The Tetrameric Molecule of Conventional Kinesin Contains Identical Light Chains†

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ABSTRACT: Conventional kinesin is a multifunctional motor protein that transports numerous organelles along microtubules. The specificity of kinesin−cargo binding is thought to depend on the type(s) of light chains that a kinesin molecule contains. We have shown previously that different isoforms of kinesin light chains are associated with different types of cargo, mitochondria and membranes of the Golgi complex. Here, we provide evidence that the two light chains present within each kinesin molecule are always of the same type. Further, we demonstrate that kinesin heavy chains interact with nascent light-chain polypeptides on ribosomes. These data suggest that incorporation of the two identical light chains into a single kinesin molecule most likely occurs cotranslationally.

Intracellular transport is a well-organized system, which is served by multiple motor proteins transporting organelles and protein complexes along either microtubules or actin filaments. Conventional kinesin (hereafter called kinesin) carries cargoes along microtubules toward their plus ends. The list of the recognized kinesin cargoes grows rapidly, and it now includes not only membranous organelles but also nonmembrane complexes, such as RNPs. Most of the newly found kinesin partners are neuronal in origin (for the recent review, see ref *1*). However, the positioning and motility of some organelles in nonneuronal cells, e.g., mitochondria, lysosomes, secretory vesicles, and vimentin filaments, have also been shown to depend on kinesin (*2*−*5*).

Kinesin has been isolated from various cells and tissues as a tetrameric complex consisting of two heavy chains (KHC)[1] and two light chains (KLC). Recent findings, however, revealed that this configuration is not obligatory. For example, kinesin isolated from fungi proved to lack KLCs (*6*), while in cultured HeLa cells, the kinesin pool contains both tetrameric molecules and KHC dimers (*7*). KHCs interact with microtubules and perform the mechanochemical work by the N-terminal motor domains. The C-terminal tail domains of KHCs bind KLCs and are also involved in the interaction with a cargo (*8*, *9*).

Movement and/or positioning in the cytoplasm of most kinesin cargoes depend on KLC. The central portion of KLC contains several long imperfect repeats, referred to as tetratrico-peptide repeats (TPR). Because TPR repeats are usually involved in protein−protein interactions the KLC TPR domain is considered to be the main candidate for the cargo-attachment site (*10*). Consistent with this idea, direct binding of kinesin to some specific cargoes has been shown to occur through the TPR repeats of KLC (*11*−*14*). Further, blocking this domain with a specific antibody releases kinesin from the membranous vesicles in extruded squid axoplasm (*15*).

Outside the TPR domain, the N-terminal portion of KLC provides the binding site for KHC (*16*). The C-terminal region of KLC is, as a rule, variable because of alternative splicing of the pre-mRNA and gives rise to multiple isoforms (*17*−*21*). The variable C-terminal region of KLC has been proposed to contribute to the discrimination between different kinesin cargoes (*17*). Indeed, various KLC isoforms have been shown to associate with different organelles, e.g., mitochondria, vimentin filaments, and Golgi membranes (*21*−*23*). This fact suggests that the variability of the light-chain C termini most likely produces the functional diversity, so that the intracellular kinesin pool comprises a mixture of molecules able to interact with different types of cargo. The question remains, however, whether a single kinesin molecule is capable of binding to just one or multiple types of cargo. We reasoned that this question could be addressed by determining whether a single kinesin molecule contains mixed KLCs or two copies of the same KLC isoform.

Here, we provide new evidence that an individual kinesin molecule always contains two identical KLC polypeptides. Further, we find that KHC dimers associate with nascent KLC polypeptides on polyribosomes and hypothesize that the kinesin molecules are assembled cotranslationally.

EXPERIMENTAL PROCEDURES

Antibodies. The rabbit polyclonal antibodies αKLC, αHCT, αB/C, and αD/E have been characterized earlier (*21*, *23*). The mouse monoclonal antibody 63-90 was a generous gift from Dr. Scott T. Brady. Mouse hybridoma cells

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[1] Abbreviations: KHC, kinesin heavy chain; KLC, kinesin light chain; TPR, tetratrico-peptide repeats; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

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producing cMyc antibody were obtained from the American Tissue Culture Collection (Rockville, MD).

Expression Constructs. pcDNA3 encoding the full-length human uKHC was kindly provided by Dr. Richard Wubbolts. To express the tagged full-length KLC isoforms in cultured animal cells, the following strategy was used.

1. The rat KLC-A was recloned from pBluescript KS+ plasmid (kindly provided by Dr. Janet Cyr and Dr. Scott Brady) into pcDNA3 vector (Invitrogen, Carlsbad, CA), using *Pvu*I site in the 5′-UTR and *Eco*RI site in the 3′-UTR; in this reaction, the *Hind*III site in pcDNA3 was eliminated so that the internal *Hind*III site could be further used for subcloning.

2. The cMyc sequence was subcloned into pGEX-3XUZ-KLC-A at the *Hind*III site located between 18 and 19 amino acid residues, so that the *Hind*III site was restored just upstream of the cMyc sequence and that the treatment with *Hind*III endonuclease formed the sequence encoding the fragment of KLC: cMyc−Gln−(19th amino acid residue)−end.

3. This tagged fragment was inserted into the pcDNA3-KLC-A instead of the untagged one, using *Hind*III and the ORF-downstream *Xho*I sites; the resulted sequence encoded the full-length rat KLC-A with the cMyc sequence between 18 and 19 amino acid residues.

