acid ethyl ester (EMS; Sigma, St. Louis, MO) to approximately 10% survival (Lawrence, 1991) and plated for single colonies on YPD at 26°C. The surviving colonies were then replica-transferred to YPD plus 5 μg/ml cycloheximide (Sigma) to identify mutants unable to segregate cycloheximide-resistant cells. Cycloheximide-sensitive mutants were then tested to identify those for which this phenotype is specific to loss of *CIN8* function. Candidates were transformed with *CIN8*-containing plasmids and tested for rescue of the cycloheximide-sensitive phenotype. pMA1257 (*CIN8 HIS3 CEN*) was used for MAY2301 derivatives and pMA1125 (*CIN8 URA3 CEN*) was used for MAY2830 and MAY2831 derivatives. Mutants that were rescued by *CIN8* were selected for further study.

Complementation was performed by crossing **a** and α *pac* mutants, selecting diploids on defined minimal medium missing adenine and lysine, and testing the diploids for their ability to segregate cycloheximide-resistant cells. Members of identified complementation groups were intercrossed to test for linkage of mutant alleles. Linkage was demonstrated for all reported complementation group mutant alleles.

Microscopy

All phenotypic analyses were performed on mutants that had been derived from backcrossing to a wild-type strain. Cultures were grown to midlogarithmic phase in YPD at 30°C and then shifted to 12°C in a shaking water bath for 24 h. For observation of nuclei, cells were fixed in 70% ethanol at 4°C and stained with 0.33 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) plus 1 mg/ml *p*-phenlenediamine to prevent fading (both from Sigma). For observation of microtubules, cells were fixed in formaldehyde, spheroplasted, and stained with the rat anti-α-tubulin monoclonal antibody YOL1/34 as described (Stearns *et al.*, 1990). To observe the budding behavior of mutant cells in the presence of the microtubule inhibitor benomyl, cultures were synchronized in G_1 with α-factor, released onto agar medium containing benomyl, and observed as previously described (Hoyt *et al.*, 1991).

Cloning and Disruption of PAC Genes

The general strategy was as follows. *pacX cin8*-Δ strains require the *CIN8*-containing plasmid pMA1208 to maintain viability and are, therefore, rendered sensitive to cycloheximide by the *CYH2* gene also carried by this plasmid. *pac* mutants with this genotype were transformed with DNA from either a *URA3-CEN* library (Rose *et al.*, 1987) or a *TRP1-CEN* library (Connelly and Hieter, unpublished observations). Replica plating to cycloheximide-containing medium was used to identify transformants capable of segregating resistant cells. For some *pac* mutants, pMA1208 was first exchanged for pMA1125 (*CIN8 URA3 CEN*), allowing the use of a *LEU2-CEN* genomic library (Spencer and Hieter, unpublished observations). In this scheme, rescued transformants were identified by their ability to segregate 5-fluoroorotic acid-resistant cells.

Plasmids that could rescue the inviability of the *pac cin8*-Δ strains were transferred to *Escherichia coli* and characterized by restriction enzyme digestion. *CIN8*-containing plasmids, an expected finding, were discarded, and plasmids containing novel inserts were transformed into the appropriate mutant strains for retesting. Subcloning and sequencing (reagents from Amersham, Arlington Heights, IL) of the inserts were performed with standard techniques (Sambrook *et al.*, 1989). Note that in many instances, only short sequences were determined because these allowed us to identify the rescuing gene as a preexisting entry in the GenBank database.

For each candidate *PAC* gene (able to rescue a *pac* mutant), linkage was used to verify that the identified locus corresponded to the original mutant locus. Specific details of these linkage experiments and the disruption of various *PAC* genes are described below.

PAC1. Subcloning and sequencing identified a previously undescribed gene capable of rescuing *pac1–1* (GenBank accession number U16827). A deletion of *PAC1* was created by removing the DNA between *Sph*I (bp 186) and *Hpa*I (bp 1035) in the open reading frame and replacing it with DNA encoding *LEU2*. MAY2897, a *pac1::LEU2 cyh2* strain, was crossed to MAY2829 (*pac1–1 cin8::HIS3 cyh2* pMA1208). In all 23 tetrads examined, all His^+ spores were either inviable or cycloheximide sensitive, indicating linkage of *pac1::LEU2* to *pac1–1*.

PAC3. Subcloning and sequencing identified the gene capable of rescuing *pac3–1* as *JNM1* (McMillan and Tatchell, 1994). Note that our unpublished sequence of the coding region of this gene very closely resembles that obtained by the *S. cerevisiae* genome project (accession number X80836), whereas the *JNM1* sequence in GenBank file Z25750 disagrees with the other two at 11 positions. A deletion of *PAC3* was created by removing the DNA between *Hin*dIII (bp 1420) and *Hin*dIII (bp 1765) in the open reading frame and replacing it with DNA encoding *URA3*. In a cross between MAY4272 (*pac3::URA3 cin8::HIS3 cyh2* pMA1208) and MAY2826 (*pac3–1 cin8::HIS3 cyh2* pMA1208), all viable spores in 17 tetrads were cycloheximide sensitive, indicating that *pac3–1* is linked to *JNM1*.

PAC7. Subcloning and sequencing identified the gene capable of rescuing *pac7–2* as *BUB2* (Hoyt *et al.*, 1991). A *bub2::URA3* construct (Hoyt *et al.*, 1991) was transformed into MAY2830 converting it to a cycloheximide-sensitive phenotype. When crossed to *pac7–1* and *pac7–2 cin8::HIS3 cyh2* (pMA1208) strains, all viable spores from eight and seven tetrads, respectively, were cycloheximide sensitive, indicating linkage of these alleles to *BUB2*.

PAC8. Subcloning and sequencing identified the gene capable of rescuing *pac8–2* as a previously described essential gene, *MPS1*/*RPK1* (Poch *et al.*, 1994; Lauzé *et al.*, 1995). A construct that marks (but does not disrupt) the *MPS1* locus with *URA3* (from E. Lauzé and M. Winey) was transformed into MAY2830. When crossed to a *pac8–1 cin8::HIS3 cyh2* (pMA1208) strain, all Ura^- spores from 15 tetrads were cycloheximide sensitive and Ura^+ spores were resistant, indicating linkage of *PAC8* to *MPS1*.

PAC9. Subcloning and sequencing identified the gene capable of rescuing *pac9–2* as *BUB3* (Hoyt *et al.*, 1991). A construct that marks (but does not disrupt) the *BUB3* locus with *URA3* (Hoyt *et al.*, 1991) was transformed into MAY2830. When crossed to a *pac9–2 cin8::HIS3 cyh2* (pMA1208) strain, all Ura^- spores from 14 tetrads were cycloheximide sensitive and Ura^+ spores were resistant, indicating linkage of *PAC9* to *BUB3*.

PAC10. Subcloning and sequencing identified a previously undescribed gene capable of rescuing *pac10–1* (GenBank accession number U29137). A PCR-based method was used to replace the entire open reading frame of *PAC10* with DNA encoding *HIS3*. Briefly, 413 bp and 215 bp of 5′ and 3′ noncoding DNAs surrounding *PAC10* were amplified by using primers that added homology to *HIS3* at one end of each. *HIS3* DNA was also amplified. All three amplified fragments were then mixed and subjected to another round of PCR, yielding *HIS3* surrounded by the 5′ and 3′ noncoding regions from the *PAC10* locus. To demonstrate linkage of the rescuing DNA to the *PAC10* locus, a *PAC10* duplication marked with *URA3* was transformed into the genome of MAY2830. When crossed to *pac10–1* and *pac10–2 cin8::HIS3 cyh2* (pMA1208) strains, all tested tetrads (eight for each cross) were parental ditypes (two Ura^-, Cyh^S spores and two Ura^+, Cyh^R spores).

