

# Characterization of the KLP68D Kinesin-like Protein in *Drosophila*: Possible Roles in Axonal Transport

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**Abstract.** This paper describes the molecular and biochemical properties of KLP68D, a new kinesin-like motor protein in *Drosophila melanogaster*. Sequence analysis of a full-length cDNA encoding KLP68D demonstrates that this protein has a domain that shares significant sequence identity with the entire 340–amin acid kinesin heavy chain motor domain. Sequences extending beyond the motor domain predict a region of alpha-helical coiled-coil followed by a globular “tail” region; there is significant sequence similarity between the alpha-helical coiled-coil region of the KLP68D protein and similar regions of the KIF3 protein of mouse and the KRP85 protein of sea urchin. This

finding suggests that all three proteins may be members of the same family, and that they all perform related functions. KLP68D protein produced in *Escherichia coli* is, like kinesin itself, a plus-end directed microtubule motor. In situ hybridization analysis of KLP68D RNA in *Drosophila* embryos indicates that the KLP68D gene is expressed primarily in the central nervous system and in a subset of the peripheral nervous system during embryogenesis. Thus, KLP68D may be used for anterograde axonal transport and could conceivably move cargoes in fly neurons different than those moved by kinesin heavy chain or other plus-end directed motors.

**M**ECHANOCHEMICAL motor proteins participate in microtubule-based transport tasks such as chromosome movement and axonal transport. One class of microtubule-based motor proteins was first defined by kinesin heavy chain (KHC)<sup>1</sup> and it now includes more than 30 kinesin-like proteins (KLPs; reviewed in Goldstein, 1993). The diversity of the kinesin superfamily suggests that the execution of different microtubule-based tasks may rely on a specific assignment of motors. Thus, understanding any particular microtubule-based movement will require analysis of the particular motors involved.

Functional studies on the kinesin heavy chain in *Drosophila* suggest that the complex processes of axonal transport might require the actions of multiple KLPs. In mutant animals lacking KHC, although larval axons are smaller than normal in size, there are no apparent defects in many processes that require active transport of materials down the axon; specifically, axonal extension, pathfinding, and syn-

apse formation (Gho, 1992). EM analysis revealed that visible synaptic vesicles were present and at apparently normal levels at the nerve terminus. Thus, kinesin alone is not likely to be responsible for all anterograde transport movements, but it might work in concert with other anterograde motors within the axon. Indeed, mutants with defects in the *unc-104* KLP in *Caenorhabditis elegans* fail to transport synaptic vesicles or their components to synapses (Hall and Hedgecock, 1991). Additional candidates for such neuronal motors include the KIF1, KIF3, and KIF5 proteins of *Mus musculus* (Aizawa et al., 1992; Kondo et al., 1994).

This paper describes the properties of KLP68D, a new kinesin-like motor in *Drosophila melanogaster*. Sequence analysis of a full-length cDNA encoding KLP68D suggests that while it is not a true homologue, KLP68D is most closely related to two previously identified kinesin-like proteins, KIF3 and KRP85 of mouse and sea urchin, respectively (Aizawa et al., 1992; Kondo et al., 1994; Cole et al., 1993). Furthermore, KLP68D is a plus end–directed microtubule motor that is expressed exclusively in the central nervous system (CNS) and in a subset of the peripheral nervous system (PNS) during embryogenesis. The analysis of KLP68D suggests that KLP68D, KIF3, and KRP85 are members of the same evolutionarily conserved family of motors that may be used for anterograde axonal transport, and could conceivably move cargoes different than those moved by KHC or by other plus end–directed motors.

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1. *Abbreviations used in this paper:* CNS, central nervous system; GST, glutathione-S-transferase; KHC, kinesin heavy chain; KLP, kinesin-like protein; PBT, PBS containing 0.1% Tween 20; PNS, peripheral nervous system.

## Materials and Methods

### Cloning and Sequencing

Using a 111-bp PCR-amplified KLP68D fragment as a probe (previously called KLP5; Stewart et al., 1991), three clones were isolated from a *Drosophila* 0–4-h embryonic cDNA library cloned in the vector pNB40 (Brown and Kafatos, 1988). By restriction analysis, all three clones appeared to be identical and to contain a 3-kb insert. A HindIII fragment from one cDNA clone, which extended from within pNB40 at the 5' end of the gene to basepair 2,920 in the 3' untranslated region of KLP68D, was subcloned into the Bluescript-SK plasmid (Stratagene, La Jolla, CA) to generate pBS68D. A series of nested deletions was created in both directions in the pBS68D cDNA insert using an ExoIII digestion kit according to directions provided by the manufacturer (Promega Corp., Madison, WI). Overlapping clones from both strands were sequenced using either the T7 or T3 primers, which flanked the insert in pBS68D. The remaining sequence, which extended from basepair 2920 through the polyA region of KLP68D, was sequenced from the pNB40-KLP68D cDNA insert using either (a) the T7 primer, which lies in the pNB40 vector at the 3' end of the KLP68D cDNA (3' to 5' direction); or (b) an internal primer synthesized in our laboratory (5' to 3' direction). Sequencing was done according to directions provided with a Sequenase kit (Stratagene).

