A New Kinesin-like Protein (Klp1) Localized to a Single Microtubule of the *Chlamydomonas* Flagellum

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Abstract. The kinesin superfamily of mechanochemical proteins has been implicated in a wide variety of cellular processes. We have begun studies of kinesins in the unicellular biflagellate alga, *Chlamydomonas reinhardtii*. A full-length cDNA, *KLP1*, has been cloned and sequenced, and found to encode a new member of the kinesin superfamily. An antibody was raised against the nonconserved tail region of the Klp1 protein, and it was used to probe for Klp1 in extracts of isolated flagella and in situ. Immunofluorescence of whole cells indicated that Klp1 was present in both the flagella and cell bodies. In wild-type flagella, Klp1 was bound tightly to the axoneme; immunogold labeling of wild-type axonemal whole mounts showed that Klp1 was restricted to one of the two central pair microtubules at the core of the axoneme. Klp1 was absent from the flagella of mutants lacking the central pair microtubules, but was present in mutant flagella from *pf16* cells, which contain an unstable C1 microtubule, indicating that Klp1 was bound to the C2 central pair microtubule. Localization of Klp1 to the C2 microtubule was confirmed by immunogold labeling of negatively stained and thin-sectioned axonemes. These findings suggest that Klp1 may play a role in rotation or twisting of the central pair microtubules.

THE kinesin superfamily of proteins has been implicated in a wide variety of microtubule-dependent motility processes, each of which requires a motor protein to provide the enzymatic link that converts the chemical energy of nucleotide triphosphates into mechanical force (for reviews, see Bloom, 1992; Skoufias and Scholey, 1993; Walker and Sheetz, 1993). Kinesin, the prototypical founding member of this family, is a microtubule plus-end–directed motor that was first isolated from squid axons (Brady, 1985; Vale et al., 1985), and has since been implicated in the movement of vesicles (Bloom et al., 1988; Vale et al., 1985), pigment granules (Rodionov et al., 1991), and cytoplasmic organelles (Dabora and Sheetz, 1988; Leopold et al., 1992; Schroer et al., 1988; Vale and Hotani, 1988). The NH_2-terminal motor domain of the kinesin heavy chain (KHC)[1] binds microtubules and generates the force required for motility (Hirokawa et al., 1989; Yang et al., 1989), while the KHC tail domain binds membranes tightly (Skoufias et al., 1994), tethering vesicles and organelles to microtubules. The tail domain of the KHC is also the region that allows kinesin to form an oligomeric protein (Hirokawa et al., 1989; Yang et al., 1989): KHC tails form homodimers, and each KHC tail binds a kinesin light chain molecule, yielding a tetrameric kinesin molecule composed of two identical heavy chains and two identical light chains (Bloom et al., 1988; Kuznetsov et al., 1988).

Since the initial molecular characterization of the *Drosophila* KHC (Yang et al., 1989), molecular techniques have greatly expanded the kinesin superfamily to include >30 members. Proteins of the kinesin superfamily are defined by a conserved motor domain that typically is 40% identical to the motor domain of the KHC (Endow and Hatsumi, 1991; Stewart et al., 1991). Though the motor domains of most kinesin-like proteins (klps) are found in the NH_2-terminal region, examples of COOH-terminal motor domains and internal motor domains have also been described (reviewed in Bloom, 1992; Goldstein, 1993*a*). Most klps are thought to drive microtubule plus-end–directed movement, but klps that are microtubule minus-end–directed motors have also been described (McDonald et al., 1990; Meluh and Rose, 1990; Walter et al., 1990). It should be noted, however, that the directionality of transport for most klps has been inferred only from the position and sequence of the motor domain; for most klps, motor activities have yet to be documented. Functional specificity is conferred to each class of klps by a tail region that is unique to members of that class (Goldstein, 1991; Goldstein, 1993*a*; Yang et al., 1989). Klps are ubiquitously distributed among organisms and cell types, and a given organism may contain genes for ≥30 different klps (Endow and Hatsumi, 1991; Stewart et al., 1991). Klps

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1. *Abbreviations used in this paper*: HMDEK, 10 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA, and 25 mM KCl; KHC, kinesin heavy chain; klps, kinesin-like proteins; KRP, kinesin-related protein; RACE, rapid amplification of cDNA ends.

have been shown to be required for meiosis and mitosis (Nislow et al., 1990, 1992; Sawin et al., 1992*a*; Walker et al., 1990; Wright et al., 1993; Yen et al., 1991, 1992; Zhang et al., 1990), as well as spindle pole body and nuclear migration in fungi (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt et al., 1992; Meluh and Rose, 1990; Roof et al., 1992; Saunders and Hoyt, 1992). In addition to microtubule-dependent motility, demonstrated in vitro activities for klps include microtubule sliding (Nislow et al., 1992), rotating (Walker et al., 1990), and bundling (Chandra et al., 1993; McDonald et al., 1990; Nislow et al., 1992). However, because most klps have been isolated and characterized solely by their homology to the head domain of kinesin, the function of most klps is unknown.

In a complementary approach to biochemical and genetic studies, antipeptide antibodies directed against highly conserved regions of the kinesin motor domain have been used to identify KHC and kinesin-related proteins (KRPs) at the protein level (Cole et al., 1992; Sawin et al., 1992*b*) and to purify a trimeric complex containing two different KRPs (Cole et al., 1993). Recently, these antibodies have been used to detect the presence of KRPs in *Chlamydomonas* flagella (Fox et al., 1994; Johnson et al., 1994; Kozminski, K., and J. Rosenbaum, unpublished observation). We have begun studies to isolate and characterize these motors to learn about their function(s) in eukaryotic flagella. Using PCR, we have isolated the gene for a klp that is present in the flagella of *Chlamydomonas*, and we have characterized its gene product, the first non-dynein force generating protein isolated from the eukaryotic flagellum. Biochemical fractionation of isolated flagella and immunolocalization using an antibody specific for this klp indicate that it is part of the central pair microtubule complex. We discuss possible roles of this protein in relation to central pair rotation and flagellar beating.

Materials and Methods

Strains and Growth Conditions

Wild-type *Chlamydomonas reinhardtii* strain *21gr* (mt^+), cell wall-less strain *cw15* (mt^+, CC-400), radial spokeless mutant *pf14* (mt^+, CC1032), central pairless mutant *pf15* (mt^+, CC1033), and the C1-defective mutant *pf16* (mt^+, CC1034) were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). Cultures were grown in R/2 medium (Kindle et al., 1989) on a 14:10 h light/dark cycle.

Molecular Cloning Procedures and Sequence Analysis

DNA clones were isolated and manipulated using standard procedures (Sambrook et al., 1989). All cloning was done into pBluescript plasmids (Stratagene, San Diego, CA). Random sequencing of the partial *KLP1* cDNA (Bankier and Barrell, 1983) was performed by the dideoxy method using Sequenase T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH). All sequences were obtained from both DNA strands. Sequence assembly and analyses were done with the GCG sequence analysis software package available from the University of Wisconsin (Madison, WI). Databases were searched using the BLAST program (Altschul et al., 1990). PCR reactions were performed in a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT).

RT-PCR Cloning

First-strand synthesis for reverse transcription PCR was performed using the cDNA Cycle Kit (Invitrogen, San Diego, CA). Poly(A) mRNA (1 μg) isolated from cells 40 min into flagellar regeneration was denatured with methylmercury as described by the supplier, and first-strand synthesis was primed with random primers. PCR primers against conserved regions of the kinesin motor domain have been described (Endow and Hatsumi, 1991), and were modified to contain restriction enzyme sites at the 5′ ends and to reflect the codon usage bias of *Chlamydomonas* (obtained via Gopher server from the *Chlamydomonas* Genetics Center, Duke University Botany Department). Primers consisted of the following oligonucleotides: primer 1 (sense primer for amino acids (I/M/L)(N/T/S)LVDLAG), atgaattc(ac)t(cg)a(acg)c(ca)t(cg)gt(cg)ga(tc)ct(cg)gc(cgt)gg; primer 2 contained a 1:1 mixture of primers 2a and 2b (antisense for amino acids PYR(D/E/K/N)SKLT), 2a: ataagctt(cg)gtcagctt(acg)ga(acg)t(ct)(acg)cggta(cg)gg 2b: ataagctt(cg)gtcagcttgct(acg)t(ct)(acg)cggta(cg)gg; primer 3 (sense primer for amino acids I(F/L)ANGQT): atgaattcat(ct)(ct)t(cg)gc(cgt)tacgg(ct)cagac. PCR reactions were performed in a volume of 50 μl using Taq or Amplitaq polymerase. Reactions contained 1/50 of the random primed cDNA pool and degenerate primers at 20 μg/ml. Reactions containing reactants (minus Taq polymerase) were heated to 97°C for 10 min and quenched on ice. Addition of Taq was followed by four cycles of 95°C, 47°C, and 72°C, and 27 cycles of 95°C, 62°C, and 72°C (all incubations were 1 min). PCR products were run on low temperature gelling agarose gels and bands of the predicted length (200 bp for primer pair 1 and 2 and 600 bp for primer pair 1 and 3) were excised. A second round of amplification was performed using aliquots of isolated fragments as templates. Conditions for the second round of PCR were 30 cycles of 95°C, 62°C, and 72°C, 1 min each. Products from the second round of amplification were gel purified and subcloned directly into the SmaI or EcoRV site of pBluescript plasmids, and individual clones were isolated and sequenced. PCR products that encoded five different kinesin-like motor domain sequences were obtained (including *KLP1*). Analysis of the non-*KLP1* clones will be described at a later date. The cDNA library prepared from *Chlamydomonas* during flagellar regeneration was kindly provided by K. Wilkerson and G. Witman (The Worcester Foundation for Experimental Biology, Worcester, MA).

