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A "Slow" Homotetrameric Kinesin-related Motor Protein Purified from *Drosophila* Embryos*

Douglas G. Cole[‡,§], **William M. SaxtonIII**[¶,‖], **Kathy B. Sheehan**[¶], and **Jonathan M. Scholey**[‡,**]
[‡]Section of Molecular and Cellular Biology, University of California, Davis, California 95616

[¶]Department of Biology, Institute for Molecular and Cellular Biology, Indiana University, Bloomington, Indiana 47405

Abstract

Pan-kinesin peptide antibodies were used to identify and isolate kinesin-related proteins (KRPs) from *Drosophila melanogaster* embryonic cytosol. These KRPs cosedimented with microtubules (MTs) polymerized from cytosol treated with AMP-PNP (adenyl-5′-yl imidodiphosphate), and one of them, KRP_{130}, was further purified from ATP eluates of the embryonic MTs. Purified KRP_{130} behaves as a homotetrameric complex composed of four 130-kDa polypeptide subunits which displays a "slow" plus-end directed motor activity capable of moving single MTs at 0.04 ± 0.01 μm/s. The 130-kDa subunit of KRP_{130} was tested for reactivity with monoclonal and polyclonal antibodies that are specific for various members of the kinesin superfamily. Results indicate that the KRP_{130} subunit is related to *Xenopus* Eg5 (Sawin, K. E., Le Guellec, K. L., Philippe, M., Mitchinson, T. J. (1992) *Nature* 359, 540-543), a member of the BimC subfamily of kinesins. Therefore, KRP_{130} appears to be the first *Drosophila* KRP, and the first member of the BimC subfamily in any organism, to be purified from native a multimeric motor complex.

Kinesin and kinesin-related proteins (KRPs)[1] comprise a family of motor proteins that play important and diverse roles in intracellular organelle transport and cell division (1-3). Kinesin was first purified from neural tissues (4, 5) and mitotic cells (6) using biochemical methods and was subsequently shown to be an asymmetric heterotetrameric complex consisting of two 110-130-kDa heavy chains (KHCs) and two 55-85-kDa light chains (7,8). At one end of the kinesin molecule, the KHCs form two globular NH_2-terminal "motor domains" (9-11) capable of ATP-dependent MT gliding activity coupled to MT-activated ATP hydrolysis (12, 13). The heavy chains are dimerized via an α-helical coiled-coil region (14). The light chains and the COOH-terminal domains of the heavy chains are found at the other end of the molecule (9). Although the role of the light chains has not been demonstrated, the carboxyl-terminal domain of the KHC is believed to be responsible for the attachment of membranous cargo to kinesin (15).

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[1]The abbreviations used are: KRP, kinesin-related protein; KHC, kinesin heavy chain; KLC, kinesin light chain; MAP, microtubule-associated protein; MT, microtubule; PC tubulin, phosphocellulose-chromatographed tubulin; PIPES, 1,4-piperazine diethanesulfonic acid; AMP-PNP, adenyl-5′-yl imidodiphosphate; GMP-CPP, α,β-methylene guanosine 5′-triphosphate.

Numerous KRPs have been identified at the nucleic acid level using genetic techniques or the polymerase chain reaction (2, 3). To complement these strategies, we prepared and used pan-kinesin antibodies against conserved kinesin motor domain peptides fort he purpose of identifying native kinesins in their natural tissues (16). A screen of sea urchin egg cytosol resulted in the purification of the first native KRP, sea urchin egg $KRP_{85/95}$, a plus-end-directed, heterotrimeric motor complex composed of 85- and 95-kDa kinesin-related polypeptides plus an uncharacterized 115-kDa subunit (16, 17).

The fruit fly *Drosophila melanogaster* has proven to be a particularly rewarding system for studying kinesins. *Drosophila* kinesin was first isolated biochemically (18) leading to the cloning and molecular analysis of the KHC gene and its expressed product (10, 12, 19). Subsequently, severafly KRPs have been characterized using genetic approach(20-25), but no KRP has thus far been purified from native fruit fly tissue. In an effort to biochemically identify and purify native *Drosophila* KRP complexes, embryonic extracts were probed with the pan-kinesin peptide antibodies. We present here the purification and characterization of one of these kinesins, KRP_{130}, a 490-kDa homotetrameric complex consisting of four 130-kDa subunits.

