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The Molecular Motor KIFC1 Associates with a Complex Containing Nucleoporin NUP62 That Is Regulated During Development and by the Small GTPase RAN[1]

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ABSTRACT

KIFC1 is a C-terminal kinesin motor associated with the nuclear membrane and acrosome in round and elongating spermatids. This location in developing spermatids is consistent with possible roles in acrosome elongation and manchette motility or both. Here we describe the association of the KIFC1 motor with a complex containing the nucleoporin NUP62. Formation of this complex is developmentally regulated, being absent before puberty and appearing only after nuclear elongation has begun. In addition, the integrity of this complex is dependent on GTP hydrolysis and the GTP state of the small GTPase RAN. Concomitant with the association of this motor with the NUP62-containing complex is an apparent reorganization of the nuclear pore with loss of NUP62 from larger complexes containing other nucleoporins. The association of KIFC1 with a component of the nuclear membrane is more consistent with a role for this motor in acrosome/manchette transport along the nuclear membrane than for a role for this motor in transport of vesicles along the outer face of the manchette.

gamete biology, manchette, molecular motor, nuclear pore, RAN, sperm, spermatid, spermatogenesis, testis

INTRODUCTION

Successful spermatogenesis is dependent on completion of a series of highly orchestrated cellular events culminating in the production of viable sperm capable of fertilization. During spermatid maturation, or spermiogenesis, these events are characterized by striking cellular reorganization and unique forms of motility including the growth and advancement of the acrosome along the nuclear surface paired with the retreat of the microtubular manchette toward the caudal end of the spermatid nucleus.

The acrosome is a specialized lysosome-like organelle containing the hydrolytic enzymes necessary for penetration of the oocyte. Spermiogenesis is divided into phases corresponding to the state of growth of the acrosome. In the initial stage of acrosome biogenesis, the Golgi phase, pro-acrosomal granules, and vesicles derived from the Golgi fuse to form a large acrosomal vesicle that then adheres to the nucleus, flattening along its surface to begin the cap phase of spermatid maturation. During the subsequent acrosome phase, the acrosome continues to extend across the surface of the spermatid nucleus while the microtubular manchette is formed around the nucleus and moves caudally. During this time, the chromatin condenses, and the nucleus is transformed into its final, species-specific shape. The mechanisms underlying both acrosome and manchette motility along the nuclear surface are unknown but are likely to involve various cytoskeletal elements of the spermatid.

Previous ultrastructural studies of wild type and mutant mouse spermatids identified proteinacous linkers between the manchette and the nuclear membrane as well as between the microtubules of the manchette [1, 2]. These authors proposed that these linkages serve to maintain a constant distance between microtubules of the manchette and the nuclear membrane and participate in the shaping of the spermatid nucleus during spermiogenesis. Their proposal is supported by observations that the nuclear membrane is flattened and oriented parallel to the manchette but is rounded in areas where the manchette is absent, either in wild-type cells or in mutants with malformed manchettes.

The molecular motors kinesin and dynein have been found associated with the manchette, making them possible candidate molecules providing the force necessary for manchette motility [3–5]. Interestingly, the size of the cross bridges described by Russell and coworkers approximates the size of kinesin motors seen to connect vesicles to microtubules in the neuronal cytoskeleton by cryoEM [6]. In addition to microtubule-based motors, the nonconventional myosin Myosin Va is associated with the acroplaxome, an actin and intermediate filament-containing plate joining the acrosome to the underlying nuclear membrane [7]. This actin-based motor was also found on vesicles associated with the cytoplasmic face of the manchette and is proposed to participate in the flow of membrane from the apical to the tail region of developing spermatids (intramanchette transport) [8–10].

We have described recently the association of the kinesin-related molecular motor KIFC1 with the nucleus of elongating spermatids [11]. KIFC1 is first seen associated with vesicles localized between the Golgi and the spermatid nucleus followed by association with the growing acrosome. In elongate spermatids, KIFC1 is associated with the caudal end of the spermatid nucleus [11]. The translocation of this motor along the nucleus suggested three different possible roles for KIFC1 in spermatid maturation: delivery of vesicles from the Golgi to the growing acrosome and continuing transport along microtubules of the manchette, recycling of vesicles back to the Golgi, and linkage of the manchette to the nuclear membrane [11]. In this paper we describe association of KIFC1 with a complex containing a resident protein of the nuclear membrane and the reorganization of this complex during spermiogenesis.

MATERIALS AND METHODS

Affinity Purification

All use of animals was approved and conducted in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research*

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and Teaching. An affinity purification column was prepared by attaching the KIFC1-targeting peptide identified previously (GKAASGASGASGRAAAIA-GRAD [12]) to a methacrylate resin (UNC Microprotein Sequencing and Peptide Synthesis Facility, Department of Microbiology, University of North Carolina at Chapel Hill). Rat testis extract was prepared as previously described [13]. Briefly, decapsulated testes from adult or prepuberal rats were homogenized in buffer (10 mM MES, pH 7.65, 1 mM EGTA, 0.5 mM $MgCl_2$, 30% glycerol) containing protease inhibitors (10 μM benzamidine, 0.1 mg/ml leupeptin, 0.1 mg/ml aprotinin, 0.1 mg/ml TAME, 3 μM PMSF) and centrifuged two times, first at 100 000 × *g* and then 130 000 × *g,* to remove cellular debris generating a high-speed supernatant (HSS) fraction. Protein concentration in testis HSS was determined by the Coomassie brilliant blue method (Bio-Rad Laboratories, Hercules, CA). The 2-ml column was equilibrated in bead-binding buffer (25 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$) supplemented with protease inhibitors. Approximately 20 mg testis lysate in 1.0 ml binding buffer were loaded onto the column and incubated, with gentle mixing, overnight at 4°C. The column was then washed with a total of 10–12 ml buffer and the flow-through collected until the OD_{280} had returned to baseline. Bound complexes were eluted from the column in 200-μl fractions first with IgG elution buffer (Pierce, Rockford, IL) followed by IgG elution buffer with 250 mM added NaCl.