5′ RACE

Rapid amplification of cDNA ends (RACE) was performed with the 5′ RACE System from Gibco BRL (Gaithersburg, MD). First-strand synthesis contained poly(A) RNA (0.75 μg) isolated from cells 40 min into flagellar regeneration and 20 pg of the *KLP1*-specific primer gaagcttcgcggacgtatg tattgctg. All PCR reactions for 5′ RACE contained the anchor primer supplied by the manufacturer and a *KLP1*-specific primer with the sequence ggaattctttgaatcctccagcactgc at concentrations of 0.4 μM. First-round PCR conditions were four cycles of 95°C, 50°C, and 72°C (1-min incubations) and 27 cycles of 95°C, 62°C, and 72°C (1-min incubations), followed by 10 min at 72°C. The major PCR product of ~400 bp was gel purified and used in a second round of PCR (95°C, 62°C, and 72°C, 1 min each for 30 cycles; 10 min at 72°C). PCR products were digested with SalI and EcoRI, gel purified, and inserted into pBluescript II KS(+). Single PCR clones were isolated from bacteria and sequenced.

RNA Analysis

RNA was isolated from *Chlamydomonas* strain *21gr*. For the RNA time course during flagellar regeneration, cells were concentrated to 1–2 × 10^7 cells/ml and deflagellated for 2 min with a mixer on setting 7 (Virtis Co., Inc., Gardiner, NY). At time points during regeneration, aliquots of cells were pelleted, frozen in liquid nitrogen, and stored at −70°C until RNA isolation. For large amounts of mRNA from cells regenerating flagella, 10 μg/ml cycloheximide was added to cultures 15 min before deflagellation to stabilize induced mRNAs (Baker et al., 1986). Flagellar regeneration was allowed to proceed for 40 min, at which time cells were pelleted for RNA isolation. RNA was isolated by resuspending cell pellets in a 1:1 mixture of buffer (0.2 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.01 M EDTA, and 1% SDS) to organic (phenol/chloroform/isoamyl alcohol. 25:24:1), followed by disruption for 1 min with the tissue grinder attachment of a homogenizer (OmniMixer; Sorvall-Du Pont, Newtown, CT) and separation of phases by centrifugation at room temperature. The aqueous phase was extracted repeatedly with organic solvent until no further material accumulated at the phase boundary during centrifugation. Poly(A) mRNA was isolated using oligo dT cellulose as described (Sambrook et al., 1989). For Northern analysis, poly(A) RNA (10 μg/lane) was fractionated on 1.0% agarose gels containing formaldehyde as described (Sambrook et al., 1989). After electrophoresis, RNA was transferred to nylon membranes (Magna NT; MSI, Westboro, MA), irradiated 35 s with UV light (Stratalinker; Stratagene, La Jolla, CA), and hybridized with probes generated by the random priming

method. Relative levels of *KLP1* and β-tubulin mRNA expression were quantitated by counting hybridization of ^{32}P-labeled probes with a Betascope 603 analyzer (Betagen Corp., Waltham, MA) and normalizing to the constitutive mRNA pcf2-40 (Youngblom et al., 1984).

Antibody Production

To produce antibody against the nonconserved portion of Klp1, the 1,500-bp FspI fragment of the *KLP1* cDNA, encoding amino acids 334–776, was inserted into the EcoRV site of pBluescript II KS(+). An insert in the correct orientation was then recloned via the BamHI and HindIII sites of the pBluescript vector into the His_6 fusion vector pQE32 (QIAGEN Inc., Chatsworth, CA). Recombinant protein was purified from *Escherichia coli* strain XL1 Blue grown at 30°C. Cell lysis and all subsequent steps were performed in the presence of 6 M urea. Binding and elution of fusion protein to Ni-NTA resin was performed as described by the supplier (QIAGEN Inc.). Purified protein was concentrated with a microconcentrator (Amicon Corp., Beverly, MA) and subjected to preparative SDS-PAGE. Gels were stained with Coomassie blue, and the band containing the 55-kD recombinant protein was excised and destained extensively with 40% methanol/10% acetic acid, followed by a brief wash with PBS. Antibody production in rabbits was performed by the Pocono Rabbit Farm and Laboratory (Canadensis, PA). Before subcutaneous injection, gel slices were ground with PBS and adjuvant. Primary injections contained ~150 μg of protein/rabbit and Freund's complete adjuvant; boosts contained ~75 μg of protein/rabbit and Freund's incomplete adjuvant. Rabbits were boosted after 4, 6, and 8 wk, and every 4 wk thereafter. Sera were collected 2 and 3 wk after each boost starting with week 10, by which time each of two rabbits had yielded significant anti-Klp1 titer. Purification of anti-Klp1 antibodies was performed using the Klp1 fragment that had been electroblotted to nitrocellulose. Binding and elution of antibody was done as described previously (Page and Snyder, 1992).

Immunoblotting and Indirect Immunofluorescence

Immunoblots were probed as described previously (Kozminski et al., 1993*a*) with affinity-purified Klp1 antibody diluted 1:10,000 in TBS/5% nonfat dry milk/0.05% Tween-20 for 16–24 h at 4°C. All subsequent steps were performed in TBS/0.05% Tween-20 at room temperature. Anti-Klp1 binding on immunoblots was detected with alkaline phosphatase conjugated to goat anti-rabbit IgG (Promega Corp., Madison, WI) developed with BCIP/NBT (Harlow and Lane, 1988). Indirect immunofluorescence was performed with the cell wall-less strain *cw15* as described (Kozminski et al., 1993*a*). Affinity-purified anti-Klp1 antibody and anti-α-tubulin serum were used at a dilution of 1:250 and bound overnight at room temperature to fixed, detergent-extracted cells. Fluorescein-conjugated goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA) was used at a dilution of 1:250 for 2 h.

Immunoelectron Microscopy

Whole mounts of axonemes were prepared essentially as described by Johnson and Rosenbaum (1992). All steps were performed at room temperature. Concentrated drops of cells were allowed to settle onto formvar-coated nickel grids, which were then inverted onto HMDEK (10 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA, and 25 mM KCl) + 0.3% NP-40. This treatment demembranates and releases the flagella from cell bodies (Dentler and Rosenbaum, 1977). Before antibody binding, grids were treated sequentially on drops containing 0.3% glutaraldehyde (3–5 min), PBS (2 × 5 min), wash buffer (PBS + 0.05% Tween-20, 2 × 5 min), and blocking buffer (wash buffer + 1% BSA [A-7906; Sigma Immunochemicals, St. Louis, MO] + 0.1% cold water fish gelatin [G-7765, Sigma Immunochemicals] + 0.02% NaN_3, 10 min). After blocking, grids were incubated in the Klp1 antibody, diluted 1:1,000 in blocking buffer, for 2–5 h. Grids were washed in wash buffer (4 × 5 min) and incubated in 12-nm colloidal gold conjugated to goat anti-rabbit IgG (Jackson Immunologicals, West Grove, PA), diluted 1:100 in blocking buffer, for 1–2 h. Grids were rinsed in wash buffer (3 × 5 min), PBS (2 × 5 min), H_2O (2 × 5 min), and then negatively stained with 2% aqueous uranyl acetate for 90 s; for a light positive stain, uranyl acetate was rinsed from the grids with H_2O (2 × 5 min).

For postembedding labeling, flagella were excised in 3 mM dibucaine and collected according to Witman (1986), then fixed in 2% glutaraldehyde on ice. The flagellar pellet was washed in 10 mM Hepes (pH 7.4), dehydrated to 90% ethanol, and embedded in LR Gold (London Resin Company, Hampshire, United Kingdom) at −20°C for 36 h. Pellets were flat embedded and polymerized at −20°C under fluorescent light with benzil (0.2%) as the catalyst. Gold sections were collected on bare and formvar-coated nickel grids, and were immunolabeled according to the procedure described above for whole mounts, except that the primary Klp1 antibody was diluted 1:500. Sections were stained with 2% methanolic (50%) uranyl acetate (10 min) and lead citrate (3 min), and viewed on an EM10 electron microscope (Carl Zeiss, Inc., Thornwood, NY) at 80 kV.

A number of controls were performed for both immunolabeling methods: omission of the primary antibody, incubation in preimmune serum, and incubation in other rabbit antisera to cytoskeletal proteins (radial spoke protein 3, actin). No specific labeling of the central pair apparatus was observed in any of these experiments (results not shown). In addition, several different size gold particles were used as probes and all gave similar results to those presented here.

Flagellar Fractionation and SDS-PAGE

Chlamydomonas cells from an 8-liter culture were collected, resuspended in 60 ml of ice-cold 5% sucrose, 10 mM Hepes, pH 7.2, and deflagellated by pH shock (Witman et al., 1972). After addition of EGTA (0.2 mM final) and PMSF (1.0 mM final), cell bodies were removed by centrifugation through a 25% sucrose cushion twice. Flagella were collected at 16,000 *g* for 20 min and resuspended in 0.5–1.0 ml of HMDEK. Membranes were solubilized from isolated flagella with 2% NP-40, and axonemes were collected by 10-min centrifugation in a microfuge at 4°C. The supernatant containing the membrane/matrix fraction was removed, and axonemes were resuspended in HMDEK. Concentrated SDS-PAGE sample buffer was added to fractions, which were then frozen. For SDS-PAGE (8.8% acrylamide), lanes contained equivalent portions of each fraction, which corresponded to ~25 μg of flagellar or axonemal protein, and significantly less (<2 μg) of membrane/matrix protein.

Results

Cloning of KLP1

The kinesin superfamily is defined by a motor domain that contains blocks of highly conserved amino acids dispersed among regions of variable sequence. Using reverse transcription PCR and oligonucleotides against conserved domains, we obtained five PCR products that encoded putative kinesin-like proteins. (A more detailed description of these clones will be presented at a later date.) These PCR products were used to screen a cDNA library prepared from *Chlamydomonas* cells undergoing flagellar regeneration, which yielded two identical partial cDNA clones encoding a kinesin-like protein, *KLP1*. The 5′ end of the *KLP1* transcript was obtained by RACE, which yielded three products, two of which had identical 5′ ends and a third that was 10 bp shorter in length. The sequences obtained by RACE were compared to cloned genomic sequences and were found to be free of error (data not shown).