4. The internal site *Not*I and the downstream site *Xho*I were utilized to substitute the C-terminal portion of the rat KLC-A with those of the hamster KLC isoforms B, C, D, E, and H (*21*).

Cell Cultures and Transfections. Green monkey kidney epithelial cells CV-1 (ATCC) and rat embryo fibroblasts REF52 (kindly provided by Dr. P. Chumakov) were maintained in DMEM (Sigma) supplemented with 10% calf serum (Biolot, St. Petersburg, Russia), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cultures were grown at 37 °C in a 5% CO_2 atmosphere.

To obtain the cell lines stably expressing cMyc-tagged KLCs, CV-1 cells were transfected with the pcDNA3 encoding the chimeric rat−hamster KLC isoforms B, C, D, E, or H (see the previous section). For selection, transfected cells were grown in the presence of 1 mg/mL geneticin (Gibco BRL, Grand Island, NY).

Cell Fractionation. Cultured cells of various lines were grown to confluence on 90-mm cell culture plates. All procedures were done on ice or at 4 °C, unless otherwise indicated.

To analyze the Triton-soluble kinesin by immunoprecipitation, cells were rinsed 3 times in phosphate-buffered saline (PBS) at room temperature and collected with a rubber scraper in ice-cold PIPES buffer containing 50 mM K^+-PIPES (pH 6.8), 1 mM $MgCl_2$, 1 mM K^+-EGTA, supplemented with 1% Triton X-100, and protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, and 1 μg/mL *N*2-*p*-tosyl-L-arginine methyl ester (TAME) (0.3−1.0 mL of the buffer per each plate). The lysate was passed 10× through a 25-gauge needle and centrifuged at 5000*g* to remove nuclei and at 250000*g* to obtain the high-speed supernatant (hereafter called S250) that was used for immunoprecipitation.

To analyze membrane-bound kinesin, cells were lysed in HEPES buffer containing 20 mM K^+-HEPES (pH 7.5), 1 mM $MgCl_2$, and 1 mM K^+-EGTA, supplemented with 0.25 M sucrose and protease inhibitors, and homogenized using a 25-gauge needle (1 mL of the buffer per plate). Homogenate was centrifuged at 5000*g*, and then organelles were pelleted at 100000*g*. The 100000*g* supernatant was used as a cytosol fraction. The pellet was suspended in 1 mL of HEPES buffer supplemented with 1% Triton X-100 and protease inhibitors, and the insoluble material was removed by centrifugation.

For polyribosomes isolation, cells were rinsed 3 times at room temperature with HEPES buffer supplemented with 0.25 M sucrose and 10 μg/mL cycloheximide (Sigma Chemical Co., St. Louis, MO), collected with the rubber scraper in HEPES buffer supplemented with 1% Triton X-100, 10 μg/mL cycloheximide, and protease inhibitors (1 mL per each plate), passed 10× through a 25-gauge needle, and centrifuged at 5000*g* and 12000*g*. The supernatant was then layered onto a cushion of 1 M sucrose in HEPES buffer supplemented with 10 μg/mL cycloheximide and centrifuged for 2 h at 250000*g* in the TLS55 rotor. The pellet containing polyribosomes was suspended in the same buffer and analyzed by immunoprecipitation and Western blots.

Microtubule Sedimentation Assay. Microtubules were polymerized from purified bovine brain tubulin isolated by the cycles of polymerization−depolymerization. Polymerized microtubules were stabilized with 20 μM Taxol. To isolate microtubule-bound kinesin, cells were lysed in PIPES buffer at pH 6.8, to provide conditions favorable for the interaction of kinesin with microtubules (*24*). S250 was immunodepleted of KLC using sequential incubations with the antibody αKLC (see below) and mixed with microtubules in the presence of either 1 mM AMP-PNP and 4 units/mL apyrase (Sigma) or 1 mM ATP; as a control, nondepleted S250 was used. After incubation for 30 min at room temperature, the mixtures were layered onto cushions of 4 M glycerol in PIPES buffer, supplemented with 5 μM Taxol and either apyrase or ATP and centrifuged at 200000*g* for 30 min at 20 °C. The pellets were analyzed in Western blots.

Gel Electrophoresis and Western Blot Analysis. Samples were analyzed by SDS−PAGE in 4−12% linear-gradient gels according to the method of Laemmli (*25*) and electroblotted onto nitrocellulose membrane (HybondECL, Amersham, Germany). After the blots were stained with Ponceau S, they were blocked for 1 h in 5% goat serum in TPBS (PBS supplemented with 0.1% Tween 20) and incubated with 1−5 μg/mL primary antibody in blocking solution for 1 h at room temperature, followed by horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PN) at 1:5000 dilution. For detection, diaminobenzidine (Sigma) was used.

To compare KHC/KLC ratios in different preparations, serial dilutions were analyzed by Western blots with antibodies αHCT and αKLC. Developed blots were scanned with Microtek ScanMaker E6, and ImageJ was used to measure the staining intensities for individual bands. The relative content of KHC and KLC in samples was determined as a ratio of the slopes of the titration curves.

