KINESIN-73 IS A PROCESSIVE MOTOR THAT LOCALIZES TO RAB5-CONTAINING ORGANELLES

Thomas M. Huckaba1, Arne Gennerich2, James E. Wilhelm3, Athar H. Chishti4, and Ronald D. Vale1,5
Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158-25171, Department of Anatomy and Structural Biology and Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY 104612, Section of Cell and Developmental Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 920313, Department of Physiology, Tufts University School of Medicine, Boston MA 021114, and Howard Hughes Medical Institute5

Running Title: Characterization of Kinesin-73

Address correspondence to: Ronald D. Vale, PhD, 600 16th Street, San Francisco, CA 94158-2517. Fax: 415-476-5233; E-mail: vale@cmp.ucsf.edu

Drosophila Kinesin-73 (Khc-73), which plays a role in mitotic spindle polarity in neuroblasts, is a metazoan-specific member of the Kinesin-3 family of motors, which include mammalian KIF1A and C. elegans Unc-104. The mechanism of Kinesin-3 motors has been controversial, since some studies have reported that they transport cargo as monomers while other studies have suggested a dimer mechanism. Here, we have performed single molecule motility and cell biological studies of Khc-73. We find that constructs containing the motor and the conserved short stretches of putative coiled-coil-forming regions are predominantly monomeric in vitro, but that dimerization allows for fast, processive movement and high force-production (7 pN). In Drosophila cell lines, we present evidence that Khc-73 can dimerize in vivo. We also show that Khc-73 is recruited specifically to Rab5-containing endosomes through its “tail” domain. Our results suggest that the N-terminal half of Khc-73 can undergo a monomer-dimer transition to produce a fast processive motor and that its C-terminal half possesses a specific Rab5-vesicle binding domain.

Microtubule motors in the kinesin superfamily transport a variety of molecular cargoes in eukaryotic cells, regulate microtubule dynamics, and organize microtubule arrays in the mitotic spindle (reviewed in 1, 2). The best characterized motor in the kinesin superfamily is Kinesin-1 (conventional kinesin), which is involved in organelle and mRNA transport (3-5). Kinesin can take over one hundred 8 nm steps along a microtubule without dissociating, a process known as processive movement (reviewed in 6). The bias for the plus-end-directed movement is thought to be driven by the docking of a mechanical element (the neck linker) to the core of the enzyme when ATP binds to the active site (7, 8).

The Kinesin-3 class is another well-established and important class of cargo-transporting kinesins. The best-studied member, KIF1A (mouse)/Unc104 (C. elegans), is involved in the transport of synaptic vesicles to the nerve terminus (9, 10). Unlike Kinesin-1, KIF1A/Unc104 and other Kinesin-3 members lack the extensive coiled-coil domain that conventional kinesin uses for dimerization. Indeed, the first characterizations of Kinesin-3 family members, mouse KIF1A and KIF1B, suggested that these motors are monomeric in vitro (10, 11) and gave rise to the hypothesis that these monomeric motors might use a biased diffusion mechanism to move along microtubules in contrast to the hand-over-hand movement of Kinesin-1 (12, 13). However, other studies have suggested that Kinesin-3 motors might dimerize through several short coiled-coil motifs when concentrated in solution or on a membrane vesicle and move by a Kinesin-1-like mechanism (14, 15). More recently, a publication has suggested that full-length KIF1A may always be a dimer both in vitro and in vivo (16). Thus the mechanism by which Kinesin-3 family members move cargo remains an open question.

An intriguing, metazoan-specific member of the Kinesin-3 family is typified by Khc-73 in Drosophila (17) and GAKIN in humans (18). While there are four Kinesin-3 family members in Drosophila, Khc-73 is unique among Kinesin-3 family members in that it contains a C-terminal
CAP-Gly (Cytoskeleton-Associated Protein Glycine-rich) domain, which is found in several microtubule binding proteins (19, 20). Khc-73 interacts with the Discs Large tumor suppressor in neuroblasts and is necessary for proper mitotic spindle orientation (21). GAKIN also binds the human homolog of Discs Large and is necessary for the enrichment of PIP3-containing vesicles at the tips of neurites (18, 22).

Since Khc-73/GAKIN remains poorly characterized and its motile properties have not been fully studied, we sought to investigate its single-molecule motility and to determine its localization when expressed in cells. Here, we show that dimeric Khc-73 motors undergo rapid (~1.5 μm/s) processive movement and generate forces comparable to conventional Kinesin-1 (~7 pN). In Drosophila S2 and BG2 cells, Khc-73 forms a specific interaction with Rab5-containing endosomes through its C-terminal tail domain. Our results also suggest that Khc-73 can dimerize both in vitro and in vivo and that the dimer is likely to be the active form of the motor.

Experimental Procedures

Cloning of Khc-73 and Rab constructs- All Khc-73 clones were amplified from the full-length Khc-73 construct generously provided by C. Doe (21). The GCN4 leucine zipper motif was amplified from a construct provided by K. Slep (23). Shorter Khc-73 constructs used for Drosophila cell line transfection were subcloned into pENTR/D-TOPO (Invitrogen) and then moved into either a Gateway C-terminal GFP or mCherry vector under the control of the copper-inducible metallothionein promoter (pMTWG and pMTWCherry; Drosophila Gateway Collection). For generation of GFP-tagged Rabs, each Rab ORF was amplified from the appropriate full-length cDNA clone (primer sequences available on request) and then subcloned into the pENTR/D-TOPO vector (Invitrogen). The Rab ORF was then moved into an N-terminal Gateway GFP vector under the control of the actin promoter (pAGW; Drosophila Gateway Collection).