PAC11. Subcloning and sequencing identified a previously undescribed gene capable of rescuing *pac11–1* (GenBank accession number U16820). A PCR-based method (Baudin *et al.*, 1993) was used to replace the entire *PAC11* reading frame with *URA3*. MAY3664, a *pac11::URA3* strain, was crossed to MAY3284 (*pac11–1 cin8::HIS3 cyh2* pMA1208). In all 16 tetrads examined, all His^+ spores were either inviable or unable to segregate Leu^- cells, indicating linkage of *pac11::URA3* to *pac11–1*.

PAC12. Subcloning and sequencing identified the gene capable of rescuing *pac12–1* as *NUM1* (Kormanec *et al.*, 1991). A *num1::URA3* construct (from M. Farkasovsky, Max Planck Institute, Göttingen, Germany) was transformed into MAY2831 rendering it sensitive to cycloheximide. When crossed to a *pac12–1 cin8::HIS3 cyh2* (pMA1208) strain, all viable progeny from 23 tetrads were cycloheximide sensitive, indicating linkage of *PAC12* to *NUM1*.

PAC13. Subcloning and sequencing identified the gene capable of rescuing *pac13–1* as *NIP100* (GenBank acession Z73530). A *nip100::URA3* construct (from G. Schlenstedt and P. Silver, Harvard University) was transformed into MAY2831 converting it to a cycloheximide-sensitive phenotype. When crossed to a *pac13–1 cin8::HIS3 cyh2* (pMA1208) strain, all viable spores from eight tetrads were cycloheximide sensitive, indicating linkage of *PAC13* to *NIP100*.

PAC14. Subcloning and sequencing identified the gene capable of rescuing *pac14–1* as *BIK1* (Berlin *et al.*, 1990). A *bik1::TRP1* construct (from D. Pellman, Harvard University) was transformed into MAY2831, but this deletion did not confer the same cycloheximide-sensitive phenotype as did *pac14–1* (see RESULTS). However, when crossed to a *pac14–1 cin8::HIS3 trp1 cyh2* (pMA1208) strain, all 14 tetrads analyzed were parental ditypes, all Trp^+ spores were cycloheximide resistant, and all Trp^- spores were cycloheximide sensitive. This indicates linkage of *PAC14* to *BIK1*.

PAC15. Subcloning and sequencing identified the gene capable of rescuing *pac15–1* as a previously identified essential gene, *IPL1* (Francisco *et al.*, 1994). A construct that marks a region close to (but not including) the *IPL1* locus with *URA3* (from C. Chan, University of Texas, Austin) was transformed into MAY2830. When crossed to a *pac15–1* strain, 13 of 15 tetrads analyzed were parental ditypes, indicating linkage of *PAC15* and *IPL1*.

Other Genes. Two *pac* mutants were found to be unable to grow in medium containing 5 μg/ml benomyl, a microtubule inhibitor. One was found to harbor a mutant allele of *CIN1*, a gene required for normal microtubule stability (Hoyt *et al.*, 1990; Stearns *et al.*, 1990). The other was defective for *PAC2*, a novel gene. This demonstration and the cloning of *PAC2* will be described elsewhere (Hoyt *et al.*, 1997). The identification of *pac5–1* as an allele of the essential *CDC20* gene will also be described elsewhere.

Deletion mutant alleles of five other genes were directly tested for the *pac* phenotype by combining each with *cin8-Δ*. *act5::HIS3* (from J. Cooper, Washington University, St. Louis) was crossed into a *cin8::HIS3 cyh2* (pMA1208) background. The resulting *act5-Δ cin8-Δ* double mutant did not segregate cycloheximide-resistant cells. In a similar manner, a *mad1::HIS3 cin8::LEU2 ura3–52* (pMA1125 = *CIN8 URA3 CEN*) strain was created (all *mad* deletion DNA constructs were from A. Murray, University of California, San Francisco). This strain was unable to segregate 5-fluoroorotic acid-resistant cells, indicating that viability could not be maintained when the *CIN8 URA3* plasmid was lost. *mad2::URA3* and *mad3::URA3* constructs were transformed into MAY2831 (Table 1). For *mad2::URA3*, cycloheximide-resistant segregants were not found. For *mad3::URA3*, we did observe cycloheximide-resistant segregants, indicating that *cin8-Δ mad3-Δ* cells are viable. A *bub1::HIS3* construct (Roberts *et al.*, 1994) transformed into a *cin8::URA3 cyh2* (pMA1208) strain caused the inability to segregate cycloheximide-resistant cells.

RESULTS

Identification of Mutants That Require CIN8

CIN8 deletion mutants are viable at all temperatures except near the highest permissible for *S. cerevisiae* growth (37°C; Hoyt *et al.*, 1992). To identify genes whose functions become essential specifically in the absence of *CIN8* function, we collected mutants that require the presence of a wild-type *CIN8* gene for viability at 26°C. *cin8-Δ* cyh2 strains, carrying plasmid pMA1208 (*CIN8 CYH2 LEU2 CEN*), were mutagenized with EMS as described in MATERIALS AND METHODS. Mutants that require *CIN8* were unable to survive plasmid loss and, because of the presence of the wild-type and dominant *CYH2* allele on the plasmid, were unable to segregate cycloheximide-resistant cells (Figure 1). Approximately 41,150 colonies were screened to identify 372 mutant strains that were unable to grow on rich medium containing cycloheximide. These were then examined to determine whether the cycloheximide-sensitive phenotype was specific to a requirement for *CIN8*. Each was transformed with another *CIN8*-containing plasmid (without *CYH2*). For 107 of the mutants (70 *MAT***a** strains and 37 *MATα* stains), this treatment restored the ability to segregate cycloheximide-resistant cells, indicating specificity for *CIN8*. These mutants were designated *pac* since they perish in the absence of *CIN8*.

Complementation Analysis

Complementation between *pac* mutants was assessed by creating *cin8-Δ/cin8-Δ pacX/pacY* diploids and examining their ability to segregate cycloheximide-resistant colonies. One mutant, *PAC5–1*, was dominant for the cycloheximide-sensitive phenotype. Of the remaining 106 *pac* mutants, 28 represented seven complementation groups containing multiple members (Table 2). Linkage analysis demonstrated that each member of these seven groups was linked to the other member(s). Note that because of the restriction of mating type, the complementation combinations performed were not complete (no single new *pac* mutant was tested against every possible other *pac* mutant). However, the large number of single-member complementation groups recovered suggests that we did not approach saturation in our mutant screen and that many genes can mutate to yield the *pac* phenotype. The seven complementation groups represented by multiple alleles and nine other well behaved *pac* mutants were chosen for further study. Many of these were chosen because they displayed *dyn1*-like mutant phenotypes (see below).

As previously described (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders *et al.*, 1995), we found that both *KIP1* and *DYN1* are required for viability in the absence of *CIN8* (Figure 1). As part of the complementation test, we determined whether any of the *pac* mutants failed to complement *KIP1* and *DYN1* deletion mutants for the cycloheximide-sensitive phenotype (Table 2). Thirteen mutants failed to complement *kip1-Δ* strains; linkage of the *pac* mutation to *KIP1* was confirmed for 6 of these. Seven *pac* mutants failed to complement *dyn1-Δ* strains; linkage analysis confirmed that all 7 were alleles of *DYN1*. The successful identification of two genes known to overlap in function with *CIN8* lends significance to other genes identified by this screen.

