Communication

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

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Pan-kinesin peptide antibodies (Cole, D. G., Cande, W. Z., Baskin, R. J., Skoufas, D. A., Hogan, C. J., and Scholey, J. M. (1992) J. Cell Sci. 101, 291-301; Sawin, K. E., Mitchinson, T. J., and Wordeman, L. G. (1992) J. Cell Sci. 101, 303-313) 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, KRP130, was further purified from ATP eluates of the embryonic MTs. Purified KRP130 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 KRP130 was tested for reactivity with monoclonal and polyclonal antibodies that are specific for various members of the kinesin superfamily. Results indicate that the KRP130 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, KRP130 appears to be the first Drosophila KRP, and the first member of the BimC subfamily in any organism, to be purified from native tissue as a multimeric motor complex.

Kinesin and kinesin-related proteins (KRPs) 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 N-terminal “motordomains” (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).

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 for the 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 KRP130 (55) 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, several fly KRPs have been characterized using genetic approaches (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, KRP130, 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 MgATP in PEM buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM MgSO₄, and 2 mM dithiothreitol) in liquid N₂ or at −80°C until needed. Preparative MTs were formed by incubating PC tubulin in 1 mM MgATP 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 MgATP, 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 PEGME buffer (100 mM PIPES, pH 6.9, 5 mM EGTA, 0.5 mM EDTA, 2.5 mM MgSO₄, 0.8 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 μl) 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 (PEGME without MgSO₄) buffer at 4°C prior to repelleting at 100,000 × g for 25 min at 4°C. The washed MT pellet was eluted with 5 ml of 10 mM MgATP, 200 μM KCN in PEGME for 6-14 h at 4°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 (1.0×80 cm) column equilibrated with 100 μM ATP, 150 μM KCN in PEGME buffer. The fractions containing kinesin and KRP130 were separately pooled and concentrated (Centriprep 30) to 1.5 ml prior to 20-min incubations with taxol MTs (PC tubulin) in 5 mM AMP-PNP,
Slow Homotetrameric KRP from Drosophila Embryos

and either 50 mM KCl (kinesin) or 125–200 mM KCl (KRP\textsubscript{130}) in PMEG buffer at 25 °C. The MTs were resuspended (100,000 × g, 20 min), followed by release of kinesin and KRP\textsubscript{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\textsubscript{130} supernatants were fractionated on linear 5–20% sucrose gradients in 100 mM 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\textsubscript{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\textsubscript{v}, was determined as described previously (16) for two different preparations of Drosophila kinesin and KRP\textsubscript{130} using the Bio-Gel A-1.5 m and Bio-Gel A-5m columns and running buffers described above. Plots of R\textsubscript{v} versus -log\textsubscript{10} K\textsubscript{w,2} (where K\textsubscript{w} = (elution volume - void volume)/total volume - void volume) generated standard linear plots (r\textsuperscript{2} = 0.964; r\textsuperscript{2} = 0.981, where r is the correlation coefficient). The standard proteins and their Stokes radii in-sucrose density gradient-purified kinesin and KRP\textsubscript{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\textsubscript{130} was measured as described previously (31) with a few modifications. A bundling activity that copurified with KRP\textsubscript{130} was purposely minimized by performing assays in 50–75 mM 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 according to Howard and Hyman (33).

** RESULTS AND DISCUSSION**

Pan-kinesin peptide antibodies (16, 34) were used to identify KRP\textsubscript{130} 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. 1B, 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 KRP\textsubscript{130} indicated that the 130-kDa polypeptide is related to Xenopus Eg5 (Fig. 1C) (35). The 120-kDa polypeptide was shown to be the kinesin heavy chain (Fig. 1D) based on its reactivity with the KHC-specific monoclonal antibody S UK4 (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 carboxy-terminal 15 amino acids of the deduced Ncd protein (data not shown; antibody provided by Drs. McDonald and Goldstein).

KRP\textsubscript{130} was purified further, based on its nucleotide-sensitive rebinding to and release from microtubules. The gel filtration fractions containing the peak of KRP\textsubscript{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\textsubscript{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 that >90% and >60% of the KRP\textsubscript{130} copelleted with MTs in AMP-PNP when the final concentration of KCI was 75 and 325 mM, respectively (data not shown), but negligible amounts of KRP\textsubscript{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\textsubscript{130} purification protocol, we rebound KRP\textsubscript{130} to MTs in the presence of AMP-PNP plus 125–200 mM KCl prior to elution with 100 mM ATP, 150 mM KCl in PMEG buffer. Fractions (3.0 ml) 23–42 were shown on the gels and blots here. The void volume corresponded to fraction 24, and the included volume corresponded to fraction 59.

FIG. 1. Bio-Gel A-1.5m gel filtration of ATP-eluted Drosophila MT-binding proteins. A. Coomassie Blue-stained SDS gel. Lane L shows the ATP-eluted MAPs that were loaded onto the column. Duplicated immunoblots were probed with "LAGSE" antibody (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 mM ATP, 150 mM KCl in PMEG buffer. Fractions (3.0 ml) 23–42 shown here refer to the void and gels bands here. The void volume corresponded to fraction 24, and the included volume corresponded to fraction 59.

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. 2A). This leads us to conclude that the 130-kDa polypeptide is the only subunit present in the KRP\textsubscript{130} complex. This is in contrast to Drosophila kinesin (Fig. 2B), which is believed to consist of two 120-kDa heavy chains and two 55-kDa light chains (the minor polypeptide band just below the KLC in Fig. 2B 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\textsubscript{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\textsubscript{130} to be approximately 9.0 and 16.2 nm, respectively. From sucrose density gradient cen-
trifugation 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 stoichiometry 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 KHC and KLC on a 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.

The 130-kDa motor subunit of KRP$_{130}$ cross-reacts with anti-Xenopus Eg5 antibody (Figs. 1C and 3B), 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 KLPL1F gene (25), a member of the BimC subfamily also known as urchin.$^2$ Disruption of the KLPL1F 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 observations 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

$^2$ P. G. Wilson and M. T. Fuller, manuscript in preparation.
Slow Homotetrameric KRP from Drosophila Embryos

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 KRP130 homotetramers. In this way, KRP130 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 KRP130 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 KRP130 are being initiated to test the hypothesis that it is indeed a bipolar assembly.

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REFERENCES