Expression, and purification of Khc-73 constructs- Bacterially-expressed constructs were cloned into pET17b with a C-terminal GFP followed by 6xHIS, expressed, and purified as described previously (14). Khc-73-positive Ni-NTA column eluates were dialyzed with BRB 80 buffer (80 mM PIPES (pH 6.8), 1 mM MgCl₂, 1 mM EGTA) supplemented with 1 mM ATP and 1 mM DTT, concentrated, aliquotted, and snap frozen in liquid nitrogen. Before use in further assays, aliquots were subjected to microtubule bind and release to purify active Khc-73 as described previously (14). Analysis of native molecular weight- The molecular weight of Khc-73 proteins in solution was determined by measuring the Stokes radius by gel filtration and the sedimentation coefficient by sucrose gradient centrifugation (24). Gel filtration was performed over a Superdex 200 column connected to an AKTA FPLC (GE Healthcare). All fractions were collected, and peak protein levels were confirmed as Khc-73 positive by SDS-PAGE followed by Coomassie blue staining. Confirmed peaks were compared to a calibration curve generated by standards of known Stokes radius (Thyroglobulin (8.5 nm), Ferritin (6.1 nm), Aldolase (4.5 nm), BSA (3.6 nm) Ovalbumin (3.1 nm), and Cytochrome c (1.6 nm)) to determine the radius of each construct. For determination of sedimentation coefficients, constructs were layered over a 12 ml, 5-40% sucrose gradient and centrifuged at 35,000 rpm (~150,000 x g) for 18 hr at 4°C in an SW40 rotor. A total of 24 fractions were collected from each sample and peak fractions were identified by Coomassie stained SDS-PAGE. Sedimentation coefficients were determined by comparing to a standard curve generated from calibration standards (Thyroglobulin, Ferritin, Catalase, Lactate Dehydrogenase, and BSA; GE Healthcare) analyzed in the same centrifugation run. The values of Stokes radii and sedimentation coefficients were used to derive the molecular weight (Mₐ) of each construct according to the following equation:

\[ M_a = 6\pi N \eta s/(1-\nu) \]

where N is Avogadro’s number, ν is the partial specific volume of the protein (estimated as 0.725 ml/g in this study), s is the sedimentation coefficient, a is the Stokes radius, and η and ρ are the viscosity (1.6 g·m⁻¹·s⁻¹) and density (1 g/ml) of water at 4°C, respectively.

Optical Trapping Assay- Stall-force and step-size measurements of single Khc-73 molecules were performed at 25 ± 1 °C with a custom-built force-clamp optical trapping microscope as previously described (25). In brief, carboxylated polystyrene
beads (0.92 mm diameter; Invitrogen) were sparsely covered with the GFP-tagged motor proteins via affinity-purified anti-GFP antibodies (14). Measurements were performed in a ~10 µl flow cell constructed by placing two strips of double-sided sticky tape between a standard microscope slide and a 160 µm thick 18 mm x 18 mm cover slip to form a channel. The assay solution consisted of 30 mM HEPES (pH 7.2), 2 mM MgAcetate, 1 mM EGTA, 1 mM MgATP, 1 mg/ml casein, 10 mM DTT, 4.5 mg/ml glucose, and an oxygen scavenger system (26). Bead displacement was detected by a quadrant photodiode and recorded at 2 kHz. Before each experiment, the trapped bead was scanned along the x-axis of the object field across the detection region to obtain the detector’s response. A trapped bead was then positioned over a rhodamine-labeled sea urchin sperm flagellar axoneme that was immobilized onto a coverslip and aligned with the x-axis of the object field (coincides with the x-axis of the position detector). Experiments were performed at dilutions at which the fraction of beads moving was \( \leq 0.3 \) to ensure measurements on a single-molecule level (27). Trap stiffness was calibrated for each trapped bead from the amplitude of the thermal diffusion. Stall forces were determined by multiplying the trap stiffness by the mean maximum distance reached. Force-feedback was operational in an area of \( \pm 200 \) nm along the x-axis of the object field. Motor steps (approximate center-of-mass movement of kinesin) were determined from the bead displacement records using a step-finding algorithm developed by Kerssemakers et al. (28). This algorithm assumes that steps are hidden in normal-distributed noise but makes no assumptions about steps sizes and durations. The resulting data were then visually screened and only those steps that could be visually separated from noise were included in the step size histograms.