### In Situ Hybridization

In situ hybridization to embryonic mRNA was done according to Tautz and Pfeifle (1989) with modifications as follows:

**Fixation.** After devitelination, embryos were transferred into 100% EtOH and then treated with a mixture of 1:1 ethanol/xylene for 5 min. Embryos were then soaked in xylene for 2 h. Another 5 min rinse in ethanol/xylene was followed by dehydration in 100% EtOH, which was then replaced with 100% MeOH. Embryos were hydrated into PBS containing 0.1% Tween 20 (PBT) through a series of graded MeOH/PBT steps: (9:1, 7:3, 5:5, 3:7, and 1:9). Proteinase K treatment (0.05 mg/ml in PBT for 4 min) was followed by several quick washes in PBT and a 5% formaldehyde treatment in PBT for 25 min.

**Hybridization.** Embryos were washed with several changes of PBT before a treatment with 1:1 PBT/hybridization solution (50% formamide, 5× SSC, 50 mg/ml heparin, 0.1 mg/ml sonicated salmon sperm DNA, and 0.1% Tween-20). Embryos were then placed into hybridization solution at 55°C. After 2 h, most of the hybridization solution was removed, and probe at a concentration of ~20 ng/ml was added. Embryos were hybridized with probe for ≥30 h before washes and detection.

**Preparation of KLP68D RNA Probe.** A kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to synthesize the digoxigenin-labeled UTP riboprobe, following the instructions provided with the kit. Anti-sense dig-UTP labeled riboprobe was transcribed from a ScaI linearized KLP68D cDNA insert in the pBS-SK vector (pBS68D) using T3 RNA polymerase. Probe was reduced in size by a treatment in 0.1 M sodium carbonate (pH 10.2), neutralized with 1/20 vol glacial acetic acid, and then EtOH precipitated before being added to hybridization solution. Sense KLP68D dig-UTP riboprobe (also using ScaI-linearized template but with T7 RNA polymerase) was used as a control for nonspecific hybridization (results not shown). For in vitro translation, RNA was prepared essentially in the same manner, but using unlabeled nucleotides. In vitro translation was done with a commercially available kit (Promega Corp.), following the instructions provided by the manufacturer.

**Probe Detection.** After probe incubation, embryos were washed in fresh hybridization solution ≥4 h at 55°C. Embryos were then brought to room temperature and put through a series of graded hybridization/PBT solutions (7:3, 5:5, and 3:7) before being washed several times in PBT alone. Anti-digoxigenin antibody was preabsorbed for 4 h by incubating with fixed, unprobed embryos, and then was added at a final concentration of 1:2,000. Developing solutions for alkaline phosphatase-conjugated antibody were provided with the kit and used at suggested concentrations.

**Developmental Analysis.** Embryos were staged according to Campos-Ortega and Hartenstein (1985). Observation was on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY); photographs were taken on Tech Pan or TMax (Eastman Kodak Co., Rochester, NY). Negatives were transferred to Photo CD and adjusted for brightness, contrast, and resolution in Adobe Photoshop on a Macintosh computer. Montages were prepared in Canvas (Software, Miami, FL) and printed on a Tektronix (Portland, OR) Phaser IISDX with a CMYK ribbon.

### Expression Constructs

Full-length KLP68D and chosen gene fragments were subcloned into either pGEX-KS (Pharmacia Fine Chemicals, Piscataway, NJ) or pRSET (Invitrogen, San Diego, CA) expression constructs. The full-length coding region, from a unique BspEI site four amino acids upstream from the start methionine and extending to the 3' HindIII site (both cut and rendered flush with Klenow) described in the pBS68D construct, was cloned in frame with the glutathione-S-transferase (GST) gene in pGEX by insertion into the HindIII site of the vector (also rendered blunt with Klenow). This cloning manipulation added amino acids G-Y-L-E at the 5' end of KLP68D. Excision of the fragment between basepair 1,880 and the end of the gene resulted in the pGEX-532 construct, which lacks sequences encoding the carboxyl-terminal 252 amino acids.

Two constructs of KLP68D lacking the motor region were subcloned in frame with gene 10 in the pRSET vector: pRSET-432 contains an XbaI fragment of KLP68D extending from amino acids 352 to 784, which includes all predicted alpha-helical coiled-coil regions and extends through the end of the gene. pRSET-138 contains the terminal portion of KLP68D extending from a BamHI site at amino acid 646.

### Expression of KLP68D Proteins and Protein Purification

**pGEX-784 and pGEX-532.** pGEX fusion constructs were transformed into either DH5- $\alpha$  or LE392 bacterial cells. Expression of both pGEX fusion proteins is induced by the addition of 0.1–0.5 mM isopropyl- $\beta$ -thiogalactopyranoside to log-phase growth cells according to directions provided with the vector, with the exceptions of fusion proteins used for motility studies (pGEX-784 and 532), which were induced at 25°C for 5 h before harvesting. Cells were harvested by centrifugation (Sorvall, Wilmington, DE) at SS34, 5,000 g for min at 4°C. After weighting the cell pellet, 1 g of cells was brought up into 2 ml of lysis buffer (20 mM PO<sub>4</sub>, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.1% Triton-X 100) at 4°C. Cells were sonicated in 2-ml aliquots on ice for 30 s total, using a microtip attachment. Lysates were cleared for 10 min in a microfuge at 4°C at the highest speed setting. 1 ml of this supernatant was incubated with 20  $\mu$ l of glutathione-coupled beads at 4°C for 30 min before elution. After rinsing protein-coupled beads three times in 4°C buffer (20 mM PO<sub>4</sub>, 100 mM NaCl, and 0.5 mM PMSF), glutathione added to a final concentration of 5 mM in the same buffer, and the beads were discarded. Recovery was monitored by SDS-PAGE electrophoresis and Western blotting; protein for motility studies was used immediately.