The *KLP1* cDNA (Fig. 1) was 2,752 bp in length, excluding the poly(A) tail. The Klp1 protein coding sequence began at nucleotide 174 and encoded a primary translation product of 776 amino acids and a relative molecular mass of 83,019. We believe translation of Klp1 initiates as indicated for the following reasons: (*a*) the ATG at nucleotide 174 was the first initiation codon of the *KLP1* cDNA, and the next in frame ATG was 240 nucleotides downstream; (*b*) the leader sequence upstream of this ATG contained stop codons in every reading frame; (*c*) the leader sequence of 173 nucleotides was similar in length to the leader sequence of many other *Chlamydomonas* transcripts (Curry, A., and M. Bernstein, unpublished compilation); and (*d*) the initiation codon was part of the sequence aaaATGg, which is identical to the pre-

A

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                                                                                TCACTTGCGTTTGATGTCCTGTTATTGTCGCTAGCTTACATTTTATACGTGGT   53

    GACTCAAGACCCCAGTGTTTGGTTTTTCGTTGTTTCGTTCTCAAGCGTGCTTGGCCTGAACGGGCTTGGTAACACACACATCTACAGCTGCTGTAGTTTCGACTGTCTTGCACATCGAAA  173

    ATGGTGAAGCAAGCTGTGAAGGTCTTCGTGAGGACGCGTCCCACAGCGACCAGTGGGAGCGGCCTAAAGCTTGGACCGGACGGGCAAAGCGTATCGGTGAATGTTCCAAAGGATCTGTCT  293
1   M  V  K  Q  A  V  K  V  F  V  R  T  R  P  T  A  T  S  G  S  G  L  K  L  G  P  D  G  Q  S  V  S  V  N  V  P  K  D  L  S

    GCGGGTCCAGTGAACAATCAGCAGGAGCAGTTCTCCTTCAAGTTTGACGGCGTGTTGGAGAATGTGAGCCAGGAGGCAGCGTACACGACTCTGGCGCATGAGGTGGTGGACAGCCTCATG  413
41  A  G  P  V  N  N  Q  Q  E  Q  F  S  F  K  F  D  G  V  L  E  N  V  S  Q  E  A  A  Y  T  T  L  A  H  E  V  V  D  S  L  M

    GCCGGATACCACGGAACTATCTTCGCGTACGGCCAGACGGGTGCTGGTAAGACGTTCACGATGTCCGGCGGCGGCACGGCGTATGCGCATCGCGGCCTCATCCCCCGCGCTATCCACCAC  533
81  A  G  Y  H  G  T  I  F  A  Y  G  Q  T  G  A  G  K  T  F  T  M  S  G  G  G  T  A  Y  A  H  R  G  L  I  P  R  A  I  H  H

    GTGTTCCGGGAGGTAGACATGCGAGCGGACAAGATGTACCGCGTTCATGTGTCGTACCTCGAGATTTACAACGAGCAACTGTACGACCTGCTGGGAGACACACCCGGGACCAGCGACGCG  653
121 V  F  R  E  V  D  M  R  A  D  K  M  Y  R  V  H  V  S  Y  L  E  I  Y  N  E  Q  L  Y  D  L  L  G  D  T  P  G  T  S  D  A

    CTGGCAGTGCTGGAGGATTCAAACAGCAATACATACGTCCGCGGCCTGACGCTGGTGCCGGTGCGCAGCGAGGAGGAGGCGCTGGCGCAGTTCTTCCTGGGCGAGCAGGGCCGCACCACT  773
161 L  A  V  L  E  D  S  N  S  N  T  Y  V  R  G  L  T  L  V  P  V  R  S  E  E  E  A  L  A  Q  F  F  L  G  E  Q  G  R  T  T

    GCCGGACACGTGCTCAACGCGGAGAGCAGCCGCTCGCACACGGTGTTCACTATTCACGTGGAGATGCGCACCAGTGATGCCGCCAGCGAGCGTGCTGTCCTCTCCAAGCTGAACCTGGTG  893
201 A  G  H  V  L  N  A  E  S  S  R  S  H  T  V  F  T  I  H  V  E  M  R  T  S  D  A  A  S  E  R  A  V  L  S  K  L  N  L  V

    GACCTGGCCGGCAGCGAGCGCACCAAGAAGACCGGCGTGACCGGCCAGACGCTGAAGGAGGCGCAGTTCATCAACCGCTCGCTGTCCTTCCTGGAGCAGACCGTCAATGCGCTCAGCCGC 1013
241 D  L  A  G  S  E  R  T  K  K  T  G  V  T  G  Q  T  L  K  E  A  Q  F  I  N  R  S  L  S  F  L  E  Q  T  V  N  A  L  S  R

    AAGGACACATACGTGCCGTTCCGCCAGACCAAGCTGACGGCGGTGCTGCGGGACGCGCTGGGCGGCAACTGCAAGACGGTCATGGTGGCCAACATCTGGGCCGAGCCGAGCCACAATGAG 1133
281 K  D  T  Y  V  P  F  R  Q  T  K  L  T  A  V  L  R  D  A  L  G  G  N  C  K  T  V  M  V  A  N  I  W  A  E  P  S  H  N  E

    GAGACCCTGAGCACGCTGCGCTTCGCATCGCGCGTGCGCACGCTGACCACCGACCTGGCGCTGAATGAGAGCAACGACCCGGGCGCTGTTACTGCGGCGGTATGAGCGCCAGATCAAGGAG 1253
321 E  T  L  S  T  L  R  F  A  S  R  V  R  T  L  T  T  D  L  A  L  N  E  S  N  D  P  A  L  L  L  R  R  Y  E  R  Q  I  K  E

    CTAAAGGCGGAGCTGGCTATGCGGGACACACTCAGCGGCAAGGGCCGTGTGTCGTACGACGACTTGACTGATGACGAGCTGCGCGAGCTGCACGCCACCTGCCGCCGCTTCCTGCACGGC 1373
361 L  K  A  E  L  A  M  R  D  T  L  S  G  K  G  R  V  S  Y  D  D  L  T  D  D  E  L  R  E  L  H  A  T  C  R  R  F  L  H  G

    GAGGCGGAGCCGGAGGACCTGCCGGCCGACTCCATGAAGCGTGTGCGGGAGACGTTCAAGGCACTGCGGGCGGTGCACGTCGCCATCAAGGCCGACATGGCCACCCAGATGGCCACATTG 1493
401 E  A  E  P  E  D  L  P  A  D  S  M  K  R  V  R  E  T  F  K  A  L  R  A  V  H  V  A  I  K  A  D  M  A  T  Q  M  A  T  L

    CGCCGGGCCACGGAGGAGGGCAGCGGAGCGGCTGCTCGCGGCGGTGACTCGGCCGGCCCCAGCGGTGTGGGCGATGTGGACCTGCGCGCCACCGGAGGCTTCACGGTGGGGCACGCACCG 1613
441 R  R  A  T  E  E  G  S  G  A  A  A  R  G  G  D  S  A  G  P  S  G  V  G  D  V  D  L  R  A  T  G  G  F  T  V  G  H  A  P

    CTGGACGCGCGGCCGCCCGTGCGCTCCGAGCTGGGCTCCCCAGGGCCGGTGCCAGCGGTGCAGAGGCACTGGGTGAGCCGCGCTCCCCCGGCGGCGGCCTGCATGCCCAGGCCAGCTCT 1733
481 L  D  A  R  P  P  V  R  S  E  L  G  S  P  G  A  G  A  S  G  A  E  A  L  G  E  P  R  S  P  G  G  G  L  H  A  Q  A  S  S

    CACACGGACGCCGGCAGCAACTGGGGCGATGCAGGGCCGCTGAGCAGTCCCGGCGGTACTCGGCTGGCGGGCATCTTCGGTGTGTCTGGCGATCGCAATGCCGTTTTCCGACGCTACAAG 1853
521 H  T  D  A  G  S  N  W  G  D  A  G  P  L  S  S  P  G  G  T  R  L  A  G  I  F  G  V  S  G  D  R  N  A  V  F  R  R  Y  K

    GTGGATGTGGGCGAGGGCCGCGAGCTGGCGGCGTCGCTCAAGGCCGCCAGCATCGCCCTGGCCGATACCAAGGCCTCCATCCGCAGCCTGGGGGCCTCCGTCAACGACGCCAAGCAGCGC 1973
561 V  D  V  G  E  G  R  E  L  A  A  S  L  K  A  A  S  I  A  L  A  D  T  K  A  S  I  R  S  L  G  A  S  V  N  D  A  K  Q  R

    ATTGACGAGCTGAGCTCGGCGCTGGCACTGCGGCGCGGCGCCACTCCGGCAGGCGGTGACGGCGAGGTGCTGGACAGCGAGGCGTACGCACTGATGCAGGAGCTGAAGTCCGCCAAGAGC 2093
601 I  D  E  L  S  S  A  L  A  L  R  R  G  A  T  P  A  G  G  D  G  E  V  L  D  S  E  A  Y  A  L  M  Q  E  L  K  S  A  K  S

    CGCTACCGCACTGACTTCGACTCGCTCAAGTCTGCGCGCGAGGAGCTTGAGCCGCAAATCCAGGCAGTGGCGGTGGCACGGGCGGGCCTGCTGGAAGCGTTCGACCGCTGGGCGGCAGCG 2213
641 R  Y  R  T  D  F  D  S  L  K  S  A  R  E  E  L  E  P  Q  I  Q  A  V  A  V  A  R  A  G  L  L  E  A  F  D  R  W  A  A  A

    CAGAGCGACACCACACTCAAGCGCATGGCCACGGCTGGCCGGGCAATGTCGGGTATTGCTCCCGGCGAGGAAGACGAGATGGATGCTGGGGAACAGTTCGAACGCATGCAGATCGCGCGC 2333
681 Q  S  D  T  T  L  K  R  M  A  T  A  G  R  A  M  S  G  I  A  P  G  E  E  D  E  M  D  A  G  E  Q  F  E  R  M  Q  I  A  R