Immunoprecipitation. This method was used in three variations: (i) For the cell extract immunodepletion of KLC, a 1-mL portion of S250 was incubated sequentially with three 10-μg portions of the antibody αKLC bound to Protein A-Sepharose beads (Sigma) for each incubation of 40−60 min; (ii) To precipitate KHC or KLC-D/E, Protein A beads

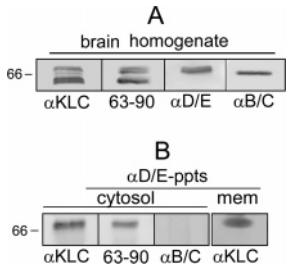

FIGURE 1: KLC isoforms of the D/E type do not combine with others. The precipitates from the cytosol and membrane fractions of the rat brain with the antibody αD/E (lower panel) was compared in Western blots with the total brain homogenate (upper panel). The position of the 66-kDa molecular mass protein marker is shown at the left.

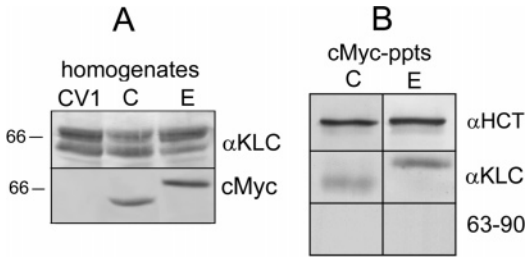

FIGURE 2: Exogenous cMyc-tagged KLC polypeptides do not combine with the endogenous KLC isoforms. The stable lines of CV-1 cells expressing cMyc-tagged KLC isoforms C and E at the moderate level (A) were fractionated to obtain S250 fractions, and kinesin was precipitated from these fractions by the cMyc antibody. The cMyc precipitates were examined in Western blots with anti-kinesin antibodies (B). The position of the 66-kDa molecular mass protein marker is shown at the left.

with 10 μg of any antibody were incubated with the cell fraction for 1 h; (iii) For immunoprecipitation of the nascent polypeptides, the 250000*g* pellet obtained by centrifugation of the postmitochondrial supernatant through the sucrose cushion was suspended in 100 μL of HEPES buffer supplemented with 1% Triton X-100, protease inhibitors, and 10 μg/mL RNAse A (Sigma). The suspension was incubated for 40 min at 25 °C, diluted with HEPES buffer to the final volume 1 mL, and passed 5 times through the syringe needle 25. The preparation was then mixed with the Protein A-Sepharose beads carrying 10 μg of the cMyc antibody and incubated overnight.

In each case, the beads were washed 3 times in lysis buffer plus 1% Triton X-100, 3 times in this buffer supplemented with 0.5 M NaCl, 3 times in buffer alone, and finally boiled in Laemmli sample buffer containing 2% SDS.

RESULTS

Both of the Light Chains within a Kinesin Molecule Are of the Same Isoform Type. To explore interactions between spliced variants of KLC, we tested whether different KLC isoforms could be coprecipitated from cell and tissue extracts using antibodies specific for individual KLC isoforms.

In the first series of experiments, KLCs were precipitated with the isoform-specific antibody αD/E and analyzed in Western blots using two pan-KLC antibodies, polyclonal αKLC and monoclonal 63-90. The antibody αD/E was raised against the unique C-terminal sequence of 56 amino acid residues shared by the KLC isoforms D and E that have been isolated from a hamster cDNA library (*21*). In Western blots of homogenates of rat brain and cultured cells of several lines, this antibody selectively recognized only some of KLCs (Figure 1A). Western blot analysis of the αD/E precipitates from cytosol fractions revealed the presence of KHC, thus indicating that this antibody pulled down the whole kinesin molecules (data not shown). Germane here is that αD/E precipitated no additional KLC polypeptides besides those that it recognized in Western blots (Figure 1B). Moreover, the antibody αB/C specific for the KLC isoforms B and C (*21*) did not detect any polypeptides of this kind in the αD/E precipitate (Figure 1B).

Upon the cell lysis, most of kinesin is found in the cytosol fraction and only a small fraction of the motor is bound to membranous organelles. To explore the cargo-associated kinesin molecules, fractions of membranous organelles were isolated from cultured cells and rat brain. A comparison between the αD/E precipitates from the cytosol and membrane fractions revealed that the D/E KLC isoforms did not mix with other types (Figure 1B; only precipitates for the rat brain fractions are shown). The same data were obtained using the cytosol and membrane fractions of cultured cells REF52 and CV-1. Thus, various KLC isoforms do not mix within a single kinesin molecule, whether the motor is bound to a membranous cargo or not.

These data were supported by the experiments with the stable cell lines. Six cMyc-tagged KLC variants, KLC-A, -B, -C, -D, -E, and -H, were stably expressed in CV-1 cells (isoforms C and E are schematically shown in Figure 3A). According to Western blots, the level of expression of the cMyc-tagged protein in the cells of each cell line was moderate, so that it was not a major KLC component (Figure 2A; the results for the lines C and E are shown). When the tagged proteins were precipitated from the cell extracts with cMyc antibody and analyzed in Western blots with αCT and αKLC, the precipitate from each cell line proved to contain, along with KHC (data not shown), only one KLC polypeptide (Figure 2B).

Another available KLC antibody, the monoclonal 63-90, did not recognize the cMyc-tagged variants, although it reacted in Western blots with the endogenous CV-1 KLCs (data not shown). The lack of interaction was probably due to the insertion of the cMyc sequence into the N-terminal region of the KLC polypeptide that could destroy the epitope. In Western blots with the cMyc precipitates, the antibody 63-90 did not detect any KLC polypeptides, thus confirming the absence of the endogenous KLCs (Figure 2B). We, therefore, concluded that each tagged KLC isoform bound to KHC but no additional KLC polypeptides entered the complex.