**pRSET-432 and pRSET-138.** pRSET constructs were transformed into BL21-DE3 cells (Novagen Inc., Madison, WI). pRSET-432 fusion protein, used for antibody generation, was induced in cells at 37°C for 1 h with 1 mM isopropyl- $\beta$ -thiogalactopyranoside. Induction from pRSET plasmid constructs results in fusion proteins consisting of gene 10 of phage T7 fused to the KLP68D peptide fragment. The resulting fusion proteins were purified via their polyhistidine metal-binding domain by immobilized metal affinity chromatography (nickel-binding column; QIAGEN Inc., Chatsworth, CA) following the manufacturer's instructions. Briefly, pRSET-432 cells were harvested by centrifugation, and were then resuspended in 6 M guanidine-HCl, pH 8. Debris was removed with a low speed spin (3,000 g for 15 min), and then the cell lysate was passed over a nickel column and rinsed with 8 M urea, 0.5 M NaCl, and 0.05 M phosphate, pH 8. Recombinant protein was then eluted in the same solution by using a pH gradient down to pH 4.

### Antisera

Purified pRSET-432 as described above was run on an 8% SDS-PAGE "curtain" gel. The recombinant protein was excised and ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was emulsified into Freund's adjuvant solution by repeated runs through a 20-gauge needle. Three rabbits were each injected with 1 ml of a solution containing ~0.3 mg of pRSET-432 fusion protein. Two further inoculations in each rabbit were separated by 2-wk periods, and after this time, rabbit sera was tested for its reaction to KLP68D proteins. Preimmune sera were obtained for use as controls.

### Motility

Motility assays were done according to the "antibody sandwich" method described by Stewart et al. (1993). Marked rhodamine labeled microtubules

were made by the method of Hyman (1991). Videoprints were scanned at 200 dpi, and were then processed in the same manner as the in situ hybridization micrographs (see above).

## Results

The KLP68D gene (previously called KLP5) was originally discovered using the polymerase chain reaction to amplify a region encoding a segment of the motor domain conserved among all known kinesin-like proteins (Stewart et al., 1991). Because Northern analysis suggested that KLP68D mRNA might be present in neuronal tissues, we considered KLP68D to be a strong candidate for a motor used in neuronal function or development. To begin testing these ideas, we isolated and analyzed KLP68D cDNA clones.

### Sequence Analysis

The nucleotide sequence of the KLP68D cDNA (pNB40-KLP68D) has a total length of 3,005 bp (Fig. 1). This is in reasonable agreement with the estimated length of the KLP68D transcript observed in Northern analysis (Stewart

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CACTTTGGAGGAGCAAGTTACAGAGAGCTGAGACAAAGAGGATTCAGGTTAGTAAATGGCTTCAAAAATAGAGCTACTGAGC 5
TTGCTTTTGTGTTAGGGAGGAGCTGCTCATCAGCAGACAGAAAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG 95
ATGATGTCAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG 185
N S A K F S R R P F G T G S S Q T F M E C V Q V V V R C R F P S
1
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N H G E S E R S P E V Y N W V F R M R G V R E L Q R N V Y G C N
61
AAGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG 455
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91
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L V N K
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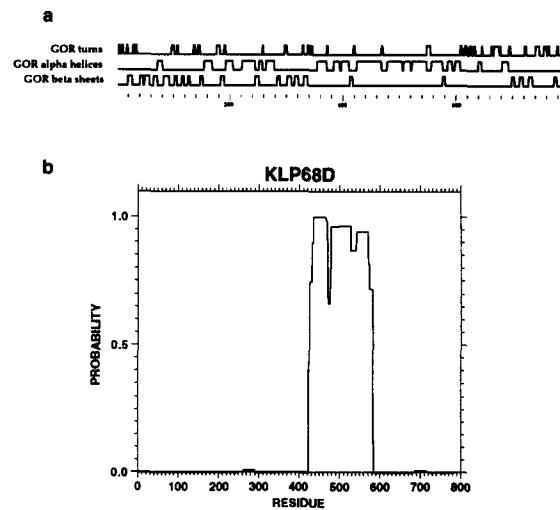
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**Figure 1.** Nucleotide and deduced amino acid sequences of KLP68D. Numbers at right correspond to nucleotides and at left to amino acids. A P-loop consensus sequence (part of the ATP-binding pocket; Saraste et al., 1990) is shown in bold, and the region predicted to form a coiled-coil structure is underlined (Lupas et al., 1991). The in-frame stop codon is denoted by an asterisk. Amino acids 19–338 were previously published as part of a multiple sequence alignment in Goldstein (1993). The nucleotide sequence data are available from EMBL/Genbank under accession No. U15974.

et al., 1991). The ATG chosen as the start of the KLP68D translated region encodes the first methionine within the longest open reading frame and is also preceded within the 5' UT by stop codons in all three frames. Starting from this methionine, a single open reading frame in KLP68D that contains 2,352 nucleotide pairs predicts a polypeptide that is 784 amino acids in length and 85 kD in size. Within the 3' UT region, a consensus polyadenylation signal starts 16 bp upstream from a poly(A) tract. In vitro translation of synthetic KLP68D RNA using a rabbit reticulocyte lysate system produces a protein whose mobility is in agreement with the predicted molecular weight (data not shown).