Proteins in eluted fraction were precipitated by addition of 1 μl 2% Na deoxycholate and 5 μl 10% TCA, vortexed for several seconds, and the samples incubated on ice for 30 min. Precipitated proteins were pelleted by centrifugation at 20 000 × *g* for 10 min at room temperature, and the supernatant was removed. Any residual TCA was then removed from the pellet by incubation with 200 μl acetone, followed by incubation at room temperature for about 10 min and centrifugation at 20 000 × *g,* 10 min at room temperature. The supernatant was removed, the acetone wash was repeated, and the samples were air-dried, resuspended in loading buffer, and resolved by PAGE. The presence of nucleoporins and the testis-specific leucine-rich-repeat (TLRR) protein in the column fraction was determined by Western blot as described next.

Western Blot and Coimmunoprecipitation

Protein samples containing equal protein prepared from prepuberal or puberal rat testis extracts, affinity purification, or sucrose gradients were separated by PAGE through 10% acrylamide gels, equilibrated in and electrophoretically transferred from the gel matrix to PVDF membrane (Bio-Rad Laboratories) in Towbin transfer buffer. Proteins were detected on the membrane with affinity-purified KIFC1 antibody, monoclonal antibodies to importin β (karyopherin β1; KPNB1; clone 23; BD Transduction Laboratories, Pharmingen, San Diego, CA), NUP62 (clone 53, BD Transduction Laboratories, Pharmingen), or FxFG nucleoporins (MAb414; Covance Research Products, Denver, PA). Immune complexes bound to the membrane were detected with horseradish peroxidase-conjugated donkey secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1:40 000 in TTBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween20) and developed with enhanced chemiluminescent reagents as described by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

Testis lysates for coimmunoprecipitation experiments were prepared as described previously. For experiments with GTP analogs, lysates were incubated with 0.5 or 1 mM GTPγS or GMP-PNP for 30 min at 37°C before immunoprecipitation. For experiments with RAN mutants, lysates were preincubated with 25 μM RAN^{T24N}, RAN^{Q69L}, or wild-type RAN protein for 30 min at 37°C (Cytoskeleton, Denver, CO). Immunoprecipitation reactions were conducted by immobilizing antibody to Protein A beads using the Seize X immunoprecipitation kit from Pierce. Twenty-five micrograms of either NUP62 or KIFC1 antibody were crosslinked to the beads before incubation with the lysate overnight at 4°C. Controls for these experiments were lysates incubated with beads crosslinked in the absence of primary antibody. Beads were then collected by centrifugation and washed with binding buffer (0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium phosphate, and 0.01 M KCl, pH 7.4) and eluted at pH 2.5. Samples were analyzed by Western blot using either KIFC1, NUP62, or KPNB1 antibody as described previously.

Immunofluorescence

Nucleoporins were detected in tissue sections essentially as previously described for the KIFC1 motor protein [11]. Testes were obtained from sexually mature rats (8–10 wk; Sprague-Dawley, Harlan, Indianapolis, IN) and immersion fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) after piercing of the capsule at the poles and orbit. In some cases, testes were perfusion fixed through the spermatic artery after removal from the animal. The organs were then incubated overnight in 0.5 M sucrose in PBS, placed in cryoprotectant, cut into 14-μm sections, and transferred to Vectabond coated slides (Vector Laboratories, Burlingame, CA). After drying at 37°C, the sections were washed with PBS and treated with 0.3% Triton X-100 for 15 min at room temperature. The tissue was blocked in 2% BSA in TBST (20 mM Tris, pH 7.5, 154 mM NaCl, 2 mM EGTA, 2 mM $MgCl_2$, 0.1% Triton X-100) and incubated sequentially with primary and secondary antibodies.

The NUP62 and MAb414 (called npc here) monoclonals were detected with a Texas Red conjugated donkey anti-mouse IgG secondary antibody (1:100 dilution; Jackson ImmunoResearch). The intracellular localization of these proteins was observed with a Nikon E600 fluorescence microscope fit with appropriate filters and images captured with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ) and analyzed with Metamorph image analysis and acquisition software (Universal Imaging Corporation, Downingtown, PA). DNA was stained with Sytox Green (Invitrogen, Carlsbad, CA). In some cases, samples were viewed with a Zeiss laser scanning confocal microscope (LSM 510 Axiovert 200M; Carl Zeiss Inc., Thornwood, NY) equipped with a 488-nm/568-nm/647-nm krypton argon laser to allow simultaneous visualization of FITC and Texas Red. The individual optical slices were merged to produce one image and exported to Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA), where the image was manipulated without alteration of data integrity.

