Suppression of the *bimC4* Mitotic Spindle Defect by Deletion of *klpA*, a Gene Encoding a KAR3-related Kinesin-like Protein in *Aspergillus nidulans*

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Abstract. To investigate the relationship between structure and function of kinesin-like proteins, we have identified by polymerase chain reaction (PCR) a new kinesin-like protein in the filamentous fungus *Aspergillus nidulans*, which we have designated KLPA. DNA sequence analysis showed that the predicted KLPA protein contains a COOH terminal kinesin-like motor domain. Despite the structural similarity of KLPA to the KAR3 and NCD kinesin-like proteins of *Saccharomyces cerevisiae* and *Drosophila melanogaster*, which also posses COOH-terminal kinesin-like motor domains, there are no significant sequence similarities between the nonmotor or tail portions of these proteins. Nevertheless, expression studies in *S. cerevisiae* showed that *klpA* can complement a null mutation in *KAR3*, indicating that primary amino acid sequence conservation between the tail domains of kinesin-like proteins is not necessarily required for conserved function. Chromosomal deletion of the *klpA* gene exerted no observable mutant phenotype, suggesting that in *A. nidulans* there are likely to be other proteins functionally redundant with KLPA. Interestingly, the temperature sensitive phenotype of a mutation in another gene, *bimC*, which encodes a kinesin-like protein involved in mitotic spindle function in *A. nidulans*, was suppressed by deletion of *klpA*. We hypothesize that the loss of KLPA function redresses unbalanced forces within the spindle caused by mutation in *bimC*, and that the KLPA and BIMC kinesin-like proteins may play opposing roles in spindle function.

THE mechanochemical protein kinesin uses the energy derived from ATP hydrolysis to generate plus end–directed movement of organelles on microtubules (for review see Vallee and Shpetner, 1990). The globular NH_2 terminus of kinesin heavy chain is sufficient for microtubule binding, ATP hydrolysis, and force generation in vitro (Yang et al., 1990), and is termed the motor domain. The remaining portion of the protein, the tail, consists of an α-helical stalk and a COOH-terminal globular domain. The COOH-terminus interacts both with kinesin light chains (Hirokawa et al., 1989), which in turn are thought to interact with the motor's cargo, and also directly with microtubules (Navone et al., 1992). This interaction with microtubules indicates kinesin can also cross-bridge microtubules and possibly provides motive force for microtubule sliding (Navone et al., 1992). Homologues of kinesin heavy chain have been identified and cloned from a variety of species and show conservation of amino acid sequence throughout the length of the proteins (Yang et al., 1989; Kosik et al., 1990; Wright et al., 1991; and Navone et al., 1992).

Several gene products known as kinesin-like proteins (KLPs)[1] have been identified from a variety of organisms. They differ from kinesin heavy chain primarily in their tail domains, each possessing a unique tail linked to a common kinesin-like motor. Functions in chromosome segregation and organelle transport have been defined for several members of this kinesin-like superfamily (for reviews see Endow, 1991; Goldstein, 1991; Sawin and Scholey, 1991). The diversity of the tail domains of the KLPs led to the proposition of the "one motor, many tails" hypothesis, which postulates that the tails are the primary determinants of functional specificity (Vale and Goldstein, 1990). Multiple genes encoding KLPs have been identified in yeast (Meluh and Rose, 1990; Roof et al., 1991; 1992; Lillie and Brown, 1992; Hoyt et al., 1992) and *Drosophila* (Yang et al., 1989; McDonald and Goldstein, 1990; Endow et al., 1990; Zhang et al., 1990; Endow and Hatsumi, 1991; Stewart et al., 1991), thus indicating that there is KLP structural and functional diversity even within a single organism.

The KAR3 (Meluh and Rose, 1990) and NCD (McDonald and Goldstein, 1990; Endow et al., 1990) proteins of *Saccharomyces cerevisiae* and *Drosophila melanogaster* form a separate subclass of KLPs. They differ from the other superfamily members in having the kinesin-like motor domain at the COOH terminus, but show no sequence similarity outside this domain. Furthermore, NCD has been shown to

1. *Abbreviations used in this paper:* KLP, kinesin-like proteins; PCR, polymerase chain reaction.

generate minus end-directed motility in vitro (McDonald et al., 1990; Walker et al., 1990). A recombinant version of *Drosophila* kinesin heavy chain with the motor domain positioned at the COOH terminus nevertheless still generates plus end–directed motility in vitro (Stewart, R. J., and L. S. B. Goldstein, unpublished observation, cited in Goldstein, 1991). Therefore, the minus end–directed motility of NCD may not be a consequence of the COOH terminal location of the kinesin-like motor, but rather almost certainly derives from the structure of the motor domain.

There are several important questions pertaining to the diversity of KLP structure and function: How is the diversity of KLP structure related to function? Is the "one motor, many tails" hypothesis adequate to describe determinants for KLP functional specificity? What cellular functions do KLPs perform? Finally, how many KLPs function in an organism, in a cell type, or in a single cellular process? In this report we attempt to provide some insight into these problems with a description of a new KLP in *Aspergillus nidulans*, which we have designated KLPA. KLPA is structurally related to KAR3 and NCD, but contains no sequence similarities to these or any other KLPs outside the motor domain. Yet, when expressed in *S. cerevisiae*, *klpA* complements a null mutation in *KAR3*. Further, a genetic interaction between *klpA* and another KLP-encoding gene in *A. nidulans*, *bimC*, suggests that these KLPs play opposing roles in the function of the mitotic spindle.

Materials and Methods

Manipulations of A. nidulans *and* S. cerevisiae

All *A. nidulans* strains are derivatives of a single isolate, FGSC4. Methods and media for the growth and maintenance of *A. nidulans* strains, transformation of protoplasts, sexual crosses, and isolation of nucleic acids were as described (O'Connell and Kelly, 1988). Nuclei were stained in glutaraldehyde fixed cells with the DNA specific dye 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) as described by O'Donnell et al., (1991). Formaldehyde fixation and immunocytochemical staining of cells with the rat anti α-tubuli mAb YOL1/34 (Sigma Chemical Co., St. Louis, MO; 1/100 detected with CY3-conjugated donkey anti-rat 1/500) (Jackson Immunochemicals, Avondale, PA) or rabbit anti γ-tubulin (MIPA) polyclonal antibody (Oakley et al., 1990; 1/500 detected with TRITC-conjugated donkey anti-rabbit 1/500) (Jackson Immunochemicals) followed the procedures of Oakley et al., (1990) and Mirabito and Morris (1993). Fixed cells were digested with 1.5% novozyme 234 + 10 μM aprotinin (Sigma Chemical Co.) for 30 min at 28°C before staining. This period of digestion, which was necessary for γ-tubulin staining, results in some fragmentation of cytoplasmic microtubules and occasional loss of DAPI staining.

General manipulations of *S. cerevisiae* strains were performed as described by Rose et al., (1990). Yeast microtubules were stained with the anti α-tubulin mAb YOL1/34 (Sera Lab, Westbury, NY). Yeast mating assays are described below.