    ATTAGCGAACGCGACCCCGACTCGCTAGCCTTCCACACGGCCCTGAAGCGCACCGGTGCCGCCGTGTCGCGGCCAGCTACGGTGGCCACGGGCGGCAACGCCAAGGCGGCGGCGATGGCC 2453
721 I  S  E  R  D  P  D  S  L  A  F  H  T  A  L  K  R  T  G  A  A  V  S  R  P  A  T  V  A  T  G  G  N  A  K  A  A  A  M  A

    ACTCGTAAGATGGAGCACACGCAGGCCGTCAACCGAGGCCTGGCGCGGTGATGCAGCGGGGCCTGCGCCGGTTGTGCAGGGTGTCGCCTGCGTGACACATCAGTGTTTTGACAGTAGGAA 2573
761 T  R  K  M  E  H  T  Q  A  V  N  R  G  L  A  R  *

    GCTCGTGAGCGTGTGCGTGAAGCATGTGTGCGCACGGGGACTGGGCTGTCGTCGGAATGAGTTGACGGGGTTGCTGACGTGGGCAAACTGACAAACAGTGATTATGTGGCGTCAGTTACT 2693

    ATGTTGCCCCAGTCCCGTGGGGGTGTCGGGCATCGGGGACTTGTTAACTGTGCCAGAGGAAAAAAAAAAAAAAAAAA 2770
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B

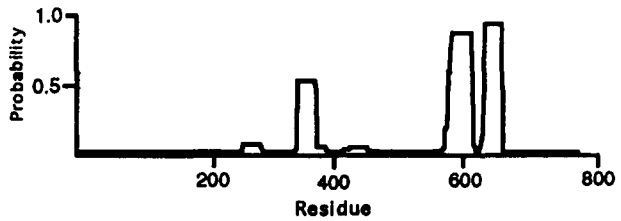


Figure 1. KLP1 sequence and coiled-coil probability. (*A*) *KLP1* cDNA sequence and deduced amino acid sequence. Underlined amino acids are tripeptide motifs shared by the Klp1 protein and fungal members of the BimC subfamily of kinesin-like proteins. Underlined nucleotides indicate the FspI site used to subclone the nonconserved carboxy-terminal portion of Klp1 for antibody production. The Klp1 protein is predicted to be 776 amino acids with a relative molecular mass 83,019 and a pI = 7.0. (*B*) Probability of coiled-coil formation at each amino acid, calculated by the program of Lupas et al. (1991). These sequence data are available from EMBL/GenBank/DDBJ under accession number X78589.

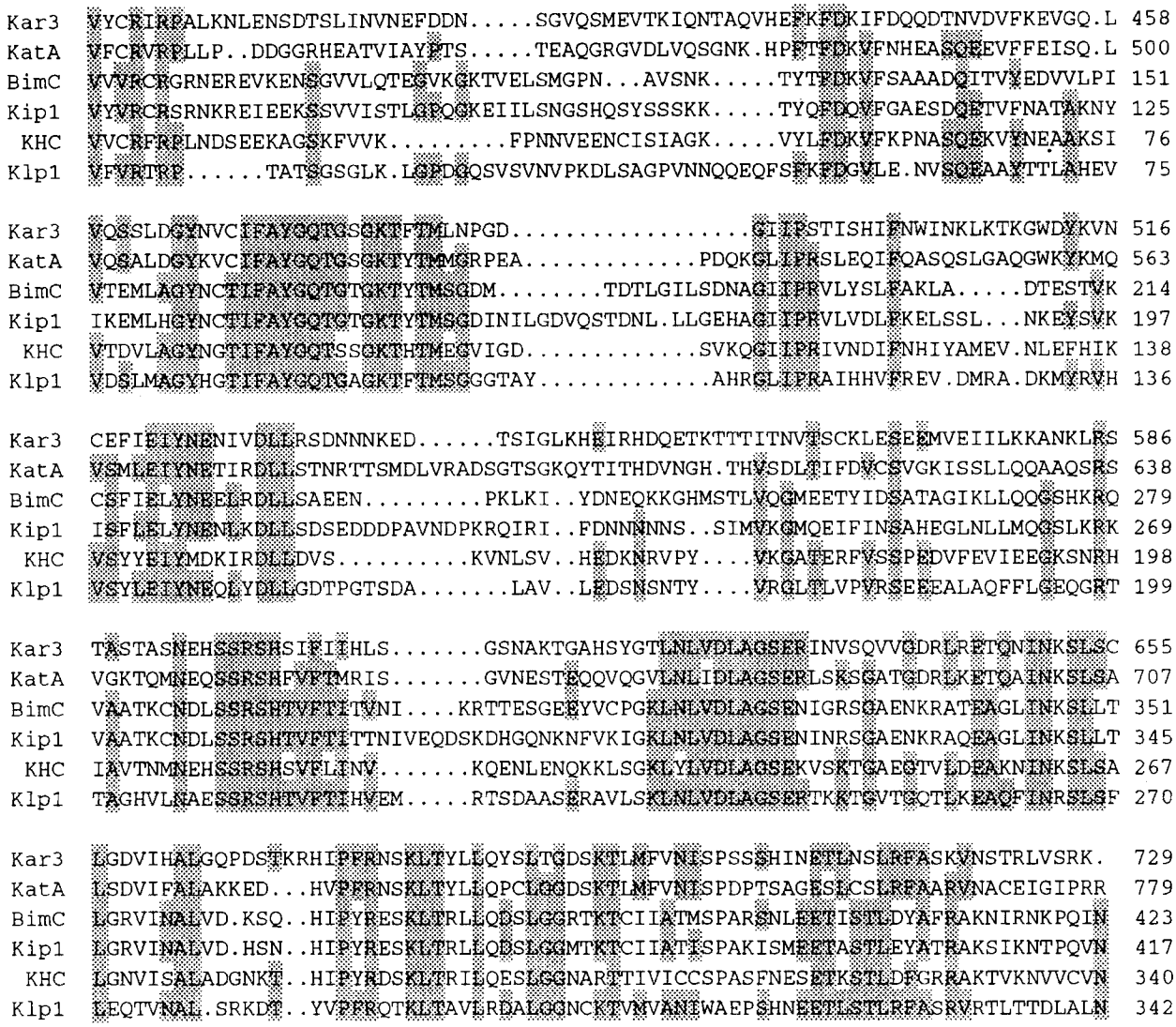


Figure 2. Sequence comparison of kinesin motor domains. Kinesin motor domains were compared using the Pileup program (GCG computer software package; University of Wisconsin). Shaded regions are positions where Klp1 is identical to at least two other proteins. Amino acid sequences were taken from the following sources: *S. cerevisiae* Kar3 (amino acids 389–728), Meluh and Rose (1990); *Arabidopsis thaliana* KatA (434-779), Mitsui et al. (1993); *Aspergillus nidulans* BimC (84-223), Enos and Morris (1990); *S. cerevisiae* Kip1 (55-417), Roof et al. (1992); *Drosophila melanogaster* KHC (15-340), Yang et al. (1989); *Chlamydomonas reinhardtii* Klp1 (8-342), see Fig. 1 *A*.

sumptive translation start site of several other *Chlamydomonas* transcripts (Franzen et al., 1989; Goldschmidt-Clermont and Rahire, 1986; Stein et al., 1993; Yu and Selman, 1988; Zimmer et al., 1988).

The NH_2-terminal portion of Klp1, from amino acids 8–342, was homologous to the motor domains of other members of the kinesin superfamily (Fig. 2). These proteins share blocks of highly conserved regions separated by sequences that are highly variable. The motor domain of Klp1 was 35–42% identical to each of the motor domains of 25 other klps whose sequences were available from the combined SwissProt, PIR, and GenPept databases via the BLAST search program (Altschul et al., 1990), which indicated that Klp1 constituted the founding member of a distinct subfamily of klp. Consistent with this interpretation was the observation that the COOH-terminal portion of Klp1 showed no significant homology to other proteins in these databases. However, as in other members of the kinesin superfamily, the COOH-terminal region of Klp1 contained sequences that have a high probability of forming coiled-coil domains (Fig. 1 *B*; Lupas et al., 1991).

Although the head region of Klp1 constituted a distinct class of kinesin motor domain, we did recognize peptide motifs within the motor domain of Klp1 that were common to fungal members of the BimC subfamily of klps (Fig. 3). These proteins include BimC from *A. nidulans*, cut7 from *Schizosaccharomyces pombe*, and Kip1 and Cin8 from *Saccharomyces cerevisiae*, which are required for the separation of spindle pole bodies before mitosis (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt et al., 1992; Meluh and Rose, 1990; Roof et al., 1992; Saunders and Hoyt, 1992; Sawin and Endow, 1993). Two tripeptide motifs (FTI at amino acids 216–218 and NAL at amino acids 276–278 of the Klp1 protein, Fig. 3) were found in Klp1 and each of the

		Motif 1			Motif 2		
D. melanogaster	KHC	SSRSH	SV	FLI	VI	SAL	A
A. thaliana	KatA	SSRSH	FV	FTM	VI	FAL	A
C. reinhardtii	Klp1	SSRSH	TV	FTI	TV	NAL	S
A. nidulans	BimC	SSRSH	TV	FTI	VI	NAL	V
S. pombe	cut7	SSRSH	SI	FTI	VI	NAL	V
S. cerevisiae	Cin8	SSRSH	TI	FTI	VI	NAL	V
S. cerevisiae	Kip1	SSRSH	TV	FTI	VI	NAL	V
S. cerevisiae	Kar3	SSRSH	SI	FII	VI	HAL	G
S. cerevisiae	Kip2	SSRSH	AI	VLI	VI	SKL	G

Figure 3. Tripeptide motifs common to the motor domains of Klp1 and the fungal BimC subfamily of klps. Boxed amino acids are tripeptide motifs that are present in the Klp1 protein and fungal members of the BimC subfamily of klps. The amino acid residues for Motifs 1 and 2 are as follows: *D. melanogaster* KHC, 208–217 and 271–276 (Yang et al., 1989); *A. thaliana* KatA, 648–657 and 711–716 (Mitsui et al., 1993); *C. reinhardtii* Klp1, 209–218 and 274–279 (see Fig. 1 *A*); *A. nidulans* BimC, 289–298 and 355–360 (Enos and Morris, 1990); *S. pombe* cut7, 270–279 and 348–353 (Hagan and Yanagida, 1990); *S. cerevisiae* Cin8, 392–401 and 454–459 (Hoyt et al., 1992); *S. cerevisiae* Kip1, 279–288 and 349–354 (Roof et al., 1992); *S. cerevisiae* Kip2, 341–350 and 401–405 (Roof et al., 1992); *S. cerevisiae* Kar3, 596–605 and 659–664 (Meluh and Rose, 1990).

fungal members of the BimC subfamily. The NAL motif is highly specific for Klp1 and the BimC subfamily members and was present in none of 34 other members of the kinesin superfamily, while the FTI motif was less restricted and was found in 6 of 31 other members of the kinesin superfamily. These highly conserved motifs, although small, may indicate regions of structural and/or functional homology among the motor domains of Klp1 and the members of the BimC subfamily of klps.

