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C-Terminal Kinesin Motor KIFC1 Participates in Acrosome Biogenesis and Vesicle Transport[1]

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ABSTRACT

We have identified a possible role for the KIFC1 motor protein in formation of the acrosome, an organelle unique to spermatogenesis. KIFC1, a C-terminal kinesin motor, first appears on membrane-bounded organelles (MBOs) in the medulla of early spermatids followed by localization to the acrosomal vesicle. KIFC1 continues to be present on the acrosome of elongating spermatids as it flattens on the spermatid nucleus; however, increasing amounts of KIFC1 are found at the caudal aspect of the spermatid head and in distal cytoplasm. The KIFC1 motor is also found in the nucleus of very immature round spermatids just prior to its appearance on the acrosome. In some cases, KIFC1 appears localized just below the nuclear membrane adjacent to the subacrosomal membrane. We demonstrate that KIFC1 is associated with importin β and colocalizes with this nuclear transport factor on curvilinear structures associated with the spermatid nuclei. These data support a model in which KIFC1, perhaps in association with nuclear factors, assists in the formation and/or elongation of the spermatid acrosome. This article represents the first demonstration of a direct association of a molecular motor with the spermatid acrosome, the formation of which is essential for fertilization.

spermatid, spermatogenesis, testis

INTRODUCTION

The successful production of mammalian sperm capable of fertilization is essential for survival of the species. Spermatogenesis involves intricate cellular change in order to transform the precursor germ cell, the spermatogonium, into a mature spermatozoon. These changes include significant remodeling of the germ cell cytoskeleton, including formation of mitotic and meiotic spindles, and creation of two microtubule structures unique to spermatogenesis: the manchette and the sperm flagella. In addition to changes in the cytoskeleton, formation and redistribution of membrane domains occur during spermiogenesis. Due to the dramatic nature of these cellular rearrangements, mammalian spermatogenesis is an extremely useful model for understanding the role of molecular motor proteins in cellular change.

The acrosome is the most visible organelle formed during spermatogenesis, first appearing early in spermatid development in the phase termed spermiogenesis. The acrosome begins its life as an acrosomal vesicle adhered to one pole of the nucleus gradually expanding to cover the majority of the spermatid nucleus by the conclusion of spermiogenesis. During the expansion of the acrosome, the underlying nucleus undergoes extensive chromatin condensation and elongation. It is known that the Golgi apparatus supplies the majority of the acrosomal membrane; however, the mechanism by which the acrosome achieves its final extended form and how this process is coordinated with nuclear changes are poorly understood.

The Golgi apparatus is the primary, but not exclusive, source of membrane and protein for the developing acrosome [1–3]. This organelle is oriented proximal to the developing acrosome early in spermiogenesis. Acrosome biogenesis begins with the formation of proacrosomal vesicles that are derived from the Golgi and contain dark acrosomal granules. After these vesicles fuse, the resultant acrosomal vesicle adheres to the nucleus, flattening upon contact. During spermatid maturation, the acrosome grows to cover most of the elongated spermatid nucleus due primarily to protein and membrane input from the Golgi [1, 2]. It has been proposed, however, that the differential delivery of proteins to the acrosome might require multiple transport pathways from the Golgi and/or endoplasmic reticulum (ER) [4].

Microtubules play a vital role in the multiple morphogenic processes of spermatogenesis, as demonstrated by multiple studies over the past 2–3 decades [5–9]. The formation and motility of the manchette, the growth of the acrosome, and the condensation and elongation of the spermatid nucleus are tightly coordinated events during spermiogenesis. In fact, the manchette has been proposed to function directly in nuclear condensation and shaping [10]. Recently, specific molecular motor proteins have been demonstrated to play important roles in these movements, including spermatid traffic within the epithelium, formation of the spindle and the manchette, and formation of the acrosome [11–14]. Due to the complexity of the cellular changes required for the formation of viable sperm, it seems likely that additional motor proteins will be identified with crucial roles in various steps in this intricate process.

This article describes an analysis of the role of the molecular motor KIFC1 in spermiogenesis. KIFC1 is a member of a highly related group of C-terminal motor proteins that are very similar to one another, diverging only in their tail domains [13]. Our initial hypothesis was that their divergent structure would result in a difference in function for these motors. In order to further examine the role of KIFC1 in the developmental program of spermatogenesis, we have localized this motor to the acrosome of developing spermatids. Prior to its localization on the acrosome, KIFC1

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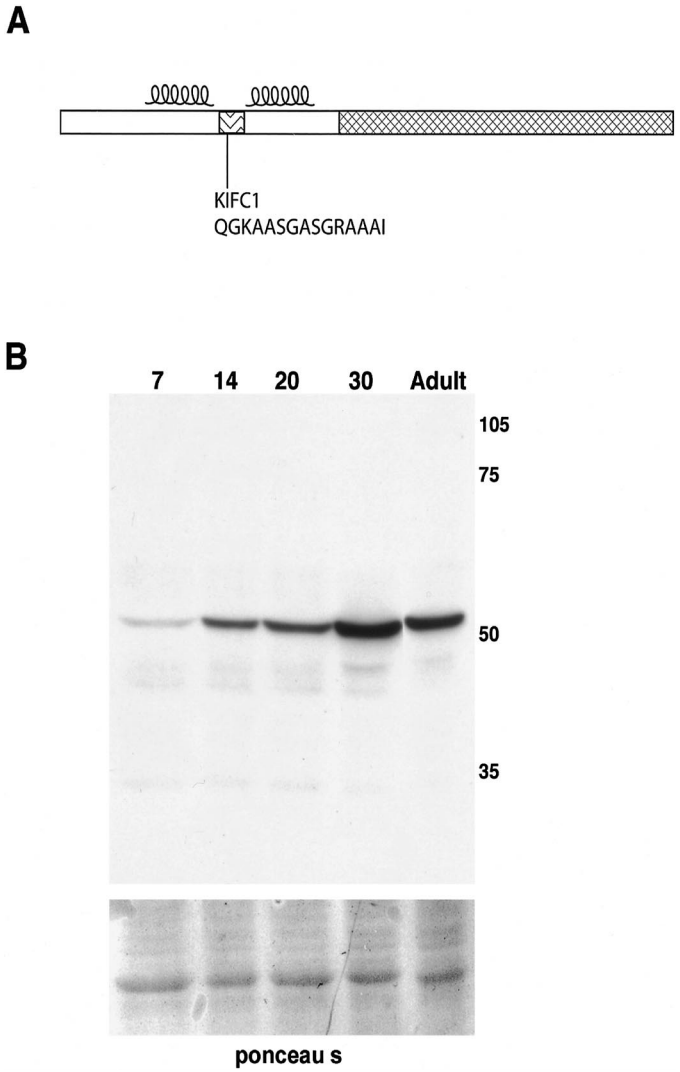

FIG. 1. KIFC1 is developmentally expressed in rat testis during acquisition of sexual maturity. **A**) A schematic of the KIFC1 protein showing the sequence unique to KIFC1 (zigzag). The hatched box indicates the motor domain and the white boxes indicate the sequences shared with motor subfamily members. **B**) KIFC1 protein was detected in extracts prepared from testis obtained from animals at 7, 14, 20, 30, and approximately 90 days (adult) after birth using the KIFC1 antipeptide antibody as described in *Materials and Methods.* The migration of molecular weight markers ($\times 10^{-3}$) is shown on the right while equal protein loading is demonstrated by ponseau staining of the blot (below).

was found on punctate structures between the Golgi and the spermatid nucleus. In addition to its location on the acrosome, this motor is also present in the nucleus during early stages of spermatid development and is associated with the nuclear transport factor importin β. These findings support a role for KIFC1 in acrosome biosynthesis and represent a link between changes in the spermatid nucleus and formation of the adherent acrosome.