We then probed the interaction within the cells of two recombinant KLCs, KLC-C and -E. These cMyc-tagged polypeptides were identical throughout the entire length except the C-terminal sequences, from which only the unique sequence of the isoform E was recognized by the antibody αD/E (Figure 3A). It has been previously reported that overexpressed KLC aggregated in the cytoplasm and that simultaneous expression of KHC could prevent KLC aggregation (*24*). Taking this finding into account, we transfected CV-1 cells with two KLC constructs and a plasmid encoding the full-length human ubiquitous KHC.

Western blots with the cMyc antibody showed that the homogenate and cytosolic fraction of transfected cells, as well as the cMyc precipitate from the cytosolic fraction,

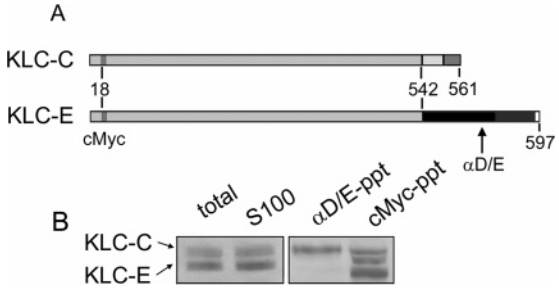

FIGURE 3: Two recombinant KLC variants do not combine within a kinesin molecule. The cMyc-tagged KLC-C and -E (A) were coexpressed with KHC in CV-1 cells. The tagged KLCs were precipitated from the cell extract with either cMyc or αD/E antibody, and precipitates were compared in Western blots with cMyc antibody (B).

contained the cMyc-tagged proteins in comparable amounts (Figure 3B). However, in contrast to cMyc antibody, αD/E precipitated from this fraction only cMyc-KLC-E but not cMyc-KLC-C (Figure 3B).

Thus, three different experimental approaches revealed that only the same KLC-isoform polypeptides combine with KHCs to form a whole kinesin molecule. This finding raises an important question of how this uniformity is achieved. We hypothesized that the KHC dimer interacts with two nascent KLCs on neighbor ribosomes translating an individual mRNA. However, this mechanism requires that a sufficient amount of KLC-free KHC dimers is present in cells. Thus, to prove the feasibility of our hypothesis, we first had to demonstrate the existence of a significant pool of KLC-free KHC in cells.

KHC Not Associated with KLC Is Present in Cells. The presence of kinesin molecules lacking the full-length KLC has been previously reported in kinesin preparations isolated from bovine brain (*26*) and HeLa cells (*7*). In the former work, however, this kinesin species was considered to result from the degradation of KLC during kinesin isolation. Therefore, we applied an immunochemical approach to examine whether KLC-free kinesin was present in CV-1 cells and whether it was a result of the KLC degradation. For this, the total cell homogenate was compared in the same Western blot with the αKLC and αHCT precipitates from the S250 fraction. Because the slopes of the dilution curves reflected the relative content of the kinesin subunits (within the linear detection range), their ratio characterized the ratio between the KHC and KLCs. The typical dilution curves that are shown at Figure 4 revealed that the KHC/KLC ratio in the cell homogenate was much higher than that in the αKLC precipitate, thus indicating the significant molar excess of KHC over KLC in cells. On the contrary, the αHCT precipitate did not differ from the cell homogenate in the KHC/KLC ratio (data not shown). The amount of the KHC excess varied from experiment to experiment, even for the same samples; however, the fraction of KLC-free kinesin could roughly be estimated at no less than 50%.

To confirm the data of the Western blot analysis, cells were lysed in the presence of Triton X-100 and the 250000*g* supernatant (S250) was immunodepleted of either KHC or KLC. According to Western blots, no KLC could be detected in S250 thoroughly depleted of KHC with the antibody αHCT (data not shown). In contrast, preparations depleted of KLC still contained substantial amounts of KHC (Figure

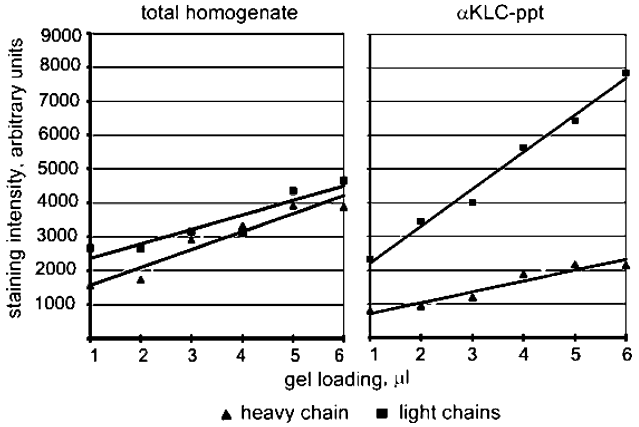

FIGURE 4: CV-1 cells contain a molar excess of KHC. The serial dilutions of the CV-1 homogenate and αKLC precipitate were compared in Western blots with αHCT and αKLC antibodies.