**In vitro motility assays**—For microtubule gliding assays, flow chambers were made as described above and 10 µl of a 0.5 mg/ml GFP antibody solution was added and allowed to attach for 5 min. Flow chambers were washed twice with motor buffer (25 mM PIPES (pH 6.8), 75 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 20 µM taxol, 5% sucrose), then incubated for 5 min with motor buffer containing 1 mg/ml casein to block non-specific binding to the coverglass. Khc-73 motors were diluted to 50 nM in motor buffer with casein and allowed to incubate for 5 min, prior to two additional washes in motor buffer. Finally, 20 µl of motility buffer (motor buffer with 1 mg/ml casein, 5 mM ATP, oxygen scavenger, and rhodamine-labeled microtubules) was added. Time-lapse imaging was performed on a Nikon Eclipse TE2000-E microscope equipped with an Andor EM CCD camera driven by Micro-Manager imaging software (www.micro-manager.org). To determine the microtubule gliding velocity, the leading edge of a microtubule was tracked through a minimum of 10 successive frames (30 seconds) and the average instantaneous velocity (sum of distances traveled in each successive frame divided by the number of frames multiplied by the time between each frame) was obtained using ImageJ. Average for each construct was determined by applying a simple one-peak Gaussian fit to a histogram of the velocities using Origin 8 software (OriginLab Corporation). Single molecule motility assays along rhodamine-labeled sea urchin axonemes were performed using a standard assay (29). Axonemes were identified using wide-field fluorescence imaging, and GFP-labeled Khc-73 constructs were imaged at 10 Hz using total internal reflection fluorescence microscopy with a 100x, 1.49 NA TIRF objective and an Andor EMCCD camera. Kymograph analysis in ImageJ was used to determine the velocity and run length for each experiment. The slope of particle movement over time was used for velocity and total distance traveled corresponded to run length. Movement frequency was generated by adding the total number of motility events and dividing by the number of axonemes in each frame and the total time of observation. Motility events were defined as movement along the length of an individual axoneme that persists through at least five individual acquisition frames. Average velocity was determined by applying a simple one-peak Gaussian fit to a histogram of the velocities for each construct, and the run length was determined by fitting an exponential decay plot to a histogram of the run lengths in Origin 8.

**Khc-73 antibody production**—The 20 amino acids at the C-terminus of Khc-73 are not found in the other Kinesin-3 family members in *Drosophila* and BLAST searches of the *Drosopila* database...
shows no significant homology of this sequence with any other expressed protein. A peptide of the final 20 C-terminal amino acids of Khc-73 was synthesized with an N-terminal cysteine residue that was conjugated to Keyhole Limpet Hemocyanin (Maine Biotechnology). Antigen was used to generate rabbit polyclonal serum against Drosophila Khc-73 (Maine Biotechnology). Serum was tested by Western blotting against a BSA-conjugated antigen peptide, with pre-immune serum and BSA used as negative controls. A portion of crude serum was further purified by incubation with protein G-Sepharose beads (Gamma-bind). Use of this total IgG fraction in Western Blots of Drosophila S2 cell lysate recognized several bands, but only one prominent band was recognized in the size range of Khc-73, which was subsequently reduced in cells treated with RNAi targeted against Khc-73 (Supplemental Figure 5).

Cell culture and RNAi- Drosophila Schneider cell line (S2) cells (Invitrogen) and Drosophila BG2 cell line (Drosophila Genomics Resource Center) were cultured as described previously (30, 31). RNAi construct generation and 7 day RNAi treatment were performed as described previously (32). Two separate dsRNA constructs used in this study. To target the 3’UTR of the Khc-73 the following primers were used: TGTACCCAAAGTGTTCGCATCAG and CATTTGCGGCATGGGGTGAGAAT. To target the coding region of the Khc-73 transcript, the following primers were used: ATTCACGAGCTTAATGACAACG and CAGCGTGTAAAACTTATGTCCG. Neither construct generates 19 nucleotide sequences with predicted off-target effects, as determined by analysis of the dsRNA sequences generated by the Genome RNAi database at the German Cancer Research Center. RNAi treatment of S2 cells with either construct led to a reduction of intensity of a single band using the Khc-73 antibody that we generated. For each experimental RNAi treatment, a sample was collected and analyzed by Western blot to determine the level of Khc-73.

Live-cell imaging and analysis- Full-length Khc-73 and shorter Khc-73 constructs were cloned into the pENTR/D-TOPO vector (Invitrogen) and moved into C-terminal Gateway GFP and mCherry vectors under the control of the metallothionein promoter described previously (33). Drosophila S2 and BG2 cells were transfected using the Effectene transfection reagent (Qiagen) using a standard protocol. 16 hr before imaging, expression was induced by the addition of 50 µM copper sulfate. 1 hr prior to imaging, S2 cells were plated on Con-A (Sigma-Aldrich) coated, glass-bottom dishes (MatTek) and allowed to spread. For analysis of Rab5-GFP motility rates, Drosophila S2 cells were treated with 7-day RNAi to knock down Khc-73 or mock treated, plated on Con-A coated coverglass and allowed to spread for one hour, then incubated with 5 µM cytochalasin D to induce cellular projections of bundled microtubules in parallel orientation as described previously (34). Time-lapse images were acquired using wide-field fluorescence on the Nikon microscope described above. Images were analyzed visually in the µManager 5D viewing frame and representative frames were chosen for documentation. Images shown were imported into Photoshop CS4 (Adobe), then enhanced by applying a linear adjustment of intensity boundaries. Individual images were inserted into corresponding channels of a single RGB image to create the merged images shown. Corresponding QuickTime movies were generated by exporting image stacks from ImageJ.

RESULTS

Analysis of the Biophysical and Biochemical Behavior of Khc-73. Previous work has shown that Unc104, a C. elegans member of the Kinesin-3 family, undergoes concentration-dependent dimerization in vitro as a result of two short helical domains that are directly C-terminal to the neck linker (14). The homologous region of the M. musculus Kinesin-3 family member, KIF1A was also predicted to form a coiled-coil (10, 16). Alignment of the Khc-73 amino acid sequence with Unc104 and KIF1A identified two potential helical regions in Khc-73 with an intermediate predicted potential for coiled-coil formation (Figure 1A). However, a 38-amino acid stretch distal to the FHA (forkhead-associated) domain (a domain characteristically found in Kinesin-3 motors) shows high coiled coil-forming probability (Figure 1B). Thus, we designed motor-containing constructs with varying portions
of these regions for further biophysical and biochemical characterization (Figure 1C).