MATERIALS AND METHODS

Antibodies and Immunofluorescence

KIFC1 was detected in tissue sections essentially as previously described for the KRP3 motor protein [14]. Testes were obtained from sexually mature rats (8–10 wk, Sprague-Dawley; Harlan, Indianapolis, IN) and immersion fixed overnight in 4% paraformaldehyde (PFA) 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 KIFC1 polyclonal antibody was affinity purified against the antigenic peptide using the Sulfolink kit according to instructions supplied by the manufacturer (Pierce, Rockford, IL). Briefly, the affinity column was prepared by conjugation of the peptide to the resin via its C-terminal cystine. Rabbit serum was then added to the column, incubated overnight at 4°C, and washed with buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 6.0). Antibody was eluted from the column with 100 mM glycine buffer, pH 2.5, and immediately neutralized with 100 mM Tris, pH 7.5. Fractions containing protein were pooled and dialyzed in PBS. Testis sections were incubated with affinity-purified polyclonal KIFC1 (1:30) in IF blocking buffer (2% BSA, 0.1% azide in TBST) for 1 h, then rinsed 3 times in TBST. The KIFC1 antiserum was prepared from rabbits by Harlan Biosciences for Science (Indianapolis, IN) using a peptide unique to this protein: QGKAASGASGRAAAIA. The primary antibody was detected with donkey anti-rabbit IgG conjugated to Texas Red (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), and microtubules were colocalized with anti-α tubulin conjugated to FITC (1:50 dilution; Sigma, St. Louis, MO). The Golgi apparatus was colocalized with KIFC1 using the anti-GM130 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ) and nuclei were stained with DAPI contained in the mounting medium (Vectasheild; Vector Laboratories). Importin β was detected with a goat polyclonal antibody (β1[C-19]; Santa Cruz Biotechnology, Santa Cruz, CA) and an FITC conjugated donkey anti-goat IgG secondary antibody. 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 Metaview image analysis and acquisition software (Universal Imaging Corporation, Downingtown, PA). Controls for these experiments included preimmune sera, omission of primary antibody, and preincubation of primary antibody with the peptide antigen. In this case, 100 μl of antibody was incubated with 20 μg antigenic peptide and 0.2 ml preabsorbent buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween-20) overnight at 4°C with mixing. The preabsorbed antibody was then diluted 1:10 in 100 μl blocking buffer and used for staining. Control sections were photographed at the same exposure settings as experimental samples.

Colloidal Gold Labeling and Electron Microscopy

Testes for immunogold analysis were removed from the animal, perfused with Earle balanced salt solution for 2 h, and the seminiferous tubules were isolated by transillumination-assisted microdissection. Tubule sections of the appropriate stage were removed to a separate dish, fixed in 4% paraformaldehyde and 0.1% gluteraldehyde, rinsed 3 times in 0.1 M sodium phosphate buffer, pH 7.4, and one time in distilled water. Samples were then dehydrated with increasing ethanol concentrations and placed in LR White (Ted Pella, Inc., Redding, CA) overnight, changed to fresh media, and transferred to gelatin capsules and polymerized at 50°C for 24 h. Samples were sectioned on a Reichert ultramicrotome and sections were collected on nickel grids for immunocytochemistry. Grids were blocked in 5% egg albumin for 2 h and then incubated with affinity-purified polyclonal antibody to KIFC1 (diluted 1:30 in 0.04 M PBS, pH 7.4, 0.5% BSA) or to monoclonal GM-130 antibody (diluted 1:20) for 1 h at room temperature. The grids were then incubated with goat anti-rabbit IgG conjugated to 30 nm gold alone or with anti-mouse IgG conjugated to 15 nm gold for double label (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:20 in 0.5% fish gelatin, 0.04 M PBS, pH 7.4, for 1 h at room temperature. The grids were then washed twice with sodium phosphate buffer, rinsed twice in distilled water, air dried for at least 30 min, and counterstained with uranyl acetate and lead citrate. Controls for these experiments included omission of primary antibody and preabsorption of primary antibody with 20–60 μg of antigenic KIFC1 peptide prior to immunocytochemistry (data not shown).

Western Blot and Coimmunoprecipitation

Protein extracts from testes obtained from prepubertal and pubertal rats were prepared as described [14]. Briefly, decapsulated testes from adult or prepubertal rats were homogenized in buffer containing protease inhibitors

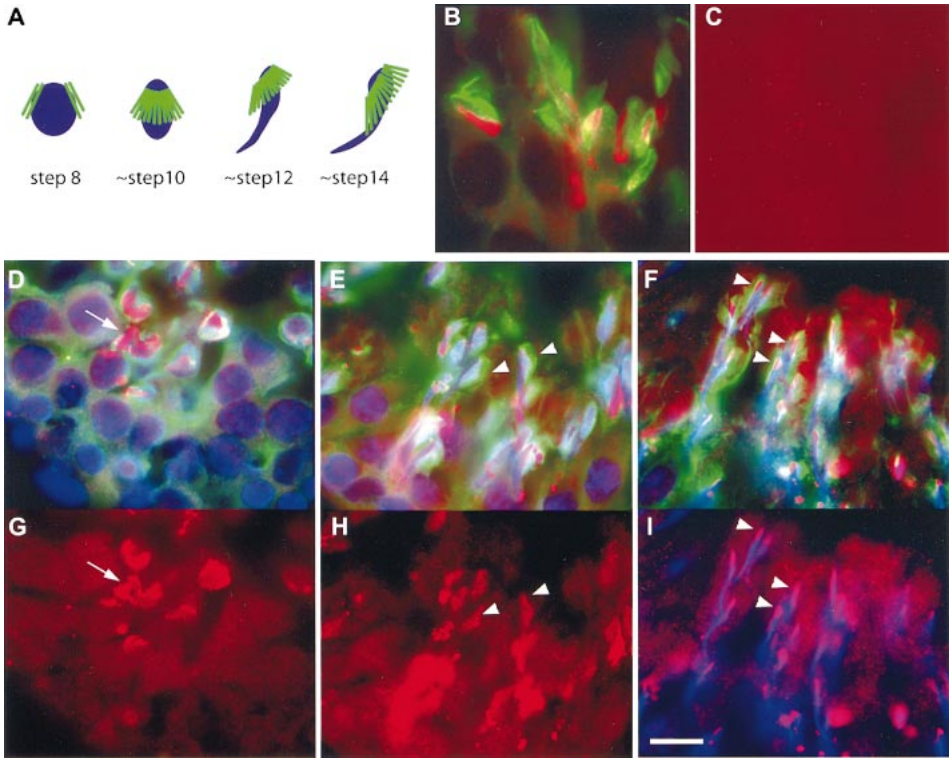

FIG. 2. KIFC1 is expressed in developing spermatids. **A**) A schematic representation showing developing spermatids during their maturation. Manchette microtubules are indicated in green, with the spermatid nucleus shown in blue. The KIFC1 antibody was incubated with fixed, frozen testis sections, detected with Texas Red conjugated secondary antibody and viewed with a confocal microscope (**B**) or by epifluorescence (**C**–**H**). Tubulin was colocalized with KIFC1 using an FITC-conjugated anti-tubulin antibody and DAPI was used to localize DNA (**D**–**F** and **I**). **D**–**F**) Triple staining (KIFC1 in red, microtubules in green, and nuclei in blue) of progressively more-mature spermatids. **G** and **H**) The KIFC1 signal for **D** and **E**, respectively, while **I** shows both DNA and KIFC1 localization for **F**. KIFC1 is found as punctate structures associated with the nucleus of early spermatids (arrows, **D** and **G**). KIFC1 is still associated with spermatid nuclei of intermediate stage (**E** and **H**), but a more diffuse staining appears near the spermatid manchette (arrowheads, **E** and **H**). In more mature spermatids, KIFC1 is located at the caudal aspect of the spermatid nucleus (arrowheads, **F** and **I**). A negative control is shown in **C**, where the affinity-purified KIFC1 antibody was preincubated with antigenic peptide as described in *Materials and Methods* prior to staining. The control section is of the same stage as shown in **B**. Bar in **I** = 10 μm.