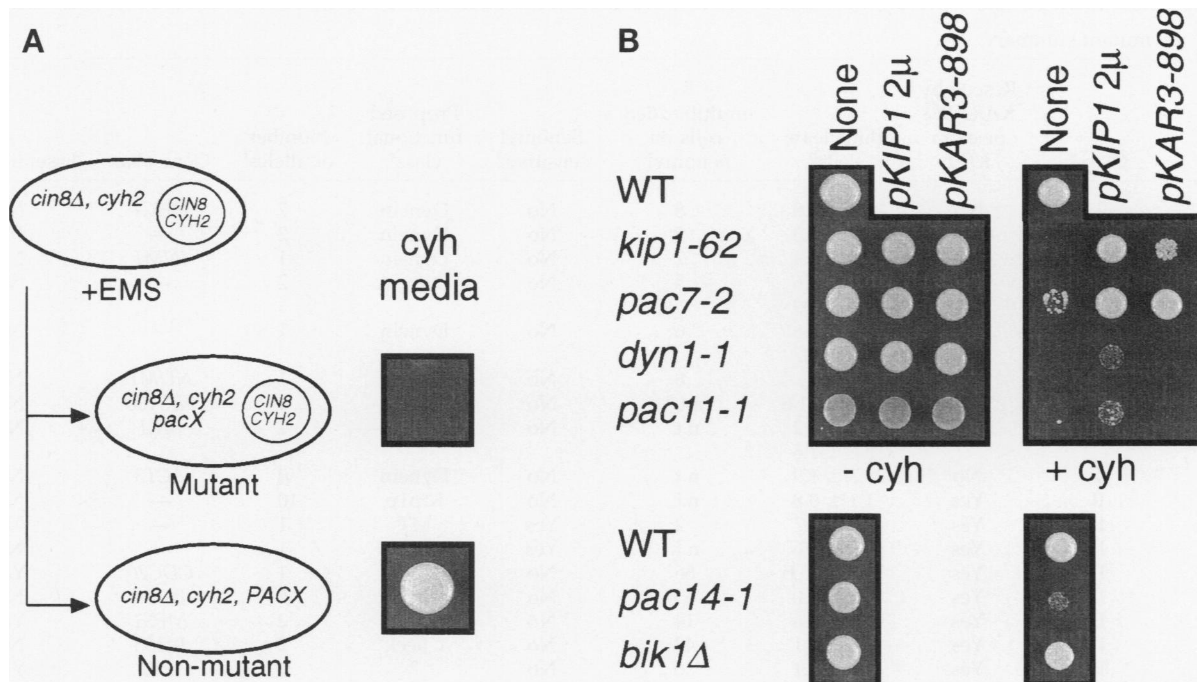

Figure 1. (A) Screen for mutants that perish in the absence of *CIN8* (*pac*). Strains bearing *CIN8* and *CYH2* on a plasmid were mutagenized with EMS. *pacX* mutants are unable to survive without the *CIN8*-containing plasmid. They are rendered sensitive to cycloheximide due to the presence of the dominant wild-type allele of *CYH2* also carried on the plasmid. Nonmutant cells are able to survive loss of the plasmid and, therefore, segregate cycloheximide-resistant cells. (B) Cycloheximide phenotypes of strains bearing *CIN8* and *CYH2* on a plasmid. Strains, with the genotype indicated on the left, were tested for their ability to segregate cycloheximide-resistant cells by spotting onto solid medium. Upper, top row, presence of a second plasmid transformed into the tested strains; None row, no second plasmid; *pKIP1*-2μ row, a high-copy plasmid containing *KIP1*; *pKAR3–898* row, a plasmid with the *KAR3–898* allele. Lower, the *bik1*-Δ strain was created from the *pac14–1* strain above it by transformation of a deletion DNA construct.

Deletion of the nonconventional actin gene *ACT5* produces similar phenotypes to a deletion of *DYN1* (Clark and Meyer, 1994; Muhua *et al.*, 1994). It has been proposed that the product of this gene functions in the dynein pathway in *S. cerevisiae*. Like *dyn1* alleles, we found that deletion of *ACT5* produces a *pac* phenotype. Complementation with *act5*-Δ deletion strains that we constructed revealed that we had not obtained mutant alleles of this gene among our 107 *pac* mutants.

Suppression of the pac Phenotype by Other Motor Genes

Evidence for overlap in function between the related Cin8p and Kip1p motors came from the observation that *KIP1* on a low-copy number (centromere containing) plasmid was able to suppress the temperature-sensitive phenotype associated with *cin8* loss-of-function alleles (Hoyt *et al.*, 1992). We examined the collection of *pac* mutants to see whether any could be suppressed by extra copies of *KIP1*, reasoning that the Cin8p-overlapping activity of Kip1p might rescue. To maximize the chance of suppression, we used a high-copy plasmid (2-μm plasmid based) carrying *KIP1*. Certain alleles of the Cin8p/Kip1p-antagonizing motor gene *KAR3* have been described that possess the unique property of being able to suppress the complete loss of both the *CIN8* and *KIP1* genes (Hoyt *et al.*, 1993). One such allele, *KAR3–898*, is dominant for this suppressing activity, which allowed us to test for suppression simply by transforming a plasmid carrying this allele into the various *pac* strains. We reasoned that *pac* mutants affected for Kip1p motor pathway function, and possibly other mitotic pathways, would be suppressed by *KAR3–898*. Indeed, transformation of the *KAR3–898* or the *KIP1* 2-μm plasmid into *cin8*-Δ *kip1* (pMA1208) strains allowed the segregation of cycloheximide-resistant cells (Figure 1).

After transformation of the *KAR3–898* and the *KIP1* 2-μm plasmids into the various *pac* mutants, we found a striking correlation in their ability to provide suppressing activity (Figure 1 and Table 2). Besides the *kip1* mutants, seven other *pac* mutants were suppressed by both plasmids. These are designated class II in Table 2. The remaining nine *pac* complementation groups (class I), which include *dyn1* and *act5*, were not

Table 2. *pac* mutant summary

Mutant allele	Class[a]	Rescue by *KAR3-898* or extra *KIP1*	% Binucleate cells[b]	% multibudded cells on benomyl[c]	Benomyl sensitive[d]	Proposed functional class[e]	Number of alleles[f]	Gene alias	Essential gene
dyn1-1	I	No	26.0 ± 1.8	8	No	Dynein	7	*DHC1*	No
pac1-1	I	No	25.7 ± 2.3	9	No	Dynein	2	—	No
pac3-1	I	No	20.6 ± 4.4	7	No	Dynein	1	*JNM1*	No
pac10-1	I	No	10.2 ± 1.6	5	No	Dynein	2	—	No
pac10-Δ			4.5 ± 1.9						
pac11-1	I	No	12.6 ± 1.6	6	No	Dynein	1	—	No
pac11-Δ			35.4 ± 0.7						
pac12-1	I	No	23.4 ± 3	8	No	Dynein	1	*NUM1*	No
pac13-1	I	No	23.1 ± 11.6	n.t.[g]	No	Dynein	1	*NIP100*	No
pac14-1	I	No	10.5 ± 2.2	n.t.	No	Dynein	1	*BIK1*	No
bik1-Δ			5.6 ± 1.6						
act5-Δ	I	No	29.3 ± 1.9	n.t.	No	Dynein	0	*ACT3*	No
kip1-Δ	II	Yes	1.1 ± 0.8	n.t.	No	Kip1p	10	—	No
pac2-1	II	Yes	3.6 ± 0.7	2	Yes	MT	1	—	No
cin1-20	II	Yes	4.3 ± 0.6	n.t.	Yes	MT	1	—	No
PAC5-1	II	Yes	5.3 ± 0.05	66	No	Check	1	*CDC20*	Yes
pac7-2	II	Yes	6.6 ± 1.0	65	No	Check	2	*BUB2*	No
pac8-2	II	Yes	5.3 ± 0.6	14	No	Check	2	*MPS1*	Yes
pac9-1	II	Yes	4.6 ± 1.1	43	No	Check	2	*BUB3*	No
pac15-1	II	Yes	6.2 ± 3.1	6	No	?	1	*IPL1*	Yes
Wild-type	Wild-type	—	0.8 ± 0.2	11	No				
cin8-Δ	*cin8-Δ*	—	8.7 ± 0.7	6.5	No				

[a]Determined from results in next column.

[b]Percentage of binucleate cells after incubation at 12°C for 24 h. For each value, 200 cells were scored in three experiments and averaged.

[c]Percentage of cell clusters on solid benomyl medium that contain three or more cell bodies; 200 cells were scored for each value.

[d]Yes indicates sensitivity to medium containing 5 μg/ml benomyl or less.

[e]Deduced function based upon phenotypes and sequence (see text). MT indicates microtubule function or stability. Check indicates mitotic spindle checkpoint function.