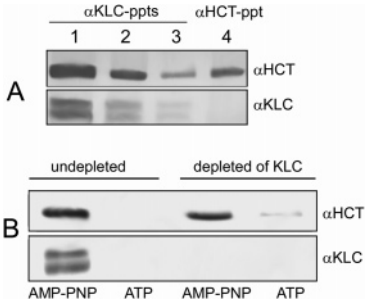

FIGURE 5: KLC-free KHC interacts with microtubules in the ATP-sensitive manner. The Triton-S250 fraction was depleted of KLCs by three successive αKLC precipitations, and the residual kinesin was either precipitated with the antibody αHCT (A) or probed for cosedimentation with microtubules (B). Precipitates and MT pellets were analyzed in Western blots with αHCT and αKLC antibodies.

5A). Using αKLC antibody for immunodepletion, this KLC-free kinesin fraction was found in the Triton extracts of various cell lines and in both cytosolic and membrane fractions (data not shown). The KLC-free kinesin was able to bind to Taxol-stabilized microtubules in the absence of ATP and release from the complex upon ATP addition (Figure 5B), thus demonstrating the features typical for the functional KHC.

Judging from the molar excess of KHC over KLC in the total cell homogenate, the KLC-free kinesin was present in cells initially and did not arise from proteolysis of KLC during cell lysis or incubation with the antibody. Nevertheless, because its content proved to be rather high, we performed control experiments to surely eliminate protein degradation. For this, cells were lysed with Triton X-100 in either HEPES buffer at pH 7.5 or PIPES buffer at pH 6.8 and incubated for up to 6 h at 4 °C. The samples taken during the incubation were compared in Western blots with the control sample obtained by boiling the whole cells in Laemmli buffer. Quantitative analysis of the blots demonstrated that the KHC/KLC ratio in these preparations remained constant throughout the incubation and was close to that of the control sample (data not shown).

These data confirmed that, in addition to tetrameric molecules, the intracellular kinesin pool contains KLC-free molecules that are most likely KHC dimers. Thus, there is enough KLC-free KHC in cells that could be involved in the assembly of the tetrameric kinesin molecules.

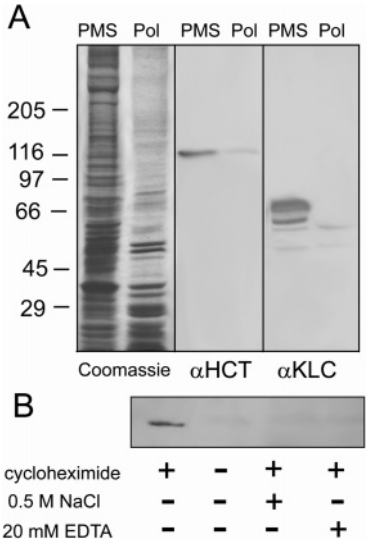

FIGURE 6: KLC-free KHC associates with polysomes. The polysome fraction (Pol) was isolated from the postmitochondrial supernatant (PMS) of CV-1 cells in the presence of cycloheximide and analyzed in Western blots with αHCT and αKLC antibodies (A). The same fractions obtained in different conditions were compared by the presence of KHC in Western blots with αHCT antibody (B). The positions of the molecular mass protein markers are shown at the left.

KLC-free KHC Associates with Polyribosomes. To test whether KHC interacts with nascent KLC peptides, cells were lysed in Triton X-100 in the presence of cycloheximide and polyribosomes were pelleted from the Triton extract by centrifugation through 1 M sucrose at 250000*g*. Western blot analysis of this fraction with αHCT antibody detected the presence of KHC (Figure 6A). This KHC polypeptide could only be the full size, because the epitope that is recognized by αHCT is located right at the C terminus of KHC (*21*). The presence of KHC in the 250000*g* pellet strongly depended on cycloheximide and was sensitive to agents that destroy ribosomes; in the presence of 20 mM EDTA or 0.5 M NaCl, KHC was not detected in Western blots of the 250000*g* pellet (Figure 6B).

Remarkably, no full-length KLC polypeptides were detected in the fraction of polyribosomes in Western blots with the antibody αKLC (Figure 6A). Two immunoreactive components of the lower molecular mass that are seen at the blot represent, presumably, the nascent KLC polypeptides.

Thus, a portion of the intracellular kinesin copurified with cycloheximide-stabilized polysomes; this polysome-associated kinesin did not contain the full-length KLCs.

KHC Interacts with the KLC Polypeptides of the Incomplete Size. To examine whether the ribosome-associated KHC interacts with nascent KLC peptides, we used the cell line stably expressing the cMyc-tagged KLC isoform E (Figure 3A). Because of the N-terminal position of the tag sequence, this 68-kDa protein could be precipitated with the cMyc antibody not only as a full-length protein but also as a nascent peptide yet attached to ribosomes. On the contrary, the αD/E antibody could be used to distinguish between the nascent and mature KLC polypeptides, because of the C-terminal position of the epitope.