MATERIALS AND METHODS

Protein Preparation

Bovine phosphocellulose-chromatographed tubulin (PC tubulin) was prepared as described previously(26) and stored in 1 mM MgGTP in PEM buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, and 2 mM dithiothreitol) in liquid N_2 or at –80 °C until needed. Preparative MTs were formed by incubating PC tubulin in 1 mM MgGTP and 20 μM taxol (Sigma) at 37 °C for 30 min. Motility assay MTs were prepared by incubating 200 μg/ml PC tubulin in 1 mM MgGTP, 10 μM taxol at 37 °C for 60 min.

Drosophila cytosolic low speed supernatant was prepared from 0–24-h embryos as described previously (18) using PMEG buffer (100 mM PIPES, pH 6.9, 5 mM EGTA, 0.5 mM EDTA, 2.5 mM $MgSO_4$, 0.9 M glycerol, and 1 mM dithiothreitol) with our standard protease inhibitor mixture (27) and stored at –80 °C. After thawing, fresh protease inhibitors were added to low speed supernatant prior to spinning at 175,000 × *g* for 45 min at 4 °C. The resulting high speed supernatant (100–150 ml) was supplemented with 1 mM GTP and 10 μM taxol, rocked for 15 min at 25 °C, then supplemented with 1 mM AMP-PNP and rocked for 20 min prior to spinning at 35,000 × *g* for 60 min at 10 °C. The MT pellet was washed with 10 ml of 10 mM EDTA in PEG (PMEG without $MgSO_4$) buffer at 4 °C prior to repelleting at 100,000 × *g* for 25 min at 4 °C. The washed MT pellet was eluted with 6 ml of 10 mM MgATP, 200 mM KCl in PMEG for 6–14 h at °C prior to repelleting at 200,000 × *g* for 20 min at 4 °C. The eluate (ATP MAPs) was concentrated to 3 ml with a Centriprep 30 (Amicon) and fractionated on a Bio-Gel A-1.5m (1.6 × 90 cm) or Bio-Gel A-5m (1.0 × 90 cm) column equilibrated with 100 μM ATP, 150 mM KCl in PMEG buffer. The fractions containing kinesin and KRP_{130} were separately pooled and concentrated (Centriprep 30) to 1.5 ml prior to 20-min incubations with taxol MTs (PC tubulin) in 2 mM AMP-PNP, and either 50 mM KCl (kinesin) or 125-200 mM KCl (KRP_{130}) in PMEG buffer at 25 °C. The MTs were respun (100,000 × *g*, 20 min), followed by release of kinesin and KRP_{130} from their respective pellets with 150 μl of 10 mM MgATP, 200 mM KCl in PMEG buffer for 30 min at 25 °C. The MTs were pelleted a final time (60,000 × *g*, 15 min), and the resulting kinesin and KRP_{130} supernatants were fractionated on linear 5-20% sucrose gradients in 100 μM ATP, 150 mM KCl in PMEG buffer formed by a piston-driven gradient former (Jule, Inc.) and spun at 300,000 × *g* for 8.5 h (4 °C).

SDS-polyacrylamide gel electrophoresis (28) and immunoblotting were done as described previously (29). The relative molecular mass of the KRP_{130} subunit was determined by using standard marker proteins; rabbit muscle myosin heavy chain (205 kDa), *Escherichia coli β*-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21.5 kDa).

Stoichiometry

The Stokes radius, R_S, was determined as described previously (16) for two different preparations of *Drosophila* kinesin and KRP_{130} using the Bio-Gel A-1.5 m and Bio-Gel A-5m columns and running buffers described above. Plots of R_S versus $-\log_{10} K_{av}^{1/2}$, where K_{av} = (elution volume – void volume)/(total volume – void volume) generated standard linear plots ($r^2 = 0.964$; $r^2 = 0.981$, where r is the correlation coefficient). The standard proteins and their Stokes radii included sea urchin egg kinesin(9.6 nm), sea urchin egg $KRP_{85/95}$ (7.9 nm, Ref. 16), yeast alcohol dehydrogenase (4.6 nm), bovine serum albumin (3.5 nm), and bovine heart cytochrome *c* (1.7 nm).