[f]Number of alleles recovered in the synthetic lethal with *cin8* (*pac*) screen.

[g]n.t., not tested.

suppressed by either plasmid. None of the mutants examined were suppressed by only one of these plasmids. For complementation groups represented by more than one allele, at least two alleles were tested for these suppression properties. In all cases, we observed phenotypic agreement among different alleles. Although the molecular mechanisms leading to suppression are not completely clear, we note with interest the close correspondence of the two *pac* mutant groups defined herein and by their nuclear migration proficiency (see below).

Many pac Mutants Are Cold-sensitive for Nuclear Migration

Loss of *DYN1* function has been previously demonstrated to cause a defect in nuclear migration that becomes particularly evident at lower incubation temperatures (Eshel *et al.*, 1993; Li *et al.*, 1993). Failure to properly position the spindle and the associated nucleus at the mother/bud neck during mitosis results in the generation of mother cells containing both progeny nuclei (a binucleate cell). We examined the *pac* mutants for the appearance of binucleate cells after a 24-h shift to 12°C (Figure 2 and Table 2). The *pac* mutants all carried *CIN8* on a plasmid. Therefore, any observed mutant phenotype could be attributed solely to the *pac* mutation. In addition, *pac* deletion alleles created in *CIN8* cells caused similar phenotypes (see below). In this study, we denoted a binucleate cell as any cell that contains two or more DAPI-staining regions in the mother cell body, regardless of whether there are any DAPI-staining areas in the daughter bud. Elevated levels of binucleate cells relative to wild type were observed in all the *pac* strains examined with one exception; strains deleted for *KIP1* were not elevated over wild type. In the binucleate cells observed, the two DAPI regions were found randomly distributed inside the mother cell body and away from the neck. Moreover, there was no obvious difference between the size or shape of the DAPI-staining regions between *pac* strains.

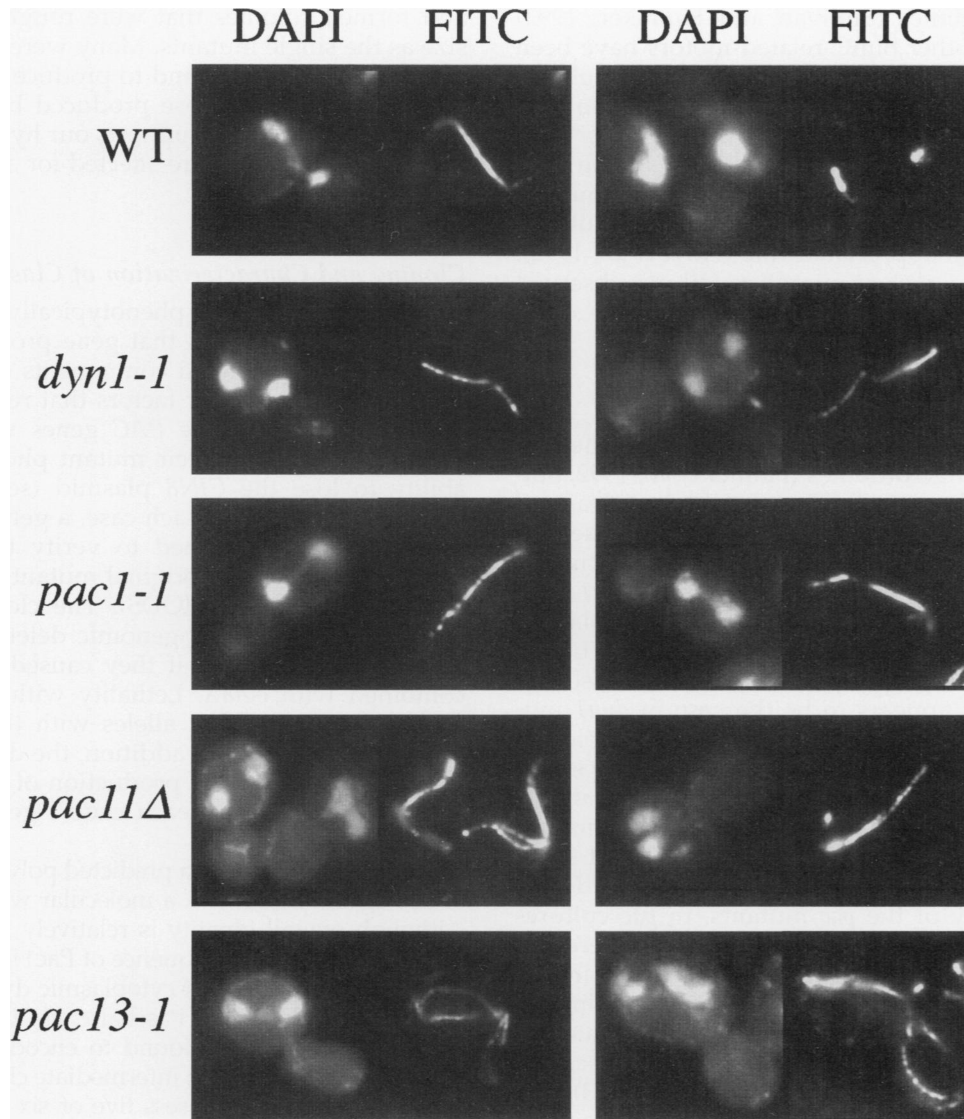

Figure 2. Immunofluorescence microscopy of *pac* mutant strains. Cells of the indicated genotype were incubated at 12°C for 24 h, fixed, and stained for DNA (DAPI) and microtubule structures (FITC).

Quantitation of the binucleate cells observed after a 24-h shift to 12°C (Table 2) demonstrated that the nuclear migration defects of the *pac* mutants closely followed the classification based upon rescue by *KAR3–898* and extra *KIP1* (see above). Most of the class I *pac* mutants, including *dyn1*, consistently formed higher numbers of binucleate cells, typically ≥20%. For *pac11*, a deletion allele of the gene produced far more binucleate cells than the original mutant allele (35.4% for the deletion versus 12.6% for *pac11–1*). This probably indicates that *pac11–1* is not null for function. In agreement with the published work of others (Clark and Meyer, 1994; Muhua *et al.*, 1994), we found that like *dyn1* mutants, a strain deleted for the nonconventional actin gene *ACT5* produced high levels of binucleate cells when grown at 12°C. Mutant alleles of *PAC10* and *PAC14* consistently produced lower numbers of binucleate cells with the deletion alleles actually producing less than the original EMS-generated alleles. This may indicate a functional difference between these two genes and the other class I genes (see DISCUSSION). The eight class II *pac* mutants produced lower numbers of binucleate cells (≤7%).

We also found that a *cin8*-Δ mutant produced elevated levels of binucleate cells (although less than the class I mutants). This was somewhat unexpected since nuclear migration in yeast is performed by the cyto-

plasmic microtubules (Sullivan and Huffaker, 1992) and Cin8p and other bimC-related motors have been localized exclusively to the spindle microtubules spanning the area between the poles (Hagan and Yanagida, 1992; Hoyt *et al.*, 1992; Roof *et al.*, 1992; Sawin *et al.*, 1992; Blangy *et al.*, 1995; Sawin and Mitchison, 1995). It is possible that a small amount of Cin8p motors act upon the cytoplasmic microtubules in a previously unappreciated role (Hoyt *et al.*, 1992). Alternatively, the nuclear migration failures observed in *cin8-Δ* cells are the indirect result of some other spindle malfunction caused by loss of Cin8p.