C-terminal GFP-tagged constructs of the Khc-73 motor were expressed in bacteria and purified. To ensure that the purified proteins were catalytically active, we performed sequential microtubule bind and release reactions as described previously (14). Analysis of the bulk activity of each construct via microtubule gliding assays showed a similar rate of microtubule motility (Table 1). To determine the processive behavior of Khc-73 motor constructs, we examined single molecules by using total internal reflection fluorescence (TIRF) microscopy. Molecules of each of the Khc-73 constructs attached to and moved along sea urchin axonemes with similar velocities in the range of 1.5 µm/s, as determined by the peak of a Gaussian curve fit to a histogram of the velocities (Table 1). A histogram of the distance traveled per microtubule encounter revealed an exponential decay constant (defined as the run length) (Table 1, Supplemental Figure 1). In addition, since experiments were performed with the same concentration of motor (50 nM), we are able to compare the relative frequency of processive movement events (construct movements along the length of the axoneme that persisted for more than five individual acquisition frames), normalized to the microtubule length. We noticed that although the velocity of each construct is similar, both the run length and movement frequency increased for the longer constructs that had more potential coiled-coil forming domains. As a positive control for dimer formation, we expressed and purified a "constitutive dimer" in which the shortest motor construct was fused to the GCN4 leucine-zipper dimerization motif (1-387-LZ-GFP). In single molecule motility assays, this construct showed the same velocity, but a longer run length and nearly 100-fold higher frequency of movement compared to the same construct without the leucine zipper. This result suggests that dimerization is needed for efficient processive movement. The finding that the truncated Khc-73 constructs undergo infrequent processive movement compared to the constitutive dimer suggests that they may be in equilibrium between pools of a predominant monomer and a dimer.

To determine the native state of the motor constructs in vitro, we performed gel filtration coupled with sucrose density gradient centrifugation (24) (Table 1). The constructs lacking the leucine-zipper motif had a calculated molecular weight close to that predicted for a monomer. In contrast, and as expected, the calculated molecular weight of 1-387-LZ-GFP was close to that predicted for a dimer of two polypeptide chains. This result suggests that the majority of the non-leucine-zipper-containing constructs are monomeric in solution, although a small amount of dimer (predicted from our single molecule motility experiments) would not likely be detected in these hydrodynamic measurements, as the dimer would likely dissociate over the time course of the gel filtration and sucrose gradient runs.

If the putative coiled-coil domains in the tail region of Khc-73 facilitate dimer formation and this dimer formation is necessary for the in vitro motility we observe, then interrupting the coiled-coil domain should abolish Khc-73 motility. Likewise, stabilization of the coiled-coil should increase the frequency of Khc-73 motility. To test this hypothesis, we created a series of mutations in the shortest (1-387) Khc-73 construct. Tomishige et al. (14) showed that two point mutations in the putative coiled-coil motif in Unc104 (I362E, L365K) abolished its in vitro motility. Since sequence alignment of Unc104 and Khc-73 (Figure 1A) shows that these residues are conserved, we made the same mutations in the Khc-73 1-387 molecule (Figure 2A, coil mutant). At the same concentration as the wild type 1-387 construct, we observed no in vitro motility (total imaging time of 12.5 minutes, 30 experiments) of the coil mutant construct (Figure 2D). In order to stabilize in vitro dimer formation, we engineered constructs with a single cysteine on either side of the single coiled-coil domain in the 1-387 construct (Figure 2A, C363 and C386). When final purification steps were carried out in the absence of a reducing agent, both the C363 and C386 constructs showed a significant population that shifted to higher molecular weight bands on a non-reducing gel compared to their migration on a reducing gel (Figure 2B). This behavior was not seen for wild-type 1-387 or the coil mutant purified in the same fashion (Figure 2D). When their motility was assayed in vitro, both C363 and C386 showed a >100-fold increase in motility frequency compared to wild type 1-387 (Figure...
These results lend further credence to the model that the Khc-73 dimer is the active form of the molecule and that dimer formation is facilitated by the predicted coiled coil helices adjacent to the Khc-73 motor domain. It also supports the notion that Khc-73 exists in a dynamic equilibrium between monomers and dimers. When diluted to nanomolar concentrations in the motility assay, the prevalent species is likely to be monomers. However, with cysteine-cysteine crosslinking, the dimer population is stable and does not dissociate to monomers upon dilution.

To determine the mechanical properties of Khc-73, we attached single dimeric 1-387-LZ-GFP molecules to latex beads and performed optical trap assays. Since optical trap studies necessitate the use of a uniform population of stable dimer and not a species that is subject to monomer-dimer equilibrium, we utilized the well-established GCN4 leucine zipper construct to ensure dimer stability. Using the laser trap to position and hold a motor-bound bead over a coverslip-attached, rhodamine-labeled axoneme, we measured the stall force (the maximal force generated by the motor before it detached from a microtubule) of the Khc-73 motor (Figure 3A). Fitting a Gaussian curve to a histogram of the individual stall forces revealed an average stall force of 6.8 ± 1.5 pN (mean ± SD, Figure 3B). Using a feedback controlled optical trap to maintain a constant load (6 pN) on the motor, we could visualize clear step-wise movement of the motor (see blown-up region of the motor trace in 3D). A histogram of the individual step sizes revealed an average step size of 7.92 ± 0.07 nm (mean ± SEM, Figure 3E), which is approximately the distance between α-β tubulin dimers and the step size of the Kinesin-1 motor (35).