Sucrose Density Gradient Centrifugation

Testis HSS was prepared as described previously, and 500 μl were applied to a 4.5-ml 5%–20% sucrose gradient prepared using a Hoefer SG 30 gradient maker. The gradient was centrifuged at 200 000 × *g* in an SW55 rotor for 16 h at 4°C. Fractions (380 μl) were collected from the bottom of the centrifuge tube, and protein was precipitated by TCA as described previously with addition of 2 μl 2% Na deoxycholate and 10 μl 10% TCA. Samples were then resuspended in SDS loading buffer for analysis by Western blot. Parallel gradients were constructed with BSA (66 kDa, 4.2 Svedberg units [S]) and β-amylase (200 kDa, 8.9S) as sedimentation markers.

RESULTS

Affinity Purification of KIFC1 Complex Using Cargo-Binding Peptide

In previous work we identified a short 19-amino-acid sequence from the tail domain of KIFC1 as necessary and sufficient to target this motor to membranes [12]. To begin to identify and characterize the proteins that interact specifically with this motor, we conducted purification of the KIFC1-containing complex from testis extract using the targeting sequence for affinity chromatography. Proteins present in the wash, flow-through, and eluted fractions were resolved by PAGE and visualized by staining and Western blot (Fig. 1). A subset of lysate proteins was found to associate with the KIFC1 peptide and was eluted with low pH (Fig. 1A, lane 1), low pH plus NaCl (Fig. 1A, lane 2), and the affinity peptide itself (Fig. 1B, lane 1) but not with a nonspecific peptide with a scrambled sequence (Fig. 1B, lane 2). A previously uncharacterized testis protein, which we have named Testis Leucine Rich Repeat protein (TLRR), was found tightly associated with this complex (Fig. 1A, bottom panels). TLRR was initially identified as a KIFC1-interacting protein using a yeast 2 hybrid screen (accession number AK076637; data not shown); therefore, its presence in the affinity-purified complex supports the specificity of this approach. The interaction of the TLRR protein with KIFC1 will be examined in a separate paper.

Since KIFC1 is found associated with the spermatid nuclear membrane, we were particularly interested to determine whether proteins integral to the nuclear membrane, the nucleoporins, might be associated with this complex. Indeed, the nucleoporin NUP62 was bound to the peptide-linked resin and was present in the fractions eluted with both low pH and low pH/high salt (Fig. 1A, lanes 1 and 2, bottom panels). We also observed that the other nucleoporins recognized by MAb414 (NUP385, NUP214, and NUP153) were not as effectively bound to the affinity column (data not shown). The elution profile of TLRR and NUP62 differed with TLRR being more tightly bound to the KIFC1 peptide and requiring both

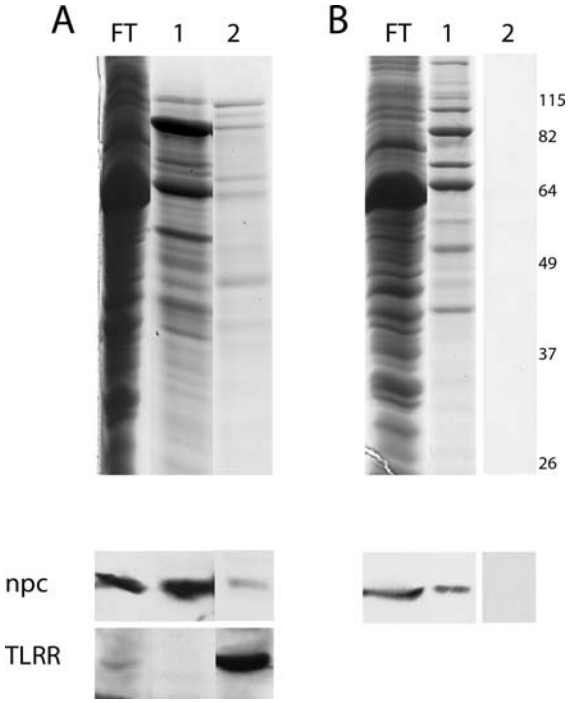

FIG. 1. Affinity purification of the KIFC1 complex. Testis lysate was prepared as described in the text and allowed to bind to an affinity column with the peptide unique to the KIFC1 motor, GKAASGASGASGRAAAIA-GRAD, attached to the resin. Flow-through, wash, and fractions eluted with (**A**) IgG elution buffer, lane 1; IgG elution buffer with 250 mM NaCl, lane 2; (**B**) the KIFC1 peptide, lane 1; or a negative control peptide with a scrambled sequence, lane 2, were collected, separated by SDS-PAGE, and either stained with Coomassie Blue-R250 (top panels) or transferred to membrane for Western blot with either MAb414 antibody to FxFG nucleoporins (npc) or affinity-purified testis-specific leucine-rich-repeat (TLRR) antibody.