Microtubules in pac Mutants

In *S. cerevisiae*, nuclear migration is accomplished by the cytoplasmic microtubules (Palmer *et al.*, 1992; Sullivan and Huffaker, 1992). The observed nuclear migration failures in the *pac* mutants could be due to a severe defect in cytoplasmic microtubule structure as exemplified by the β-tubulin mutation *tub2–401* (Sullivan and Huffaker, 1992). In contrast, the cytoplasmic microtubules may appear intact by microscopy but be unable to function properly due to loss of an essential interaction. This appears to be the case in *dyn1* mutants where cytoplasmic microtubules are visible but the spindle is often misoriented (Eshel *et al.*, 1993; Li *et al.*, 1993). The microtubule structures of *pac* mutants from each of the 15 groups were examined by immunofluorescence microscopy. After incubation at 30°C, there was no obvious difference from the wild-type observed for any of the *pac* mutants. In *pac* cultures that were shifted to 12°C for 24 h, the only differences in microtubules that were detected occurred in cell bodies that contained at least two DAPI-staining regions (Figure 2). These cells possessed misoriented or multiple spindles similar to that previously described for *dyn1* mutants (Eshel *et al.*, 1993; Li *et al.*, 1993). The misoriented spindles contained apparently intact cytoplasmic microtubules that often extended into the daughter bud, suggesting that the nuclear migration defect of the *pac* strains is not due to simple loss of cytoplasmic microtubule structure.

Class I pac Double Mutants Are Viable

The striking phenotypic similarities exhibited by the class I *pac* mutants suggested that the class I gene products act within a common functional pathway. It is possible, however, that mutants within this group are affected for different nuclear migration pathways whose absence causes similar phenotypes. In this latter case, we might expect to see additive effects in double mutant strains. We have created many of the possible class I double mutant combinations using deletion alleles (i.e., *dyn1-Δ pac1-Δ*, *dyn1-Δ pac3-Δ*, *dyn1-Δ pac10-Δ*, *dyn1-Δ pac11-Δ*, *dyn1-Δ act5-Δ*, and *pac3-Δ pac11-Δ*). All combinations tested were viable and formed colonies that were roughly of the same size as the single mutants. Many were examined after growth at 13°C and found to produce binucleate cells at levels similar to those produced by class I single mutants (≥15%). This supports our hypothesis that all of the class I mutants are affected for a common pathway of function.

Cloning and Characterization of Class I PAC Genes

Class I *pac* mutants are phenotypically similar to *dyn1* mutants. This suggests that gene products from this class may be additional components of the cytoplasmic dynein complex or factors that regulate its localization or activity. The *PAC* genes were cloned by complementation of their mutant phenotype, the inability to lose the *CIN8* plasmid (see MATERIALS AND METHODS). In each case, a genetic linkage experiment was performed to verify that the cloned DNA represented the original mutant locus (see MATERIALS AND METHODS). The cloned DNA was used to create marked genomic deletions, and these were examined to see if they caused lethality when combined with *cin8Δ*. Lethality with *cin8Δ* was observed for all deletion alleles with the exception of *pac14-Δ* (see below). In addition, the deletion mutants were examined for the production of binucleate cells (Table 2). We discuss each class I gene individually below.

PAC11. *PAC11* encodes a predicted polypeptide product of 533 amino acids with a molecular weight of 58 kDa. Although overall identity is relatively low (22.4%), the predicted amino acid sequence of Pac11p is significantly similar to the 74-kDa rat cytoplasmic dynein intermediate chain 1A (Figure 3; Paschal *et al.*, 1992). Regions of higher similarity were found to encode structural features common to dynein intermediate chains. All dynein intermediate chains possess five or six WD (or β-transducin-like) repeat units in their carboxyl termini (Wilkerson *et al.*, 1995). These degenerate repeats are believed to be involved in protein–protein interactions (Neer *et al.*, 1994). Six WD repeats were found in the carboxyl half of Pac11p (Figure 3, solid regions) with the repeat ending at $W^{465}D^{466}$ being most highly conserved with the deduced consensus (Neer *et al.*, 1994). This region of Pac11p displays the highest level of similarity to the rat intermediate chain. The amino termini of cytoplasmic dynein intermediate chains have a heptad repeat sequence predicted to form coiled α-helical coils, including a region rich in amino acids of mixed charge (Vaughn and Vallee, 1995). Both of these features were found in the amino terminus of the Pac11p sequence, although the coiled region appears to have been split into two smaller coils. After the coiled-coil domains of cytoplasmic dynein intermediate chains is a serine-rich region. A 21-amino acid region of Pac11p (amino acids 141–161) that contains five serines may correspond to this serine-rich

Figure 3. Comparison of the amino acid sequence of Pac11p and the rat cytoplasmic dynein intermediate chain. The WD repeat region and the amino-terminal (putative) coiled-coil region are the most closely related by sequence. Serine-rich regions in both polypeptides are also indicated, although they display weak sequence relationship.

region. The sequence of the intermediate chain from rat brain was used as a probe in a computer homology search of the entire translated *S. cerevisiae* genome. Among the gene products showing the highest similarity, only Pac11p displayed the sequence features described above. Cells deleted for *PAC11* were viable.

PAC13. *PAC13* was found to be equivalent to *NIP100*, previously identified via a "two-hybrid assay" (Fields and Song, 1989) designed to screen for genes whose products may interact with Nup1p, a nuclear pore component (Schlenstedt and Silver, personal communication). The 100-kDa predicted *PAC13* gene product shows sequence and structural similarity to the largest polypeptide component of the dynein-activating dynactin complex often referred to as $p150^{Glued}$. Sequence identity between Pac13p and $p150^{Glued}$ is very strong only within a 40-amino acid amino-terminal region determined to be responsible for microtubule binding (Figure 4; Riehemann and Sorg, 1993; Waterman-Storer *et al.*, 1995). Interestingly, this conserved motif was found in *PAC2* and *PAC14* (=*BIK1*) as well (see below). Outside of this small region, similarity of sequence is low. In a comparison to the *Neurospora ro-3* gene product, a $p150^{Glued}$ homologue (Tinsley *et al.*, 1996), overall identity was found to be 25.1%. However, the locations of predicted coiled α-helical coil regions appear to be quite similar in the two polypeptides (Figure 4). Note also, that the product of the *Drosophila melanogaster glued* gene and characterized homologues from other species are larger in size; typically close to 145 kDa. The sequence of the *Neurospora ro-3* gene product was used as a probe in a computer search of the entire translated *S. cerevisiae* genome. This revealed that Pac13p is the *S. cerevisiae* gene product closest to the ro-3 protein. Cells deleted for *PAC13* were viable.

PAC1. *PAC1* encodes a predicted polypeptide of 492 amino acids with a molecular weight of 54 kDa. Examination of the Pac1p sequence identified five WD repeats in the carboxyl-terminal two-thirds of the molecule and a potential coiled α-helical coil region in the amino terminus (Figure 5). Pac1p shares these features and high amino acid identity throughout its entire sequence with a human gene required for normal brain development, *LIS-1* (Reiner *et al.*, 1993; 33% identical), and an *Aspergillus nidulans* gene implicated in dynein function, *nudF* (Xiang *et al.*, 1995; 31% identical; see DISCUSSION). The conservation is not confined to the WD repeat regions but extends throughout the entire sequences and includes two amino-terminal regions of very high similarity. Deletion of *PAC1* revealed that this gene is not essential for viability.

PAC3. PAC3 was identified as *JNM1*, a gene previously implicated in a genetic pathway responsible for localizing or activating cytoplasmic dynein (McMillan and Tatchell, 1994). McMillan and Tatchell observed a similar cold-sensitive binucleate phenotype for *dyn1* and *jnm1* mutants and found that double mutants are no more severely affected than single mutants. Deletion of *PAC3* (McMillan and Tatchell, 1994; this work) revealed that this gene is not essential for viability.

PAC12. The complementing activity for *pac12–1* was found to correspond to *NUM1*, a nonessential gene required for normal nuclear migration (Kormanec *et al.*, 1991; Farkasovsky and Küntzel, 1995). Similar to dynein-deficient cells, *num1*-Δ cells were found to often contain misaligned mitotic spindles with elongated cytoplasmic microtubules (Farkasovsky and Küntzel, 1995).