Analysis of the KLP68D sequence reveals that it is likely to be composed of three domains. First, the homology to the KHC motor region is included within the NH<sub>2</sub>-terminal 340 amino acids of the KLP68D protein (Yang, 1990). Amino acid identity shared between KLP68D and other KLPs, with the exceptions described below, is exclusively restricted to this region. In addition, multiple sequence analysis (data not shown) using the UWGCG program PILEUP (Devereux et al., 1984) indicates that the motor region of KLP68D is most closely related to the motor regions of KIF3 from mouse, KRP85, and a fragment of KRP95 from sea urchin (Aizawa et al., 1992; Cole et al., 1993).

A second domain of KLP68D, extending from amino acids 350 through 580, is predicted to be an almost unbroken stretch of alpha helix (Fig. 2 a). A structure prediction program designed to estimate the probability that a given sequence will form an alpha-helical coiled-coil structure (Lupas et al., 1991) predicts that within this second domain, KLP68D has a high probability of forming a coiled-coil structure from residues 424 through 583 (Fig. 2 b). Thus,



**Figure 2.** Secondary structure predictions for the KLP68D protein. (a) The secondary structure of KLP68D as predicted by the method of Garnier (GOR) or Chou-Fasman (CF) contains a region of virtually unbroken alpha helix extending from amino acids 350 to 610. Regions predicted to be alpha helices, beta sheets, or beta turns are indicated by elevated lines. Graphic representation is a Plotstructure output of the Peptidestructure program as implemented in the UWGCG system (Devereux et al., 1984). (b) The probability that each residue of KLP68D will participate in a coiled-coil structure is represented in a bar graph, as calculated by the algorithm of Lupas et al. (1991).

native KLP68D, like KHC (de Cuevas et al., 1992), may form a dimer via an alpha-helical coiled-coil interaction.

Although many known KLPs contain a predicted coiled-coil region, such regions, except those that are similar among homologues, vary greatly in both size and sequence. KLP68D is a notable exception to this observation since it shares an atypically high sequence similarity with a comparable region of both mouse KIF3 and sea urchin KRP85. This is best illustrated by dot matrix analysis. When KHC is compared to KLP68D (Fig. 3 *a*) no obvious similarity beyond the first ~370 amino acids, i.e., the predicted motor domains, is seen. Instead, a scatter pattern typical of comparisons between unrelated alpha-helical coiled-coil regions is apparent. This same pattern is found in alignments of KLP68D with other KLPs, with the exception of the comparisons between KLP68D and KIF3 (Fig. 3 *b*) and KLP68D and KRP85 (Fig. 3 *c*). A stretch of similarity beyond the motor domain falls within a region predicted to form a coiled-coil structure in all three molecules. Close inspection of the predictions for all three proteins indicates that KIF3 and KRP85 are predicted to form two coiled-coil regions. In KRP85, the first region runs from amino acids 353–389, and it is then followed by a region containing numerous glycines and a proline. This likely “break or hinge” region is then succeeded by a second coiled-coil region running from amino acids 415–594 (Cole et al., 1993). A comparable pattern is seen in KIF3 (Goldstein, L.S.B., unpublished observations). It is this second and longer coiled-coil region whose sequence is similar to the coiled-coil region of KLP68D. Strikingly, the predicted length of this second coiled-coil region in KLP68D, KIF3, and KRP85 is very similar (Cole et al., 1993; and data not shown), which is also an atypical feature of known KLPs.

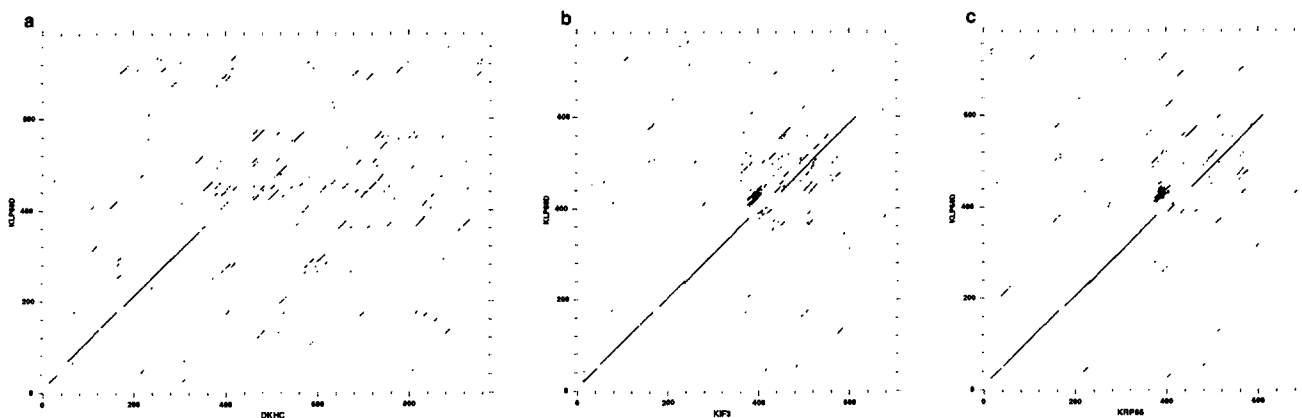
The third structural domain of KLP68D (beyond the predicted coiled-coil region) is predicted to encode a globular tail domain, whose most notable characteristic is that it is highly basic (pI = 11). Basic tail regions with disparate sequences have been found in KHC and many other members of the superfamily. While such regions have no demonstrated function at present, it has been suggested that these regions