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 (Biorad, Hercules, CA) and samples containing equal protein were separated by polyacrylamide gel electrophoresis (PAGE) through 10% acrylamide gels, equilibrated in and electrophoretically transferred from the gel matrix to polyvinylidene difluoride membrane (Bio-Rad) in Towbin transfer buffer [15]. Proteins were detected on the membrane with affinity-purified KIFC1 or monoclonal importin β antibody (BD Biosciences). Immune complexes bound to the membrane were detected with horseradish peroxidase-conjugated donkey secondary antibody diluted 1:20 000 in TTBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween20) (Jackson ImmunoResearch) and developed with enhanced chemiluminescent reagents as described by the manufacturer (Amersham Pharmacia Biotech).

Testis lysates for coimmunoprecipitation experiments were prepared as described above. Testis HSS was precleared with Sepharose-4B Protein A (Zymed, South San Francisco, CA) and the KIFC1 antigen captured by incubation of lysate with either 1 μg or 10 μg KIFC1 antibody overnight at 4°C. Protein A-conjugated Sepharose 4B beads were added, incubated at room temperature for 1 h with gentle shaking, pelleted, and washed twice with co-IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40). Samples were analyzed by Western blot using importin β antibody as described above.

RESULTS

KIFC1 Is Expressed During the Development of Sexual Maturity in the Rat

The molecular motor KIFC1 was initially identified in a reverse transcription-polymerase chain reaction screen for motor proteins in embryonic mouse brain [16]. Additional molecular analysis showed that this protein is a member of a group of highly related C-terminal motors and is expressed in the testis [13]. A schematic of KIFC1 is shown in Figure 1A, where the zigzag indicates a sequence found only in KIFC1, whereas the motor domain (hatched box) and parts of the stalk and tail domains (open boxes) are common to this subfamily of motors. In order to examine the role of this motor in spermatogenesis, we produced a polyclonal antiserum using a peptide specific to the KIFC1 protein (indicated in Fig. 1A). KIFC1 was analyzed by Western blot of testis extracts during the development of sexual maturity in young animals. KIFC1 is barely detectable at the earliest stage examined, 7 days postpartum (dpp), where only spermatogonia and Sertoli cells are found in the testis (Fig. 1B). However, the amount of KIFC1 increases steadily as spermatogenesis proceeds, reaching a maximum at 30 dpp when the amount of KIFC1 is comparable with that found in the adult. In fact, KIFC1 appears more abundant at 30 dpp compared with that seen in fully mature testis. This is likely due to the fact that the cell population at 30 days is the result of about one, almost synchronous, cycle and is more homogeneous than that in the adult. This increase in KIFC1 coincides with the appearance of spermatids in the epithelium and suggests that this motor may be involved in some aspect of spermiogenesis. The increase in KIFC1 protein at 14 days may indicate a secondary role for the KIFC1 protein in meiotic cells or

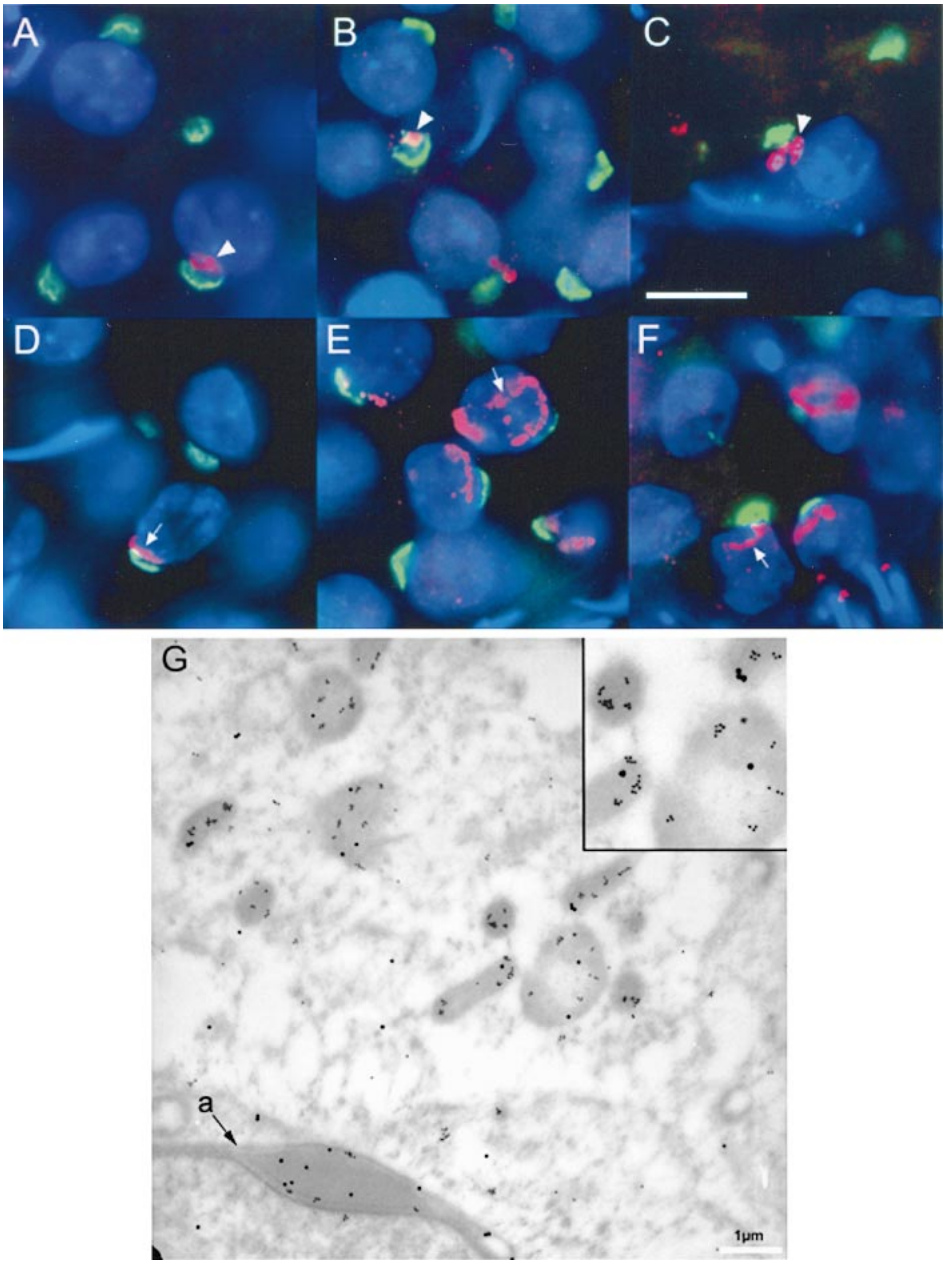

FIG. 3. KIFC1 transits from the medulla to the nucleus of early spermatids. KIFC1 was colocalized with the Golgi in frozen rat testis using the KIFC1 polyclonal antibody, visualized with a Texas Red-conjugated secondary antibody; and GM130 anti-Golgi matrix monoclonal antibody, visualized with an FITC-conjugated secondary antibody. **A**–**C**) Three examples of step 1 spermatids triple stained for KIFC1 (red), the Golgi (green), and nuclei (blue), with arrowheads indicating KIFC1 staining (bar in **C** = 10 μm). **D**–**F**) Spermatids of approximately step 7, triple stained as for **A**–**C**, with arrows indicating KIFC1 staining. **G**) Immunogold localization of KIFC1 (30 nm gold) and GM130 (15 nm) in thin sections of rat testis; a indicates the acrosome. The inset shows a higher magnification of the cluster of MBOs in the center of the field. GM130 is abundant on structures that also stain for KIFC1.

early translation of the KIFC1 message. The fact that this protein is not abundant in 7-day-old testis, where spermatogonia are actively dividing, indicates that this motor is not involved in cell division.