PAC10. *PAC10* is predicted to encode a polypeptide of 199 amino acids with a molecular weight of 22 kDa. The region between I^{134} and L^{176} is predicted to form a coiled α-helical coil. Two proteins in the GenBank database exhibit high sequence similarity to Pac10p: YAS7 (GenBank accession number Q10143; 43% identical), a *Schizosaccharomyces pombe* gene product of unknown function and VBP-1 (GenBank accession number U56833; 43% identical), a human protein that associates with the von Hippel-Lindau tumor suppressor gene product (Tsuchiya *et al.*, 1996). The significance of these similarities is unknown. We note that there is no gene product with close relationship to the von Hippel-Lindau tumor suppressor encoded by the *S. cerevisiae* genome. Haploid spores deleted for *PAC10* displayed a germination defect; all 34 *pac10*-Δ spores from 17 tetrads failed to grow into colonies on rich medium. *PAC10* was not essential for vegetative

A

```
Pac13p              30 VKFIGETQFAKGIWYGIELDKPLGKNDGSANGIRYFDIDL 69
                       V+++GET FA G W GIELD+P GKNDGS  G RYF+ ++
N. crassa ro-3      24 VRYVGETAFAPGTWVGIELDEPSGKNDGSVQGERYFNCEM 63
CAP-Gly consensus          G      G W GI L     GKN GS  G  YF
                                  F   L I     H   T
                                  Y   V V         A
                                      M M
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B

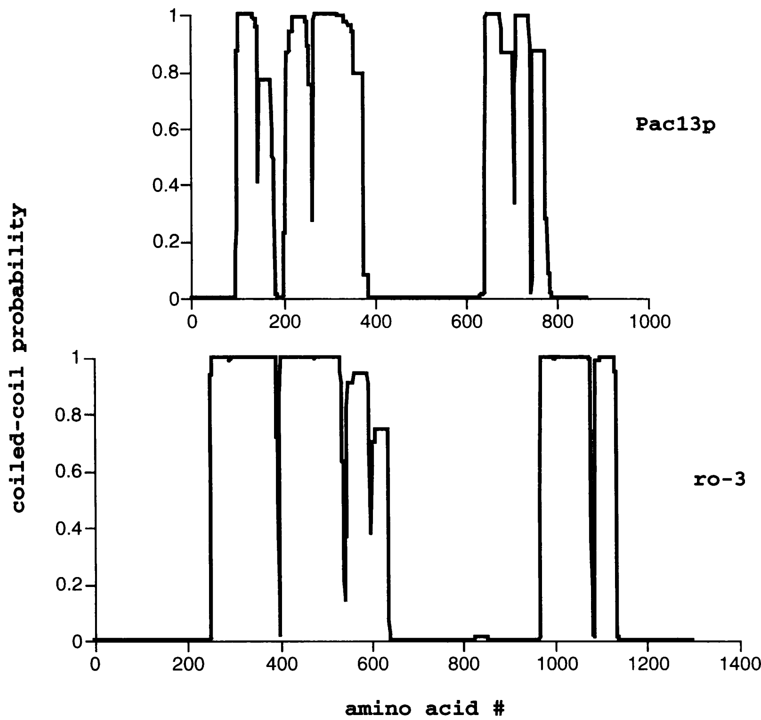

Figure 4. Comparison of the amino acid sequence of Pac13p and the ro-3 protein, a p150^{Glued} homologue from *Neurospora*. (A) Sequence comparison of the amino-terminal region predicted to encode a microtubule-binding domain (CAP-Gly). The consensus for this region is from Riehemann and Sorg (1993). (B) Coiled-coil probability determined by the program of Lupas *et al.* (1991) using a window of 28 amino acids.

growth, however. A *pac10*-Δ strain carrying a *PAC10* plasmid germinated well and was also able to survive loss of the plasmid, although the resulting *pac10*-Δ cells grew at a reduced rate on rich medium. Like *PAC14* (below), a *pac10*-Δ strain produced less binucleate cells than a *pac10–1* strain. The deletion of *PAC10* caused lethality when combined with *cin8*-Δ.

PAC14. The complementing activity for *pac14–1* was found to correspond to *BIK1*, a nonessential gene encoding a microtubule-associated protein (Berlin *et al.*,

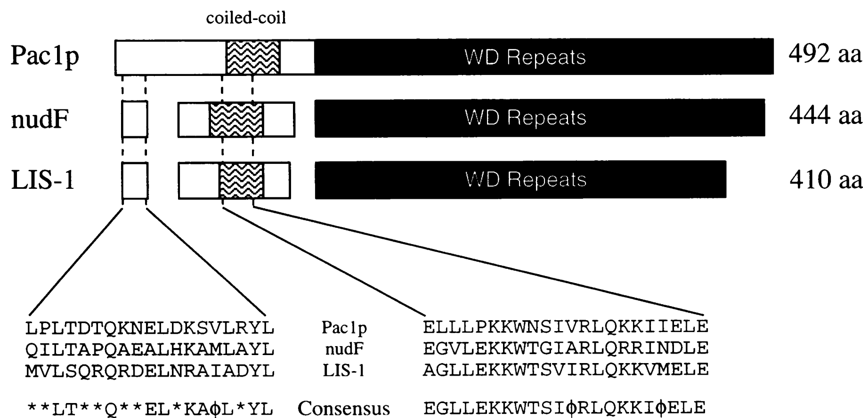

Figure 5. Comparison of the amino acid sequence of Pac1p, nudF from *Aspergillus*, and human LIS-1. Two amino-terminal regions of high similarity are indicated. The consensus sequences for these regions were determined by agreement at each position between at least two of the polypeptides. ϕ, a hydrophobic amino acid.

1990). Like *pac14–1*, a deletion of *BIK1* was found to cause an accumulation of binucleate cells that increased at lower growth temperature (Berlin *et al.*, 1990; Table 2). However, the *pac14–1* mutant produced about twice the number of binucleate cells as the *bik1*-Δ mutant and both produced less than the typical class I mutant. Surprisingly, we found that a deletion of *BIK1* did not cause lethality when combined with *cin8*-Δ. A *cin8*-Δ *pac14–1* strain originally unable to segregate cycloheximide-resistant cells was subsequently able to do so after transformation with a DNA construct that deleted the *BIK1* gene (Figure 1). Growth of the resulting *cin8*-Δ *bik1*-Δ cells was slow relative to *cin8* or *bik1*-Δ cells, however.

Cloning and Characterization of Class II PAC Genes

The mutants in class II were rescued by the *KAR3–898* and *KIP1*-2-μm plasmids and in general produced lower levels of binucleate cells. Alleles of *KIP1* were found among the class II mutants. Class II *PAC* genes were cloned by the same protocol as described for the class I genes. Our findings suggest that the class II genes act in a few distinct functional pathways.

Mitotic Spindle Checkpoint Genes. *PAC7*, *PAC8*, and *PAC9* were found to correspond to genes implicated in mitotic spindle checkpoint regulation; *BUB2*, *MPS1*, and *BUB3*, respectively. Defects in these and related genes cause an inability to properly halt the cell cycle in M phase in response to impaired spindle function (Hoyt *et al.*, 1991; Li and Murray, 1991; Weiss and Winey, 1996). *BUB2* and *BUB3* are nonessential genes (Hoyt *et al.*, 1991). In contrast to BUB^+, *bub2* and *bub3* mutants continue their budding cycles in the presence of benzimidazole compounds that depolymerize microtubules. *MPS1* encodes a protein kinase that is essential for viability and required for mitotic duplication of the spindle pole bodies (Poch *et al.*, 1994; Lauzé *et al.*, 1995). Temperature-sensitive *mps1* mutants also exhibit a mitotic checkpoint defect; at elevated temperatures they continue budding even in the presence of microtubule-depolymerizing treatments (Weiss and Winey, 1996).

In light of these findings, representative *pac* mutants were examined for continued budding in the presence of benomyl, a phenotype characteristic of a mitotic checkpoint defect (Table 2). After release from α-factor synchronization in G_1, most *pac* mutants arrested as large-budded cells when placed on medium containing benomyl. In contrast, after 6 h on benomyl, the *pac7*, *pac9*, and *PAC5* mutants had formed extra buds. The *pac8* mutants tested did not show elevated extra budding. We note, however, that unlike the temperature-sensitive *mps1* alleles that displayed conditional aberrant budding (Weiss and Winey, 1996), the *pac8* alleles that we isolated were not temperature sensitive for growth. Despite the failure to observe extra budding, we have assigned the *pac8* mutants to the checkpoint class in Table 2 based on the published properties of *MPS1*. The dominant *PAC5–1* mutant was determined to harbor a novel allele of *CDC20*, an essential gene required for progression through mitosis (E.J.S. and M.A.H., unpublished observations). A checkpoint role for *CDC20* had previously not been appreciated. Neither the remaining class II mutants nor any of the class I mutants tested displayed the extra budding phenotype.

These finding also prompted us to test whether other spindle checkpoint genes display the *pac* phenotype. Double mutants combining *cin8*-Δ with *bub1*-Δ (Roberts *et al.*, 1994), *mad1*-Δ, *mad2*-Δ, or *mad3*-Δ (Hardwick and Murray, 1995) were created (see MATERIALS AND METHODS). Of these, all were inviable except for the *cin8*-Δ *mad3*-Δ combination. However, we observed that deleting *MAD3* in a *cin8–3* strain resulted in increased temperature sensitivity. This suggests a synthetic deleterious interaction of *cin8* with *mad3* as well.

Microtubule Stability Genes. Two of the *pac* mutants were found to be extremely sensitive to the antimicrotubule compound benomyl (Hoyt *et al.*, 1997). Complementation and linkage analysis revealed one to be an allele of *CIN1*, previously demonstrated to act in a pathway required for normal microtubule stability (Hoyt *et al.*, 1990; Stearns *et al.*, 1990). The second, *pac2*, was found to be a novel gene. A *pac2*-Δ allele caused a similar benomyl-sensitive phenotype. Like other class II mutants, the *pac* phenotypes of *cin1* and *pac2* were suppressed by the *KIP1* and *KAR3–898* plasmids, and both mutants produced only slightly elevated numbers of binucleate cells (Table 2). Evidence was obtained indicating that Pac2p and Cin1p operate in a pathway leading to normal microtubule stability along with Cin2p and Cin4p (M.A.H. and J.R.G., unpublished data).