The cells were fractionated in the presence of cycloheximide and Triton X-100, to obtain polysomes and the postribosomal 250000*g* supernatant (S250). The tagged polypeptides were then precipitated from these two fractions using cMyc antibody. Western blots of the precipitates with the αHCT antibody showed that KHC was pulled down from both S250 and polysomes (Figure 7). In the control experiment with the ordinary CV-1 cells, the cMyc antibody did not precipitate KHC from the polysome fraction (data not shown); therefore, it was unlikely that any other component of the translating complex, besides the cMyc-containing nascent peptides, could be responsible for the KHC binding. Moreover, the same results were obtained when the polysome-bound mRNA was subjected, prior to immunoprecipitation, to the limited treatment with RNAse A. Centrifugation of the RNAse-treated preparation in the 0.5−1.5 M sucrose gradient proved that such treatment induced dissociation of polysomes to monoribosomes but did not destroy ribosomes themselves (data not shown). Additionally, the beads with cMyc precipitates were washed in buffer containing 0.5 M NaCl, so that the association of KHC with the precipitates proved to be resistant to high ionic strength. This is typical for KHC−KLC binding (*27*) but not for ribosomes that dissociate to individual components under these conditions (*28*). Thus, we concluded that KHC specifically interacted with the cMyc-containing peptides.

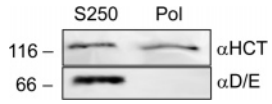

FIGURE 7: Ribosome-associated KHC is bound to KLC polypeptides lacking the C termini. The cells of the stable cell line expressing the cMyc-tagged KLC-E were fractionated to S250 and polyribosome fractions, and the cMyc-containing polypeptides were precipitated from both fractions using cMyc antibody. The precipitates were equalized by the KHC contents and analyzed in Western blots with the antibody αD/E. The positions of the molecular mass protein markers are shown at the left.

To analyze these peptides, the samples were equalized in the KHC content and probed in Western blots. The precipitate from S250 contained a 68-kDa component that was recognized by αD/E (Figure 7); additionally, it reacted in Western blots with αKLC antibody (data not shown). Thus, it was regarded as the full-length KLC-E. In the cMyc precipitate from the ribosome suspension, the antibody αKLC interacted with a few peptides of the lower molecular mass (data not shown) and neither αD/E (Figure 7) nor αKLC detected a polypeptide with the molecular mass of cMyc-KLC-E.

These results indicate that the ribosome-associated KHC is bound to nascent KLC peptides that already have N-terminal regions (containing the cMyc sequence) but lack the C-terminal portion.

DISCUSSION

There are 3 genes (*klc1*−*3*) that encode KLCs in mammals (*29*, *30*). It has been reported that the products of two genes KLC1 and KLC2 never coprecipitate (*29*). Further, neuronal kinesins containing KLC1 and KLC2 (*31*) and testis kinesin containing KLC3 (*30*) all differ in their properties. This clearly demonstrates that the structural diversity stipulates the functional variety of the motor molecules. At the same time, kinesin diversity is not confined within combinations of different gene products, because the transcripts of the KLC genes have been shown to undergo alternative splicing, which

gives rise to multiple variants of the protein with unique C termini (*17*, *21*, *24*, *32*). We show in this study that different endogenous KLC isoforms do not combine with each other, as well as with an exogenous KLC variant. Further, two recombinant KLC isoforms, differing only in a short C-terminal sequence, do not form the joint molecules within the cell. Thus, our data reveal that each heterotetrameric kinesin molecule always contains two identical KLC isoforms.

Data presented here allow us to propose a particular mechanism that would ensure the identity of two KLCs during kinesin assembly. We suggest that the KHC dimers associate with nascent KLC polypeptides, so that two KLCs that are being synthesized on a single mRNA become incorporated into a single kinesin molecule.

There are two major conditions that must be met for such a mechanism to work. First, there must be a pool of kinesin molecules that contain the heavy but no light chains. The presence of such KLC-free molecules in the kinesin preparations has been reported previously. For example, the KHC/KLC ratio in kinesin purified from bovine adrenal medulla was determined as 2:1 (*33*). Further, KLC-free fractions of KHC have been described in gel filtrates of kinesin isolated from bovine brain (*26*, *34*) and sea-urchin embryos (*35*). Using immunochemical analysis of the kinesin pool, we also found in various cultured animal cells a noticeable fraction of kinesin that does not include the light chains. Thus, the first condition of our model, the existence of KLC-free kinesin dimers, is met in cells.

In the aforementioned studies, the absence of KLCs was explained by specific degradation of the light-chain polypeptides. Finding the specific PEST sequences in KLC that form sites of likely protease attack (*19*) supported the idea of preferential KLC degradation. We tested this suggestion and found that, at least in the case of cultured cells, KLC degradation could not be the source of the KLC-free kinesin molecules. Our data are in agreement with the study of De Luca and co-workers who recently isolated dimeric KHCs from mitotic HeLa cells (*7*). Importantly, the KLC-free KHCs are present in cells as full-length intact polypeptides, capable of binding to microtubules in the ATP-sensitive manner (our data) and promoting their gliding in vitro (*7*). When taken together, these data reveal that the KLC-free kinesin molecules found in various cultured cells represent functional KHC dimers that possess motor properties of conventional kinesin.

Further studies will elucidate if this KLC-free kinesin fulfills motor functions. Recent data revealed that some kinesin cargoes, as well as the putative kinesin receptor kinectin, bind directly to KHC (*36*−*39*). Additionally, KHC has been shown to be involved in transporting mRNA, e.g., *oskar* in *Drosophila melanogaster* oocytes in the KLC-independent manner (*9*). This raises a concern that our polyribosome fractions can be contaminated with KHC because of the interaction between KHC and mRNAs unrelated to KLC. However, the fact that the ribosome-associated KHC coprecipitates with nascent KLC polypeptides indicates that these particular kinesin molecules play a specific nonmotor role in the light chains folding even though it does not exclude the possibility that some kinesin associates with ribosomes independently of KLCs.