KIFC1 Is Associated with Developing Spermatids

To begin analysis of KIFC1 in spermatogenesis, this protein was localized in testis sections using indirect immunofluorescence with the antipeptide antibody. Figure 2A represents a simplified diagram of the types of cells present in the seminiferous epithelia during spermatid development, highlighting the dynamics of the manchette and the nucleus. The phases, or steps, in spermatid development are defined by the numerous cellular changes, particularly in the acrosome, that take place during transformation from round spermatid (step 1) to elongated spermatozoon (step 19). The manchette microtubular structure begins to form around the nucleus at spermatid step 8 and is disassembled at step 14. During this time, the nucleus undergoes dramatic condensation and elongation. KIFC1 was found associated with the nucleus near the manchette in a punctate manner in intermediate spermatids (Fig. 2B). This punctate staining was also visible on the nucleus in more immature spermatids at a time when the manchette is just beginning to form (arrow, Fig. 2, D and G). At this stage, KIFC1 staining sometimes resembled beads on a string looping near the surface of the spermatid nucleus (arrow, Fig. 2, D and G; also Fig. 3, E and F). A more diffuse staining was seen associated with the manchette or with a region of the nucleus adjacent to the manchette in approximately step 12 spermatids (arrowheads, Fig. 2, E and H). In more mature spermatids, of about step 14, shown in Figure 1E, KIFC1 was discernible at the caudal aspect of the spermatid nucleus (especially evident in 2I). This distribution of KIFC1 appeared unevenly localized with respect to the manchette (arrowheads, Fig. 2F). In more mature spermatids after manchette dissolution, KIFC1 is no longer associated with the nucleus (unpublished observations). Negative controls

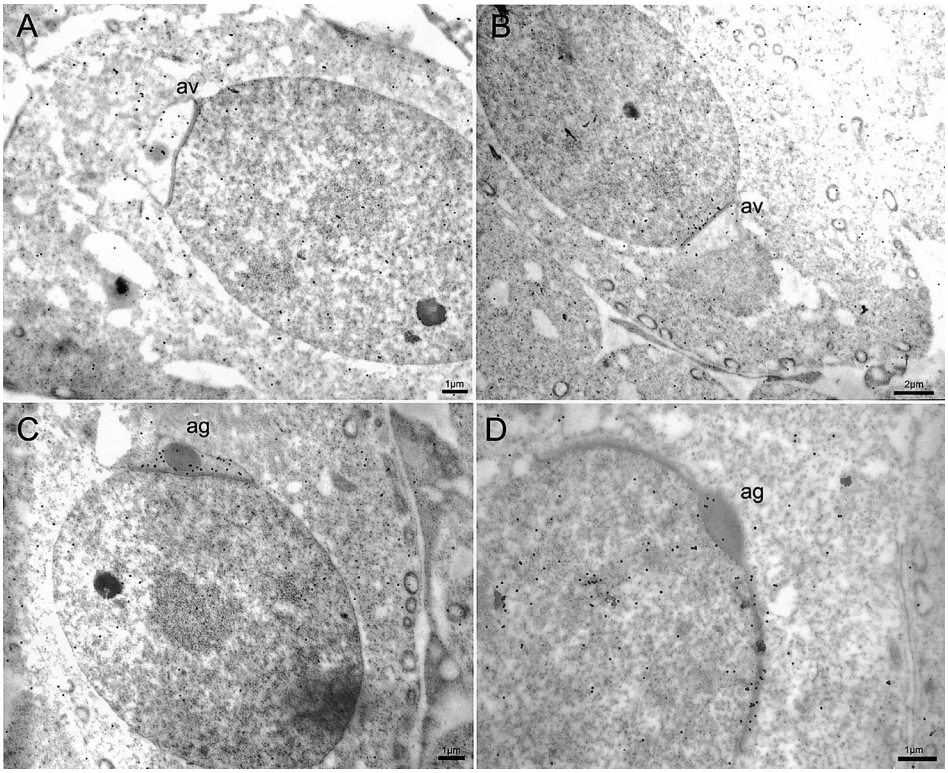

FIG. 4. Ultrastructural localization of KIFC1 in step 4–7 spermatids. **A**–**C**) Immunogold localization of KIFC1 in approximately stage 4 spermatids after contact of the acrosomal vesicle with the nuclear membrane. **D**) A slightly older spermatid with flattened acrosome. av, Acrosomal vesicle; ag, acrosomal granule.

for these experiments included no primary antibody, incubation with preimmune rabbit serum, or preabsorption with the antigenic peptide (Fig. 2C); all showed negligible signal compared with the experimental sample.

KIFC1 Locates near the Golgi Apparatus in Early Spermatids

Early in spermiogenesis, developing spermatids become polarized, with both the acrosome and Golgi apparatus located at the anterior pole of the nucleus and the centrioles and developing flagella at the caudal pole. Because KIFC1 staining is also polarized with respect to the nucleus (see Fig. 2, B and D), we wanted to determine the relative orientation of KIFC1 staining structures with respect to the Golgi apparatus. The Golgi matrix protein GM130 (BD Biosciences) was colocalized with KIFC1 in testis sections using indirect immunofluorescence (Fig. 3). In very early spermatids at approximately step 1, KIFC1 is often seen as a cluster of staining between the Golgi and the nuclear membrane (Fig. 3, A–C). This pattern changes in older spermatids between steps 7 and 8, where KIFC1 appears as curvilinear punctate structures associated with the nuclear membrane (Fig. 3, D–F). Even though more dispersed, KIFC1 staining is still concentrated toward the acrosomal pole of the spermatid nucleus. Notably, it is at this developmental stage that the microtubule manchette begins to form.

The location of KIFC1 and GM130 in the medulla of round spermatids was further examined in thin sections of testis by double-label immunogold colocalization. Consistent with immunofluorescence, KIFC1 (visualized with 30 nm gold) was found on structures near the nucleus (Fig. 3G). Many MBOs in this region contain primarily GM130 (inset, Fig. 3G) while a subset also contains KIFC1 as indicated by 30 nm gold. In addition, both KIFC1 and the Golgi protein are found on the acrosome. Our finding that a Golgi protein is present on the acrosome is consistent with the observations of others [1]. The colocalization of KIFC1 with the Golgi marker in the medulla and on the acrosome is suggestive of a role for this motor in acrosome biogenesis, perhaps by transport of MBOs bound for the acrosome.