PAC15. *PAC15* was found to correspond to *IPL1*, identified in a screen for genes involved in regulation of chromosomal ploidy (Chan and Botstein, 1993). *IPL1* encodes an essential protein kinase whose actions appear to be opposed by the type 1 protein phosphatase encoded by the *GLC7* gene. Plasmid pCC418 carrying a dominant-negative truncated allele of *GLC7* relieves the temperature sensitivity of *ipl1* mutations (Francisco *et al.*, 1994). Consistent with this finding, we observed that pCC418 could suppress the lethality of the *cin8*-Δ *pac15–1* combination. Although the specific role of Ipl1p is unknown, it is clear that its action is required for proper execution of mitosis. *cin8* defective alleles were identified in a screen for mutants that cause lethality when combined with *ipl1–2* (Chan, personal communication), results that complement our findings.

DISCUSSION

BimC-related Cin8p appears to be the most important spindle-pole–separating motor in *S. cerevisiae*. Loss of Cin8p causes the greatest reduction to anaphase pole separation proficiency relative to loss of the two other motors that contribute to this process, Kip1p and Dyn1p (Saunders *et al.*, 1995). In addition, in the absence of Cin8p, the two normally nonessential Kip1p and Dyn1p motor pathways both become essential for viability (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Saunders *et al.*, 1995). We have identified mutant genes that cause lethality only in cells deficient for Cin8p function. Among the mutants that perish in the absence of *CIN8* (or *pac*), we found the expected *kip1* and *dyn1* defective alleles. In addition, we found 18 other genes capable of mutating to the *pac* phenotype. All are involved in aspects of the mitotic division process. Below we propose that the products of these genes act in at least four distinct cellular pathways of function (Table 2): 1) Kip1p, 2) the dynein pathway (class I genes *DYN1, PAC1, PAC3/JNM1, PAC10, PAC11, PAC12/NUM1, PAC13/NIP100, PAC14/BIK1,* and *ACT5*), 3) microtubule stability (*PAC2* and *CIN1*), and 4) mitotic spindle checkpoint function (*PAC5/CDC20, PAC7/BUB2, PAC8/MPS1, PAC9/BUB3, BUB1, MAD1,* and *MAD2*. [It is not clear into which group to place *PAC15/IPL1* (see below).] In this discussion, we consider the nature of these gene products and their interaction with Cin8p.

The simplest mechanism leading to the synthetic lethality of a double mutant combination is redundancy for an essential function (Guarente, 1993). This is the most reasonable explanation for *cin8 kip1* and *cin1 dyn1* lethality because overlap in spindle assembly and pole-separating activities has been demonstrated. Other *PAC* gene products may act within the Kip1p or Dyn1p pathways or define other pathways of function required when Cin8p is deficient. Those that act within the Kip1p or Dyn1p motor pathways may include structural components of the force-generating motor assembly, regulators of activity or localization, or any other component required for proper motor function. The *PAC* gene products that act in pathways distinct from Kip1p and Dyn1p may define other mitotic motor activities. However, as described below, our findings do not suggest that the *PAC* genes act in motor pathways beyond Kip1p and Dyn1p. On the other hand, at least two nonmotor pathways appear to become essential in the absence of Cin8p; a microtubule-stabilizing pathway and a mitotic spindle checkpoint pathway(s).

We have divided 17 of the *pac* mutants into two classes based upon their ability to be rescued by multicopy *KIP1* and *KAR3–898*. A perfect correspondence of these two phenotypes was observed. *dyn1* and phenotypically similar mutants constituted class I and failed to be suppressed by these treatments. *kip1* mutants fell into class II. In the case of the class II mutants, we presume that suppression by Kip1p reflects its ability to provide a Cin8p-like function. The *KIP1* gene was originally detected in this laboratory as an extra-copy suppressor of *cin8* mutants (Hoyt *et al.*, 1992). The correspondence of the *KAR3–898* effect suggests that suppression is achieved by a related mechanism. The *KAR3–898* allele was originally detected by its ability to bypass the requirement for either *CIN8* or *KIP1* for viability (Hoyt *et al.*, 1993). The failure of extra *KIP1* to suppress the class I mutants indicates that Cin8p and Kip1p are not completely functionally redundant. In the absence of Cin8p and Dyn1p or other class I gene products, an essential activity appears to be missing that cannot be compensated by an increased amount of *KIP1* genes. A likely source of this functional difference is the great sequence divergence between Cin8p and Kip1p outside of their highly conserved motor domains.

The Class I PAC Genes Act in the Dynein Pathway

Our findings suggest that all of the class I *pac* mutants affect a common pathway of function. All class I mutants failed to be suppressed by multicopy *KIP1* and *KAR3–898*, and most (with the exception of *pac10* and *pac14*) displayed very high levels of binucleate cells when incubated in the cold. Microtubule structures appeared to be normal with the exception that spindles were frequently misoriented from the mother-bud axis, a phenotype characteristic of *dyn1* mutants (Eshel *et al.*, 1993; Li *et al.*, 1993). In addition, a screen for cold-sensitive mutants that form a high number of binucleate cells identified defective alleles of *DYN1, PAC1, PAC3/JNM1, PAC13/NIP100,* and *PAC14/BIK1* (Yamamoto and Koshland, personal communication). None of the class I *PAC* genes were essential for viability except at colder incubation temperatures. Many class I double-deletion mutants were created and all were viable and grew at rates comparable to the single mutants. This would be expected if the combined mutations affected the function of the same cellular pathway. In experiments to be reported elsewhere (J.R.G. and M.A.H., unpublished data), we have identified a *dyn1* allele that strongly inhibits cell growth. In cells expressing this mutant form of dynein, elimination of *PAC1, PAC11,* or *ACT5* completely suppressed the growth inhibition. This indicates that these genes are required for the deleterious action of this Dyn1p mutant form. Finally, the sequences of three class I gene products display similarity to polypeptides associated with dynein in other eukaryotic cells. Pac11p and Pac13p/Nip100p display weak, but significant, similarity to the cytoplasmic dynein intermediate chain and the p150^{Glued} subunit of the dynein-activating dynactin complex, respectively. As

described by others, Act5p is a nonconventional actin related to the form found in the dynactin complex (Clark and Meyer, 1994; Muhua *et al.*, 1994). Therefore, it is likely that all the class I *pac* mutants are affected for dynein activity. However, we have yet to demonstrate a physical interaction of any of the *PAC* gene products with dynein.

Three of the class I mutants, *pac3/jnm1*, *pac12/num1*, and *act5*, have previously been found to display a nuclear migration defect similar to that of *dyn1* (Clark and Meyer, 1994; Farkasovsky and Küntzel, 1995; McMillan and Tatchell, 1994; Muhua *et al.*, 1994). Our findings provide further evidence that the products of these genes function in concert with dynein in *S. cerevisiae*. Like *ACT5*, *JNM1* may encode a dynactin component. We and others (K. Tatchell, personal communication) have noted the similarity in predicted structure between the *JNM1* gene product and the p50 dynactin component. Although sequence similarity is too low to be detected by homology search algorithms, both p50 and Jnm1p possess three small potential coiled α-helical coil regions located at similar positions. *num1* mutants display genetic interactions with tubulin mutants and Num1p is associated with the cell cortex (Farkasovsky and Küntzel, 1995). Num1p may participate in forming a cortical structure that serves as a site from which dynein acts upon the cytoplasmic microtubules.