Polymerase Chain Reaction Amplification, Cloning, Sequencing, and Mapping of klpA

Polymerase chain reaction (PCR) amplification of *klpA* sequences was performed with primers designed on the basis of regions conserved between the driven amino acid sequences of *Drosophila* kinesin heavy chain (Yang et al., 1989), *A. nidulans* BIMC (Enos and Morris, 1990) and *S. cerevisiae* KAR3 (Meluh and Rose, 1990). The forward primer corresponded to amino acids TIFAYGOT with a 1,536-fold degenerate sequence of ACCCCGGGA-TCCACNAT(CTA)TT(CT)GCNTA(CT)GGNCA(AG)AC, and the reverse primer corresponded to amino acids HIPYRESN with a 1,536-fold degenerate sequence of CTGCAGCTGTA(CT)TTNGA(TC)TC(TC)TANGG-(AGT)AT(GA)TG (where N = any nucleotide and parentheses indicate alternatives at these positions). Reactions were carried out using a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) and GeneAmp PCR kit (recommended buffer conditions). Amplifications were from wild-type *A. nidulans* genomic DNA with the following protocol for 30 cycles: 1 min at 94°C, 2 min at 45°C, and 3 min plus 5 s per cycle at 72°C. Amplified products were gel purified, cloned into pBluescriptKS(+) (Stratagene Inc., La Jolla, CA) and their nucleotide sequence determined using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH). One cloned product, designated pPCR52, contained an open reading frame which predicted a KLP. pPCR52 was used as a probe to isolate several cDNAs from a library constructed in λgt10 (Osmani et al., 1987). One clone, which was judged to be full length by comparison with the size of the corresponding mRNA detected on Northern blots, was retained for DNA sequence analysis. The insert of this clone was subcloned into pBluescriptKS(+), and the resulting plasmid was designated pKLPA1. The nucleotide sequence of this cDNA (EMBL database accession number X64603), derived from a gene designated *klpA*, was determined from overlapping regions of the entirety of both strands using either subclones derived from pKLPA1 or oligonucleotides corresponding to internal *klpA* sequences. The 5′ end of *klpA* in pKLPA1 is at the SacI end of the pBluescript multiple cloning site. The longest open reading frame was translated to derive the predicted KLPA amino acid sequence. Computer manipulations of sequence data were performed using the University of Wisconsin GCG package (Devereux et al., 1984). The *klpA* locus was mapped to chromosome 1 using a Southern blot of whole chromosomes (provided by Dr. G. May) probed with pKLPA1. Subsequently, parasexual analysis using diploid strains made between strains in which the *klpA* locus was marked through transformation by heterologous markers (see below) and FGSC483 (*fpaB7 galD5 suA1adE20 SulA1 riboA1 anA1 pabaA1 yA2 adE20 biA1*) showed *klpA* to be on the left arm of chromosome I, distal to *SulA*.

Plasmid Constructions

PCR was used to insert unique XbaI and KpnI restriction sites at nucleotide 140 (see Fig. 1 *A*) of the *klpA* cDNA in pKLPA1 using the following primers: forward - GCTCTAGAGGTACCGCACAGTCGTCGGTGA (XbaI and KpnI restriction sites underlined); reverse - GGGCCAGTTGAGGCACTGCAG-TGG (PstI site at nucleotides 292–297 (see Fig. 1 *A*) underlined. This PCR product was digested with XbaI and PstI and used to replace the 5′ end of the *klpA* cDNA in pKLPA1, with the XbaI site provided by vector sequences. The accuracy of the PCR reaction was confirmed by DNA sequencing and the resulting plasmid was designated pKLPA2. The insert of pKLPA2 was subcloned as a KpnI fragment into the *alcA* expression plasmid pKK12 (Kirk, K. E., 1992) to generate plasmids pKK12-27 (reverse orientation or *klpA* relative to the *alcA* promoter) and pKK12-29 (forward orientation).

To express *klpA* in *S. cerevisiae*, the following plasmids were constructed. pKLPA3 was generated by insertion of a BamHI linker into the 5′ XbaI site and a BglII linker into the 3′ EcoRV site (in vector multiple cloning site) of pKLPA2. The insert of pKLPA3 was subcloned as a BamHI–BglII fragment into yeast vectors containing the *GAL1* promoter (Johnston and Davis, 1984) derived from pMR79 (Rose and Fink, 1987) to generate plasmids pMR2388, pMR2389, pMR2390, and pMR2391 (see Fig. 6). pMR1682 (Fig. 6) contains nucleotides −71 to +2751 of the published *KAR3* sequence (Meluh and Rose, 1990). pMR820 (Fig. 6), which contains a genomic subclone of *KAR3* has been described previously (Meluh and Rose, 1990).

Genomic clones of *klpA* were obtained from a library which was constructed using the partial end fill procedure in the lambda replacement vector LambdaGem-11 as suggested by the manufacturer (Promega Corp., Madison, WI). Standard subcloning procedures were used to construct a plasmid designated pKLPA4, which contains the 7-kb XbaI–ClaI fragment of the *klpA* region in pBluescriptKS(+). pKLPA4 was digested with BglII and EcoRI (removing 3.1 kb) and end filled. EcoRI adapters were ligated to these blunt ends and the orotidine-5′-phosphate decarboxylase encoding *pyr4* gene of *Neurospora crassa* (Buxton and Radford, 1983) was inserted as a 2.1-kb EcoRI fragment derived from pGM1 (May et al., 1985), generating pΔKLPA1. Subsequently, the 1.8-kb ClaI genomic fragment 3′ to *klpA* was added to pΔKLPA1 to generate pΔKLPA2 (see Fig. 3).

Construction of a klpA *Null Allele*

A *klpA* null allele was constructed by transformation mediated two-step gene replacement. A two-step procedure was used due to very low frequency of plasmid integration at the *klpA* locus (<1 in 200 transformants). *A. nidulans* strain SJ035 (*pyrG89; wA2; chaA1*, supplied by Dr. S. James,

UMDNJ-RWJMS, Piscataway, NJ) was transformed with pΔKLPA2, and PyrG$^+$ transformants were selected as uracil + uridine prototrophs. After purification of transformants by three rounds of streaking for single colonies, DNA was prepared and subjected to Southern blot analysis (not shown). One transformant (ΔK22) was found in which pΔKLPA2 had integrated by a single event 5′ to *klpA*. This transformant was crossed to FGSC767 (*pyrG*89; *nicA*2), and PyrG$^+$ progeny were selected for Southern analysis. One strain, ΔK13 (*klpA*1 *pyrG*89; *nicA*2), was found in which the *klpA* coding region was deleted. This null allele, which is a deletion extending from 0.5 kb 5′ to the coding region through to amino acid 726, was designated *klpA*1.

Construction of the klpA*1;* bimC*4 Double Mutant*

In matings between a *klpA*1 strain (*klpA*1 *pyrG*89; *nicA*2) and a *bimC*4 strain (*pabaA*1 *biA*1; *argB*2; *bimC*4), ~25% of the progeny show an intermediate level of growth at 42°C compared with the parent strains. This phenotype is 100% linked (n = 50) to the presence of the *klpA*1 allele which can be scored either by Southern blot analysis or by presence of the *N. crassa pyr4* marker which gives reduced growth on unsupplemented minimal media compared to Pyr$^+$ strains. That the *bimC*4 allele was also present in these strains was confirmed by backcrossing putative double mutants to both parents and a wild-type strain.