KIFC1 Is Associated with the Acrosome at the Beginning of Its Development

The KIFC1 antibody was used to initiate an ultrastructural analysis of KIFC1 localization during spermatid maturation. Figure 4, A–C, shows immunoelectron micrographs of round spermatids just after the acrosomal vesicle contacts the nucleus. In each case, numerous gold particles are located in and around the acrosomal vesicle. Of particular interest is Figure 4B, where KIFC1 appears adjacent to the inner nuclear membrane just underneath the acrosomal vesicle. In a slightly older spermatid, in which the acrosome has described an almost 180° angle relative to the center of the nucleus, numerous gold particles can be seen associated with the acrosome (Fig. 4D). Many particles are also seen in the nucleus. The distribution of particles to one side of the acrosome in Figure 4D may be due to the angle of section or may reflect an inherent polarity of the structure.

KIFC1 Is Present on Both the Acrosome and Manchette of Condensing and Elongating Spermatids

In order to determine whether the distribution of KIFC1 changes during the macromolecular transformations of spermiogenesis, we examined the location of this motor during nuclear condensation and shaping in rat spermatids. As was seen in younger spermatids, KIFC1 is localized to the acrosome in approximately step 9 spermatids (Fig. 5, A and C, with more magnified views in Fig. 5, B and D,

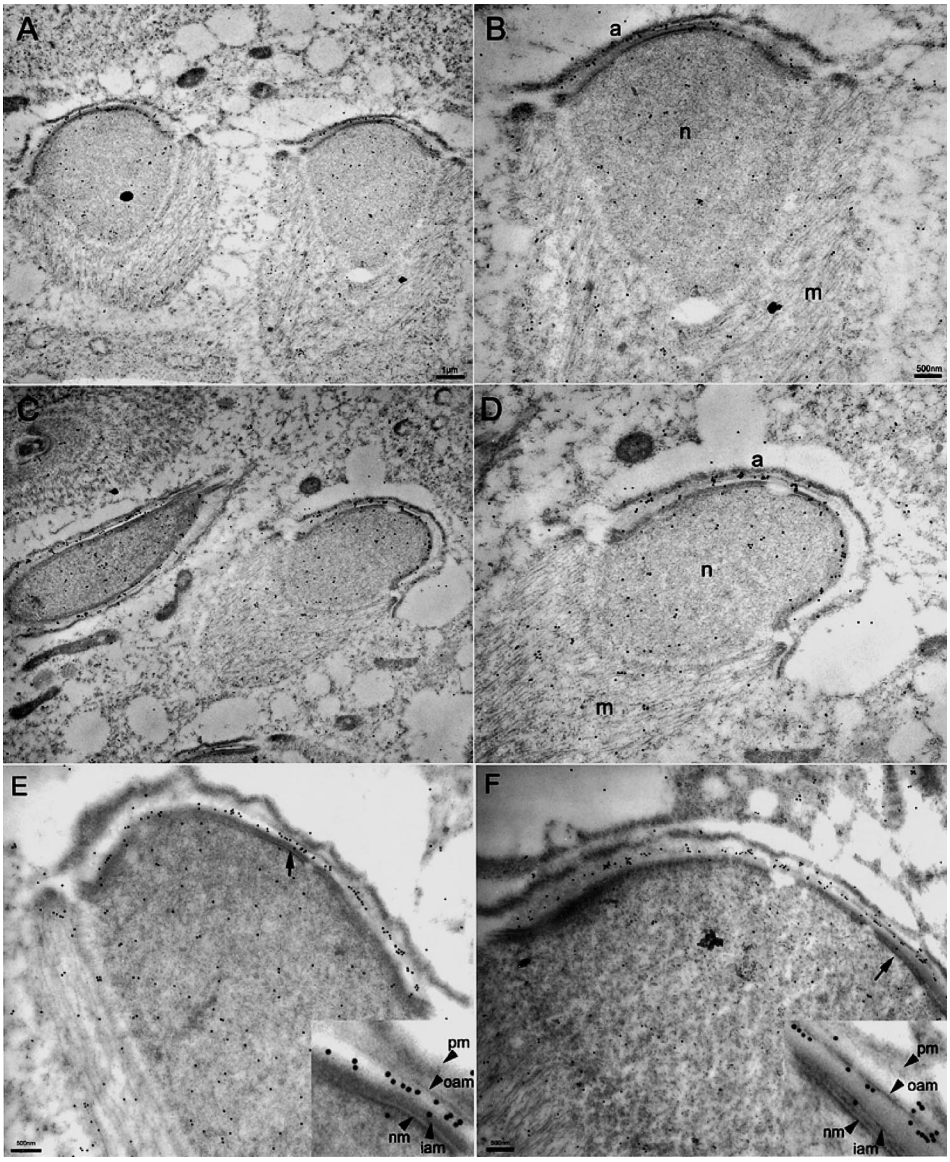

FIG. 5. Ultrastructural localization of KIFC1 in approximately step 9 spermatids. **A** and **C**) Localization of KIFC1 in the acrosome, nucleus, and manchette of intermediate stage spermatids. **B** and **D**) A higher magnification of **A** and **C**, with **a** indicating the acrosome, **m** indicating the manchette, and **n** indicating the nucleus. **E**–**F**) Nucleus and acrosome of approximately step 9 spermatids with inset (taken from area indicated by arrow) enlarging the acrosome. pm, Plasma membrane; oam, outer acrosomal membrane; iam, inner acrosomal membrane; nm, nuclear membrane.

respectively). In addition, gold particles are enriched on the spermatid manchette and are also seen in the nucleus. Higher magnification of additional spermatids at this stage (Fig. 5, E and F) shows gold particles primarily in the acrosome itself. Closer examination (insets, Fig. 5, E and F) supports the idea that, at this stage of development, KIFC1 is found within the acrosomal space and may be preferentially associated with the inner face of the outer acrosomal membrane. More elongated spermatids of approximately step 12 also show KIFC1 localization in the acrosome, nucleus, and manchette (Fig. 6, A–D). Gold particles are found clustered along microtubules of the manchette (inset, Fig. 6B). The relative amount of the KIFC1 motor in these structures appears consistent with that seen in more immature cells; however, the amount of KIFC1 found in the distal cytoplasm (asterisk, Fig. 6B) is increased compared with earlier spermatids. In addition to labeling of germ cells with the KIFC1 antibody, we observe staining of mitochondria in both Sertoli cells (Fig. 6B) and germ cells (Fig. 9).

KIFC1 Is Present in the Nucleus of Early Spermatids

Analysis of spermatids from early to late stages of development suggested that KIFC1 might be present in the nucleus of spermatids during their maturation. Indeed, KIFC1 is enriched in the nucleus of round spermatids of about step 2 (arrowheads, Fig. 7). The more immature cells present at this spermatogenic stage (stage II) include pachytene spermatocytes (arrows, Fig. 7, B and D) and spermatogonia (arrows, Fig. 7, A and C). Both of these cell types display much less nuclear staining than do round spermatids. This finding supports the results of the developmental Western blot shown in Figure 1B and suggests that KIFC1 might perform an important function in the transformation of the nucleus and associated structures during spermiogenesis.