mediate interactions with other proteins or cargoes (reviewed in Goldstein, 1993) or microsomal membranes (Skoufias et al., 1994). BLAST searches (Altschul et al., 1991) of the EMBL and Genbank sequence databases with the KLP68D tail sequence revealed no significant similarity to any other known proteins. In addition, direct comparison of the tail regions of KLP68D with those of KRP85 and KIF3 revealed no obvious sequence similarity, although the tail regions of KRP85 and KIF3 are substantially similar in sequence to each other (65% identical over 108 amino positions; data not shown).

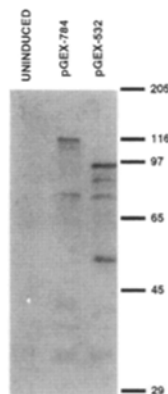
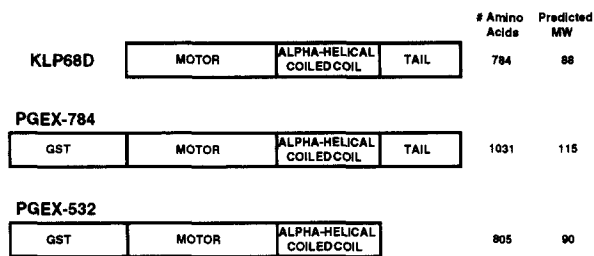
### Motility

To determine whether KLP68D is a microtubule-based motor and to define its direction of movement, we constructed two expression plasmids encoding the predicted motor region of KLP68D. Constructs consisting of full-length KLP68D (pGEX-784), or a protein missing the carboxyl terminal 252 amino acids of KLP68D (pGEX-532) were expressed as polypeptides fused to the carboxyl terminus of the GST protein (Guan and Dixon, 1991). Upon induction, pGEX-784 expresses a fusion product of 115 kD and pGEX-532 expresses a product of 85 kD; both react with anti-KLP68D antisera (Fig. 4). Both proteins were purified from crude bacterial lysates on immobilized glutathione for motility studies.

We tested the ability of the KLP68D protein to move purified bovine brain microtubules in an *in vitro* motility assay (Schnapp, 1986) using the “antibody sandwich” technique (Stewart et al., 1993). Glass surfaces coated with either of the GST-KLP68D fusion proteins supported microtubule gliding (Fig. 5, *a–c*). The motor activity, like that of KHC, is MgATP dependent. The rate of microtubule movement for either fusion protein was ~0.3 μm/s. In control experiments using expressed GST protein alone, no microtubules were found bound to the glass surface. Since fusion proteins encoded by both pGEX-786 and pGEX-532 are microtubule motors, the motor capacity resides, as predicted, within at least the first 532 amino acids of the

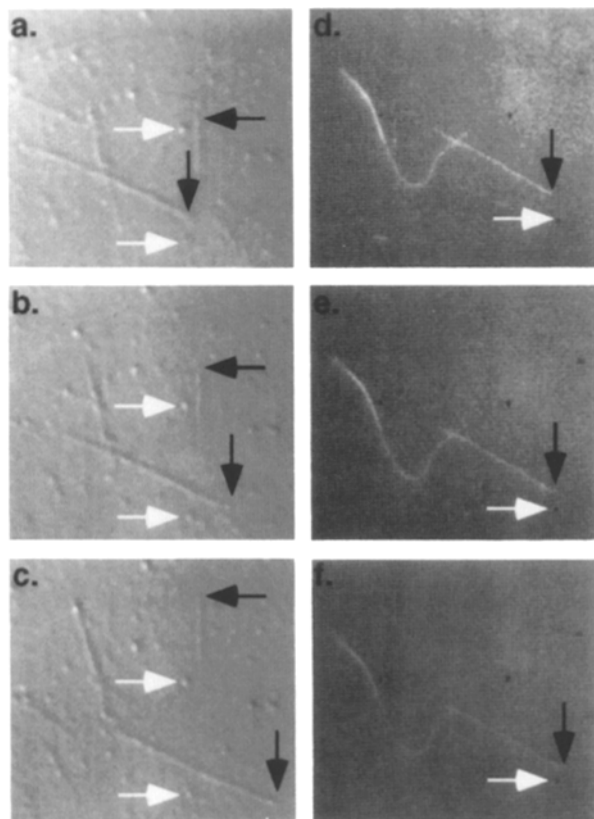


**Figure 3.** Comparison of KLP68D to other kinesin-like proteins. Dot matrix sequence comparisons of KLP68D with KHC (*a*), KIF3 (*b*), and KRP85 (*c*). All three comparisons reveal a large amount of sequence similarity within the NH<sub>2</sub>-terminal motor domains (extending from approximately amino acids 1 to 370 in each comparison). In *b* and *c*, a second stretch of similarity occurs in the tail domains of KLP68D, KIF3, and KRP85, including the regions predicted to form alpha-helical coiled-coils. Comparisons were done using the UWGCG program COMPARE (window = 30, stringency = 15), and were then plotted using DOTPLOT (Devereux et al., 1984).



