

# Comparative Analysis of Two C-Terminal Kinesin Motor Proteins: KIFC1 and KIFC5A

Yuguo Zhang and Ann O. Sperry\*

*Department of Anatomy and Cell Biology, Brody School of Medicine at East Carolina University, Greenville, North Carolina*

We have taken advantage of the close structural relationship between two C-terminal motors, KIFC5A and KIFC1, to examine the sequence requirements for targeting of these two motors within the cell. Although KIFC5A and KIFC1 are almost identical in their motor and stalk domains, they differ in well-defined regions of their tail domains. Specific antisera to these motors were used to determine their localization to distinct subcellular compartments, the spindle for KIFC5A or membranous organelles for KIFC1. In addition to defining the intracellular localization of KIFC1, the reactivity of the KIFC1 antibody demonstrates that this motor contains a frame shift with respect to KIFC5A and is likely the product of a separate gene. The divergent tail domains of these motors are predicted to harbor specific information that directs them to their correct intracellular targets. In order to define the sequences responsible for the differential localization of these two motors, GFP was fused to motors with various tail deletions and their localization visualized after transfection. We were able to identify distinct sequences in each motor responsible for its unique cellular localization. The KIFC5A tail contains a 43 amino acid sequence with both nuclear localization and microtubule binding activity while KIFC1 contains a 19 amino acid sequence sufficient to target this motor to membrane-bounded organelles. *Cell Motil. Cytoskeleton* 58:213–230, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** kinesin-related molecular motor; mitosis; vesicle transport

## INTRODUCTION

Growth and cellular change are critically dependent upon proper targeting of numerous cellular constituents during the dramatic remodeling of the cytoskeleton. A number of kinesin-related motors function in the cytoskeletal rearrangements required for formation and maintenance of microtubule superstructures including the spindle apparatus and the neuronal cytoskeleton. In addition to functioning to create microtubule structures, other classes of kinesins participate in transport of organelles including mitochondria, lysosomes, and synaptic vesicles [reviewed in Hirokawa, 1998; Kamal and Goldstein, 2000].

A complex microtubule-based machine, the spindle is designed to separate chromosomes faithfully at mitosis. One critical component of this process is the formation of two discreet spindle poles from which radiate the microtubule arrays that form the spindle. Minus end-directed motors, both dynein and kinesins, and the non-

centrosomal spindle component NuMA have been shown to be essential for the formation and functioning of the spindle pole and are thought to tether the microtubules to the pole [Gordon et al., 2001; Merdes et al., 2000]. In addition to this tethering function, minus end-directed kinesins are proposed to crosslink microtubules as they move toward microtubule minus ends via microtubule binding sites in their tail domains, thus focusing and

Contract grant sponsor: NIH; Contract grant number: GM60628.

\*Correspondence to: Ann O. Sperry, Department of Anatomy and Cell Biology, Brody School of Medicine at East Carolina University, Greenville, NC 27858. E-mail: sperrya@mail.ecu.edu

Received 21 October 2003; accepted 2 March 2004

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cm.20008

TABLE I. PCR Primers for KIFC5A and KIFC1 Constructs

Name	Sequence	Purpose
KIFC5Aa	TAAAGCTTATGGACGTGCAGGCGCAGAGGCCA	5', KIFC5A full-length
Insert1b	TAGGATCCCTGGTCAGGACCCCTCTTGAG	3', insert 1
Insert2a	TAAAGCTTATGGACCAGCAGAGGCTGGAGACG	5', insert 2
Insert2b	TAGGATCCGGTACCCAGACATTCCTCC	3', insert 2
KIFC5AGFPA	GCAAGCTTATGGAGGATGCCTTGAGCCTGCA	5', 129; minus insert 1
KIFC5AGFPC	GCAAGCTTATGAGGAAAGGCTGCTTCAGGAGCTT	5', 669 minus insert 1 and 2
KIFC5AGFPD	TAGGATCCCTTCTTATTAGCCTGAGCAGTACC	3', KIFC5A full-length
KIFC1A	ACAAGCTTATGGAGGATGCCTTGAGGCC	5', KIFC1 full-length
KIFC1B	CTGGATCCGACTTCACTTCTTATTAGCC	3', KIFC1 full-length
KIFC1D	TAAAGCTTATGACACTGAGCACCCAGCTGGA	5', KIFC1 535 deletion

stabilizing the spindle poles [Matuliene et al., 1999; Walczak et al., 1997]. These cross-linking and tethering functions appear to be performed redundantly by components of the spindle lattice and motor proteins.