Yeast Mating Assays

Qualitative mating assay was performed as previously described (Rose et al., 1990). Transformants (plasmid bearing derivatives of MS918: *MATα, ura3-52, Leu2-3, leu2-112, ade2-101 kar3-102::LEU2*) were pregrown on SC-uracil/2% glucose or SC-uracil/2% galactose (to induce *klpA* expression). Matings to MS914 (*MATα, ura3-52, leu2-3, leu2-112, trpl-Δ1, kar3-102::LEU2*) tester were on YPD for 20 h or YPGal for 38 h at 23°C. Diploids were selected on SDmin + uracil.

Quantitative matings (see Table I) were performed on YPGal at 23°C for 8–10 h essentially as described by Rose and Fink (1987), using 10^7 cells of each parent. Each parent 1 (plasmid bearing derivatives of MS918) was pregrown to mid-logarithmic phase in SC-uracil/2% galactose to induce p*GAL1* expression plasmids. Parent 2 was pregrown to mid-logarithmic phase in YPGal. Parent 2 genotypes were: MS241: *MATα, ura3-52, leu2-3, leu2-112, trpl-Δ1, lys2-801, cyh*^{R}; MS2835: *MATα, ura3-52, leu2-3, leu2-112, trpl-Δ1, cyh*^{R}, *kar3-102::LEU2*.

Results

Identification and Cloning of klpA *Gene of* A. nidulans

Mutation of the *bimC* gene of *A. nidulans*, which encodes a KLP, results in failure of spindle pole body separation at mitosis. *bimC*-related sequences have been detected by low stringency Southern blot analysis, suggesting the existence of multiple KLPs in *A. nidulans* (Enos and Morris, 1990). Using PCR, we identified a sequence related to *bimC* in the region of the kinesin-like motor domain (see Materials and Methods). This PCR product, used to probe Northern blots of wild-type mRNA, detected a single message of ~2.8 kb

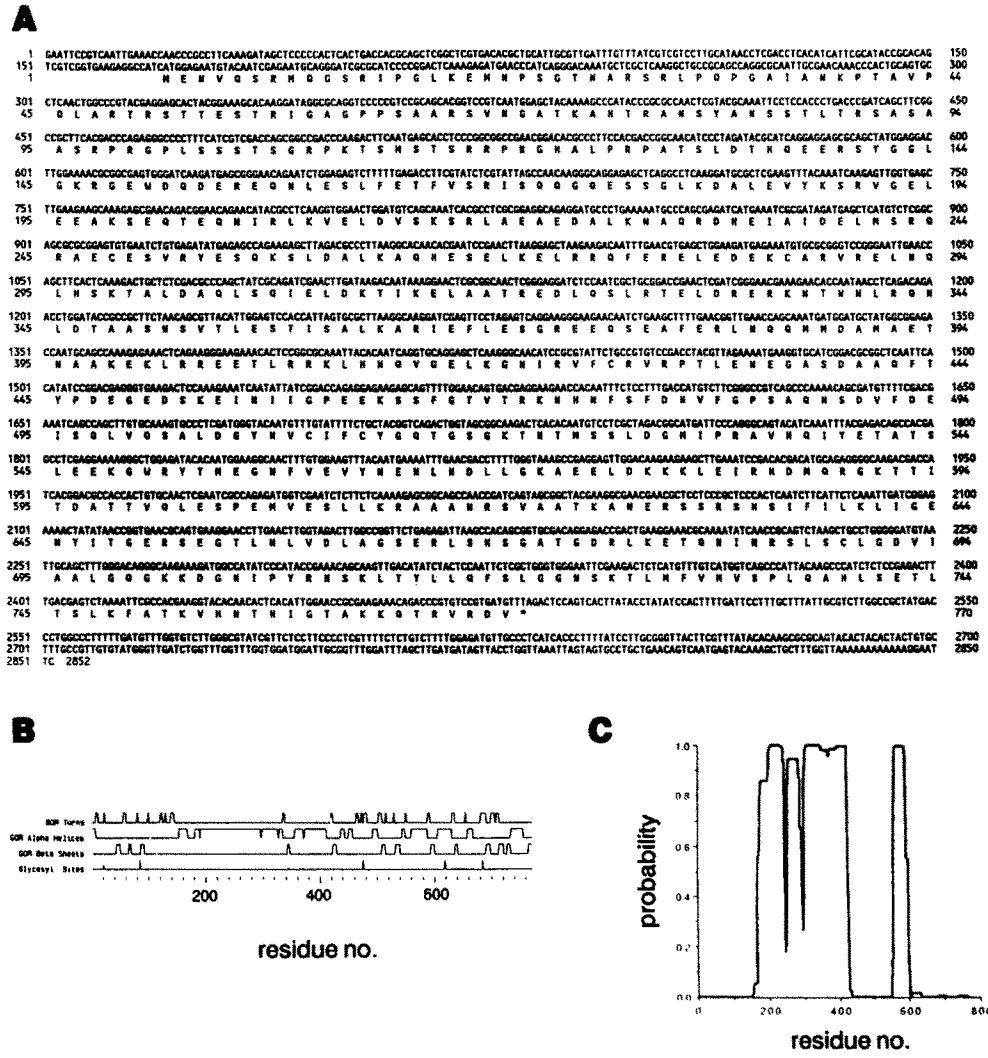

Figure 1. Structural features of the *A. nidulans klpA* cDNA and the predicted KLPA polypeptide. (*A*) Nucleotide sequence of the *klpA* cDNA and derived amino acid sequence of KLPA. (*B*) Structural features of KLPA as predicted by the method of Garnier et al. (1978) determined and graphically presented using the UWGCG programs Peptidestructure and Plotstructure (Devereux et al., 1984). (*C*) The probability of each residue of KLPA forming a coiled-coil structure was determined using the program described by Lupas et al., (1991) using a window of 28. These sequence data are available from EMBL/GenBank/DDBJ under accession number X64603.

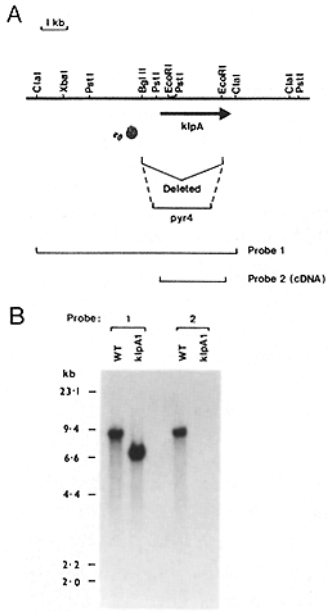

Figure 2. Construction of a null allele of *klpA*. (*A*) Restriction map of the *klpA* region. The location of the *klpA* coding region is indicated as is the region deleted and replaced by *N. crassa pyr4* (see Materials and Methods). Probes for Southern blot analysis are indicated. Probe 1 is the 8-kb ClaI fragment. Probe 2 is nucleotides 1 to 2,339 (an EcoRI fragment; see Fig. *1 A*) of the *klpA* cDNA. (*B*) Southern blot analysis of the *klpA*1 deletion. DNA was extracted from wild-type *A. nidulans* (FGSC767; *pyrG*89; *nicA*2) and a strain carrying the *klpA*1 deletion (ΔK13: *klpA*1; *nicA*2), digested with ClaI, and tandem samples electrophoresed on a 0.8% agarose gel. The gel was transferred to Zeta-Probe membrane (BioRad Laboratories) and hybridized with the probes indicated in *A*. The mobility shift of the probe 1 hybridizing band in *klpA*1 is due to the difference in size between the 3.1-kb BglII–EcoRI fragment deleted from pΔKLPA1 and 2.1-kb *pyr4* fragment (see Materials and Methods for plasmid constructions). This mobility shift in *klpA*1, and the absence of hybridization of *klpA*1 DNA with probe 2 indicates a chromosomal deletion of the 3.1-kb BglII–EcoRI fragment in this strain.