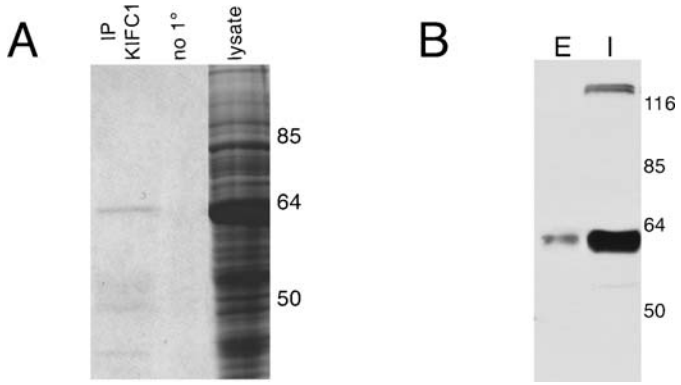

FIG. 2. NUP62 is a major protein associated with KIFC1 in testis lysate. **A**) Lysate containing 200 μg total protein was incubated either with KIFC1 antibody-linked beads (first lane) or control beads without crosslinked antibody (second lane) and immune complexes eluted from the beads as described in *Materials and Methods,* separated by electrophoresis and stained with Coomassie Blue-R250. Total proteins in lysate is shown. **B**) Coimmunoprecipitation of NUP62 with KIFC1 antibody was performed as described in **A** and eluted complex or input protein separated in lanes E and I, respectively, transferred to membrane, and blotted with the Mab414 antibody, which recognizes Nup385, Nup214, Nup153, and NUP62 in rat liver [33].

low pH and salt for elution. NUP62 was also effectively eluted using the peptide itself (Fig. 1B, lane 1, bottom panels) but not with a scrambled peptide (Fig. 1B, lane 2, bottom panels), further demonstrating the specificity of this complex.

KIFC1 Associates with a NUP62-Containing Complex in a Developmentally Regulated Manner

To confirm that KIFC1 and NUP62 reside in the same protein complex in the testis, we conducted coimmunoprecipitation experiments of these proteins from testis lysates. Importantly, NUP62 is a major component of the complex precipitated with the KIFC1 antibody as visualized by Coomassie staining (Fig. 2A). Furthermore, Western analysis with the MAb414 antibody confirms that NUP62 is the primary nucleoporin associated with this complex, although Nup153 is visible on longer exposure (our unpublished results; Fig. 2B). NUP62 appears to be more abundant in the testis than the higher-molecular-weight nucleoporins, a pattern that differs from that reported for other tissues, such as liver [14]. Based on comparison of input NUP62 protein (Fig. 2B, lane I) with bound NUP62 (Fig. 2B, lane E), we estimate that less than 5% of total testicular NUP62 is part of the KIFC1 complex. This result is consistent with the amount of NUP62 bound tightly to the KIFC1 peptide affinity column.

To determine whether the formation of the KIFC1/NUP62-containing complex is developmentally regulated, we immunoprecipitated this complex from lysates prepared from differently aged rats. In this case, KIFC1 was immunoprecipitated with an anti-NUP62 antibody. At the developmental time points selected, the developing testis is enriched in specific germ cell types: mitotically dividing spermatogonia at 7 days after birth and meiotically dividing spermatocytes at 14 and 20 days and round spermatids at around 30 days. Significantly, although both of these proteins are expressed on and after Day 14 ([11]; Fig. 3A), no association is detected by coimmunoprecipitation until Day 30 (Fig. 3B), a time when KIFC1 first appears associated with the nucleus of round spermatids [11]. This interaction is maintained throughout the remainder of development and in the adult animal (Fig. 3B).

KIFC1 Association with the NUP62-Containing Complex Is Regulated by GTP Hydrolysis on RAN

We next wanted to determine whether formation of the KIFC1-containing complex is regulated by GTP hydrolysis on the small GTPase RAN, as has been shown for other C-terminal motors [15]. If complex formation is regulated by the GTP-bound state of RAN, we would predict that coimmunoprecipitation would require GTP hydrolysis. Testis lysates were incubated with nonhydrolyzable analogs, either GTPγS or GMP-PNP, followed by immunoprecipitation with the NUP62 antibody (Fig. 4A). Both analogs interfere with immunoprecipitation, especially at higher concentrations. These results indicate that GTP hydrolysis is required for KIFC1 to interact with the complex containing nucleoporin NUP62.

We next determined whether dominant negative RAN proteins could interfere with formation of the motor complex in adult rat testis lysates. For these experiments, we incubated lysates with either the constitutively active mutant of the RAN GTPase, RAN^{Q69L}, locked in a GTP-bound state, or an inactive mutant, RAN^{T24N}, unable to bind GTP, followed by coimmunoprecipitation of NUP62 with the KIFC1 antibody. While incubation with RAN^{Q69L} completely disrupted KIFC1 binding to the NUP62-containing complex, a portion was stable in the presence of the inactive RAN^{T24N} mutant (Fig. 4B). KPNB1 (importin β) is the direct downstream effector of RAN in multiple pathways, including spindle formation, nuclear transport, and nuclear envelope formation. We have previously