KLP1 Gene Expression

In response to environmental stresses, such as low pH or mechanical sheer, *Chlamydomonas* cells shed their flagella (Randall et al., 1967; Rosenbaum et al., 1969) through an active process that requires a second messenger (Cheshire and Keller, 1991; Quarmby et al., 1992) and the action of the Ca^{2+}-dependent contractile protein, centrin (Sanders and Salisbury, 1989). Immediately after flagellar excision, cells upregulate genes encoding flagellar proteins and use newly synthesized proteins, as well as preexisting precursors, to regenerate full-length flagella in 80 min (Lefebvre and Rosenbaum, 1986). Fig. 4 *A* illustrates that *KLP1* encoded a transcript of ~3,000 nucleotides (consistent with the length of the *KLP1* cDNA) that was upregulated during flagellar regeneration. As with many other genes encoding flagellar proteins, a low level of *KLP1* message was detectable in nondeflagellated cells. With kinetics similar to tubulin (Fig. 4 *B*) and other flagellar proteins (Curry et al., 1992; Youngblom et al., 1984), the *KLP1* message was upregulated within 10 min of flagellar removal, peaked at 45 min into regeneration, and returned to basal levels within 90–120 min after deflagellation. These results provided the first evidence that Klp1 was a flagellar protein.

Anti-Klp1 Antibody

As a probe for Klp1 protein, we raised antisera directed against amino acids 334–776 of the nonconserved tail region

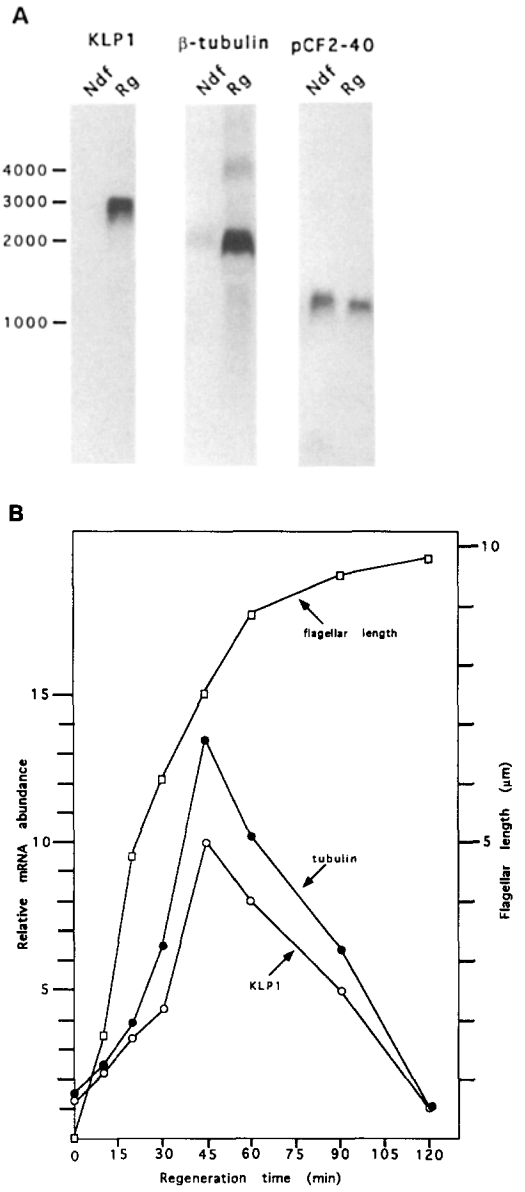


Figure 4. mRNA analysis. (*A*) mRNA probed with *KLP1*, β-tubulin (Youngblom et al., 1984), and clone pCF2-40 (Youngblom et al., 1984). Each lane contains 10 μg of poly(A) RNA isolated from either nondeflagellated (*Ndf*) cells or cells regenerating flagella (*Rg*; 40 min after deflagellation). A single nylon filter, probed and stripped sequentially, was used for all three probes. The culture medium of cells used to prepare Rg RNA contined 10 μg/ml cycloheximide to stabilize mRNA during regeneration. (*B*) Upregulation of mRNA during flagellar regeneration. The relative levels of expression for *KLP1* (*open circles*) and β-tubulin mRNA expression (*closed circles*) at times after flagellar removal were determined by quantifying the amount of transcript present at each time point and normalizing that value to the level of expression for the constitutive clone, pCF2-40 (Youngblom et al., 1984). The mean flagellar length at each time point (*boxes*, $n = 30$) is also shown.

of Klp1 protein expressed in bacteria (see Materials and Methods). We would expect these antisera to recognize only Klp1 or a Klp1 homologue, but not other members of the kinesin superfamily. Though we report on only one antiserum in this work, two independent sera gave identical results

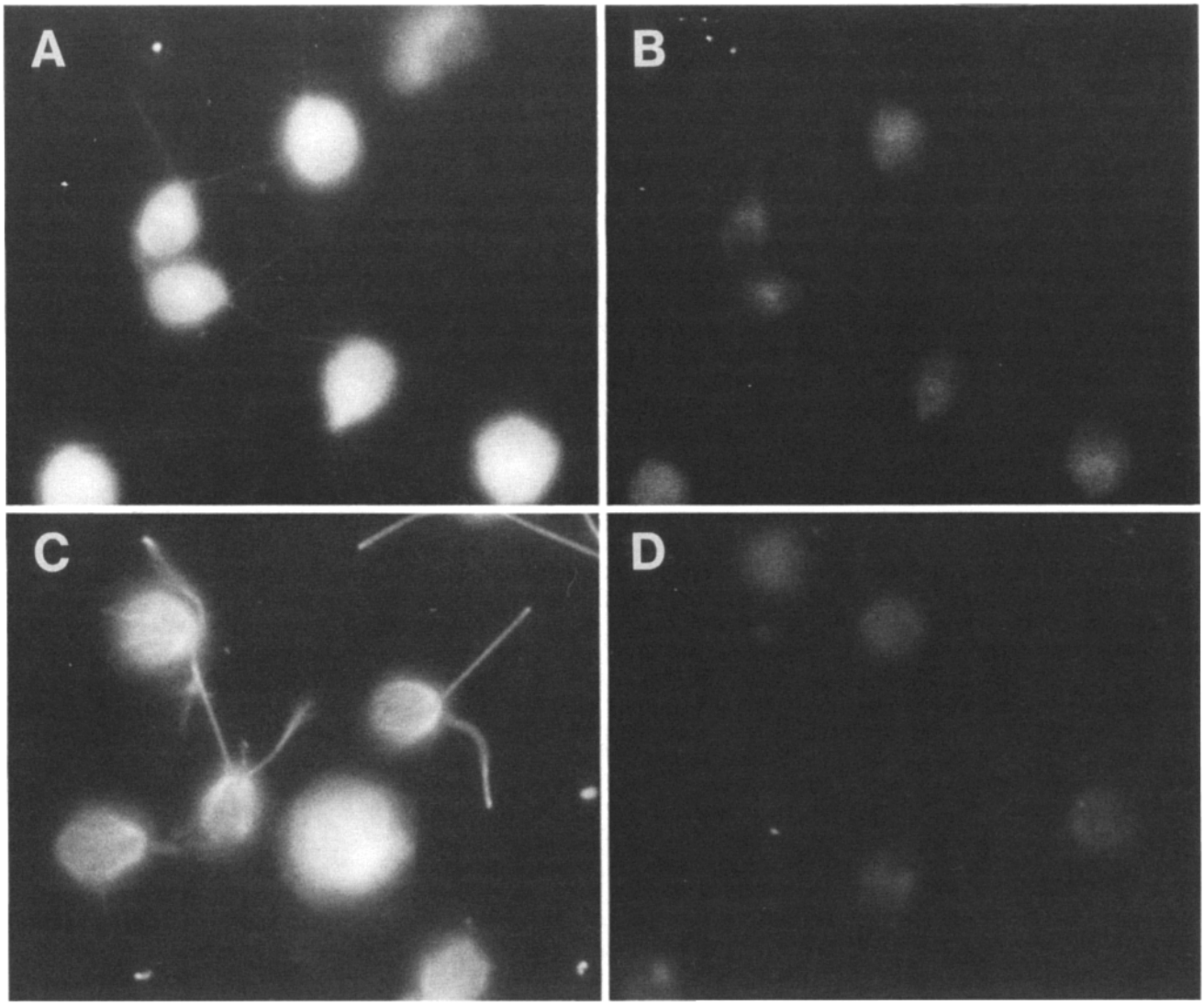


Figure 5. Indirect immunofluorescence. The cell wall-less strain *cw15* was probed using primary antibodies against Klp1 or α-tubulin (Silflow and Rosenbaum, 1981) and a fluorescein-labeled secondary antibody. Klp1 antibody (*A* and *B*) stains the flagella and cell bodies. *B* shows that the cell body associated Klp1 is concentrated slightly around the nucleus and in the region between the nucleus and flagellar base. α-Tubulin antiserum (*C*) reveals heavy labeling of the flagella and cytoplasmic microtubules. (*D*) Minus primary antibody control. Exposure times for photographing indirect immunofluorescence were: Klp1, 60 s; α-tubulin, 30 s; and minus primary antibody control, 60 s. *A* and *B* are prints from a single negative with *B* printed to reveal the concentration of Klp1 in the anterior region of the cell bodies.

in all the experiments described. All experiments used antibody that had been affinity purified against bacterially expressed Klp1 tail.