Several members of the KIN C family of C terminal motors have been identified from different species with roles in maintaining the integrity of spindle poles. *Xenopus* XCTK2 is required for the formation of well-focused asters and bipolar spindles in *Xenopus* egg extracts [Walczak et al., 1997]. HSET is required, in combination with NuMA, for the formation of asters in cell-free systems and chromosomal movement in cultured cells [Gaglio et al., 1997; Gordon et al., 2001]. However, ablation of the activity of these motors alone does not disrupt spindle formation significantly. The situation is made more complex by the identification of several highly related C-terminal motors in the mouse and in the human. The mouse genome contains 4 loci related to KIFC1, a mouse homologue of HSET, while two isoforms of HSET have been identified, variable at their C terminus [Mountain et al., 1999; Navolanic and Sperry 2000]. In addition, at least two distinct HSET sequences are detected in databases. The purpose of these multiple motors in microtubule structures has not been determined.

Along with their vital role in the construction of microtubule structures, kinesins are essential for polarized vesicle motility in cells. Conventional kinesin has a well-established role in anterograde movement of vesicles in the secretory pathway [reviewed in Kamal and Goldstein, 2000]. Furthermore, specific interactions have been detected between individual motor subtypes and vesicles containing defined contents [Nakagawa et al., 2000; Noda et al., 2001; Setou et al., 2000]. While anterograde transport is mediated primarily by kinesins, transport of vesicles in the opposite direction has been the domain of cytoplasmic dynein. The only known candidate for a kinesin involved in retrograde transport, a distant relative of the C-terminal kinesins called tbKIFC1, is found in the trypanosome and thought to be

responsible for retrograde transport of acidic vesicles in the blood forms of this parasite [Dutoya et al., 2001].

This report compares the structure and function of two closely related C-terminal kinesins, KIFC1 and KIFC5A. Although these motors are 87% identical along their entire length, they display quite different subcellular localizations. We have identified domains in the KIN C member KIFC5A that target this motor to the nucleus and enable it to bind microtubules. Interestingly, KIFC1, although almost identical to KIFC5A, is localized to membranous structures rather than to the spindle apparatus. These studies have identified unique sequences on these highly related motors that direct them to drastically different cellular targets: the spindle and cytoplasmic vesicles.

## MATERIALS AND METHODS

### DNA Manipulations

Fragments from the tail domain of KIFC5A were amplified using the PCR primers listed in Table I in order to produce GFP fusion proteins. Amplification products were ligated to pGEM-T (Promega, Madison, WI) and recognition sites incorporated into the 5' primer (Hind III) or the 3' primer (BamHI) were used to subclone each fragment into the GFP fusion vector pEGFP-N2 (Clontech, Palo Alto, CA) such that the C-terminus of the motor tail was fused in frame with EGFP. RT-PCR reactions were conducted essentially as previously described [Sperry and Zhao, 1996]. The original KIFC5A cDNA clone in Bluescript was used as a template for all reactions to amplify KIFC5A tail fragments while the KIFC1 cDNA was cloned from mouse 3T3 cell RNA using the KIFC1 primers. To produce cDNA, 10  $\mu$ g RNA was mixed with random primers, annealed by slow cooling to room temperature from 65°C, and extended with MMLV-RT at 37°C in the provided buffer (Stratagene, La Jolla, CA). After heat denaturation, the cDNA was ready for amplification.

Typical PCR reactions contained 1–3  $\mu$ l of the cDNA reaction mixture or 0.5–1  $\mu$ g KIFC5A or KIFC1 plasmid, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM  $MgCl_2$ , 0.2 mM each dNTP, 1  $\mu$ M each primer in a volume of 25  $\mu$ l. The reactions were preheated to 95°C for 5 min, and 2.5  $\mu$ l Taq polymerase (Gibco-BRL, Rockville, MD) added as the reaction cooled to 60°C for 5 min. Cycling conditions were 72°C, 1–2 min; 93°C, 1 min; 42°–58°C (primer dependent), 1 min; for 25 cycles. Amplification products were separated by electrophoresis in low melting point agarose (Gibco-BRL, Rockville, MD) and the DNA fragments purified by extraction from the gel using the Qiaex kit from Qiagen (Valencia, CA). After ligation into pGem-T, the purified subclones were digested with the appropriate restriction enzymes and ligated to Bluescript II SK. Recombinants were sequenced with universal primers using the fmol sequencing kit from Promega.