The *PAC1* gene product exhibits a high sequence similarity to *nudF* from *Aspergillus nidulans* and *LIS-1* from humans. *nudF* mutations were found to cause a defective nuclear migration phenotype similar to that caused by mutations in the cytoplasmic dynein heavy chain gene *nudA* (Xiang *et al.*, 1994, 1995). The correspondence of our findings in *S. cerevisiae* with those from *A. nidulans* suggests a conserved functional relationship between Pac1p/NudF and the cytoplasmic dynein heavy chain. Defects in human *LIS-1* cause Miller-Dieker lissencephaly, a disease characterized by massive underdevelopment of the cerebral cortex (Reiner *et al.*, 1993). The migration of neural cells, an essential aspect of the development of cortical architecture, is thought to cease abnormally at about 10–14 wk of gestation (Dobyns *et al.*, 1993). The migration of neurons in the cerebral cortex and the cerebellum has been found to be accompanied by specific nuclear movements (perikaryal translocation; Book and Morest, 1990; Book *et al.*, 1991; Hager *et al.*, 1995). Therefore, it is possible that the primary defect in *LIS-1* mutant cortical neurons is loss of a dynein-mediated nuclear motility event essential for proper migration of the cells.

The roles of the two remaining class I genes, *PAC10* and *PAC14*, are less clear. Defects within these two genes failed to produce the extremely high binucleate cell levels characteristic of the other class I genes. This finding, and the other differences noted herein, suggest that the products of these two genes are not as closely associated with dynein function as the remainder of the class I genes. The *PAC10* deletion mutants created were unique in their poor spore germination and slow growth. *pac14–1* was found to be an unusual allele of *BIK1*. Two *pac14–1* phenotypes, lethality with *cin8*-Δ and binucleate cell production, were found to be more extreme than a *BIK1* deletion. Bik1p is a nonessential microtubule-associated protein that was localized primarily to the nuclear microtubules (Berlin *et al.*, 1990). The observed properties of *pac14–1* suggest that this mutant form of Bik1p interferes with the action of dynein. Since dynein is probably performing its role on the cytoplasmic microtubules (Yeh *et al.*, 1995; Bloom, personal communication), it is possible that Bik1p also acts outside of the nucleus. Alternatively, *BIK1* or *PAC10* may be involved in a nuclear migration pathway distinct from that in which dynein acts but whose absence causes similar phenotypes. Note, however, that *dyn1*-Δ *bik1*-Δ and *dyn1*-Δ *pac10*-Δ double mutants are viable (our unpublished observations).

The Class II PAC Genes Represent Different Mitotic Pathways

When we initiated this screen, we expected to find alleles of *KIP1* due to functional overlap with the *CIN8*-encoded motor. We also expected to find mutant alleles of other genes required for the normal function of the Kip1p pathway. In *CIN8* cells, *kip1* loss-of-function mutations do not cause detectable phenotypes (Hoyt *et al.*, 1992; Roof *et al.*, 1992). Except for the *kip1* alleles that we recovered, we were able to detect mutant phenotypes in *CIN8* cells for all of the *pac* mutants recovered in this study. This suggests that none were uniquely affected for Kip1p function. In this regard, a more significant negative result is our inability to find, by exhaustive screening, well-behaved mutations that cause lethality when combined with *kip1*-Δ other than those that affect the *CIN8* gene (L.J.T. and M.A.H., unpublished observation). Thus, these findings suggest that neither the Cin8p or the Kip1p pathways contain additional nonessential gene products uniquely specific for the function of each pathway (i.e., a light chain that functions uniquely with Cin8p). This conclusion is consistent with the observation that *Drosophila* BimC motor KLP61F (also known as KRP130) can be purified as a homotetramer with no associated accessory polypeptides (Cole *et al.*, 1994; Kashina *et al.*, 1996a,b).

The remaining class II genes appear to represent at least two different mitotic functional pathways: a pathway required for normal microtubule stability (*PAC2* and *CIN1*) and a checkpoint pathway required for response to mitotic spindle defects (*PAC5*, *-7*, *-8*, and *-9*). Loss of *CIN1* or *PAC2* causes a dramatic

destabilization of all vegetative microtubule structures; microtubules become extremely sensitive to depolymerizing agents and cold incubation temperatures (Hoyt *et al.*, 1990; Hoyt *et al.*, 1997). It seems likely, therefore, that the lethality of the *cin8 pac2* combination reflects a reduced proficiency of the remaining motors to operate on unstable microtubules. Alternatively, Cin8p may make a contribution to microtubule dynamics that overlaps with a function provided by Cin1p and Pac2p. Other kinesin-related proteins have been observed to influence the dynamic properties of microtubules (Endow *et al.*, 1994; Walczak *et al.*, 1996).

Mitotic-spindle checkpoint-defective mutants are sensitive to treatments that perturb the function of the spindle. They are unable to promote M-phase delay or arrest required to respond to spindle damage (Hoyt *et al.*, 1991; Li and Murray, 1991; Weiss and Winey, 1996). We observed that *BUB1*, *-2*, and *-3*, *MAD1* and *-2*, and *MPS1* were required for viability in the absence of *CIN8*. This suggests that cells require these genes to respond to the spindle defect caused by *cin8*-Δ. Perhaps cell cycle delay is required so that the remaining motors can accomplish spindle assembly and elongation. We have found that the M-phase arrest caused by *cin8* mutations at 37°C requires the actions of *BUB1* and *BUB2* (E.J.S. and M.A.H., unpublished observation). The dominant-acting *PAC5–1* mutation also permitted us to identify a previously unknown mitotic checkpoint function for *CDC20*.

It is not clear whether *PAC15/IPL1* acts in one of the previously described mitotic pathways or represents a new pathway. Temperature-sensitive alleles of the essential gene *IPL1* cause massive mitotic nondisjunction, indicating that its protein kinase product may be an important regulator of mitotic processes (Chan and Botstein, 1993; Francisco *et al.*, 1994). Lethality with *cin8*-Δ would be expected if one of its roles is the activation of either the Kip1p or Dyn1p motors.

A *cin8*-Δ synthetic lethal approach identified a number of *S. cerevisiae* genes whose products participate in various mitotic activities. These findings illustrate the complexity of the mitotic process that requires the coordination of numerous structural, mechanical, and regulatory activities. The screen performed was particularly useful for identifying members of the cytoplasmic dynein motor pathway. This approach, along with the genetic studies of dynein pathway genes in the filamentous fungi (Plamann *et al.*, 1994; Morris *et al.*, 1995), should permit a new and powerful level of analysis of this complex motor pathway (Lupas *et al.*, 1991).

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