Immunofluorescence

Affinity-purified Klp1 antibody was used to localize Klp1 in fixed and permeabilized *Chlamydomonas* cells by indirect immunofluorescence. As predicted from the finding that *KLP1* is upregulated during flagellar regeneration, we observed Klp1 staining in the flagella of cells (Fig. 5 *A*). We also observed Klp1 fluorescence within the cell body, with a higher level of staining in the regions surrounding the nucleus and between the nucleus and flagellar base (Fig. 5, *A–B*). However, Klp1 was not concentrated on the flagellar roots (compare to Fig. 5 *C*), as might be expected if Klp1 is bound to cytoplasmic microtubules. The Klp1 observed in the cell body could represent precursors to flagellar-localized Klp1, since precursors of many flagellar proteins are found in the cytoplasm and transported to the flagella during flagellar regeneration. Alternatively, like the tubulins, Klp1 could be localized stably within both the cell body and the flagella.

Klp1 in the Flagellum and Cell Body

When used to probe immunoblots of *Chlamydomonas* flagella, the affinity-purified Klp1 antibody recognized two proteins with an apparent molecular weight of 96 kD (Fig. 6 *A*). In every case examined, we observed this doublet, suggesting that at least a fraction of the primary Klp1 translation product had undergone posttranslational modification, or that the *KLP1* gene, which is present in only one copy per

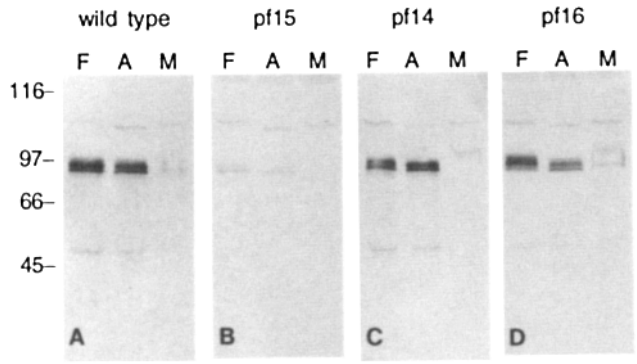


Figure 6. Klp1 in flagellar fractions. Fractions from whole flagella (*F*), axonemal pellets (*A*), and membrane/matrix fractions (*M*) were immunoblotted and probed with Klp1 antibody. Fractions were prepared from (*A*) wild-type flagella (*B*) *pf15* (9 + 0, central pairless) mutant flagella (*C*) *pf14* (radial spokeless) mutant flagella and (*D*) *pf16* (C1-deficient) mutant flagella. All lanes contain flagellar fractions from starting material of ~2 × 10^8 cells, which corresponds to ~25 μg of protein for the flagellar and axonemal fractions, and <2 μg of protein for the membrane matrix fractions. Positions of protein standards (*kD*) are shown on the left.

cell (data not shown), had undergone differential mRNA splicing. The apparent molecular weight of 96 kD was greater than the size of 83 kD predicted from the *KLP1* cDNA. However, other members of the kinesin superfamily have also been shown to migrate slower in SDS-PAGE than their predicted molecular weight (Chandra et al., 1993; Roof et al., 1992), indicating that this may be a general feature of klps. As expected, we also found Klp1 in cell bodies from which the flagella had been detached (data not shown). By comparing Klp1 signals in fractions containing equivalent numbers of cell bodies and flagella, we estimated that Klp1 was enriched at least 20-fold in flagella versus cell bodies. However, we estimated that the cell body contained more total Klp1 protein than the flagella (data not shown).

Flagellar Fractionation and Localization of Klp1

To localize Klp1 in the flagellum, we isolated flagella and extracted them with the nonionic detergent NP-40, which yielded a detergent-soluble membrane/matrix fraction and an insoluble axonemal fraction. After detergent extraction of wild-type cells, the majority of Klp1 within flagella remained bound to the axoneme (Fig. 6 *A*). Furthermore, Klp1 remained attached to axonemes after incubation in HMDEK + 5 mM ATP (data not shown). These results indicated that Klp1 was neither tightly associated with the flagellar membrane nor in the soluble matrix fraction.

We also assayed for the presence of Klp1 in flagella defective for specific axonemal structures. In flagella from *pf15* cells (Adams et al., 1981; Warr et al., 1966), which are missing the central pair microtubules and their attached projections (9+0 axonemes), the level of Klp1 protein was severely reduced relative to wild-type flagella (Fig. 6 *B*). We also examined other flagella with the 9+0 phenotype (*pf18*, *pf19*, and *pf20*) (Adams et al., 1981; Warr et al., 1966) and found that the level of Klp1 was also low in flagella from these strains (data not shown).

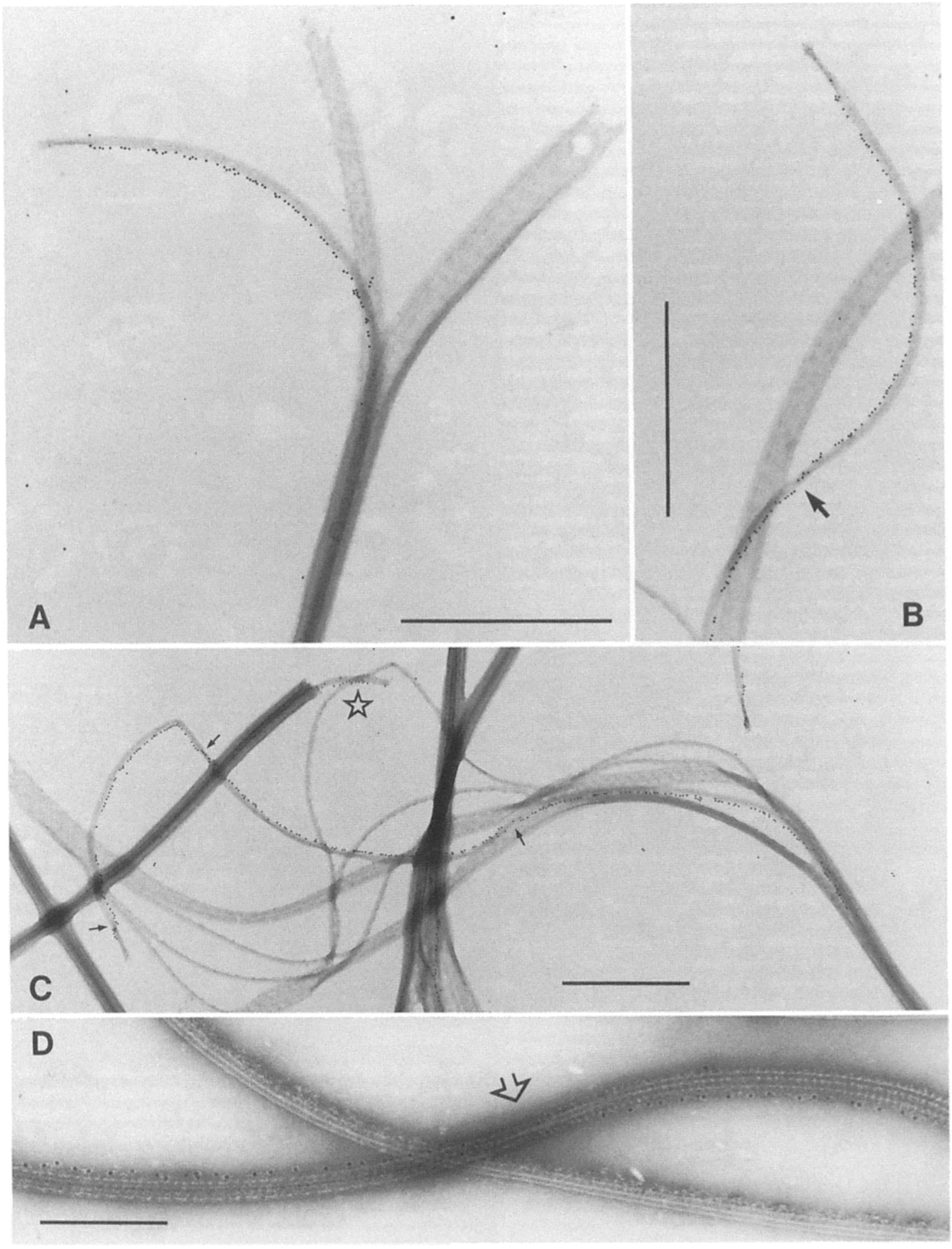
In *Chlamydomonas* flagella, as in other types of eukaryotic flagella, projections attached to the central pair microtubules are in close proximity to radial spokes (Goodenough and Heuser, 1985; Hopkins, 1970), which extend from the A tubules of the outer doublet microtubules. It is possible that the central pair projections interact with the radial spokes to form a bridge between the central pair and outer doublet microtubules (Goodenough and Heuser, 1985; Hopkins, 1970; Warner and Satir, 1974). To determine if radial spokes were required for proper localization of Klp1, we examined flagella from the radial spokeless mutant, *pf14* (Piperno et al., 1981; Witman et al., 1978), and found that Klp1 localization to the flagellum was unaffected (Fig. 6 *C*) and, as in flagella from wild-type cells, Klp1 was not extracted from *pf14* axonemes by ATP treatment (data not shown).

In *Chlamydomonas* and other species, the two central pair microtubules of the flagellum are not structurally or biochemically equivalent (Dutcher et al., 1984; Linck et al., 1981). Attached to each central pair microtubule are characteristic projections that differ between the C1 and C2 microtubules (Adams et al., 1981; Hopkins, 1970), and a *Chlamydomonas* mutant (*pf16*) has been isolated, in which the C1 central pair microtubule is unstable relative to the C2 central pair microtubule (Dutcher et al., 1984). We examined flagellar fractions from *pf16* cells by immunoblotting, and found that the localization of Klp1 in these mutant flagella was identical to wild-type flagella (Fig. 6 *D*), suggesting that, at least in these mutant flagella, Klp1 was capable of quantitative localization and stable binding to the C2 microtubule.