KIFC1 Associates with Importin β

We have shown that KIFC1 is present in spermatid nuclei, particularly in immature spermatids. Previously, we demonstrated that members of the nuclear transport machinery are also found in spermatid nuclei, some in a polarized fashion [14]. In order to determine whether the KIFC1 motor associates with the nuclear transport machinery, we chose to examine the interaction of KIFC1 with one member of this pathway, the nuclear import factor importin β, by coimmunoprecipitation and indirect immuno-

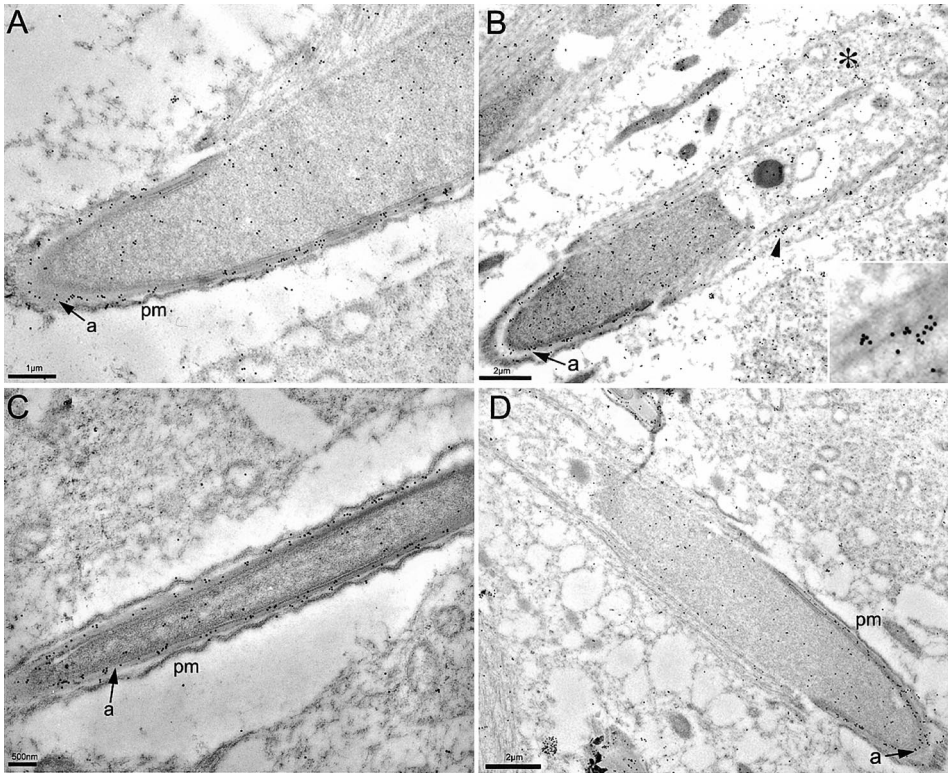

FIG. 6. Ultrastructural localization of KIFC1 in approximately step 12 spermatids. **A**–**D**) Localization of KIFC1 in condensing spermatids. pm, Plasma membrane; acrosome, a, indicated by the arrows. Inset in panel **B** is enlargement of an area of the manchette indicated by the arrowhead. Residual cytoplasm, heavily labeled by KIFC1, is indicated by the asterisk in **B**.

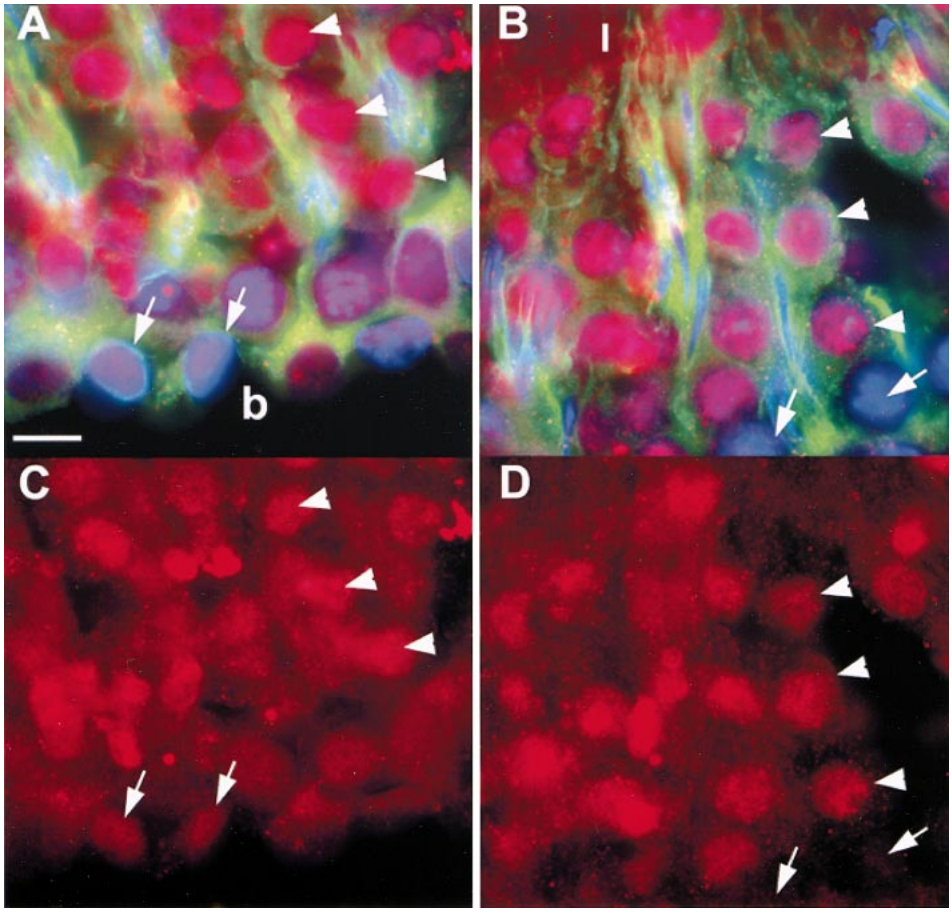

FIG. 7. KIFC1 is located in the nuclei of round spermatids. **A**–**B**) Triple staining of frozen rat testis with anti-KIFC1 (red), anti-tubulin (green), and nuclei (blue). b, Basal area of the epithelium; l, lumenal area. **C**–**D**) KIFC1 staining corresponding to **A** and **B**. Arrowheads indicate round spermatid nuclei, while arrows indicate spermatogonia (**A** and **C**) or spermatocyte (**B** and **D**) nuclei. Bar = 10 μm.