**Figure 4.** KLP68D protein expression. Two KLP68D constructs in the pGEX vector (Pharmacia Fine Chemicals) encoding GST fused to the full-length coding sequence (pGEX-784), or the predicted motor and stalk regions alone (pGEX-532), were used to produce KLP68D protein for motility studies. At the right is a Western blot of *E. coli* lysates containing the proteins produced; the blots are probed with an anti-KLP68D polyclonal antibody.

KLP68D protein. One other kinesin-like protein (*ncd*) was previously found to bundle (cross-link) microtubules in this assay; the second microtubule-binding site appeared to be in the tail region of that protein (McDonald et al., 1990). pGEX-784 protein does not appear to share this property, nor is there any perceptible difference in the rate or behavior of purified microtubules moved by the pGEX-532 “tail-less” construct.



**Figure 5.** Microtubule-based movement in the presence of KLP68D protein. (a–c) KLP68D protein expressed and purified from pGEX-784 (or pGEX-532) will support microtubule gliding. Micrographs were taken at 1, 55, and 120 s. The black arrows are placed at the ends of two moving microtubules; the white arrows mark immobile particles stuck to the glass. (d–f) A rhodamine-labeled microtubule moving in the presence of KLP68D protein with the brightly labeled minus end (denoted by black arrow) leading; immobile particles are marked with white arrow.

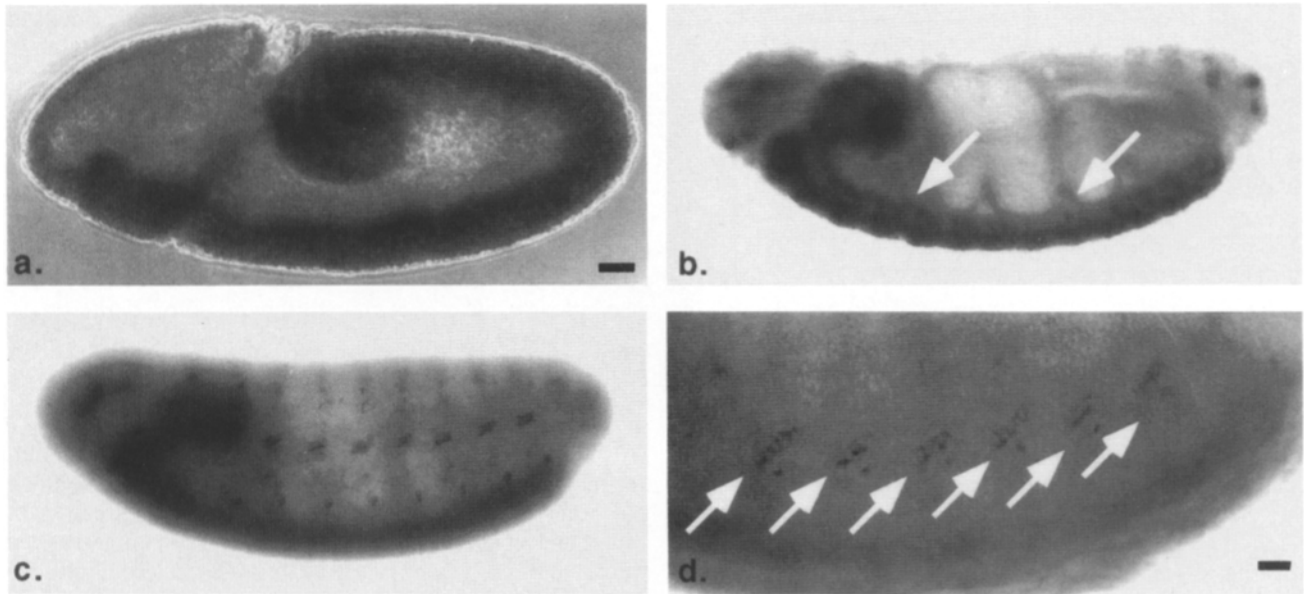
Understanding the function of any microtubule-based motor requires knowing the direction of force production along the polarized microtubule. We analyzed the direction of movement of the KLP68D motor using rhodamine-labeled microtubules (Hyman, 1990). In this assay, microtubules are polymerized from a brightly labeled “seed” segment of rhodamine labeled tubulin. Polymerization, which occurs more rapidly from the plus end, is then allowed to continue in the presence of a mixture of labeled and unlabeled tubulin. The net result is a mostly dim microtubule with a brighter segment near or at the minus end. KLP68D moved these microtubules with their bright ends (minus) leading and their plus ends trailing (Fig. 5, d–f). Based on this analysis, we conclude that KLP68D is a plus end-directed motor.