The sedimentation coefficient $S_{20,W}$ was determined using two separate kinesin and two separate KRP_{130} preparations on 5-ml 5-20% linear sucrose gradients in 100 pr ATP, 150 μM KCl in PMEG. The gradients were overlaid with a solution containing kinesin or KRP_{130} and 100 μg of bovine liver catalase (11.3 S), 50 μg of bovine serum albumin (4.4 S), and 100 μg of ovalbumin (3.66 S). The gradients were centrifuged at 300,000 × *g* for 8.5 h. A graph of $S_{20,W}$ *versus* the distance traveled through the gradient for the two KRP_{130} preparations yielded standard linear plots ($r^2 = 0.997$; $r^2 = 0.998$). The native molecular weights of kinesin and KRP_{130} were calculated from the measured *s* values and Stokes radii as described previously (16, 30).

Motility Assays

MT gliding over a glass coverslip coated with sucrose gradient-purified kinesin and KRP_{130} was measured as described previously (31) with a few modifications. A bundling activity that copurified with KRP_{130} was purposely minimized by performing assays in 20-μl flow cells that produced widely dispersed MTs (32). In addition, MTs attached to the KRP_{130}-coated coverslips displayed either intermittent or no motility unless the motility buffer (9 μM MgATP in PMEG) was supplemented with 50-75 μM KCl or NaCl. The video image was enhanced with an Argus-10 Image Processor (Hamamatsu). The polarity of MT gliding was determined with fluorescent “marked” MTs prepared using rhodamine-tubulin, *N*-ethylmaleimide-modified tubulin, GMP-CPP, and oxygen scavengers (all a generous gift from R. D. Vale) according to Howard and Hyman (33).

RESULTS AND DISCUSSION

Pan-kinesin peptide antibodies (16, 34) were used to identify KRPs in fractions obtained during the purification protocol outlined under “Materials and Methods.” A number of polypeptides in the ATP MAPs fraction reacted with the peptide antibodies (Fig. 1*B*, *lane L*); the three with the greatest reactivity migrated at 130, 120, and 90 kDa (Fig. 1, *A and B*). Probing Western blots of the gel filtration column fractions with antibodies that are specific for various KRPs indicated that the 130-kDa polypeptide is related to *Xenopus* Eg5 (Fig. 1*C*) (35). The 120-kDa polypeptide was shown to be the kinesin heavy chain (Fig. 1*D*) based on its reactivity with the KHC-specific monoclonal antibody SUK4 (29). We believe that the 90-kDa polypeptide is the protein product of the *ncd* gene (20,21) based on its cross-reactivity with an Ncd-specific antibody raised against a peptide corresponding to the

carboxyl-terminal 15 amino acids of the deduced Ncd protein (data not shown; antibody provided by Drs. McDonald and Goldstein).

KRP_{130} was purified further, based on its nucleotide-sensitive rebinding to and release from microtubules. The gel filtration fractions containing the peak of KRP_{130} were pooled and concentrated to 1.5 ml, then mixed with AMP-PNP and MTs formed from bovine brain PC tubulin. Critical to the purification of KRP_{130} was the finding that it bound to MTs in PMEG buffers containing AMP-PNP supplemented with significant amounts of KCI. In preliminary MT pelleting experiments, it was striking tha >90% and >60% of the KRP_{130} copelleted with MTs in AMP-PNP when the final concentration of KCl was 75 and 325 mM, respectively (data not shown), but negligible amounts of KRP_{130} cosedimented with MTs in ATP. By comparison, only 50% and <10% of *Drosophila* kinesin cosedimented with AMP-PNP MTs under the same conditions. Consequently, in the KRP_{130} purification protocol, we rebound KRP_{130} to MTs in the presence of AMP-PNP plus 12.5-200 mM KCl prior to elution with 100-200 μl of PMEG containing 10 mM MgATP and 200 mM KCl; these conditions optimized the removal of contaminating polypeptides and served to concentrate KRP_{130} in the eluates.