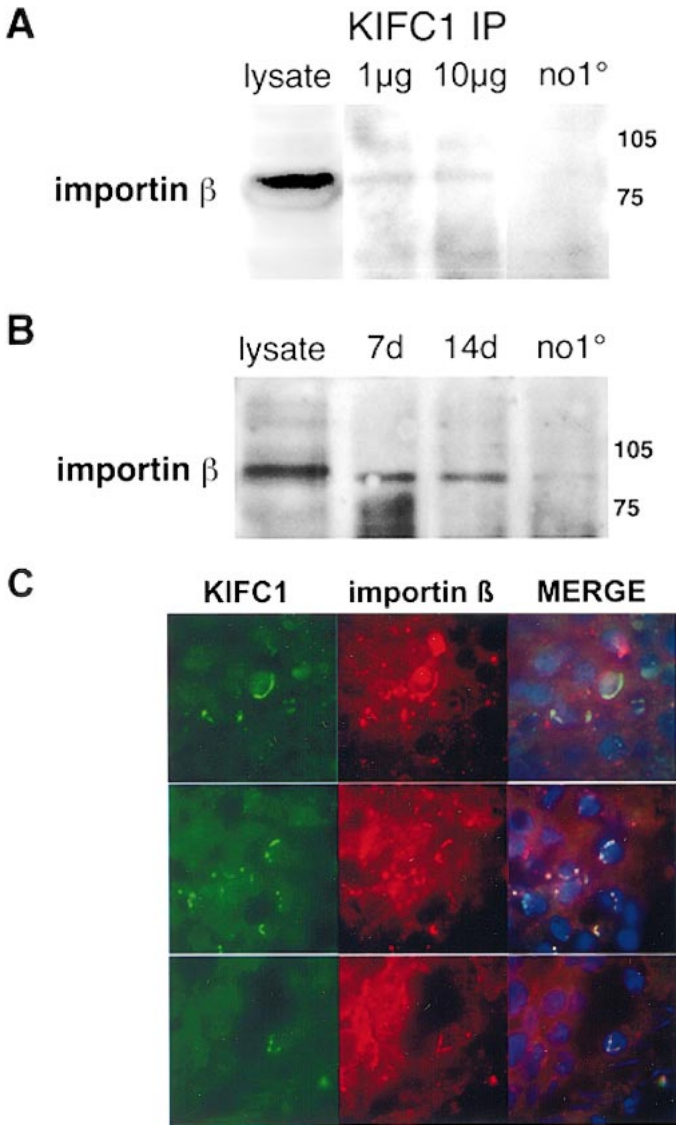

FIG. 8. The KIFC1 motor associates with importin β. Lysates were made from adult testes (**A**) or prepubertal testes (**B**) as described in *Materials and Methods.* **A**) One microgram and 10 μg KIFC1 antibody was used for immunoprecipitation and the blot was probed with anti-importin β. **B**) Five micrograms KIFC1 antibody was used for immunoprecipitation from 7-day- and 14-day-old testes and the blot was probed with anti-importin β. The lysate lane in both **A** and **B** was made from adult testes as a marker for the location of importin β. **C**) Frozen sections of adult testes were incubated with antibody to KIFC1 (detected with FITC-conjugated secondary antibody), and importin β (detected with Texas Red-conjugated secondary antibody), and the triple-stained image on the right with both antibodies and the DNA stained with DAPI. Bar = μm.

fluorescence. A small but reproducible fraction of total importin β found in the adult testis is immunoprecipitated with the KIFC1 antibody (Fig. 8A). In comparison, when lysates from prepubertal testis (before the onset of spermiogenesis and appearance of the acrosome) were used for immunoprecipitation, more importin β was found associated with KIFC1 (Fig. 8B). Consistent with these results, KIFC1 was colocalized with importin β on round spermatids in frozen sections of adult rat testes (Fig. 8C). Although the importin β antibody gave a higher background than KIFC1, perhaps reflecting the multiple roles of this transport factor, there is clear colocalization between these two proteins in step 6–7 spermatids (Fig. 8C) in what appear to be the nuclear-associated curvilinear structures previously noted (Fig. 3, E–F).

KIFC1 Is Discarded in Residual Bodies

Because KIFC1 seemed increased in the distal cytoplasm of elongate spermatids (Fig. 6B), we examined this area in later stage spermatids. This portion of spermatid cytoplasm accumulates cellular debris destined to be removed through phagocytosis by Sertoli cells giving rise to residual bodies. Immunogold localization revealed that KIFC1 is enriched in distal cytoplasm and is associated with the numerous membrane-bounded organelles, including mitochondria, found in this region (Fig. 9, A and B). This localization is consistent with KIFC1 immunostaining of droplets at the lumenal face of stage XIII and IX tubules (unpublished observations). The presence of KIFC1 in distal cytoplasm suggests that either KIFC1 may be actively transporting cargo to this area for disposal or passively being directed here for elimination.

DISCUSSION

The subcellular reorganization of the male germ cell that occurs during spermatogenesis is perhaps one of the most striking developmental changes in biology. In addition to dramatic changes in the germ cell cytoskeleton and nucleus, membrane domains of the spermatid undergo significant redistribution with the formation of the sizable acrosome and the subsequent removal of residual cytoplasm to produce the streamlined sperm. Although the morphological changes involved in acrosome formation have been well described, the underlying molecular events required for acrosome biogenesis remain unclear.

We have identified a molecular motor protein, KIFC1, which associates with the spermatid acrosome. The KIFC1 motor was originally identified in embryonic mouse brain but later was demonstrated to be expressed in the testis. Of the tissues we examined, KIFC1 is found at its highest level in the testis but is also abundant in ovary, spleen, and liver [13, 17] (unpublished observations). The presence of KIFC1 in these other tissues suggests multiple roles for this protein in cellular transport. Indeed, in addition to its localization on the acrosome, we observe this motor associated with mitochondria in germ cells and Sertoli cells.

KIFC1 is a member of the C-terminal class of kinesin-related proteins characterized by the mitotic motors ncd and KAR3; however, this group has become quite divergent recently with the identification of new members and probably consists of evolutionarily diverged subfamilies [18]. Previous molecular analysis identified three motors related to KIFC1 in the mouse [13]. Comparison of these related motors revealed a sequence unique to KIFC1, which was used to develop a specific antibody to this protein. We report here that the KIFC1 motor is associated with the acrosome from the initial stages of its development. In fact, this motor appears to translocate from a region near the Golgi apparatus in early spermatids to the residual cytoplasm of late spermatids during sperm development.

In step 1 spermatids, KIFC1 is localized to the medulla and soon afterward becomes localized to the acrosome. This relocation of KIFC1 is consistent with a role for this motor in vesicle trafficking from Golgi to the acrosome. As nuclear elongation proceeds, KIFC1 remains on the acrosome; however, a significant portion relocalizes to the caudal area of the elongated spermatid head to an area occupied by the spermatid manchette. Thereafter, increasing

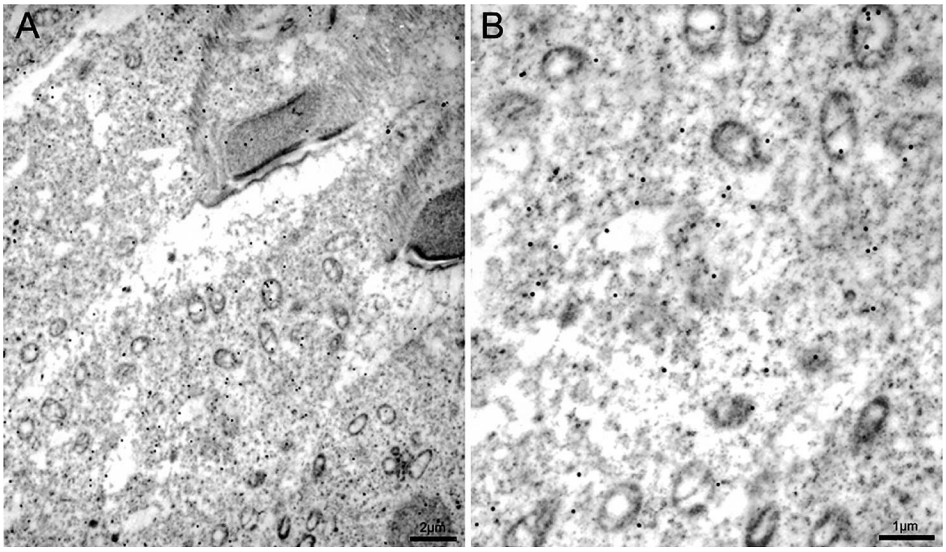

FIG. 9. KIFC1 is enriched in distal cytoplasm. **A**) The cytoplasmic lobes of three spermatids are shown labeled by the KIFC1 antibody. **B**) A higher magnification of this region of a spermatid is shown with evident cytoplasmic and vesicular staining.

amounts of KIFC1 are found in spermatid distal cytoplasm and KIFC1 is then found in residual bodies at the lumenal surface of the epithelium.