Khc-73 is Enriched at the Ends of Microtubules and is Recruited to Rab5-containing Vesicles. Khc-73 has been shown to have a role in mitotic spindle orientation in Drosophila neuroblasts (21), but its interphase localization has not been explored. We expressed full-length Khc-73 in Drosophila S2 cells and saw two distinct localizations, the first enriched at the peripheral tips of microtubules, and the second on motile puncta in the cytosol (Figure 4A, Supplemental Movie 1). A subset of microtubule-associated proteins known as +TIPs bind to the polymerizing plus ends of microtubules (36). This is particularly relevant to Khc-73, as it contains a C-terminal CAP-Gly domain, which is present in some +TIP proteins and in certain cases has been shown to be sufficient to endow plus-end tracking behavior if the CAP-Gly domain is present as a dimer (23, 37). To test whether the Khc-73 CAP-Gly domain might account for its accumulation at microtubule plus ends, we expressed and localized the CAP-Gly domain as a monomer and a dimer (fused to a leucine zipper, CAP-Gly-LZ). The monomeric form (CAP-Gly) exhibited diffuse cellular localization, while the dimeric form (CAP-Gly-LZ) bound non-specifically along the microtubule lattice (Supplemental Figure 2). Thus, the microtubule tip enrichment of Khc-73 is apparently not due to CAP-Gly-dependent plus end tracking.

Full-length Khc-73 might also enrich at microtubule plus ends through its motor activity and might be carrying a cargo to the tip. Since many kinesins associate with membrane vesicles, we examined whether Khc-73 co-localized with distinct Rab GTPases, which are good markers for different intracellular membrane vesicles (38, 39). We created a collection of GFP-tagged Rab GTPases (Rabs 5, 6, 8, 9, 10, 11, 14, 18, 19, 23, 30, 32, and 40) and performed pairwise co-transfections of Drosophila S2 cells with each individual construct and mCherry-labeled full-length Khc-73. Although most co-transfections yielded fluorescence signals in non-overlapping compartments (Supplemental Figure 3), we found that Khc-73-mCherry exhibited nearly complete overlap with Rab5-GFP, a well-characterized marker of early endosomes (40-42) (Figure 4B, Supplemental Movie 2). Since Khc-73 has been previously characterized in Drosophila neuroblasts (21), we also co-expressed Rab5-GFP and Khc-73-mCherry in the Drosophila neuronal BG2 cell line and saw similar co-localization both at the periphery and in motile puncta (Figure 4B, Supplemental Movie 3). Thus, in Drosophila cells lines, Khc-73 exhibits a very specific localization to Rab5-containing early endosomes.

Knockdown of Endogenous Khc-73 Reveals Dimerization and Rab5-containing Vesicle Recruitment Domain.

To determine the portion of Khc-73 that is responsible for binding Rab5-containing vesicles, we co-expressed mCherry-tagged constructs of
Khc-73 with Rab5-GFP and examined their localization. To our surprise, all constructs (many of which were non-overlapping) were recruited to Rab5-containing vesicles, with the exception of 1-432 which showed diffuse cytosolic localization (Figure 5, Supplemental Figure 4). This result might suggest that the Khc-73 molecule has non-overlapping N- and C-terminal domains that target the motor to Rab5-containing vesicles. However, another possibility is that some of the expressed constructs were localizing to Rab5 vesicles indirectly through an association (e.g. dimerization) with endogenous Khc-73. To test this hypothesis, we depleted the endogenous Khc-73 by performing RNAi targeted to the 3’UTR of Khc-73 in BG2 cells (Supplemental Figure 5), then co-expressed Rab5-GFP and the same set of mCherry-tagged Khc-73 constructs. Under these conditions where endogenous Khc-73 was depleted, we found that all of the N-terminal constructs (containing the motor up to the coiled-coil motif C-terminal to the FHA domain) no longer co-localized with Rab5-GFP (Figure 5, Supplemental Figure 6). The requirement of endogenous Khc-73 for these N-terminal constructs to localize with early endosomes suggests that these constructs may co-dimerize with endogenous motor (see Discussion). Under conditions of endogenous Khc-73 depletion, N-terminal constructs still accumulated at microtubule-rich regions at the periphery without colocalization with Rab5 (Figure 5, Supplemental Figure 6), which might reflect dimerization and processive movement of these constructs towards microtubule plus ends.

In contrast to the N-terminal constructs, all of the C-terminal constructs (containing the portion of the tail between the coiled-coil and the CAP-Gly domain) co-localized with Rab5-GFP in the absence of endogenous Khc-73 (Figure 5, Supplemental Figure 6). However, there were distinct differences in constructs that contained or did not contain the CAP-Gly domain. A C-terminal construct (443-1806) that lacked the CAP-Gly domain co-localized with Rab5-GFP in its normal punctate pattern (Figure 5). However, inclusion of the CAP-Gly domain in this construct (443-1921) resulted in strong microtubule binding and altered the normal localization of Rab5-GFP in BG2 cells, recruiting these membranes all along the microtubule lattice (Figure 5). The fact that the expression of full-length Khc-73 does not show this strong microtubule binding and abnormal Rab-5 localization suggests that the presence of the motor domain in the wild-type protein masks this behavior. These results indicate that the C-terminal half of Khc-73 contains a cargo binding site for Rab5 early endosomes and that the CAP-Gly domain is not needed for directing Khc-73 to these membranes.