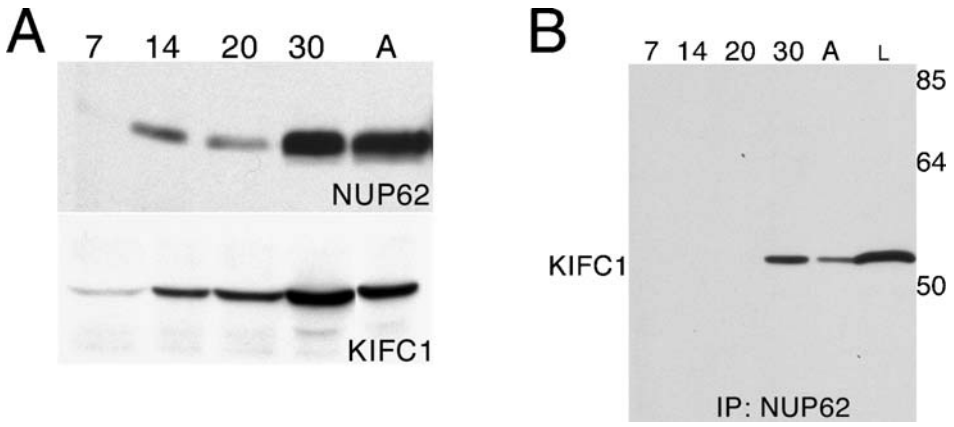

FIG. 3. KIFC1 association with NUP62 is developmentally regulated during spermatogenesis. **A**) Testis lysates were prepared from animals at the indicated ages and adult (lane A) and aliquots containing 50 μg total protein were separated by electrophoresis, transferred to membrane, and probed with either NUP62 antibody or KIFC1 antibody. **B**) Lysate aliquots containing 200 μg total protein at each time point were immunoprecipitated using NUP62 antibody-linked beads and the bound complexes eluted as described in *Materials and Methods,* separated by electrophoresis, transferred to membrane, and probed with KIFC1 antibody. Total testis lysate was loaded into lane L to show position of the KIFC1 protein. This experiment was repeated three times with comparable results.

shown that KIFC1 interacts with KPNB1 in adult testis lysate and colocalizes with this protein on the spermatid nucleus by immunofluorescence [11]. Similar to the situation with NUP62, RAN^{Q69L} eliminated association of KPNB1 with the motor-containing complex, while RAN^{T24N} was less effective (Fig. 4C). These results taken together support the idea that formation of a complex containing KIFC1, NUP62, and KPNB1 requires binding and hydrolysis of GTP on the small GTPase RAN.

The Complex Containing NUP62 Reorganizes During Spermiogenesis

The adult testis is composed of a mixed population of germ cells in various stages of maturation. To better evaluate the role of the KIFC1/NUP62 complex during spermiogenesis, we compared the status of this complex in the adult testis, rich in elongating spermatids, with that present in 30-day-old animals where nuclear elongation has not yet commenced using sucrose density centrifugation. We reasoned that if the KIFC1/NUP62-containing complex is involved in later event in spermiogenesis, such as acrosome/manchette motility, it should be abundant in the adult testis while precursor complexes might be found in peripuberal testis. Proteins in gradient fractions were separated by electrophoresis, and the presence of KIFC1, nucleoporins, and KPNB1 was determined by immunoblot.

The sedimentation profile of NUP62-containing complexes changes during development shifting from larger complexes in peripuberal testis to smaller complexes in the adult. The amount of NUP62 reaches a peak in fractions 9–11 in 30-day testis compared to fractions 5–7 in the adult (Fig. 5). NUP62 in 30-day testis comigrates with other nucleoporins including NUP153 (Fig. 5A) and higher-molecular-weight nucleoporins NUP214 and NUP385 (visible at higher exposure; data not shown). In contrast, the other nucleoporins do not comigrate with the bulk of NUP62 in the adult (Fig. 5B). In addition, fractions containing KIFC1 and NUP62 overlap more extensively in the adult than in the peripuberal testis at a developmental time point where the two protein coimmunoprecipitate.

NUP62 Redistributes in Male Germ Cells Differently Than Other FxFG Nucleoporins

Previous studies have demonstrated that nuclear pores redistribute during spermatogenesis, moving away from the