This movement suggests one possible model for KIFC1 function in acrosome biogenesis with KIFC1 transporting vesicles to the growing acrosome (Fig. 10, model **a**). This model is consistent with the sequential placement of KIFC1 first between the Golgi and the developing acrosome and then on the acrosome itself. Once at the acrosome, this motor appears to reside either on the acrosome or within the acrosomal space in elongating spermatids. KIFC1 then translocates toward the caudal end of the spermatid head along microtubules of the manchette, perhaps carrying cargo destined for incorporation into the spermatid tail or to be discarded in the residual body. There is ample precedent for manchette-mediated transport in spermatids (reviewed in [19]). Spermatid proteins that translocate from the apical to the caudal aspect of the spermatid head and onto components of the sperm tail include Odf1-Spag4 and Sak57, among others [20, 21]. In none of these cases has a molecular motor protein been shown to be directly involved.

Although the above scheme is attractive, the presumed minus-end directed nature of KIFC1 conflicts with some aspects of this model. The motor domain of KIFC1 resides at its C-terminus; all kinesins with this arrangement so far characterized move toward microtubule minus ends. In somatic cells, microtubule minus ends are organized at the microtubule-organizing center (MTOC) near the Golgi apparatus with their plus ends radiating toward the cell periphery. The spermatid is a unique polarized cell lacking a conventional MTOC; however, evidence supports the idea that vesicle transport in spermatids is similar to that of its somatic counterparts [2] with some deviations. Multiple pathways appear responsible for vesicle trafficking to the acrosome [22], and the acrosome is resistant to the fungal metabolite, Brefeldin A [2, 3]. However, if microtubules are indeed organized with their minus ends at the Golgi in spermatids as in somatic cells, KIFC1 would move vesicles away from the acrosome, not toward it, as depicted in model **a** of Figure 10. In this case, KIFC1 could be responsible for recycling of membrane back to the Golgi.

Another model (Fig. 10, model **b**) is based on the observation that KIFC1 is localized in the nucleus of round spermatids just prior to its appearance near the Golgi. KIFC1 is not detectable in the nuclei of spermatogonia, spermatocytes, or later stage spermatids. Instead, KIFC1 accumulates in the nucleus in round spermatids just prior to its appearance near the Golgi. Perhaps the KIFC1 motor is sequestered in the nucleus in an inactive state until the proper stage of spermatid development. Upon activation, we propose that KIFC1 exits the nucleus through nuclear

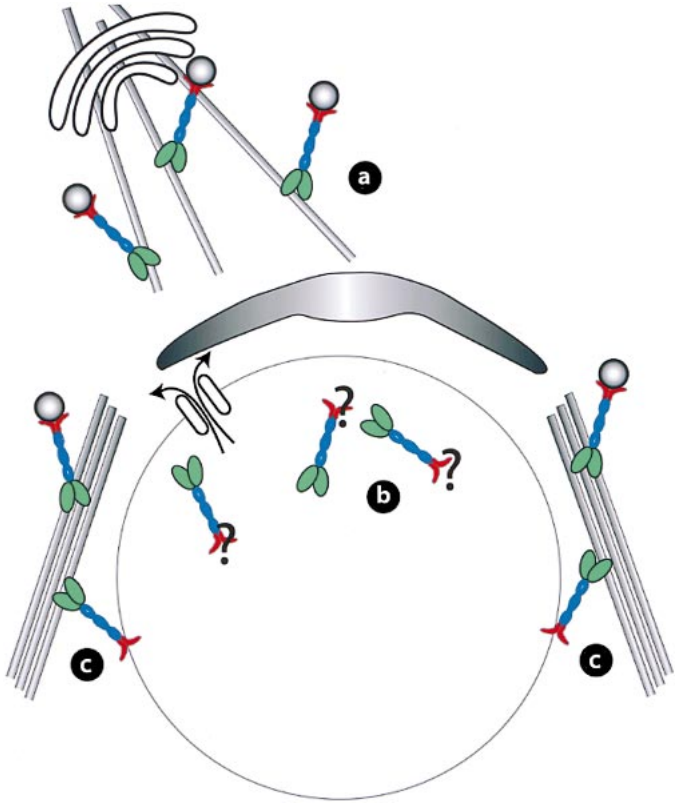

FIG. 10. Models for KIFC1 function in acrosome formation. Model **a** depicts KIFC1 transporting acrosome-bound vesicles from the Golgi and continuing along manchette microtubules to the caudal aspect of the spermatid. Model **b** shows an alternative, where nuclear KIFC1, possibly inactivated by association with importin β or another protein (indicated by ?), is released from inhibition to exit the nucleus in order to recycle vesicles to the Golgi. Model **c** shows KIFC1 linking the nucleus to microtubules of the manchette, thus powering its movement along the surface of the nucleus.

pores, becoming associated with vesicles returning to the Golgi or with the acrosome during its elongation. This scheme would be consistent with the polarity of this motor. In support of this idea, KIFC1 can be found localized at the nuclear membrane just beneath the acrosome in some sections of early spermatids (Fig. 4E).

A third possible function for KIFC1 (Fig. 10, model **c**) is in manchette-based motility. Colocalization of KIFC1 with the manchette in elongating spermatids and its translocation to the caudal aspect of the nucleus is consistent with a role for this motor in the movement of the manchette along the spermatid nucleus. In addition, the directionality of KIFC1 is in accordance with the proposed polarity of manchette microtubules. In this model, KIFC1 molecules, attached to a receptor on the nuclear membrane would move along manchette microtubules toward their minus ends at the perinuclear ring. This movement would result in the simultaneous transportation of the nucleus through the manchette as the manchette is moved caudally along the nucleus.

Previously, we demonstrated that components of the nucleocytoplasmic machinery are found in spermatid nuclei and are arranged in a polarized manner [14]. Interestingly, Ran is found in a discrete location in spermatocyte nuclei and subsequently near the acrosome of young spermatids, while RCC1, Ran's exchange factor, localizes to the spermatid manchette in condensing spermatids. Because importin β plays a crucial role in several nuclear events, including transport, nuclear membrane formation, and spindle construction, it was of interest to determine whether this molecule physically interacts with KIFC1 during spermatid development. Indeed, we found that KIFC1 interacts with importin β and that this interaction is more prominent at early stages of development when KIFC1 is not yet found on the acrosome. However, by immunofluorescence, the most visible association appears to be not in the nucleus but on structures near the nuclear membrane. Further investigation is needed to determine the relationship between KIFC1, importin β, and structures associated with the nuclear envelope, including the acrosome and microtubules of the developing manchette.

Recently, evidence has been obtained supporting a functional link between nuclear activities, vesicle trafficking, and acrosome formation [23, 24]. The scaffolding protein Eps15 provides a framework for endocytosis and vesicle trafficking via interaction of its Eps15 homology domains with multiple cellular targets (reviewed in [25]). One such target is Hrb (also known as hRIP or RAB), a nucleoporin-like protein originally identified as a cellular cofactor for HIV-1 Rev that enhances its nuclear export [26, 27]. Surprisingly, homozygous deletion of this gene from mouse resulted in a complete absence of an acrosome in these animals along with disruption of Eps1 protein:protein interactions [28].

We provide evidence that the C-terminal motor KIFC1 may play an important role in formation of the mammalian acrosome. The acrosome is a unique organelle whose biogenesis is critical for fertility; however, the KIFC1 motor functions in other cell types, as its expression is not restricted to the testis. In somatic cells, KIFC1 may play a related role in the association of cytoplasmic membranes with the nucleus (unpublished observations). In addition, we document the association of KIFC1 with the nuclear transport factor importin β. This finding provides additional evidence of a link between nuclear activities and the control of vesicle transport. Along with our recent description of novel N-terminal motors that are also localized near the acrosome, the identification of KIFC1 will provide the unique opportunity to examine the roles of two oppositely directed motor proteins in acrosome biogenesis [14].

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