Immunolocalization of Klp1

The low level of Klp1 in 9+0 flagella suggested that Klp1 was bound specifically to the central pair apparatus of the axoneme. To test this hypothesis, we used anti-Klp1 antibody for immunogold electron microscopy of axonemal whole mounts (Fig. 7). On every axoneme in these preparations, we found that where the axoneme had splayed open, Klp1 labeled a single microtubule of the central pair microtubules, which were

Figure 7. Klp1 labeling of axonemal whole mounts. Electron micrographs of axonemes labeled with Klp1 antibody and 12-nm gold particles. (*A–C*) light-positive stain; (*D*) negative stain. (*A*) A typical axoneme showing the frayed distal end and the arc of the central pair. Labeling is restricted to one side of the central pair microtubules; note the absence of labeling at the distal 200–250 nm. Bar, 1 μm. (*B*) More of the central pair is exposed revealing a twist that is matched by a cross-over of the Klp labeling (*arrow*) from one side of the central pair to the other. Bar, 1 μm. (*C*) An axoneme showing labeling along the entire length of the central pair microtubules and three points of cross-over (*arrows*). Note labeling of the central pair protruding from the basal end of an adjacent axoneme (*star*). Bar, 1 μm. (*D*) Negative stain reveals the two microtubules of the central pair, and that Klp1 staining is along only one microtubule. The side view of the central pair at the point of twist (*arrow*) shows projections from a single microtubule. Also visible is an outer doublet of microtubules with dynein arms projecting towards the top of the page. Bar, 250 nm.



identified by their characteristic arc in frayed axonemes, striated appearance, and distinctive plus-end cap (Dentler and Rosenbaum, 1977; Hopkins, 1970; Johnson and Rosenbaum, 1992). In regions where the central pair microtubules twisted and crossed over each other, the Klp1 signal also crossed from one side to the other of the central pair microtubules (Fig. 7, *B–C*). Interestingly, gold labeling was always observed on the microtubule oriented towards the inside of the curved central pair microtubules. In regions where the axoneme had frayed extensively, or where the central pair microtubules had been released from the outer doublet microtubules, labeling was observed along the length of the central pair microtubule. However, labeling was rarely observed over the distal end of the central pair microtubules (~0.25 μm), a region that appears to lack central pair projections (Dentler and Rosenbaum, 1977). No labeling was observed in regions where the axoneme had remained intact, similar to what has been observed with other proteins that are found within the cylinder of the axoneme (Johnson and Rosenbaum, 1992). Labeling of negatively stained whole mounts confirmed that Klp1 was associated with a single central pair microtubule (Fig. 7 *D*) and suggested that Klp1 labeling was in register with the central pair projections. However, the resolution of this technique is insufficient to determine if Klp1 is attached to these projections or directly to the central pair microtubule. As controls, the Klp1 antibody showed no labeling over 9+0 axonemes, and preimmune whole serum showed no labeling of axonemes above background (data not shown). Thus, in wild-type cells, Klp1 was localized asymmetrically to the central pair apparatus, and was confined to only one of the two central pair microtubules or a structure associated with only one of the two central pair microtubules.

Postembedding labeling of thin sections was performed to determine which of the central pair microtubules binds Klp1. As predicted, Klp1 antibody efficiently labeled the central pair apparatus in axonemal thin sections (30% of the transverse sections labeled, $n = 200$), and Klp1 labeling was rarely observed over other regions of the axoneme (1% of the transverse sections, $n = 200$). Control samples lacking primary antibody failed to label any axonemal structures and antibodies directed against other axonemal components, e.g., radial spoke proteins, yielded only background label over the central pair microtubules (data not shown). The central pair microtubules can be distinguished from each other by the size of their projections, which in transverse section are ~18 nm in length on the C1 microtubule and ~8 nm on the C2 microtubule (Dutcher et al., 1984; Hopkins, 1970; Witman et al., 1978). In fixed and embedded axonemes, the projections on the C1 microtubule could be resolved, which allowed us to determine that Klp1 labeling was always ($n = 32$) associated with the C2 microtubule (Fig. 8). Usually, only a single gold particle was present on each of the labeled transverse sections, and these particles were located on or near the C2 microtubule on the side away from the C1 microtubule. In <10% of the labeled transverse sections, two or more particles were seen, also closely associated with the C2 microtubule (Fig. 8 *F*). Since the C2 projections were not apparent in our preparations, we could not say if Klp1 was present on the C2 microtubule or on the C2-associated projections.

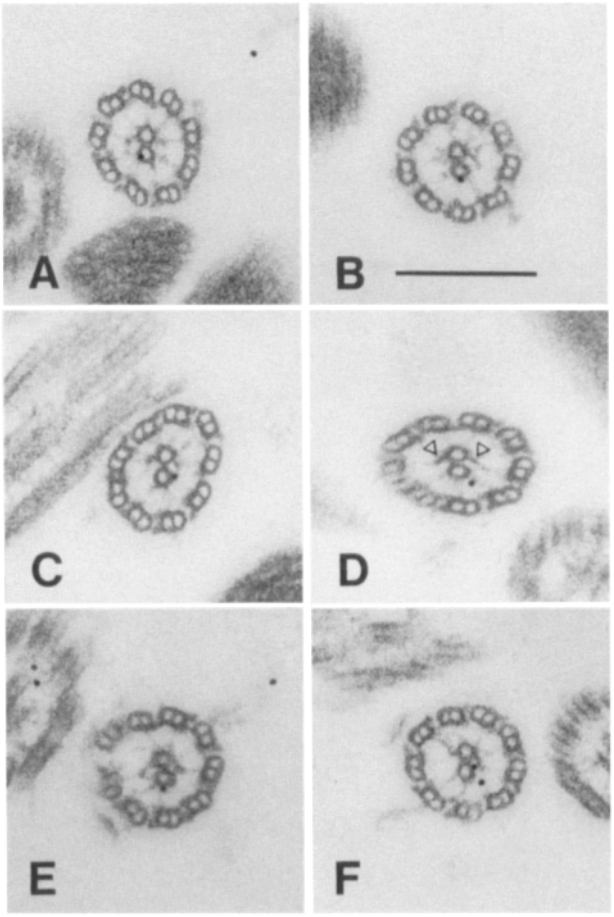


Figure 8. Klp1 labeling of embedded and sectioned axonemes. Electron micrographs of axonemes embedded in LR Gold, sectioned and labeled with Klp1 antibody and 12-nm gold particles. Axonemes are shown in transverse section, viewed from the proximal end of the flagellum with the C1 microtubule on top. The C1 microtubule is distinguished by its prominent projections, particularly evident in *D* (*arrowheads*). Gold particles are always associated with the C2 microtubule. A total of 32 axonemes were scored in which labeling was present and the central pair microtubules could be distinguished from one another. 26 of these showed labeling on the side of C2 that is furthest away from C1 (e.g., *B* and *E*) or on the right-hand side of C2 (e.g., *C*, *D*, and *F*); the other six were labeled on the left side of C2 and, in five of these six, the gold particle was within ~20 nm of the position shown in *B* and *E* (e.g., *A*). Bar, 200 nm.

Discussion

Proteins of the kinesin superfamily are ubiquitous and have varied functions. We have cloned a gene from *Chlamydomonas, KLP1*, which encodes a protein with a typical kinesin motor domain. Using a Klp1-specific antibody, we have detected the Klp1 protein in flagella, the first kinesin superfamily member of the eukaryotic flagellum to be cloned and characterized. Analysis of paralyzed flagellar mutants indicated that Klp1 is part of the central pair apparatus, and immunogold localization showed that Klp1 is bound specifically to central pair microtubule C2 or to projections that are attached to C2.

Klp1 Protein Structure

The general design of Klp1 is similar to many other members of the kinesin superfamily. The conserved kinesin motor domain at the NH_2-terminal region of Klp1 suggests that Klp1, like most other characterized members of the kinesin superfamily, may encode a plus-end–directed microtubule dependent motor. The COOH-terminal tail domain of Klp1 is novel, but as found in other members of the kinesin superfamily, it contains regions that have a high probability of forming coiled-coil structures. Thus, Klp1 may form homodimers, as has been demonstrated for the *Drosophila* KHC polypeptide (de Cuevas et al., 1992; Kuznetsov et al., 1988), bind light chains (Gauger and Goldstein, 1993; Kuznetsov et al., 1988), or associate with other KRPs in a heteromeric complex (Cole et al., 1992, 1993). On immunoblots, Klp1 appears as a 96-kD doublet, indicating that Klp1 exists in multiple isoforms. Southern blot analysis showed that Klp1 is a single copy gene in *Chlamydomonas* (unpublished data), indicating that isoforms arise either from alternative splicing of *KLP1* RNA or posttranslational modification of the Klp1 protein. Many *Chlamydomonas* flagellar proteins are modified, including isoforms that are phosphorylated (Piperno et al., 1981; Williams et al., 1989) or acetylated (L'Hernault and Rosenbaum, 1985), suggesting that Klp1 also undergoes posttranslational processing.

The head region of Klp1 shares two tripeptide motifs with the fungal members of the BimC subfamily of kinesins (Fuller and Wilson, 1992; Goldstein, 1993*b*). Though small, the motifs found among Klp1 and the BimC subfamily of proteins suggest that these proteins share conserved structural and/or functional features at these positions, as it has been shown that as few as three amino acids can constitute a structurally and functionally conserved motif. For example, the tripeptide sequence of N-X-S/T is necessary and sufficient to act as a site for addition of N-linked oligosaccharides to a protein (Kornfeld and Kornfeld, 1985), and peptide sequences of four amino acids have been shown to have a conserved structure that is sufficient to act as an internalization signal for cell surface receptors (Collawn et al., 1990, 1991).