**DISCUSSION**

In this study, we have performed the first in vitro characterization of the motility of Khc-73, a member of the Kinesin-3 family. Khc-73 is well conserved in metazoans, but not found in lower organisms, and appears to have important functions in neuronal development (17, 18, 22), although it likely serves additional roles as well. Although it has been controversial whether Kinesin-3 family members are monomeric or dimeric (10, 11, 14, 16), our results suggest that truncated Khc-73 constructs are in an equilibrium between monomers and dimers and that the dimer is a fast and highly processive motor that generates forces comparable in magnitude to Kinesin-1. We also provide evidence that Khc-73 strongly associates with early endosomes in vivo, an interaction mediated through its non-motor domain. Collectively, these findings provide new insight into the biophysical mechanism and cell biological roles for this poorly understood kinesin.

**Evidence that Kinesin-3 can form dimers in vitro and in vivo.** The Kinesin-3 class of motors was originally considered to be “monomeric” based upon the initial biochemical characterization of KIF1A as a monomer (10) and the finding that Kinesin-3 members have lower propensity of predicted coiled coil formation compared to other kinesin classes. In addition, single molecule studies suggested that monomeric KIF1A might transport cargo by a biased diffusion mechanism (12). In this work, a series of Khc-73 constructs of the N-terminal, motor-domain containing half of the protein behave predominantly as monomers in hydrodynamic analysis. However, a weak dimer might dissociate to a monomer during these prolonged separation techniques. Indeed our in vitro motility experiments are most consistent with Khc-73 being in equilibrium between a monomer and dimer. In support of this idea, Khc-73
dimerized constitutively by fusion to a C-terminal leucine zipper shows longer run lengths and a much higher (~20-fold) frequency of processive movement than a comparable construct without the leucine zipper (LZ), but the velocities of the leucine zipper and non-leucine zipper constructs were nearly identical. These results are most easily explained by a model in which the majority of the expressed Khc-73 molecules are monomers that do not exhibit processive movement, but a subpopulation of Khc-73 forms a dimer with identical motility properties to Khc-73-LZ. Interestingly, comparably truncated Unc104 constructs do not exhibit processive motility unless LZ-dimerized (14). Unc104/KIF1A possess a long flexible hinge between two helices next to the motor domain, which is thought to create an autoinhibited conformation through the formation of an intramolecular coiled coil that must be broken to form a processive motor dimer (43). Khc-73 appears to lack this flexible loop (Fig. 1A), which may allow these helices to more readily form intermolecular coiled coils.

Our transfection data also suggests that Khc-73 can dimerize in cells. In the presence of endogenous Khc-73, a variety of different length Khc-73 constructs all co-localize with Rab5-GFP. However, when cells are incubated with RNAi constructs knocking down the endogenous Khc-73 protein, none of the motor-containing constructs that lack the central domain between the coiled-coil and the CAP-Gly domain co-localize with Rab5-GFP. This endogenous Khc-73–dependent localization result suggests that endogenous Khc-73 is responsible for recruiting the expressed motor constructs to the surface of Rab5-containing vesicles. While we cannot rule out a scenario in which a higher order protein complex dependent on full-length Khc-73 is responsible for this recruitment, it seems most likely that the expressed proteins are recruited to Rab5-containing vesicles by forming heterodimers with the endogenous full-length protein.

Collectively our data suggest that in vitro and in vivo Khc-73 exists in equilibrium between a monomer and dimer. Our biochemical analysis of purified Khc-73 showing a predominant monomer species is in general agreement with previous results with KIF1A by the Hirokawa lab (11, 13). However, the Hirokawa lab did not report or suggest the possibility that a subpopulation of this kinesin can self-associate to create a dimer. In a contrasting story, Hammond et al. propose that KIF1A is constitutively dimerized, based upon co-immunoprecipitation from lysate, chemical crosslinking, intracellular FRET, co-migration with conventional kinesin by gradient centrifugation, and single molecule motility (16). It is important to note, though, that while our current in vitro work with Khc-73 and the previous work on KIF1A used bacterial- and baculovirus-expressed constructs (11, 13), the recent study by Hammond et al. used KIF1A from cell lysate from cell lines expressing KIF1A constructs for in vitro studies. This presents the possibility that there is a cellular component that stabilizes the Kinesin-3 dimer, which is consistent with our in vivo data with Khc-73. Future studies are targeted toward identifying this potential cellular factor.
moves early endosomes, thereby accounting for their continued motility after Khc-73 knockdown. Another possibility that cannot be excluded is that residual Khc-73 after RNAi treatment (Supplemental Figure 5) might suffice for early endosome motility.

A critical question that remains is how the biophysical properties and endosomal localization of Khc-73 might be related its biological functions in the living animal. The clearest biological activity ascribed to Khc-73 is its mitotic role in spindle orientation in Drosophila neuroblasts (21). Through its interaction with Discs Large, Khc-73 induces the cortical polarity of the Pins/Gai complex that is necessary for asymmetric cell division. The Khc-73 homolog in mammals also has been found to bind to membrane scaffolding proteins (18, 45). In our study, Khc-73 also localizes to early endosomes at the tips of microtubules that are very close to the cell cortex. In summary, there are now several results implicating Khc-73 with functions of microtubules/cargo at the cell cortex, but its exact activity and mechanism remain unclear and constitutes an interesting topic for future investigation.
REFERENCES


**FOOTNOTES**

The authors would like to thank Chris Doe and Kevin Slep for providing constructs, Nico Stuurmann for microscopy assistance, and Andrew Carter for assistance with the manuscript. This work was supported by National Institutes of Health grants NIH 38499 to R.D.V and CA094414 to A.H.C. T.H. is a Howard Hughes Medical Institute fellow of the Damon Runyon Cancer Research Foundation (DRG 1962-07).