(not shown). Sequencing of a full length cDNA isolated using this PCR product as a probe identified an open reading frame that predicted an 85.8-kD protein of 770 amino acids (Fig. 1 *A*). The gene identified by this cDNA has been designated *klpA* (*k*inesin-*l*ike *p*rotein) and its protein product KLPA. Computer-generated structural predictions of the derived KLPA amino acid sequence indicated KLPA consists of globular NH_2- and COOH termini, separated by a potential α-helical, coiled-coil region (Fig. 1, *B* and *C*). As with KAR3 and NCD, the kinesin-like motor domain of KLPA is at the COOH terminus. Computer comparisons showed KLPA to have sequence similarity to KLPs between amino acids 400 and 770. In this region, KLPA is most similar to KAR3 (55.7% identical, 70.6% similar) and NCD (48.2% identical, 65.4% similar). Comparison of KLPA to *D. melanogaster* kinesin heavy chain in this region shows 40.3% identity (59.7% similarity). The potential coiled-coil region separating the NH_2 and COOH termini suggests the functional KLPA protein may be oligomeric. No significant sequence similarity between the nonkinesin-like portion of KLPA and any sequence on the GenBank database (release 71.0) has been found. The *klpA* locus was mapped to the left arm of chromosome 1 (see Materials and Methods).

Construction of a Null Allele of klpA

To investigate functional roles for KLPA in *A. nidulans*, a strain carrying a *klpA* null mutation (*klpA*1: a deletion in which sequences from 0.5 kb 5′ to the start codon to amino acid 726 are replaced by the *Neurospora crassa pyr4* gene), was constructed by transformation mediated gene replacement (see Materials and Methods; Fig. 2). This strain was examined for defects in microtubule-mediated processes. The *klpA*1 strain showed no defects in cell differentiation, vegetative growth rates, nuclear movement, or asexual spore production and viability. Mitosis, assayed by staining of nuclei, appeared to be normal both in gross morphology and frequency. Sexual development and meiosis assayed by ascospore yield and viability (Kirk and Morris, 1991) were also unaffected. Because *S. cerevisiae* strains carrying *kar3* mutations show altered sensitivity to antimicrotubule drugs and mutagens (Roof et al., 1991; P. Meluh and M. Rose, unpublished observations) we also tested *klpA*1 for these phenotypes. *klpA*1 showed wild-type sensitivity to the antimicrotubule drugs benomyl or thiabendazole and to the mutagens methanesulfonate (EMS and MMS) or ultraviolet light. Staining of *klpA*1 cells with antibodies against α-tubulin and γ-tubulin showed these cells to have wild-type microtubule and spindle pole body morphologies (not shown).

Deletion of klpA *Suppresses Mutation in* bimC

Spindle pole bodies fail to separate in strains carrying the recessive, temperature sensitive *bimC*4 mutation when incubated at 42°C, resulting in germlings with a single, enlarged nucleus possessing multiple unseparated spindle pole bodies. Therefore, it is likely that the *bimC*-encoded KLP is required for spindle pole body separation (Enos and Morris, 1990). To assess whether KLPA and BIMC interact, a *klpA*1; *bimC*4 double mutant was constructed. The double mutant exhibited significant (but not wild-type) growth at 42°C, whereas at this temperature the *bimC*4 parent failed to grow (Fig. 3). The growth of the double mutant at the restrictive temperature for *bimC*4 indicates partial suppression of *bimC*4 by loss of KLPA function. DAPI staining of the double mutant when grown at 42°C showed that, unlike *bimC*4 strains, there are multiple nuclei present in the germlings (not shown).

Overexpression of klpA *Blocks Nuclear Division*

Plasmids were constructed in which the *klpA* cDNA was placed under the control of the *alcA* promoter, together with the *argB* gene as a selectable marker for transformation (see Materials and Methods). Expression from this promoter is strongly induced in the presence of ethanol and repressed in the presence of glucose (Lockington et al., 1985). These plasmids were transformed into wild-type *A. nidulans* and strains in which a single copy of this construct was integrated at the *argB* locus on chromosome 2 as judged by Southern

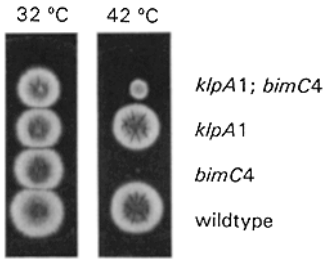

Figure 3. Suppression of *bimC*4 by *klpA*1. Strains were grown on *Aspergillus* supplemented complete medium at 32°C for 2 d, or at 42°C for 3 d. The relevant genotypes of strains are shown. Full genotypes are: *klpA*1; *argB*2; *nicA*2; *bimC*4 (*klpA*1; *bimC*4), *klpA*1 *pabaA*1; *argB*2 (*klpA*1), *argB*2; *nicA*1; *bimC*4 (*bimC*4), *pabaA*1; *argB*2 (wild-type, SJOO8 provided by Dr. S. James).