#### **KLP68D RNA Expression in *Drosophila* Embryos**

To probe the biological role of KLP68D, we examined its pattern of expression. We previously found that a single 3,100-nucleotide KLP68D transcript was present in ovaries, testes, heads, and the S2M3 embryonic cultured cell line (Stewart et al., 1991). Because the tissues examined contained multiple cell types, this analysis did not reveal if expression of KLP68D is limited to any specific developmental stage or cell type. This issue was particularly intriguing because we had previously observed that KHC mRNA expression was virtually ubiquitous during embryogenesis (Pesavento, P. A., and L. S. B. Goldstein, unpublished observations). To analyze spatial and temporal expression of KLP68D during early development, in situ hybridizations were performed on 0–16-h *Drosophila* embryos using anti-sense KLP68D RNA as a probe.

Maternal KLP68D transcript is ubiquitous at fertilization and apparently disappears during cellularization (stage 4) so that it is no longer visible by the beginning of stage 5. Presumed zygotic KLP68D mRNA first appears throughout the presumptive ectodermal layer during gastrulation movements (stage 8, Fig. 6 a), but it becomes restricted to segmental clusters of cells as germ band extension ends in stage 11. Expression during germ band retraction and in all later stages of embryogenesis is restricted to cells of the nervous system.

In the CNS KLP68D transcript is best visualized in late stages, where it is contained within the condensed ventral nerve chord and the brain hemispheres (stage 16, Fig. 6 b). Because of the signal intensity in these regions, it is difficult to assess whether expression is restricted to neurons or in-



**Figure 6.** KLP68D RNA localization visualized in developing embryos by in situ hybridization analysis. Embryos are staged according to Campos-Ortega and Hartenstein (1985); anterior is to the left and ventral is down. Presumed maternal KLP68D RNA is ubiquitously present in the syncytial embryo until the onset of cellularization (stage 4). At stage 4, staining is last visible in the cortex and then disappears completely by the end of cellularization. Zygotic RNA is first seen in stage 8 embryos (*a*), where it appears to be confined to the outer, ectodermal layer of cells (ventral, posterior, and dorsal surfaces). CNS staining persists through germ band retraction, and is best seen in later embryos in the condensed ventral nerve chord and cephalic lobes (*b*, stage 16; note intense staining in ventral region, *arrows*, that then turns dorsally near the anterior end); *c* shows the same embryo that appears in *b*, but with a lateral focal plane. The row of cell clusters appearing in the midlateral region are neurons of the chordotonal organs. These neurons have a characteristic pattern within both the abdominal and thoracic segments. In the abdominal segments, one neuron from each cluster is offset laterally (*d* is a higher magnification view of *c*, *arrows* mark clusters of chordotonal neurons). KLP68D is also expressed in a row of ventrolateral cells that are probably ventral sensilla, and in small patches within the head and tail (*b* and *c*). We were not able to determine whether these patches correspond to sensory organs that are present in these regions. Bars, 50  $\mu\text{m}$  in *a-c*; 25  $\mu\text{m}$  in *d*.

cludes support cells of the central nervous system. KLP68D expression within cells of the PNS, however, is more easily interpreted.

While cells of the CNS are highly condensed in the later stages of embryogenesis, cells of the PNS are relatively sparse and predictably simple in their pattern. For example, beginning at stage 14, each abdominal segment has a lateral cluster of neurons with a characteristic pattern of one chordotonal neuron set apart from a group of four other chordotonal neurons (Ghysen et al., 1986). KLP68D expression appears in these neuronal cells of the chordotonal organs (Fig. 6, *c* and *d*), but not apparently in other cells of the PNS. KLP68D RNA contained within the thoracic segments also reflects the pattern of the chordotonal neurons in this region. Although the chordotonal organs begin differentiation as early as stage 11 (Hartenstein, 1988), KLP68D expression does not appear in these sensilla cells until stage 14. Thus, in the chordotonal neurons, KLP68D expression does not appear to correlate with cell differentiation. It does, however, correspond to the onset of axonogenesis in these neurons (Giniger et al., 1993). Other cells that appear to contain KLP68D RNA (Fig. 6 *c*) are single lines of segmentally repeated ventrolateral neurons that correspond in their position to either the chordotonal or external sensory neurons of the ventral cluster (Ghysen et al., 1986). We have attempted to localize the KLP68D polypeptide in these cells using specific antisera, thus far without success (Pesavento, P. A., and L. S. B. Goldstein, unpublished observations).

## Discussion

In this paper, we report the analysis of the sequence, motility, and expression of KLP68D. Our findings permit us to draw several conclusions about the potential functions of this new motor protein.

Our sequence analysis suggests that *Drosophila* KLP68D, mouse KIF3 (Aizawa et al., 1992), sea urchin KRP85, and sea urchin KRP95 (Cole et al., 1993) are all likely to be members of the same evolutionarily conserved family of kinesin-like motors within the kinesin superfamily. This conclusion initially derives from the multiple sequence analysis, which demonstrates that the motor domains of these proteins are all more similar to each other than they are to other members of the kinesin superfamily. In addition, although no sequence outside of the motor has yet been reported for KRP95, the predicted alpha-helical coiled-coil region of KLP68D shows significant similarity in sequence to comparable regions of KIF3 and KRP85. This finding is particularly striking since, with the exception of members of the kinesin heavy chain family (and possibly some short stretches of conserved sequence in the tail domains of members of the bimC family; Heck et al., 1993), no long stretches of sequence similarity have yet been seen outside of the motor regions among different kinesin-like proteins (reviewed in Goldstein, 1993).