The abbreviations used are: Cap-Gly, Cytoskeleton-Associated Protein, Glycine-rich; FHA, Forkhead Associated; CC, Coiled-coil; LZ, Leucine Zipper; TIRF, Total Internal Reflection Fluorescence; PH, Pleckstrin Homology

**FIGURE LEGENDS**

Fig. 1. Khc-73 domain analysis and construct design. A) Sequences of *D. melanogaster* Khc-73, *C. elegans* Unc104, and *M. musculus* KIF1A were aligned using the CLUSTALW multiple sequence alignment tool at EMBL-EBI. Dark green shaded regions indicate sequence identity, while light green shaded regions indicate sequence similarity. B) *D. melanogaster* Khc-73 sequence was analyzed for coil-forming probability by the COILS program (www.ch.embnet.org). Probabilities shown are the output using windows of 21 amino acids. C) Schematic of Khc-73 constructs used in this work. Label numbers refer to the Khc-73 amino acid boundaries of each construct.

Fig. 2. The coiled-coil domain of Khc-73 facilitates processive movement. A) Sequences of the regions surrounding helix 1 in wild type, coil mutant (I367E, L370K), and cysteine insertions before (C363) and after (C386) helix 1. B) Side-by-side preparations of wild type, coil mutant, C363 and C386 constructs were boiled in SDS sample buffer containing or omitting reducing agent (+DTT and –DTT, respectively), and run on the same gel. C) *In vitro* motility assays were performed as described above and the frequency of processive motility was calculated by adding the total number of events and dividing by the number of axonemes observed and the total time of imaging. Experiments were performed after incubating each construct in the presence and absence of 5 mM TCEP for 6 hr at 4° C (reducing and non-reducing conditions, respectively). n.d. = not detected (in 12.5 minutes of observation, 30 experiments). D) Sample kymographs of Khc-73 construct motility on individual axonemes. Distance is in the x-coordinate and time is in the Y-coordinate. Diagonal lines indicate processive movement of single molecules along an axoneme.
**Fig. 3.** Force generation and processive movement of single dimeric Khc-73 molecules in the optical trapping microscope. The Khc-73 1-387-LZ construct was used for each of the optical trap experiments. A) Displacement of a single kinesin molecule at 1 mM ATP in a fixed (nonfeedback) optical trap showing successive motor detachments and stalling events (trap stiffness: k=0.057 pN/nm). B) Stall force distribution (n = 187). C) Processive microtubule plus-end directed motion of Khc-73 under a constant 6 pN opposing load (force-feedback mode) in the presence of 1 mM ATP (trap stiffness: k=0.057 pN/nm). The bead displacement is shown in the upper trace and the trap position in the lower trace. The outlined portion of the bead displacement trace is enlarged in panel D. D) Enlargement of outlined trace in C. The raw data are shown in black and the steps detected by the step-finding program are shown in gray. E) Histogram of step sizes for microtubule plus-end-directed movement under 6 pN opposing load in the presence of 1 mM ATP (n = 370).

**Fig. 4.** Khc-73 is enriched at the distal ends of microtubule and co-localizes with Rab5-GFP. A) *Drosophila*  S2 cells were transiently transfected with constructs containing mCherry-tagged α-tubulin and GFP-tagged, full-length Khc-73 under the control of the metallothionein promoter (see Material and Methods). Images shown are an individual frame of a time-lapse movie provided as Supplemental Movie 1. Merged panel shows enlargement of boxed regions in the image with Khc-73 puncta at the tips of microtubules. Top left corner corresponds to the box on the lower left, and top right corner corresponds to the boxed region in the lower right. B) *Drosophila* S2 and BG2 cells were transiently transfected with constructs containing mCherry-tagged Khc-73 under the control of the metallothionein promoter and GFP-tagged Rab5 under the control of the actin promotor. Images shown are individual frames of time-lapse movies provided as Supplemental Movies 2 and 3. C) Enlargement of boxed region shown in the merged image of the BG2 cell shown in B. Arrowhead points to a motile puncta where both Khc-73-mCherry and Rab5-GFP co-localize.