The final purification step involved sucrose density gradient centrifugation (Fig. 2). The 130-kDa polypeptide does not cosediment with significant amounts of any other polypeptides greater than 20 kDa (Fig. 2*A*). This leads us to conclude that the 130-kDa polypeptide is the only subunit present in the KRP_{130} complex. This is in contrast to *Drosophila* kinesin (Fig. 2*B*), which is believed to consist of two 120-kDa heavy chains and two 55-kDa light chains (the minor polypeptide hand just below the KLC in Fig. 2*B* may represent a modified light chain; Ref. 36). Unlike bovine and sea urchin egg kinesins, we detected no KLC-depleted fly kinesin (15, 37).

The properties of *Drosophila* KRP_{130} are compared with those of fly kinesin in Table I (the quaternary structure of fly kinesin has not been reported previously). We determined the Stokes radii of *Drosophila* kinesin and KRP_{130} to be approximately 9.0 and 16.2 nm, respectively. From sucrose density gradient centrifugation performed under the same buffer conditions as the gel filtration, we estimate the sedimentation coefficients of kinesin and KRP_{130} to be 9.1 and 7.6 S, respectively (data not shown). Using the method of Seigel and Monty (30), we estimate the relative molecular masses of kinesin and KRP_{130} to be 340 and 490 kDa, respectively. The calculated molecular mass of kinesin agrees closely with the value of 337 kDa predicted from cDNA cloning of fly KHC (19) and KLC (36), demonstrating the validity of our technique. Considering that each subunit of KRP_{130} is approximately 130 kDa, we calculate a subunit to complex ratio of 3.8 to 1, suggesting that KRP_{130} is composed of four 130-kDa kinesin-related motor subunits. Our attempts to visualize KRP_{130} by rotary shadowing and electron microscopy have thus far not revealed any consistent structure. Identical conditions produced high quality micrographs of *Drosophila* kinesin, suggesting that KRP_{130} may be too fragile for this imaging technique. However, the presence of four motor subunits in one complex is consistent with a bipolar structure consisting of two antiparallel 130-kDa dimers.

To test the ability of KRP_{130} to act as an MT-based motor, standard MT gliding assays were used (see “Materials and Methods”). The purified KRP_{130} complex acts as a “slow” ATP dependent plus-end-directed motor (0.04 ± 0.01 μm/s). This motility and a bundling activity copurify with the KRP_{130} on the sucrose gradients. The motility was labile; the activity apparent in “fresh” KRP_{130} deteriorated during storage of KRP_{130} on ice over a 24-h period.

The 130-kDa motor subunit of KRP_{130} cross-reacts with anti-*Xenopus* Eg5 antibody (Figs. 1*C* and 3*B*), suggesting that KRP_{130} may represent a *Drosophila* homolog of Eg5 (35, 38)

and may therefore be a member of the BimC subfamily of kinesins (3). This hypothesis is supported by the observations that slow plus-end-directed motility is a property of both purified KRP_{130} (0.04 ± 0.01 μm/s) and bacterially expressed *Xenopus* Eg5 (0.035 μm/s; Ref. 35).

Members of the BimC subfamily have been identified genetically across a wide range of organisms and appear to play important roles in the formation and maintenance of the mitotic spindle (25, 35, 38–45). It is possible that the 130-kDa polypeptide of KRP_{130} is the product of the *Drosophila* KLP61F gene (25), a member of the BimC subfamily also known as *urchin*.[2] Disruption of the KLP61F gene results in failed spindle pole separation during mitotic prophase (25). Similar defects in spindle assembly result from fungal *bimC* (39) and *cut7* (40) mutations.