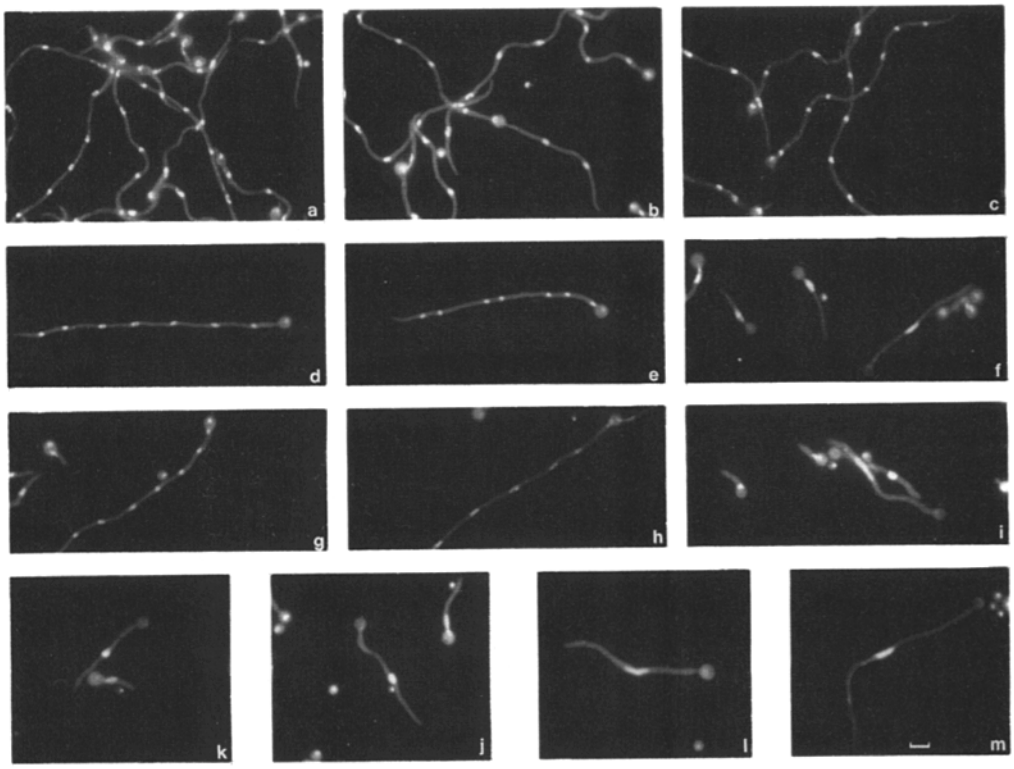

Figure 4. Overexpression of *klpA* blocks nuclear division in *A. nidulans*. All strains are derivatives of SJOO8 (*pabaA1*; *argB2*, provided by Dr. S. James) carrying single plasmid integrations at *argB*. Transformed plasmids are: (*a*, *d*, and *g*) pKK12 (vector only); (*b*, *e*, and *h*) pKK12-27 (reverse orientation of *klpA* coding region on the *alcA* promoter); and *c*, *f*, *i–l*, and *m*) pKK12-29 (forward orientation of *klpA* on the *alcA* promoter). See Materials and Methods for details of plasmid constructions. Media contained 2% glucose (*a–c*) or 2% ethanol(*d–m*) as the added carbon source. Strains were grown in petri dishes containing liquid medium with cover slips at the bottom of the plate for 12 h at either 25°C for glucose grown cultures, or at 37°C for ethanol grown cultures. Cover slips were removed and the cells were fixed in glutaraldehyde and stained with DAPI. Bar, 10 μm.

blot analysis (not shown) were chosen for further analysis. These strains failed to grow significantly on ethanol medium, but showed wild-type growth when glucose was the added carbon source. Control strains transformed with either vector alone or with the *klpA* cDNA in the reverse orientation grew normally under identical inducing conditions (not shown). Staining of nuclei in the *klpA*-overexpressing strains revealed that they were arrested with a single nucleus, which showed enhanced DAPI staining and was frequently enlarged compared to controls (Fig. 4). Such enlarged nuclei

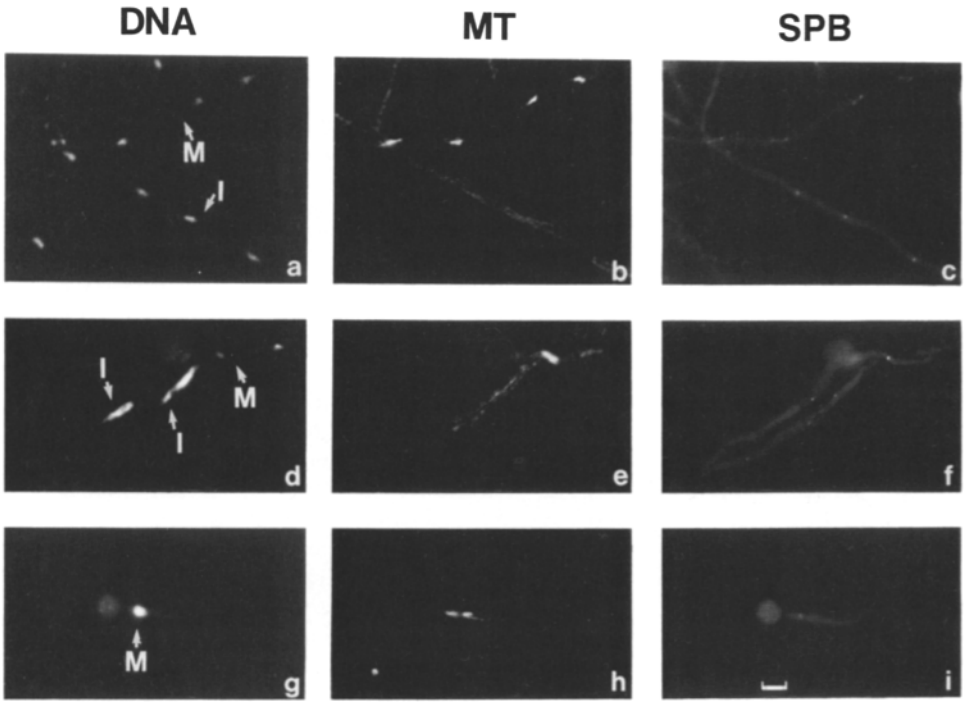

Figure 5. Microtubule and spindle pole body morphology in *klpA*-overexpressing strains. Panels depict DAPI stained nuclei (*DNA*), anti α-tubulin stained microtubules (*MT*) and anti γ-tubulin stained spindle pole bodies (*SPB*). Cells in mitosis (*M*) or interphase (*I*) are indicated in the DNA panel. (*a–c*) pKK12 transformant (vector only.) (*d–i*) pKK12-29 transformant (*palcA::klpA*). Cells were grown in ethanol medium for 18 h and stained as described in Materials and Methods. Mitotic cells overexpressing *klpA* show condensed chromatin and an aberrant monopolar spindle. Bar, 10 μm.

are also seen in mutants of *A. nidulans* (*benA*33, *bimC*4, *mipA*d1 and *bimB*3) and also in benomyl treated wild-type cells, which are defective for chromosome segregation and exhibit DNA replication in the absence of nuclear division (Oakley and Morris, 1981; Enos and Morris, 1990; Oakley et al., 1990; May et al., 1992). Thus it is likely that the nuclei in the *klpA* overexpressing strains also have an increased DNA content. The *klpA*-overexpressing cells had an approximately wild-type mitotic index of 6% ($n = 205$; vector only transformant: 4%, $n = 218$), as judged by chromatin condensation and microtubule morphology (Fig. 5). However, the spindles in the *klpA*-overexpressing strains were enlarged and associated with only a single focus of γ-tubulin staining (Fig. 5, *d–i*). Vector only transformed control cells showed wild-type spindle morphology with γ-tubulin staining at both ends (Fig. 5, *a–c*). It is not clear from this resolution whether the γ-tubulin staining pattern is due to single spindle pole body or multiple unseparated spindle poles. However, as mitotic figures are not accumulated, it is likely that these cells enter mitosis and exit without segregating their chromosomes. Although it is possible that *klpA*-overexpression could nonspecifically affect microtubule mediated processes, this would be confined to the nuclear compartment as nuclear movement, which is dependent on cytoplasmic microtubules (Oakley and Morris, 1980) is unaffected.