Though the head domain of a klp generates force, it is the tail region of each protein that is thought to provide the functional specificity of a given motor protein by binding to a particular substrate (Goldstein, 1991; Goldstein, 1993*a*). The tail region of Klp1 is novel and has no known homologue, suggesting that Klp1 function is unique among the known members of the kinesin superfamily. To be functional, the tail domain of a klp must bind its substrate tightly, as is the case for the tail region of kinesin, which binds lipids with a high affinity and allows vesicles to be tethered to and moved along microtubule tracks (Henson et al., 1992; Leopold et al., 1992; Skoufias et al., 1994; Vale et al., 1985). The localization of Klp1 on a central pair microtubule and a lack of vesicles within the core of the flagellum make it unlikely that the tail of Klp1 binds to membranes. ATP hydrolysis dissociates the motor domain-microtubule interaction of kinesins (Chandra et al., 1993; Vale et al., 1985) leading to the extraction of some, though not all (Chandra et al., 1993; McDonald et al., 1990; Nislow et al., 1992; Sawin et al., 1992a; Sawin et al., 1992b; Yen et al., 1992), klps. Since ATP treatment does not release Klp1 from the axoneme, it is probable that Klp1 is bound tightly to the axoneme via its tail domain.

Multiple Kinesin Like Protein within the Chlamydomonas Flagellum

A single microtubule array, such as the mitotic spindle, may contain multiple classes of motor proteins (Sawin and Endow, 1993; Skoufias and Scholey, 1993). Similarly, in addition to the well characterized flagellar dyneins, the flagella of *Chlamydomonas* contain proteins that are putative members of the cytoplasmic dynein, myosin, and kinesin superfamilies (Kozminski, K., and J. Rosenbaum, unpublished). Among these non-dynein motor proteins are multiple members of the kinesin superfamily. We have obtained two PCR clones, distinct from *KLP1*, that encode members of the kinesin superfamily and which recognize mRNAs that are upregulated during flagellar regeneration (data not shown), implying that the klps encoded by these genes are flagellar proteins. Recently, other workers have used immunological and biochemical techniques to identify putative klps in the flagella of *Chlamydomonas*, though the identification of these proteins as bona fide members of the kinesin superfamily remains to be established. An antibody against the head domain of *Drosophila* KHC (Rodionov et al., 1991) has been used to identify a 110-kD flagellar protein (Johnson et al., 1994) and, using antipeptide antibodies against conserved regions of the kinesin motor domain (Cole et al., 1992; Sawin et al., 1992*b*), Fox and co-workers (1994) identified a different 110-kD protein, a 125-kD protein, and a 97-kD protein, which was shown to bind brain microtubules in vitro. It is unlikely that this 97-kD protein corresponds to Klp1 because the 97-kD protein of Fox et al. (1994) reacted with an antipeptide antibody directed against the sequence HIPYRESKLT (Fox et al., 1994; Sawin et al., 1992*b*), whereas Klp1 contains the sequence YVPFRQTKLT at this position and does not react with the HIPYR antibody (data not shown). These results indicate that the flagella of *Chlamydomonas* contain at least five members of the kinesin superfamily.

Targeting of Klp1

Our results showed that in wild-type cells, Klp1 was localized to the C2 central pair microtubule at the core of the flagellum. In cells lacking central pair microtubules, the amount of Klp1 within the flagellum was severely reduced, indicating that in the absence of these structures, Klp1 either failed to become concentrated within the flagellum or was degraded upon delivery to flagellum. In the absence of central pair microtubules, Klp1 may lack a site of attachment, and so does not accumulate in the flagellum. Alternatively, in the central pairless mutants examined, Klp1 may fail to be transported into the flagellum. Radial spoke proteins are thought to form partially assembled complexes within the cytoplasm of the cell body before transfer and complete assembly within the flagellum (Tao, W., D. Diener, and J. Rosenbaum, unpublished observation). A mutation in one radial spoke protein, rsp3, prevents transport and flagellar assembly of all radial spoke proteins (Piperno et al., 1977; Witman et al., 1978), while mutations in other radial spoke proteins, rsp4 and rsp9, block transport and flagellar assembly of the subset of radial spoke proteins that form the spoke head (Huang et al., 1981; Piperno et al., 1977). Proteins associated with the central pair microtubules may also form preassembly complexes within the cell body, and only one of the proteins within such a complex needs to have a flagellar targeting signal. If complexes fail to assemble because of

mutations in any member of the assembly, then the localization of all members of the complex that lack their own localization signal would be blocked. Such a model is consistent with the failure of Klp1 to localize in all mutants that lack central pair microtubules. A 110-kD flagellar klp (Johnson et al., 1994) is also targeted to the central pair apparatus. However, in contrast to Klp1, the central pairless mutations do not block flagellar accumulation of this 110-kD protein, suggesting that either Klp1 and the 110-kD protein assemble into different structures of the central pair complex, or that the 110-kD protein contains an active flagellar localization signal.

Klps have been shown to be concentrated in specific regions of microtubule arrays, such as the midzone (Nislow et al., 1990, 1992; Yen et al., 1992), kinetochore (Yen et al., 1992), or spindle pole regions (Sawin et al., 1992*a*) of the mitotic spindle. Klp1 is also associated with microtubules. However, Klp1 shows a much more highly restricted localization pattern, being bound to only a single microtubule within the flagellum, which contains a total of 20 microtubules (nine outer doublet microtubules and two central pair microtubules). In *Chlamydomonas*, all microtubules are composed of identical α/β tubulin dimers (James et al., 1993; Youngblom et al., 1984). Yet, Klp1 is bound specifically to the C2 microtubule, or to a C2-associated structure, indicating that Klp1 (or a complex that contains Klp1) is capable of discriminating the C2 microtubule from the other 19 microtubules of the axoneme. Other axonemal structures are also bound to a subset of microtubules, e.g., the beaks that are found within the B tubule of three of the nine outer doublet microtubules and the bridge structure that connects a single pair of outer doublet microtubules (Hoops and Witman, 1983). Other structures, though not restricted to a single microtubule or microtubule doublet, are assembled along the axoneme with highly characteristic spacings, e.g., radial spokes (Goodenough and Heuser, 1985; Hopkins, 1970; Warner and Satir, 1974; Witman et al., 1978), and dynein arm complexes (Goodenough and Heuser, 1985; Mastronarde et al., 1992; Warner, 1983).

Function of the Klp1 Protein

We localized Klp1 to one of the two central pair microtubules of the flagellum, where, presumably, it functions by exerting mechanical force. However, the dynamic result of Klp1 activity remains undefined. Kinesin, the founding member of the kinesin superfamily, traverses the length of microtubules by moving along a single protofilament (Gelles et al., 1988; Kamimura and Mandelkow, 1992; Ray et al., 1993) and, via interactions through its tail domain (Skoufias et al., 1994), transports vesicles towards the plus-end of microtubules. Although the inside of the axonemal cylinder, where the central pair microtubules are localized, contains no vesicles, it is known that axonemal precursors are transported to and assembled at the distal end of the axoneme (Johnson and Rosenbaum, 1992; Rosenbaum et al., 1969; Witman, 1975). In central pairless mutants lacking Klp1, however, flagellar assembly is normal, indicating that Klp1 is unlikely to be required for the transport of axonemal precursors.

To convert the sliding of outer doublet microtubules into flagellar beating, the dynein motors that drive sliding must be coordinately regulated (Warner and Satir, 1974). Two lines of evidence indicate that the central pair microtubules may be important in this process. Though the flagella of the central pairless mutants of *Chlamydomonas* are paralyzed, the dynein arms present on the outer doublet microtubules of these mutants are still capable of generating the forces required for microtubule sliding (Witman et al., 1978), which indicates that these flagella are paralyzed because they fail to coordinate dynein activity. Secondly, rotation and twisting of the central pair microtubules have been documented in a variety of organisms (Melkonian and Preisig, 1982; Omoto and Kung, 1979, 1980; Omoto and Witman, 1981; Tamm and Horridge, 1970), including *Chlamydomonas* (Kamiya, 1982), and in some cases has been found to show a strong correlation with the position of the ciliary beat (Omoto and Kung, 1980; Tamm and Horridge, 1970), suggesting that the orientation of the central pair apparatus may regulate dynein arm activity (Omoto and Kung, 1980; Warner and Satir, 1974). It is unclear, however, whether central pair rotation is a general property of all flagella (Tamm and Tamm, 1981). Given its position on the central pair apparatus, it is possible that Klp1 could function in the rotation or twisting of central pair microtubules. In either of these cases, it would appear necessary that Klp1 move or apply force radially between adjacent protofilaments of a central pair microtubule, rather than linearly along a single protofilament, as is the case for kinesin (Gelles et al., 1988; Kamimura and Mandelkow, 1992; Ray et al., 1993). This interpretation is consistent with the possibility that Klp1 is part of projections attached to C2. Interestingly, in an in vitro motility assay, one klp, the minus-end–directed motor ncd has already been shown to generate a torque that twists microtubules (Chandra et al., 1993; Walker et al., 1990).

A second 110-kD klp in *Chlamydomonas* (Johnson et al., 1994) has a localization pattern that is strikingly similar to Klp1, i.e., association with a single microtubule of the central pair apparatus. We have no direct evidence on whether Klp1 and this 110-kD protein bind to the same or to a different central pair microtubule. We did note, however, that in axonemal whole mounts Klp1 labeling was always observed on the microtubule oriented towards the inside of the curved central pair microtubules, while the labeling associated with the 110-kD protein was always oriented towards the outside of the central pair microtubules, suggesting that the proteins are on different microtubules. If both Klp1 and the 110-kD protein are required for central pair rotation or twisting, they could encode redundant or overlapping functions, or distinct functions that could be either synergistic or antagonistic. It will be interesting to determine how these klps move microtubules in vitro.

In the future, molecular cloning will identify the other klps present in the *Chlamydomonas* flagellum. These proteins may be associated with the central pair apparatus or they may be associated with the outer doublet microtubules, where they could be required for other dynein-independent movements within the flagellum. In particular, a number of movements have been described that are associated with the flagellar membrane, including gliding motility (Bloodgood, 1981; Lewin, 1952), bead movement (Bloodgood, 1977), tipping of glycoproteins during mating (Goodenough and Jurivich, 1978), and submembranous intraflagellar transport (Kozminski et al., 1993*b*). Additional members of the kinesin superfamily remain likely candidates for the motors that drive these processes.

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