The second condition of our model is that KHC dimers must be able to bind nascent KLC polypeptides. In this respect, the binding site for the ubiquitous KHC has been mapped close to the N terminus of KLC (*16*). Further, although depletion of cell extracts with αKLC antibody never brings down all KHC, the residual KHC is capable of forming a complex with nascent light chains on ribosomes. Importantly, in the presence of cycloheximide, this complex remains detectable even after the RNAse treatment that destroys mRNA and induces disassembly of polyribosomes to individual ribosomes. Furthermore, the complex is resistant to high ionic strength that is characteristic for the isolated heterotetrameric kinesin (*40*, *41*), as well as for the interaction of the recombinant KHC and KLC in vitro (*16*, *27*).

An important question is whether two KLCs synthesized on the same mRNA interact with KHC independently or if they first dimerize cotranslationally and then join with the heavy chains to form a complete molecule. While, admittedly, our work does not provide direct evidence to differentiate between these two possibilities, there are multiple circumstantial indications that the former is more likely. In this regard, it has been noticed that, in transiently transfected animal cells, the overexpressed KLC requires extra amounts of KHC to avoid aggregation (*24*). This observation can be easily explained if we assume that KHC cotranslationally interacts with the growing KLC polypeptides, similar to the formation of the intermolecular bonds between the heavy and light chains of immunoglobulins G in the mouse myeloma cells (*42*). Further, the fact that despite our best efforts we were unable to detect KHC-free KLCs using immunodepletion argues against KLC dimerization.

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REFERENCES

1. Vale, R. D. (2003) The molecular motor toolbox for intracellular transport, *Cell 112*, 467−480.
2. Nakata, T., and Hirokawa, N. (1995) Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport, *J. Cell Biol. 131*, 1039−1053.
3. Tanaka, Y., Kanai, Y., Okada, Y., Nonaka, S., Takeda, S., Harada, A., and Hirokawa, N. (1998) Targeted disruption of mouse conventional kinesin heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria, *Cell 93*, 1147−1158.
4. Prahlad, V., Yoon, M., Moir, R. D., Vale, R. D., and Goldman, R. D. (1998) Rapid movements of vimentin on microtubule tracks: Kinesin-dependent assembly of intermediate filament networks, *J. Cell Biol. 143*, 159−170.
5. Varadi, A., Tsuboi, T., Johnson-Cadwell, L. I., Allan, V. J., and Rutter, G. A. (2003) Kinesin I and cytoplasmic dynein orchestrate glucose-stimulated insulin-containing vesicle movements in clonal MIN6 β-cells, *Biochem. Biophys. Res. Commun. 311*, 272−282.
6. Kirchner, J., Woehlke, G., and Schliwa, M. (1999) Universal and unique features of kinesin motors: Insights from a comparison of fungal and animal conventional kinesins, *Biol. Chem. 380*, 915−921.
7. DeLuca, J. G., Newton, C. N., Himes, R. H., Jordan, M. A., and Wilson, L. (2001) Purification and characterization of native