**Fig. 5.** RNAi-mediated knockdown of endogenous Khc-73 identifies Rab5 vesicle targeting domain in Khc-73. *Drosophila* BG2 cells were subjected to 7-day RNAi treatment with an RNAi construct targeting the 3'-UTR of Khc-73 or mock treated. On day 4, cells were transfected with constructs containing the mCherry-tagged portion of Khc-73 shown under the control of the metallothionein promoter and GFP-tagged Rab5 under the control of the actin promotor. On day 6, cells were incubated with 50 mM copper sulfate for 16 hr. On day 7, time-lapse imaging was performed.
Table 1. Motile and Hydrodynamic Behavior of Khc-73 Motor-containing Constructs

<table>
<thead>
<tr>
<th>Khc-73 Construct</th>
<th>Microtubule gliding velocity (μm/sec)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Single Molecule Motility&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Sedimentation coefficient (x10&lt;sup&gt;−13&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Stokes Radius (Å)</th>
<th>Molecular Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD shown for &gt;50 measurements.</td>
<td>Mean ± SD shown for &gt;40 measurements.</td>
<td>Each data set comes from multiple experiments using protein from a single preparation.</td>
<td>Molecular weight based on the calculation described in Experimental Procedures.</td>
<td>Molecular weight based on the sum of amino acids in the Khc-73 construct, linker, GFP, and 6xHIS affinity tag.</td>
</tr>
<tr>
<td>1-387-GFP</td>
<td>1.45 ± 0.07</td>
<td>1.57 ± 0.26</td>
<td>0.45 ± 0.09</td>
<td>3.0</td>
<td>42.5</td>
</tr>
<tr>
<td>1-432-GFP</td>
<td>1.51 ± 0.02</td>
<td>1.58 ± 0.12</td>
<td>1.30 ± 0.49</td>
<td>0.15</td>
<td>3.0</td>
</tr>
<tr>
<td>1-547-GFP</td>
<td>1.62 ± 0.10</td>
<td>1.33 ± 0.23</td>
<td>0.76 ± 0.11</td>
<td>0.13</td>
<td>3.0</td>
</tr>
<tr>
<td>1-623-GFP</td>
<td>1.58 ± 0.04</td>
<td>1.54 ± 0.46</td>
<td>1.04 ± 0.11</td>
<td>0.28</td>
<td>3.0</td>
</tr>
<tr>
<td>1-387-LZ-GFP</td>
<td>N.D.</td>
<td>1.56 ± 0.15</td>
<td>1.51 ± 0.06</td>
<td>6.77</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SD shown for >50 measurements.  
<sup>b</sup>Mean ± SD shown for >40 measurements.  
<sup>c</sup>Each data set comes from multiple experiments using protein from a single preparation.  
<sup>d</sup>Molecular weight based on the calculation described in Experimental Procedures.  
<sup>e</sup>Molecular weight based on the sum of amino acids in the Khc-73 construct, linker, GFP, and 6xHIS affinity tag.
Figure 1

A) D.m. Khc-73 356 AVNEDPNARIIRHVEVETLRSMLKATGSPGDVQ-
C.e. Unc104 351 AVNEDPNALIREELNEVIKLRSLLDKGIDVTIDVQ
M.m. KIF1A 358 AKINEDPNAILIREKEVLRSLLDKGIDVTDQ

--- Helix 1 ---

D.m. Khc-73 394 TPGKHKKGPKLPAHVH---EQLF
C.e. Unc104 389 TPGKHKGKPPLPAHVH---EQLF
M.m. KIF1A 397 ALVGMSPPSSSALSSRAASVSSLHERILFAPGSEAIF

--- Inter-Helix Hinge ---

D.m. Khc-73 395 KLAESLMLKQISQTwEKLVIKTTERIQNERQQALEKKG
C.e. Unc104 409 KLESEKLMAEIGKTWEQKTEarthEIRKQREEELRDMGL
M.m. KIF1A 436 RLKETEKLAEINETWEEKLRRTEAIRMERELLAEGKV

--- Helix 2 ---

B) Coil-forming Probability

Domain: H1 H2 FHA CC

C) Full Length

Motor H1 H2 FHA CC CAP-Gly

Figure 2

A) 
- WT 1-387: VNEDP-NARIIRELHVEETLRSMLKHAT-GS 387
- Coil mutant C363: VNEDP-NARIIREKHLHVEETLRSMLKHAT-GS 387
- C386: VNEDP-CNARIIRELHVEETLRSMLKHAT-GS 388

B) 
- WT 1-387
- Coil mutant
- C363
- C386

C) 

<table>
<thead>
<tr>
<th>Movement Frequency (events/axoneme/min)</th>
<th>Reducing</th>
<th>Non-Reducing</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT 1-387</td>
<td>0.17</td>
<td>0.11</td>
</tr>
<tr>
<td>Coil mutant</td>
<td>(n.d.)</td>
<td>(n.d.)</td>
</tr>
<tr>
<td>C363</td>
<td>4.1</td>
<td>23.8</td>
</tr>
<tr>
<td>C386</td>
<td>5.0</td>
<td>19.4</td>
</tr>
</tbody>
</table>

D) 

- WT 1-387
- Coil mutant
- C363
- C386

Helix 1
Figure 3

A) Force (pN) vs. Time (s)

B) Stall Force (pN) vs. # of stalls

C) Distance (nm) vs. Time (s)

D) Distance (nm) vs. Time (s) for another dataset

E) # of steps vs. Step size (nm)
Figure 4

A) Rab5-GFP Khc-73-mCherry

B) Rab5-GFP Khc-73-mCherry

S2

BG2

C) Rab5-GFP Khc-73-mCherry

Downloaded from http://www.jbc.org/ by guest on January 1, 2018
<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Khc-73 RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khc-73 construct</td>
<td>Rab5</td>
<td>Rab5</td>
</tr>
<tr>
<td>1-387</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>443-1806</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>443-1921</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Kinesin-73 is a processive motor that localizes to Rab5-containing organelles
Thomas M. Huckaba, Arne Gennerich, James E. Wilhelm, Athar H. Chishti and Ronald D. Vale

J. Biol. Chem. published online December 18, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.167023

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/12/18/M110.167023.DC1