Complementation of the S. cerevisiae kar3Δ *Karyogamy Defect by* klpA

KLPA is structurally related to KAR3 and NCD, but is most similar in sequence to KAR3 (56% identical, 71% similar) in the putative motor domain. Computer comparisons showed that there is no significant region of amino acid sequence similarity between KLPA and KAR3 outside this domain. Although the "one motor, many tails" hypothesis suggests that function is specified by the tail regions, in light of the high degree of relatedness between the motor domains of KLPA and KAR3, we wished to test if they were functionally homologous. In *S. cerevisiae*, KAR3 is essential for karyogamy; in matings between two *kar3* null strains, diploid formation is extremely rare. Both the NH_2 and COOH termini of KAR3 have been localized by immunocytochemistry to microtubles in α-factor treated cells (Meluh and Rose, 1990). Taking these observations together with the likely motility function for KAR3 and the phenotypes of *kar3* mutants, it has been proposed that in zygotes KAR3 cross-bridges antiparallel cytoplasmic microtubules and then provides the motive force to draw the nuclei together (Meluh and Rose, 1990). The *klpA* cDNA was expressed from the *GAL1* promoter in *S. cerevisiae* strains carrying the *kar3-102::LEU2* deletion (Meluh and Rose, 1990). These strains were then mated to *kar3-102::LEU2* strains of opposite mating type to determine mating proficiency. Qualitative mating assays showed *klpA* to complement the *kar3* defect in a galactose- and orientation-dependent manner (Fig. 6). However, the efficiency of diploid formation in these matings was below that of both wild-type *KAR3* and p*GAL1::KAR3* expressing strains. Quantitative mating assays showed that p*GAL1::klpA* dependent diploid formation in strains carrying pMR2388 (2 μm based) occurred at a frequency ~10% of that directed by p*GAL1::KAR3*. This is however at least three orders of magnitude higher than that observed for matings

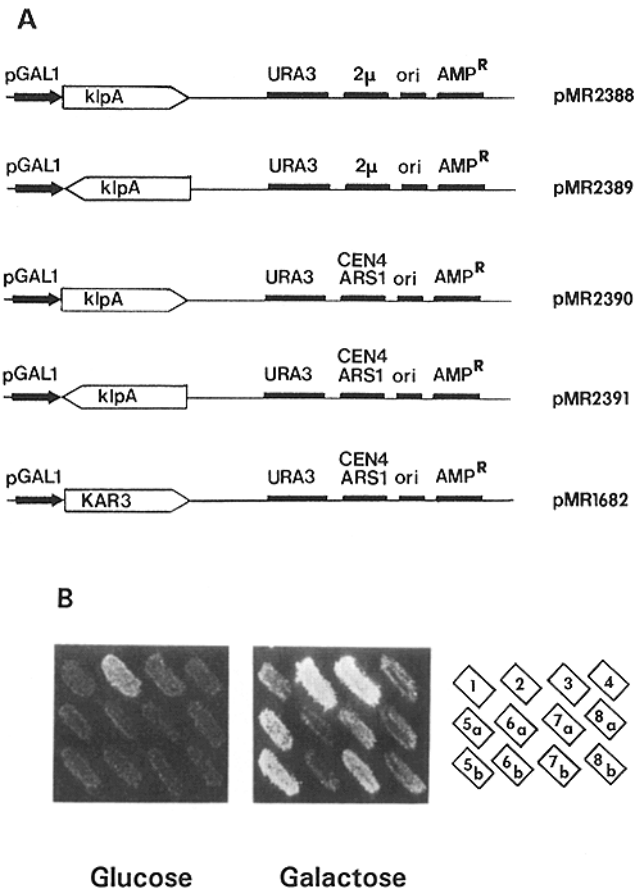

Figure 6. Complementation of the *kar3-102::LEU2* karyogamy defect by the *A. nidulans klpA* gene. (*A*) Expression plasmids constructed for this study. *klpA* constructs contain nucleotides 140 to 2852 (Fig. 1 *A*). pMR1682 contains residues −71 to +2751 of the published *KAR3* sequence (Meluh and Rose, 1990). Details of plasmid constructions are given in Materials and Methods. (*B*) Qualitative assay formating between various *MATa kar3-102::LEU2* transformants and a *MATα kar3-102::LEU2* tester strain on glucose and galactose medium. Transformants contain: (*1* and *4*) pMR79 (Rose and Fink, 1987; vector alone); (*2*) pMR820 (Meluh and Rose, 1990; *KAR3*); (*3*) pMR1682; (*5*) pMR2388; (*6*) pMR2389; (*7*) pMR2390; and (*8*) pMR2391. Two isolates (*a*, *b*) containing each *klpA* construct are shown. On galactose medium, *klpA* expression supported diploid formation in an orientation dependent manner.

between two *kar3-102::LEU2* null strains (Table I). The higher frequency of diploid formation in strains carrying pMR2388 (2 μm based) over those with pMR2390 (centromere based) is presumably a result of higher *klpA* expression levels.

Microscopic investigation of p*GAL1::klpA* zygotes was used to confirm and extend the quantitative mating assays by determining the percentage of cells that had undergone karyogamy as judged by nuclear and microtubule morphology (Fig. 7; Table I). The frequency of Kar$^+$ zygotes (12 versus 99% for *KAR3* and <0.03% for *kar3-102::LEU2*) was comparable with the relative frequency of diploid colony formation (9.5%). Furthermore, in some early zygotes, uninterrupted microtubule staining between two separate nuclei was observed (Fig. 7, *b* and *e*). Such a staining pattern, which is common in wild-type matings, is never observed in matings between two *kar3-102::LEU2* strains (Meluh and Rose, 1990).

Table I. Quatitation of Karyogamy Proficiency

Cross	Parent 1	Relevant genotype	Parent 2	Relevant genotype	% Diploid formation*	Relative % diploid formation§	KAR+ zygotes‖
1	MS918 [pMR1682]	*kar3-102::LEU2*, CENp*GAL1::KAR3*	MS2835	*kar3-102::LEU2*	34	100	121/146 (83%)
2	MS918 [pMR2388]	*kar3-102::LEU2*, 2μp*GAL1::klpA*	MS2835	*kar3-102::LEU2*	3.22	9.5	35/297 (12%)
3	MS918 [pMR2390]	*kar3-102::LEU*, CENp*GAL1::klpA*	MS2835	*kar3-102::LEU2*	1.03	3.0	17/312 (5.4%)
4	MS918 [pMR79]	*kar3-102::LEU2*, CEN vector only	MS2835	*kar3-102::LEU2*	0.014‡	0.041	0/313 (<0.3%)
5	MS918 [pMR820]	*kar3-102::LEU2*, CEN*KAR3*	MS2835	*kar3-102::LEU2*	32.4	95	140/159 (88%)
6	MS918 [pMR79]	*kar3-102::LEU2*, CEN vector only	MS241	*KAR3*	40.5	119	84/85 (99%)

In crosses between various transformants and either a wild-type or *kar3-102::LEU2* tester, karyogamy was quantitatively measured by the frequency of diploid formation. In a separate experiment, zygotes were examined microscopically for karyogamy. Quantitative mating assays are described in Materials and Methods. Microscopic examination of cells is described in the legend to Fig. 7.

* Percent diploid formation was calculated as a percentage of viable cells that formed colonies on SDmin + uracil (selective for diploids). Data represent the average of three experiments.

‡ This value is likely to be an overestimate as a large proportion of such selected colonies from other matings have been shown not to be bona fide diploids (P. Meluh, unpublished observations).