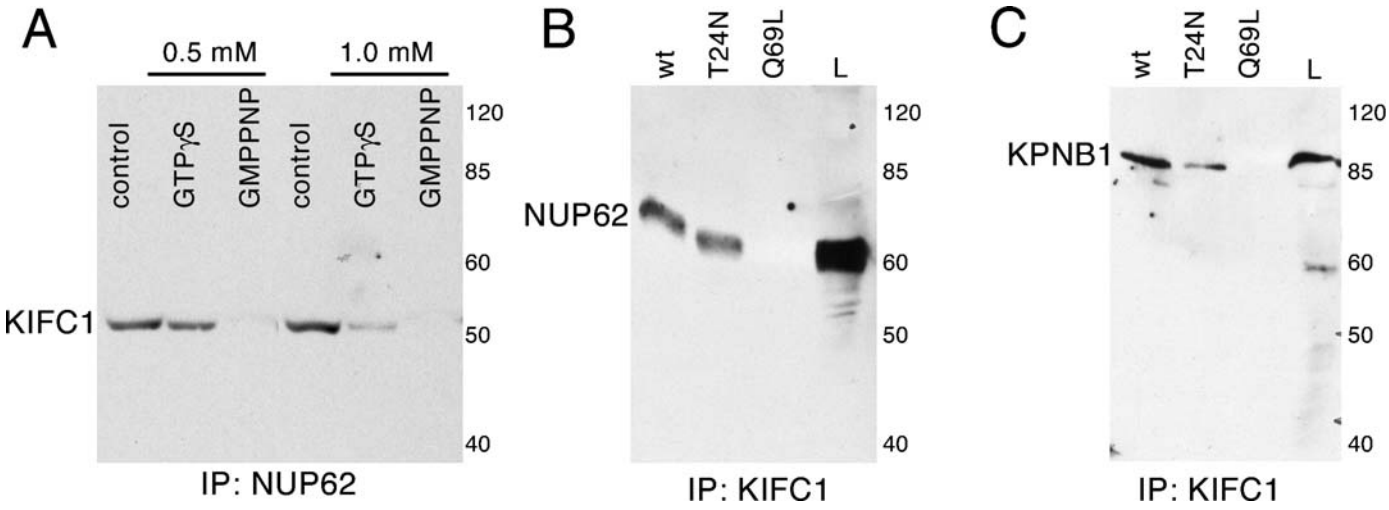

FIG. 4. KIFC1 motor complex formation requires GTP hydrolysis on RAN GTPase. Adult rat testis lysate was preincubated with GTP analogs (**A**) or recombinant RAN mutants or wt protein (**B** and **C**). Immunoprecipitations were conducted as described in *Materials and Methods* using either NUP62 antibody-linked beads (**A**) or KIFC1 antibody-linked beads (**B** and **C**) and bound complexes separated by electrophoresis, transferred to membrane, and probed with KIFC1 antibody (**A**), NUP62 antibody (**B**), or KPNB1 (importin β) antibody (**C**). The migration of the protein in total testis lysate is indicated in the lane labeled L. These experiments were repeated three times with comparable results.

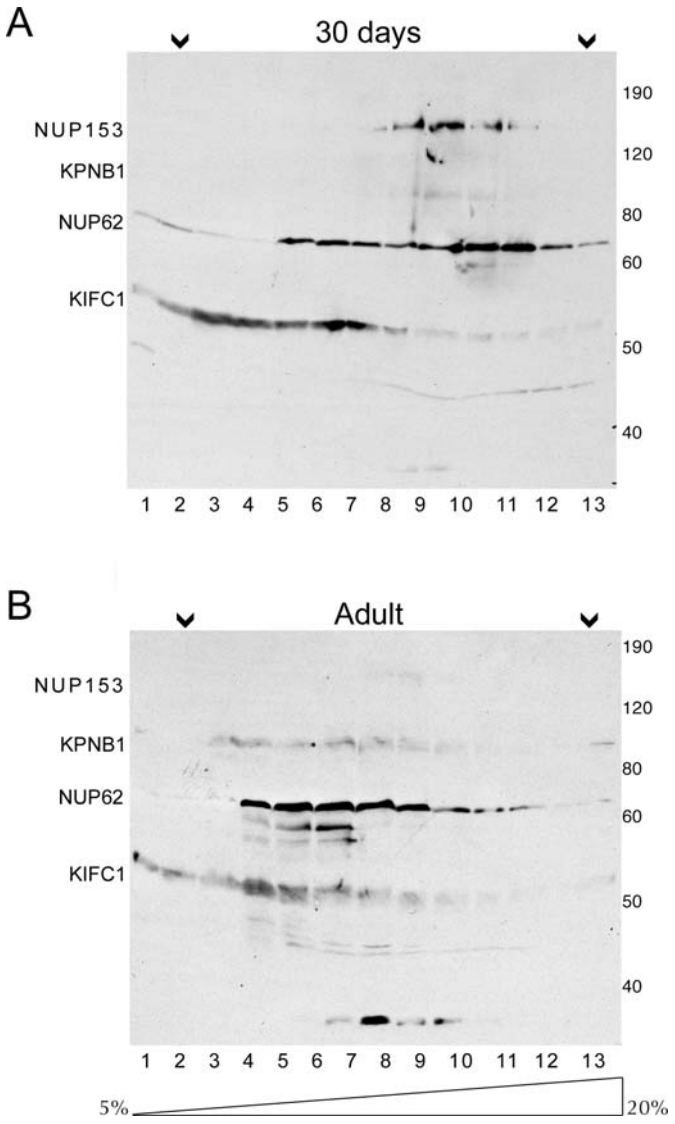

FIG. 5. NUP62 is part of high-molecular-mass complexes that redistribute during spermatogenesis. Lysates from either 30-day rat testis (**A**) or adult testis (**B**) were separated on 5%–20% sucrose gradients, and fractions were separated by electrophoresis, transferred to membrane, and incubated with a mixture of anti-importin β (anti-KPNB1), anti-KIFC1, and anti-nucleoporin (MAb414) antibodies. Arrows over profiles indicate BSA (4.2S) and β-amylase (8.9S) as sedimentation markers.

acrosome to cluster at the caudal end of the nucleus [16–20]. We next wanted to determine whether the distribution of NUP62 differs from that of the other nucleoporins, as was suggested by the sucrose gradient results mentioned previously (Fig. 5); therefore, we used two different antibodies to detect NUP62, one specific for this protein and MAb414 that recognizes several nucleoporins. Both nucleoporin antibodies stain the nuclear membrane of cultured cells in a manner typical for nuclear pores (Fig. 6C) and stain the nuclear membrane of pachytene spermatocytes in a similar pattern (Fig. 6A, arrows). However, in round spermatids, the NUP62 antibody stains a structure within the nucleus of these cells that is not detected with the MAb414 (Fig. 6A, open arrowheads), while MAb414 continues to stain the nuclear membrane sometimes in a polarized manner (filled arrowheads). We also observe cytoplasmic staining in both germ cells and cultured cells.

The NUP62-specific structures seen in the spermatid nuclear interior are visualized with both a mouse monoclonal antibody and a hamster polyclonal antibody to NUP62 (data not shown) and do not appear to localize to the nucleolus. Although KIFC1 is found in the nucleus of round spermatids, we do not visualize colocalization of KIFC1 with the NUP62 positive nuclear structures ([11]; data not shown). This result may represent the limits of the sensitivity of immunoflourescence since only a small fraction of NUP62 is associated with KIFC1 (Fig. 2B). The differential localization of NUP62 and other nucleoporins is consistent with the observed shift of NUP62 from larger complexes containing other nucleoporins in lysates prepared from 30-day-old testis to slower-migrating complexes lacking other Mab414 reactive nucleoporins in the adult testis as detected by density gradients (Fig. 5).