conventional kinesin, HSET, and CENP-E from mitotic hela cells, *J. Biol. Chem. 276*, 28014−28021.
8. Bi, G. Q., Morris, R. L., Liao, G., Alderton, J. M., Scholey, J. M., and Steinhardt, R. A. (1997) Kinesin- and myosin-driven steps of vesicle recruitment for Ca^{2+}-regulated exocytosis, *J. Cell Biol. 138*, 999−1008.
9. Palacios, I. M., and Johnston, D. S. (2002) Kinesin light chain-independent function of the Kinesin heavy chain in cytoplasmic streaming and posterior localisation in the Drosophila oocyte, *Development 129*, 5473−5485.
10. Gindhart, J. G., and Goldstein, L. S. (1996) Tetratrico peptide repeats are present in the kinesin light chain, *Trends Biochem. Sci. 21*, 52−53.
11. Bowman, A. B., Kamal, A., Ritchings, B. W., Philp, A. V., McGrail, M., Gindhart, J. G., and Goldstein, L. S. (2000) Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein, *Cell 103*, 583−594.
12. Verhey, K. J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B. J., Rapoport, T. A., and Margolis, B. (2001) Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules, *J. Cell Biol. 152*, 959−970.
13. Rietdorf, J., Ploubidou, A., Reckmann, I., Holmstrom, A., Frischknecht, F., Zettl, M., Zimmermann, T., and Way, M. (2001) Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus, *Nat. Cell Biol. 3*, 992−1000.
14. Semiz, S., Park, J. G., Nicoloro, S. M., Furcinitti, P., Zhang, C., Chawla, A., Leszyk, J., and Czech, M. P. (2003) Conventional kinesin KIF5B mediates insulin-stimulated GLUT4 movements on microtubules, *EMBO J. 22*, 2387−2399.
15. Stenoien, D. L., and Brady, S. T. (1997) Immunochemical analysis of kinesin light chain function, *Mol. Biol. Cell 8*, 675−689.
16. Diefenbach, R. J., Mackay, J. P., Armati, P. J., and Cunningham, A. L. (1998) The C-terminal region of the stalk domain of ubiquitous human kinesin heavy chain contains the binding site for kinesin light chain, *Biochemistry 37*, 16663−16670.
17. Cyr, J. L., Pfister, K. K., Bloom, G. S., Slaughter, C. A., and Brady, S. T. (1991) Molecular genetics of kinesin light chains: Generation of isoforms by alternative splicing, *Proc. Natl. Acad. Sci. U.S.A. 88*, 10114−10118.
18. Wedaman, K. P., Knight, A. E., Kendrick-Jones, J., and Scholey, J. M. (1993) Sequences of sea urchin kinesin light chain isoforms, *J. Mol. Biol. 231*, 155−158.
19. Beushausen, S., Kladakis, A., and Jaffe, H. (1993) Kinesin light chains: Identification and characterization of a family of proteins from the optic lobe of the squid *Loligo pealii*, *DNA Cell Biol. 12*, 901−909.
20. Fan, J., and Amos, L. A. (1994) Kinesin light chain isoforms in *Caenorhabditis elegans*, *J. Mol. Biol. 240*, 507−512.
21. Khodjakov, A., Lizunova, E. M., Minin, A. A., Koonce, M. P., and Gyoeva, F. K. (1998) A specific light chain of kinesin associates with mitochondria in cultured cells, *Mol. Biol. Cell 9*, 333−343.
22. Liao, G., and Gundersen, G. G. (1998) Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin, *J. Biol. Chem. 273*, 9797−9803.
23. Gyoeva, F. K., Bybikova, E. M., and Minin, A. A. (2000) An isoform of kinesin light chain specific for the Golgi complex, *J. Cell Sci. 113* (part 11), 2047−2054.
24. Verhey, K. J., Lizotte, D. L., Abramson, T., Barenboim, L., Schnapp, B. J., and Rapoport, T. A. (1998) Light chain-dependent regulation of Kinesin's interaction with microtubules, *J. Cell Biol. 143*, 1053−1066.
25. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
26. Hackney, D. D., Levitt, J. D., and Wagner, D. D. (1991) Characterization of α 2 β 2 and α 2 forms of kinesin, *Biochem. Biophys. Res. Commun. 174*, 810−815.
27. Gauger, A. K., and Goldstein, L. S. (1993) The Drosophila kinesin light chain. Primary structure and interaction with kinesin heavy chain, *J. Biol. Chem. 268*, 13657−13666.
28. Spirin, A. S. (2000) *Ribosomes*, Kluwer Academic/Plenum Publishing, New York.
29. Rahman, A., Friedman, D. S., and Goldstein, L. S. (1998) Two kinesin light chain genes in mice. Identification and characterization of the encoded proteins, *J. Biol. Chem. 273*, 15395−15403.
30. Junco, A., Bhullar, B., Tarnasky, H. A., and van der Hoorn, F. A. (2001) Kinesin light-chain KLC3 expression in testis is restricted to spermatids, *Biol. Reprod. 64*, 1320−1330.
31. Rahman, A., Kamal, A., Roberts, E. A., and Goldstein, L. S. (1999) Defective kinesin heavy chain behavior in mouse kinesin light chain mutants, *J. Cell Biol. 146*, 1277−1288.
32. McCart, A. E., Mahony, D., and Rothnagel, J. A. (2003) Alternatively spliced products of the human kinesin light chain 1 (KNS2) gene, *Traffic 4*, 576−580.
33. Murofushi, H., Ikai, A., Okuhara, K., Kotani, S., Aizawa, H., Kumakura, K., and Sakai, H. (1988) Purification and characterization of kinesin from bovine adrenal medulla, *J. Biol. Chem. 263*, 12744−12750.
34. Sadhu, A., and Taylor, E. W. (1992) A kinetic study of the kinesin ATPase, *J. Biol. Chem. 267*, 11352−11359.
35. Johnson, C. S., Buster, D., and Scholey, J. M. (1990) Light chains of sea urchin kinesin identified by immunoadsorption, *Cell Motil. Cytoskeleton 16*, 204−213.
36. Diefenbach, R. J., Diefenbach, E., Douglas, M. W., and Cunningham, A. L. (2002) The heavy chain of conventional kinesin interacts with the SNARE proteins SNAP25 and SNAP23, *Biochemistry 41*, 14906−14915.
37. Skoufias, D. A., Cole, D. G., Wedaman, K. P., and Scholey, J. M. (1994) The carboxyl-terminal domain of kinesin heavy chain is important for membrane binding, *J. Biol. Chem. 269*, 1477−1485.
38. Ong, L. L., Lim, A. P., Er, C. P., Kuznetsov, S. A., and Yu, H. (2000) Kinectin−kinesin binding domains and their effects on organelle motility, *J. Biol. Chem. 275*, 32854−32860.
39. Setou, M., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M., and Hirokawa, N. (2002) Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites, *Nature 417*, 83−87.
40. Bloom, G. S., Wagner, M. C., Pfister, K. K., and Brady, S. T. (1988) Native structure and physical properties of bovine brain kinesin and identification of the ATP-binding subunit polypeptide, *Biochemistry 27*, 3409−3416.
41. Kuznetsov, S. A., Vaisberg, E. A., Shanina, N. A., Magretova, N. N., Chernyak, V. Y., and Gelfand, V. I. (1988) The quaternary structure of bovine brain kinesin, *EMBO J. 7*, 353−356.
42. Bergman, L. W., and Kuehl, W. M. (1979) Formation of intermolecular disulfide bonds on nascent immunoglobulin polypeptides, *J. Biol. Chem. 254*, 5690−5694.

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