§ Relative percent diploid formation was calculated against the percent diploid formation from cross 1.

‖ Kar+ zygotes were quantified microscopically based on nuclear and microtubule morphology (see Fig. 7). Data presented as a ratio of Kar+/total zygotes, with percentages in parentheses.

Discussion

In this paper we present the identification by PCR and preliminary functional analysis of a new *A. nidulans* KLP protein, KLPA. KLPA is most closely related to KAR3 of *S. cerevisiae* and NCD of *Drosophila* by virtue of its COOH terminal motor domain. However, as with sequence comparisons between most KLPs identified thus far, there is no significant amino acid sequence similarity outside the kinesin-like motor domains of these proteins.

An *A. nidulans* strain was constructed in which the *klpA* gene was deleted. This allele (*klpA*1) exerted no discernable mutant phenotype. Although *klpA* was identified on the basis of homology rather than function, Northern blot analysis showed that the gene is expressed. Thus, the function of KLPA either is cryptic or it is redundant with other as yet unidentified proteins. Precedent for redundant KLP function has been seen in *S. cerevisiae*, where the *KIP1* and *CIN8* encoded KLPs form a redundant pair, either one of which is sufficient for the assembly of the mitotic spindle (Roof et al., 1992; Hoyt et al., 1992).

There is much evidence to support the idea that multiple motor proteins, including KLPs, function in the mitotic spindle (for reviews see Sawin and Scholey, 1991; McIntosh and Pharr, 1991). Immunological studies using polyclonal antibodies made against peptides conserved between KLPs have identified multiple reactive proteins in the spindle (Sawin et al., 1992). Furthermore, a minus end-directed motor, cytoplasmic dynein, has been immunolocalized to the spindle and kinetochores (Pfarr et al., 1990; Steuer et al., 1990) and both plus and minus end-directed motor activities at the kinetochore have been detected by in vitro motility assays (Hyman and Mitchison, 1991). The *bimC*4 mutation in *A. nidulans* prevents the separation of the spindle pole bodies (Enos and Morris, 1990). Similar phenotypes were seen in *cut7* mutants of *Schizosaccharomyces pombe* (Hagan and Yanagida, 1990) and in the *kip1, cin8* double mutants of *S. cerevisiae* (Roof et al., 1992; Hoyt et al., 1992). The CUT7, KIP1, and CIN8 proteins have been localized to the spindle by immunocytochemistry (Haigan and Yanagida, 1992; Roof et al., 1992; Hoyt et al., 1992). BIMC, CUT7, KIP1, and CIN8 are structurally similar to each other, and to a *Xenopus* KLP Eg5 (Le Guellec et al., 1991), but lack significant sequence similarity outside the kinesin-like motor domain. Although these proteins are structurally similar and may be functionally homologous, there have been no reports of cross-species tests to confirm this. Mutants defective in the NCD and NOD proteins of *Drosophila* also show spindle defects (Hatsumi and Endow, 1992; Theurkauf and Hawley, 1992). *S. cerevisiae* strains carrying null *kar3* mutations show a mitotic phenotype with slow colony growth and production of inviable cells blocked in the G2/M phase of the cell cycle, which is consistent with a defect in spindle elongation (Meluh and Rose, 1990). In addition, deletion of *KAR3* is synthetically lethal with *bub* (Hoyt et al., 1991) and *mad* (Li and Murray, 1991) spindle checkpoint mutations (Roof et al., 1991). As there are multiple motors of both polarities acting at the spindle, correct spindle function would appear to require that they be regulated in terms of magnitude, duration and polarity of the force they produce.

The genetic suppression of the *bimC*4 allele by *klpA*1 provides evidence for a functional role for KLPA in the mitotic spindle. Since there is ample evidence that multiple motors act within the spindle, and as the *bimC*4 mutation results in a block in spindle pole body separation, one obvious model to explain the observed phenotype of the double mutant would be that BIMC and KLPA are motors which provide opposing forces on the mitotic spindle. Under this model, inactivation of BIMC by the *bimC*4 mutation could disturb the balance of forces in the spindle and prevent its assembly. Removal of KLPA by the *klpA*1 deletion allele in the double mutant would then partially redress this imbalance and hence allow spindle formation to proceed. Interestingly, Saunders and Hoyt (1992) have observed a potentially analo-

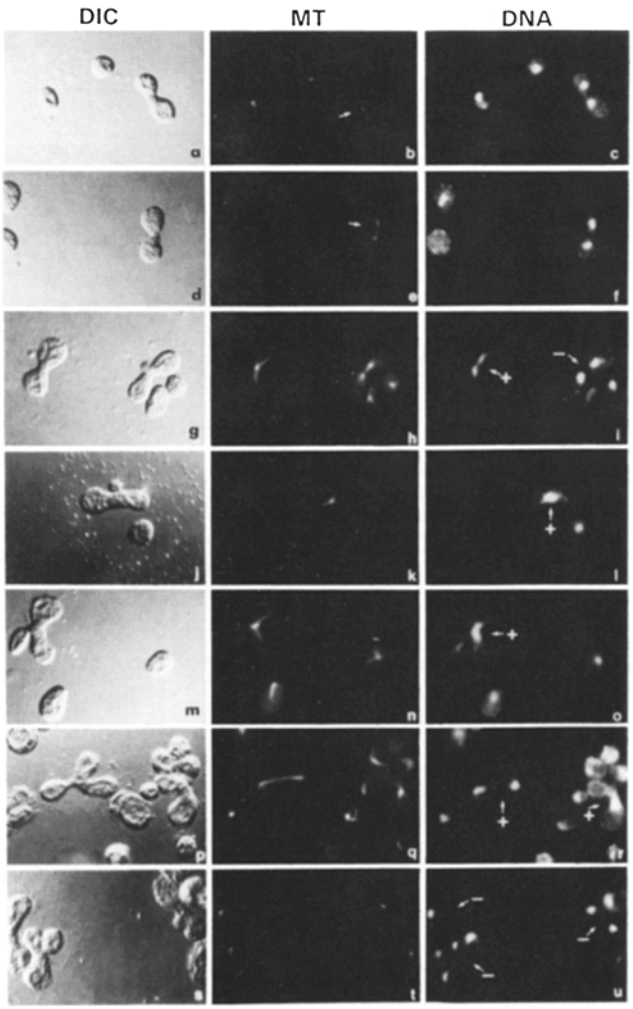

Figure 7. Microscopic analysis of *kar3-102::LEU2* zygotes expressing *klpA* (pMR2388). Panels depict whole cells (*DIC*), microtubules (*MT*) stained with the anti α-tubulin mAb YOL1/34 (Sera-Lab) and nuclei (*DNA*) stained with DAPI. Arrows in *MT* column (*b* and *e*) indicate early zygotes with cross-bridged microtubules. Arrows in the DNA panel indicate zygotes at various stages which have (+) or have not (−) undergone karyogamy. Kar^+ zygotes are distinguished by a single nucleus and a single focus of MT staining (e.g., *k*, *l*, *n*, and *o*). Cells were prepared as described for the quantitative mating assay (Materials and Methods). Staining techniques were as described by Rose et al., (1990). Quantitation of these events is presented in Table I.

gous genetic interaction in *S. cerevisiae* between *KAR3* and the redundant gene pair *KIP1* and *CIN8*. Specifically, loss of KAR3 function was found to partially suppress the temperature sensitive phenotype of a *cin8-3* (ts) *kip1*Δ strain. As the *klpA* deletion by itself has no phenotype, we have proposed that in *A. nidulans* there is at least one other protein possessing a function redundant with that of KLPA. This may also explain why *klpA*1 only partially suppresses the growth inhibition caused by the *bimC*4 mutation. If KLPA function is essential but not required due to functional redundancy, then inactivation of redundant functions in a *klpA*1 background would be lethal. However, conditional inactivation of a second KLPA-like function in a *klpA*1; *bimC*4 background may lead to complete suppression of *bimC*4 by balancing forces on the spindle and hence result in a viable strain. We are currently looking for this function.