DISCUSSION

KIFC1 is associated with the spermatid nucleus throughout spermiogenesis. This motor protein first appears in the nucleus of round spermatids followed by localization on the surface of the spermatid nucleus in a punctate fashion at the acrosomal pole [11]. The KIFC1 motor then translocates over the nuclear surface in close proximity to the microtubule manchette until it is situated ultimately at the extreme caudal end of the spermatid nucleus [11]. We have previously proposed several models for KIFC1 function/regulation in spermatogenesis: transport of vesicles from Golgi to acrosome and along manchette microtubules, recycling of vesicles to the Golgi, translocation of the manchette along the nuclear surface, and regulation of motor activity by sequestration in the spermatid nucleus [11]. Here we report that KIFC1 is part of a complex that contains components of the nuclear pore. These findings are not consistent with a role for this protein solely in vesicle transport to and from the Golgi or along the outer face of manchette microtubules. Rather, the data presented here suggest a role for KIFC1 in the dramatic reorganization of the spermatid nucleus through interaction with a nucleoporin. In addition, our findings suggest that the composition of the nuclear pore may change during the morphological changes of spermiogenesis.

KIFC1 is a member of a group of highly related C-terminal motor proteins with divergent tail domains, the kinesin-14 subfamily [12, 21, 22]. We hypothesize that these different regions confer specific cargo-binding properties to the individual motors in this group. In support of this idea, antibodies specific to KIFC5A and KIFC1 stain distinct subcellular structures with KIFC5A localized to the mitotic spindle, while KIFC1 was found on membranes in cultured cells and on the nucleus in developing spermatids [11, 12]. KIFC1 contains a short, 19-amino-acid peptide not found in the other motors that is responsible for specifically directing this motor to intracellular membranes [12]. To begin to define possible cargo molecules of the KIFC1 motor, we used the specific KIFC1 peptide for affinity purification. A set of proteins was found to specifically associate with the KIFC1 cargo-binding peptide. One of these was a novel TLRR also detected in a yeast two-hybrid screen using the 19-amino-acid sequence as bait, while another was identified as nucleoporin NUP62. The interaction of the TLRR protein with KIFC1 is currently being investigated.

NUP62 is found in a complex in the testis that also contains KIFC1, as demonstrated by coimmunoprecipitation. The formation of this complex is developmentally regulated and appears at a time when the spermatid nucleus is undergoing dramatic shape change. The function of this complex is not known; however, we can envision several possibilities based