Based on our analysis, we suggest that KIF3 and KRP85 are true homologues of each other. Furthermore, while



KLP68D is likely to be a member of this family of proteins, we believe that it is not actually a homologue of KIF3 and KRP85. There are three reasons for this latter point of view: (a) Although KLP68D, KIF3, and KRP85 all share significant sequence similarity in a region predicted to form alpha-helical coiled-coil region, KIF3 and KRP85 share an additional short region predicted to form alpha-helical coiled-coil immediately adjacent to the conserved motor region; KLP68D lacks this region. (b) KIF3 and KRP85 share similar globular tail region sequences; KLP68D has a longer tail sequence whose sequence is different. (c) Recent analyses of KLP64D in *Drosophila* (Perez, S., L. Goldstein, and H. Steller, manuscript in preparation) indicates that KLP64D shares a tail sequence with KIF3 and KRP85 that is not shared with KLP68D; hence, KLP64D may be the *Drosophila* homologue of KRP85 and KIF3.

The shared nonmotor sequences raise the possibility that members of the KIF3/KLP68D family interact with the same classes of cargo in vivo. Alternatively, the conserved sequences could be used to form interactions with other kinesin-like proteins rather than with cargo per se. This suggestion arises from recent observations in several different systems. (a) KRP85/95 copurify from sea urchin eggs along with a third, as yet uncharacterized, protein (Cole et al., 1993). (b) KIF3 from mouse also appears to copurify as a protein complex containing two, possibly different, kinesin-like proteins (Kondo et al., 1994). (c) Recent sequence analysis of KLP64D from *Drosophila* indicates that it, too, is a member of the KIF3/KLP68D family that possesses a region with significant sequence similarity to the alpha-helical coiled-coil region of KLP68D (Perez et al., manuscript in preparation). The finding of two such proteins with similar alpha-helical coiled-coil tail sequences in one organism, the fly, raises the possibility that KLP64D and KLP68D (and perhaps other family members) might form heterodimers through a coiled-coil interaction similar to that which is found in the leucine zipper class of transcription factors (Landshulz et al., 1988). A similar suggestion was made by Cole et al. (1993), who suggested that KRP85 might dimerize with KRP95 through an alpha-helical coiled-coil interaction. This property might generate additional functional diversity of kinesin-like motors through mix-and-match mechanisms. The prediction is that sea urchin KRP95, for which only partial sequence data are available, will have a domain with sequences very similar to the alpha-helical coiled-coil domain of KRP85, and that the association with KRP85 will be through a parallel coiled-coil interaction. We also predict that the mouse will have multiple members of this family with shared nonmotor sequences.

Another important question is whether the obvious evolutionary conservation of protein structure in this family is accompanied by a corresponding conservation of function. At present, our information about the likely functions of KLP68D comes from biochemical and expression analysis. This analysis indicates that KLP68D is expressed primarily in the developing ventral nerve chord, cephalic lobes, and a subpopulation of cells within the peripheral nervous system. These tissues contain KLP68D RNA during a time in which both axonal and dendritic outgrowth are occurring and in which neuronal synapses are defined. These data, in combination with the motility data showing that KLP68D is a plus end-directed microtubule motor, suggest that KLP68D

could function in anterograde axonal transport in the cells in which it is expressed. Mouse KIF3 is also a plus end-directed microtubule motor expressed most prominently in brain, which also accumulates on the proximal side of sites of axonal ligation (Kondo et al., 1994). These data are consistent with KIF3 having a function in anterograde axonal transport. Information about the expression of KRP85/95 is still lacking, although it is clearly expressed in early sea urchin embryos, similar to KLP68D expression in early fly embryos. Biochemical analysis of KRP85/95 reveals that it also is a plus end-directed microtubule motor, perhaps one with a microtubule-bundling activity (Cole et al., 1992). This latter activity has not been seen with KLP68D or KIF3, but, at least in the case of KLP68D, the protein was expressed in a heterologous system so that normal associated proteins are not present. Taken together, however, the data suggest that this conserved family of kinesin-like motors with similar structures may all have roles in anterograde axonal transport, and perhaps additional roles in the early embryo.

The likelihood that members of the KIF3/KLP68D family are anterograde axonal transporters fits well with recent work on mutations in the genes encoding kinesin heavy chain and *unc-104*. In *Drosophila* and *Caenorhabditis*, kinesin heavy chain is not obviously required for the movement of synaptic vesicle components in axons (Gho et al., 1992; Hall, D., J. Plenefisch, and E. Hedgecock. 1991. *J. Cell Biol.* 115:389a.). In *C. elegans*, however, *unc-104* is required for axonal transport of some synaptic vesicle components, but not obviously for other axonal materials (Hall and Hedgecock, 1991). Thus, if KLP68D and other members of the KIF3 family are axonal transport motors as suggested, they might act along with other KLPs in addition to KHC. In fact, the data suggest that three different families (KHC, KIF3, and *unc-104*) of evolutionarily conserved kinesin-like motors may exist and function in axonal transport. This raises the question of why there might be so many families of motors carrying out such an apparently simple process. One possibility is that each motor translocates a different class of cargo to the nerve terminus. Alternatively, each motor might be specialized for exclusive or preferred use in particular cell types within the nervous system. Finally, it is possible that these various motors each participate in the movement of all or overlapping types of cargo, in which case they might be redundant. Definitive resolution of these issues awaits further genetic and biochemical analysis.

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