Our observation that KRP_{130} is a homotetramer may be relevant to the mechanism of spindle pole separation mediated by members of the BimC subfamily. In interphase, duplication of the centrosome occurs, so that during prophase, cells contain two spindle poles lying side by side with arrays of MTs emanating from them (plus ends of MTs distal to the poles). We speculate that KRP_{130} (and other members of the BimC subfamily) could cross-link MTs emanating from one pole to parallel MTs emanating from the neighboring pole, and could cause the attached MTs to "slide" with their minus ends leading, thereby exerting "pushing" forces on the attached poles. If we assume that the heads of KRP_{130} can swivel to permit MT motility in any direction (as found for kinesin by Hunt and Howard (Ref. 46)), then such a protein assembly would be expected to "self-organize" into a metaphase-like array, consisting of separated spindle poles linked by overlapping arrays of antiparallel MTs cross-linked in the region of MT overlap by KRP_{130} homotetramers. In this way, KRP_{130} homotetramers could function as the microtubule cross-linking motors described in a recent model of spindle pole separation (see Fig. 3 of Ref. 47). Our hypothesis for KRP_{130} function is based on the notion that its four subunits are organized into a bipolar array capable of crosslinking adjacent microtubules. Additional studies of the structure and function of KRP_{130} are being initiated to test the hypothesis that it is indeed a bipolar assembly.

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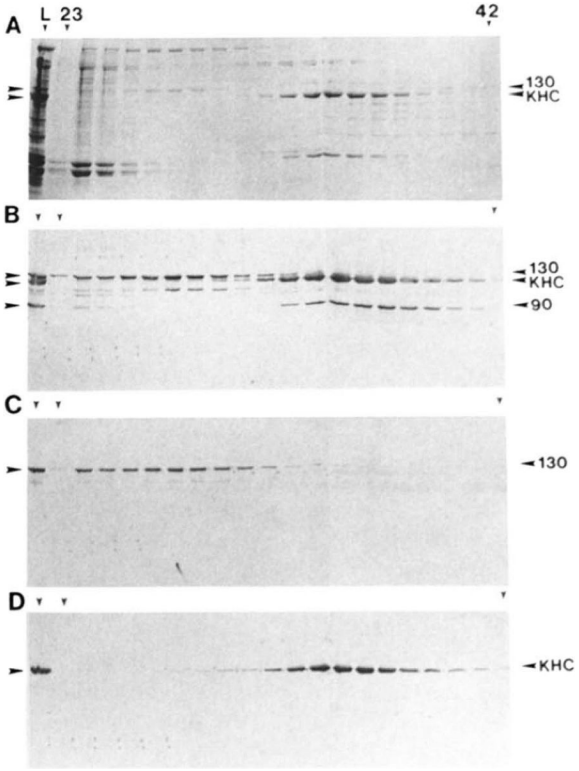

FIG. 1. Bio-Gel A-1.5m gel filtration of ATP-eluted *Drorophila* MT-binding proteins

A, Coomassie Blue-stained SDS gel. *Lane L* shows the ATP-eluted MAPs that were loaded onto the column. Duplicate immunoblots were probed with "LAGSE" antihody (similar results were obtained using other pankinesin antibodies 16, 34)) (*B*), anti-Eg5 antibody (*C*), and SUK4 antibody (*D*). The column (1.6 × 85 cm) was equilibrated with 100 μ_M ATP, 150 m_M KCl in PMEG buffer. Fractions (3.0 ml) 23–42 are shown on the gels and blots here. The void volume corresponded to fraction 24, and the included volume corresponded to fraction 59.