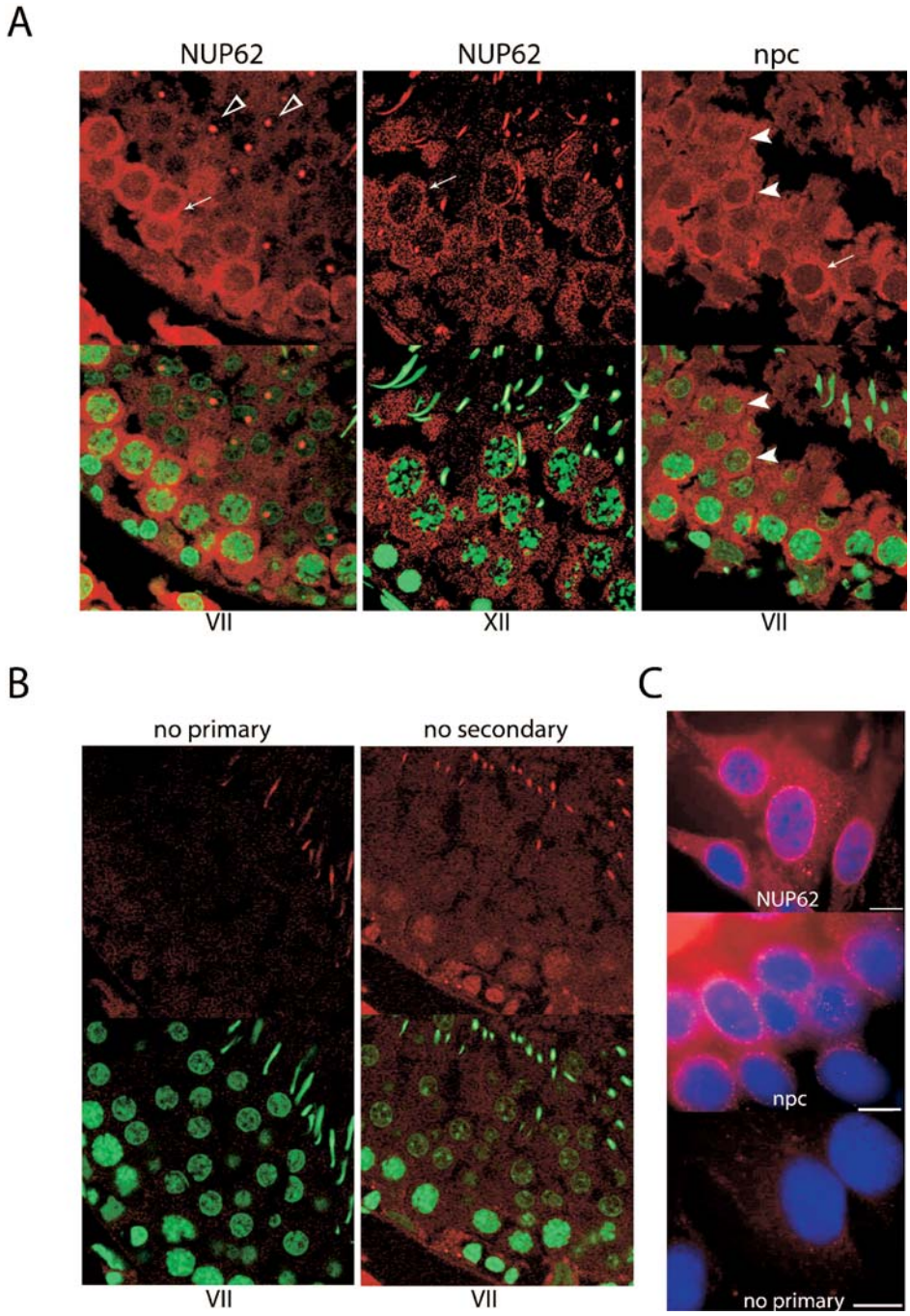

FIG. 6. Comparison of NUP62-specific staining with that of FxFG nucleoporins in testis sections. **A**) Scanning confocal fluorescence micrographs of testis sections showing tubules of approximately stage VII and stage XII containing round spermatids and spermatocytes stained with an antibody specific to NUP62 or Mab414 (npc) that recognizes a number of nucleoporins. Open arrowheads indicate NUP62 staining in round spermatids, arrows point to spermatocytes, and filled arrowheads indicate round spermatids stained with anti-npc antibody. **B**) Control staining of approximately stage VII tubules showing results of incubation with no primary antibody or no secondary antibody (NUP62 antibody alone). The same scanning settings were used in **B** as in **A**. Nucleoporin antibodies were visualized with a Texas Red conjugated donkey secondary, while bottom panels in **A** and **B** show the merged image with DNA stained with Sytox Green. **C**) NIH/3T3 cells stained with antibodies to NUP62, npc, or no primary antibody as control. DNA was stained with DAPI. Bars = 10 μm.

on our data. The association of KIFC1 with nuclear membrane proteins and the microtubular manchette suggests a possible role in manchette movement. In addition to a direct involvement in motility, KIFC1 could provide a platform, through interaction with scaffolding proteins such as the TLRR protein, for placement of regulatory proteins at the appropriate site (i.e., the spermatid nuclear membrane) to control key events in spermiogenesis, including acrosome spreading and manchette movement. Such a role for the manchette in positioning of regulatory proteins has been previously proposed by Kierszenbaum [23]. There is precedent for such an involvement of kinesin motors, including interaction of the light chain of Kinesin-1 with the JIP family of scaffolding proteins for mediation of the JNK signaling pathway [24, 25]. In Drosophila, the costal-2 kinesin interacts directly with kinases important in the hedgehog signaling pathway [26–28].

We demonstrate that association of KIFC1 with the NUP62-containing complex in the testis is controlled, in part, by the GTP-bound state of by RAN. RAN is a multifunctional GTPase with roles in nucleocytoplasmic transport, microtubule nucleation and spindle formation, and, of particular importance to this work, the dynamics of the nuclear membrane, including nuclear pore formation and nuclear membrane assembly, and may have distinct roles in the testis [29]. Our laboratory and others have shown that RAN is associated with different intracellular compartments during spermatogenesis, including the nucleus of spermatocytes and round spermatids, manchette microtubules, and the centrosome, and may participate in spermatid-specific forms of motility, such as intramanchette transport, manchette motility, and acrosome spreading [10, 13]. We consistently observe a difference in the effect of the two RAN mutants on complex formation: the RAN^{Q69L} mutant,

where GTP cannot be hydrolyzed to GTP, completely abolishes association of KIFC1 with the NUP62-containing complex; however, we detect complex formation with the inactive T24N mutant of RAN. In contrast, RAN^{T24N} also completely blocks nuclear import. Our results may reflect differential interaction of RAN with downstream effectors in the distinct functional pathways and/or interruption of different steps in this pathway. Even though the two mutants have phenotypically similar effects on protein import, the underlying molecular consequences are distinct. For example, the Q69L mutant abolishes both docking to the nuclear pore and transport, while the T24N mutant still allows docking to the pore [30–32]. In addition, the two RAN mutants have a disparate effect on binding of the tail domain of a related C-terminal motor, XCTK2, to its cargo, importin α/β [15].

The molecular organization and function of the nuclear pore complex in late-stage spermatids is not well defined. Our results, both from sucrose density centrifugation and from immunofluorescence, indicate a change in the composition of the nuclear pore in developing male germ cells. In 30-day testis that lacks any developmental stage beyond round spermatids, NUP62 shows nuclear rim staining in spermatocytes, similar to its location in somatic cells, and is part of a large complex comigrating with other nucleoporins and transport factors (Fig. 5A). In contrast, in the adult testis where elongating spermatids are predominant, NUP62 migrates as a smaller complex, depleted in higher-molecular-weight nucleoporins and comigrating with KIFC1 (Fig. 5B). Given the dramatic decrease in nucleocytoplasmic transport in condensing spermatids, it is plausible that the function of NUP62 (and other components of the nuclear pore) might be altered during spermiogenesis from a transport to a more structural role.

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