An alternative possibility is that lack of KLPA leads to the induction of other motor activities that compensate for loss of BIMC function. However, consistent with the model of balancing forces is the observed block in nuclear division and correct mitotic spindle assembly seen in *A. nidulans* when *klpA* is overexpressed. We can not rule out pleiotropic effects through altered regulation, mislocalization, or titration of interacting proteins of such overexpression of *klpA* interfering with a range of microtubule-based processes, although the effects of overproduction of KLPA appear to be confined to the nucleus as nuclear movement on the cytoplasmic microtubules (Oakley and Morris, 1980) is unaffected. Once we are able to generate mutations in a gene(s) whose product is functionally redundant with KLPA, it will be possible to test directly whether KLPA plays important roles in spindle function, or for that matter, any other cellular processes.

In *S. cerevisiae* matings, KAR3 is essential for karyogamy. Analysis of KAR3 function during mating suggests it acts to cross-bridge the antiparallel microtubules emanating from the parental nuclei of the early zygote, and then provides the motive force to draw them together (Meluh and Rose, 1990). The karyogamy defect in matings between two null mutants was complemented by *klpA* when expressed from the *GAL1* promoter: diploids were produced at ~10% the level seen in wild-type matings. This was at least three orders of magnitude higher than matings between two *kar3-102::LEU2* controls. Microscopic analysis detected uninterrupted microtubule staining between the nuclei in early zygotes expressing *klpA*, which is a phenomenon not seen in matings between two *kar3* null parents (Meluh and Rose, 1990).

Does complementation of *kar3-102::LEU2* by p*GAL1::klpA* imply functional identity between KLPA and KAR3? The microtubule staining seen in early zygotes indicates that, at a minimum, the *klpA* complementation is likely to involve microtubule cross-bridging, one of the proposed karyogamy functions for KAR3. However, the successful completion of karyogamy in *kar3-102::LEU2* strains expressing *klpA* suggests that KLPA also provides motor activity required to pull the nuclei together before fusion. The lower frequency of KLPA dependent diploid formation compared to that directed by KAR3 is not surprising in light of the phylogenetic distance between these two species. The partial complementation by KLPA may be due to inefficient interactions between KLPA and *S. cerevisiae* proteins involved in the subcellular targeting and/or function of KAR3. We can not however rule out the possibility that KLPA might perform only a subset of KAR3 functions, for example in microtubule cross-bridging, sufficient to facilitate karyogamy.

Would any KLP complement loss of KAR3 function? Two observations lead us to believe this complementation is specific to *klpA*. First, several other KLPs have been identified in *S. cerevisiae* (Roof et al., 1991; 1992; Hoyt et al., 1992; Lillie and Brown, 1992) and, despite an extensive search, none of these or any new KLPs were found as high-copy suppressors of the karyogamy defect associated with *kar3* mutations. Second, hybrid molecules consisting of the KAR3 tail domain and the *D. melanogaster* kinesin motor domain do not complement mutations in *KAR3* (P. Meluh and M. Rose, unpublished observations).

The finding that the *A. nidulans klpA* gene can substitute for the *S. cerevisiae KAR3* gene in karyogamy represents the first demonstration of conserved KLP function between spe-

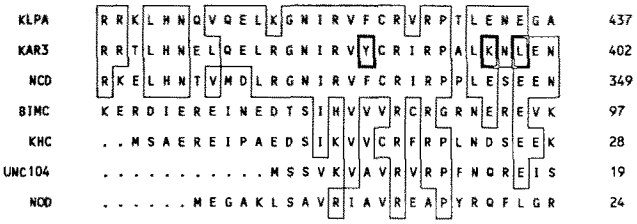

Figure 8. A sequence motif conserved between KLPA, KAR3, and NCD. Numbers to the right refer to the final amino acid shown. Sources of sequence data: *S. cerevisiae* KAR3 (Meluh and Rose, 1990); *D. melanogaster* NCD (McDonald and Goldstein, 1990; Endow et al., 1990); *A. nidulans* BIMC (Enos and Morris, 1990); *D. melanogaster* kinesin heavy chain (KHC, Yang et al., 1989); *Caenorabditis elegans* UNC104 (Otsuka et al., 1991); and *D. melanogaster* NOD (Zhang et al., 1990).

cies. This result also implies that primary sequence conservation between the tails of KLPs is not necessary for conserved function. If the tails are the determinant of conserved functional specificity, as suggested by the "one motor, many tails" hypothesis (Vale and Goldstein, 1990), then this must depend upon either tertiary structure or relatively small and extremely subtle motifs in the tails rather than upon extensive primary amino acid sequence similarity.

The kinesin-like motor itself, either through tertiary structure or primary amino acid sequence, may be important for determining functional specificity. In support of this, the KLPA motor domain is most closely related in sequence to that of KAR3, and we have demonstrated conserved function between these proteins. The KLPA, KAR3, and NCD subclass of KLPs possess a small motif at the NH_2 terminus of their motor domains which is absent from the other KLPs that may have functional significance (Fig. 8; Roof et al., 1991). NCD is known to generate minus-end directed motility in vitro (McDonald et al., 1990; Walker et al., 1990), and in vitro experiments performed with *Drosophila* kinesin heavy chain indicate both the motive force and its direction are properties of the motor domain itself (Yang et al., 1990; Stewart, R. J., and L. S. B. Goldstein, unpublished results, cited in Goldstein, 1991). It is not yet known whether KLPA and KAR3 also possess minus-end directed motor activity or if NCD is functionally homologous with KLPA and KAR3. Further, the BIMC, CUT7, KIP1, CIN8, and Eg5 KLPs also form a subclass that are separated from other KLPs by motor domain sequence and potentially by function (Le Guellec et al., 1991; Roof et al., 1991, 1992; Hoyt et al., 1992). Finally, although yet to be described, interacting proteins analogous to the kinesin light chains may be an important determinant of functional specificity.

Although it is not yet clear how many KLPs function in a cell, it is likely that multiple KLPs are involved in different aspects of single cellular processes, for example, assembly of the mitotic spindle. The relative contributions of the motor, tail and accessory proteins to the functional specificity of individual KLPs remains to be determined. However, our results have shown that conservation of the primary amino acid sequence of the tails is not required for two KLPs to be able to carry out the same function in vivo. With the description of multiple KLPs in organisms amenable to genetic analysis, we anticipate that further progress will be made to describe the determinants of KLP functional specificity.

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