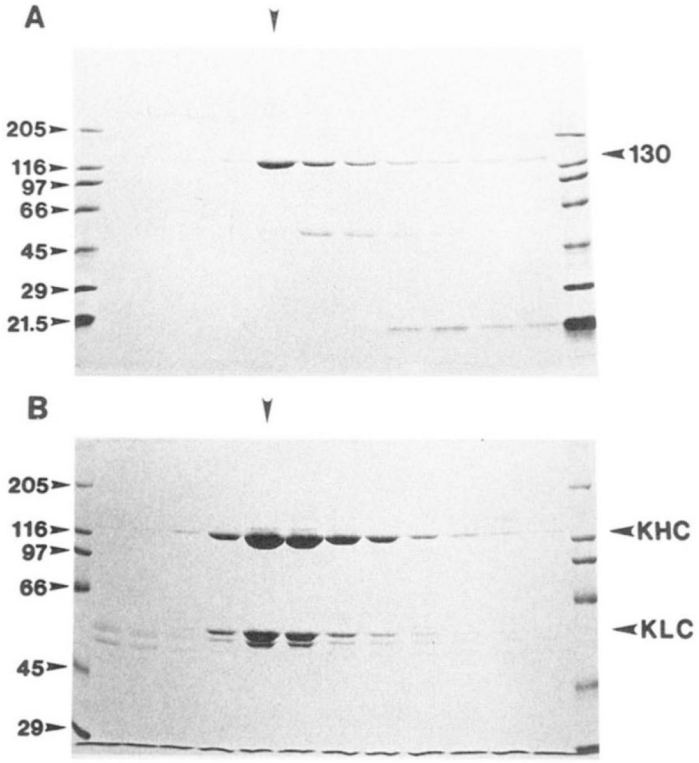

FIG. 2. Fractionation of *Drosophila* kinesin and KRP_{130} by sucrose density gradient centrifugation

A, KRP_{130}. Coomassie Blue-stained 5-20% acrylamide gradient SDS gel of a 5-20% sucrose gradient shows that KRP_{130} sediments as a single 7.6 S peak. No detectable polypeptides greater than 20 kDa coelute with the 130-kDa polypeptide. *B*, kinesin (KHC and KLC). Coomassie Blue-stained 7.5% acrylamide SDS gel of 5-20% sucrose gradient shows that kinesin sediments as a single 9.1 S peak. *Vertical arrowheads* indicate that peak KRP_{130} and kinesin fractions.

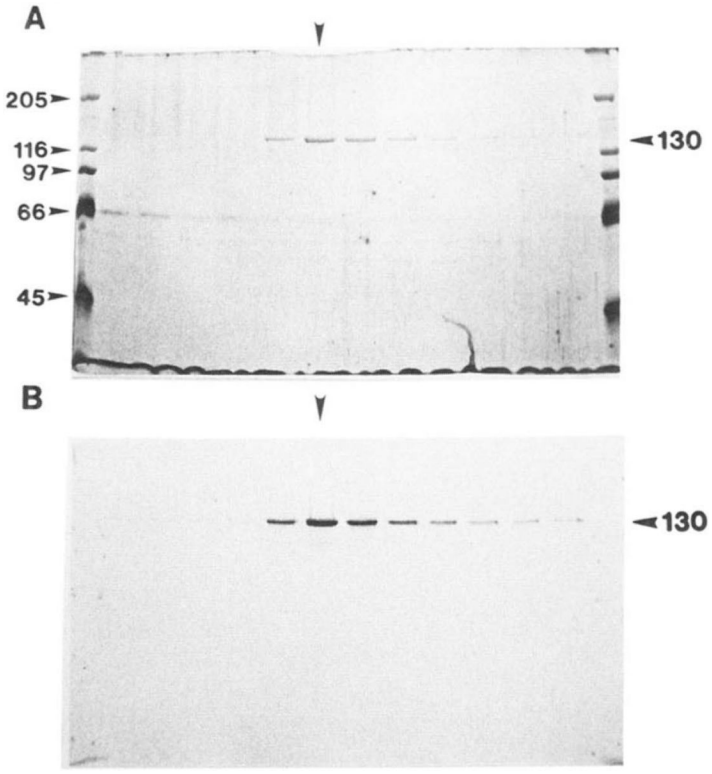

FIG. 3. Immunoreactivity of KRP_{130} with anti-Eg5 antibody

A silver-stained SDS gel of a 5-20% sucrose gradient shows that KRP_{130} sediments as a single peak. *B*, duplicate immunoblot probed with anti-Eg5 antibody. *Vertical arrowheads* indicate that peak KRP_{130} fraction.

Table I

Comparison of Drosophila KRP_{130} and Drosophila kinesin

	Kinesin	**KRP_{130}**
Motility	Plus-end. 0.8 ± 0.06 μm/s	Plus-end, 0.04 ± 0.01 μm/s
MT binding	Strong: AMP-PNP	Strong: AMP-PNP
	Weak: ATP	Weak: ATP
M_r (subunits)	120 kDa, 55 kDa	130-kDa
R_S (complex)	9.0 nm	16.2 nm
Sedimentation Coefficient	9.1 S	7.6 S
Molecular mass (complex)	340 kDa	490 kDa
Stoichiometry	2 × 120 kDa	4 × 130 kDa
	2 × 55 kDa	