### Western Blot

Protein extracts were prepared from tissue culture cells or mouse tissues by modification of published protocols [Bellve et al., 1977; Neely and Boekelheide, 1988]. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) through 10% acrylamide gels, and equilibrated in and electrophoretically transferred from the gel matrix to PVDF membrane (BioRad Laboratories, Hercules CA) in Towbin transfer buffer [Towbin et al., 1979]. Proteins were detected on the membrane with primary polyclonal antibodies to KIFC5A and KIFC1. The KIFC5A antisera was prepared using the peptide DQAQRPPLEVK not found in KIFC1 and KIFC1 antisera was prepared against QGKAASGAS-GRAAIA, a peptide unique to this protein (see Fig. 1). The KIFC5A anti-peptide antibody was prepared by Aves Labs (Tigard, OR) from serum derived from 2 injected chickens. The immunofluorescent staining pattern and Western blot were identical for both sera. The KIFC1 anti-peptide antibody was prepared by Harlan (Indianapolis, IN) from serum derived from 2 rabbits. Anti-KIFC1 antibodies were also generated in chickens (sera from both rabbits and chickens gave identical immunofluorescence patterns and Western blot). In each case, antibodies were affinity purified by binding to peptide-conjugated columns produced using the Sulfolink kit from Pierce (Rockford, IL). Characterization of the KIFC1 antibody has been previously published [Yang and Sperry, 2003]. Immune complexes bound to the membrane were detected with horseradish peroxidase-conjugated donkey secondary antibody diluted 1:20,000 in TBST (20 mM Tris, pH 7.5, 154 mM NaCl, 2 mM EGTA, 2 mM  $MgCl_2$ , 0.1% Triton X-100) (Jackson ImmunoResearch Inc., West Grove, PA) and developed with enhanced chemiluminescent reagents as described

by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

### Cell Culture and Immunofluorescence

Mouse 3T3 cells (American Type Tissue Collection, Manassas, VA) were seeded onto coverslips and grown approximately 24 h to 75% confluency. Culture media were removed by dipping each coverslip several times in PBS, and were drained and immersed immediately in a 50:50 mixture of acetone/methanol at room temperature for 5 min. The coverslips were removed from the acetone mixture and rinsed 2 times with PBS. Coverslips were then flooded with antibody solution and incubated at room temperature for 30 min. The following antibodies were used for these experiments: anti-kinesin H2 monoclonal, diluted 1:1,000 (kind gift of Dr. Scott Brady), anti-KIFC5A affinity purified polyclonal diluted 1:10 in PBS with 3% (w/v) Bovine Serum Albumin and anti-KIFC1 affinity purified polyclonal. Following incubation with primary antibody, coverslips were rinsed 3 times with PBS and incubated 30 min at RT with anti-rabbit IgG (or anti-chicken) conjugated to Texas Red (1:200), and anti-tubulin conjugated to FITC (1:200; monoclonal). After incubation, cells were washed 3 times with PBS and coverslipped with vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA) and observed with a Nikon E600 fluorescence microscope fit with appropriate filters and images captured with an Orca II CCD camera from Hamamatsu (Bridgewater, NJ) and analyzed with Metamorph image analysis and acquisition software from Universal Imaging Corporation (Downingtown, PA). Controls for these experiments included a nonspecific antibody (anti HIS6 monoclonal, Qiagen), omission of primary antibody, and normal rabbit sera.

### DNA Transfection

Two days before transfection, 3T3 cells were seeded onto coverslips in 6-well plates in complete growth medium (DMEM, 10% calf serum). The cells were then incubated at 37°C in a CO<sub>2</sub> incubator until about 70% confluent. For each transfection, about 1.5  $\mu$ g of DNA and 10  $\mu$ l of Lipofectamine reagent (Invitrogen, Carlsbad, CA) was diluted into 100  $\mu$ l DMEM, mixed gently and incubated at room temperature for 30 min to allow DNA:liposome complexes to form. After complex formation, 0.8 ml of DMEM was added and mixed gently. Cells were rinsed with DMEM once and the complex solution overlaid onto cells followed by incubation for 3–6 h at 37°C. Following incubation, 1 ml of growth medium containing twice the normal concentration of serum was added. The cells were then incubated at 37°C and observed with a fluorescent microscope at following time points: 2, 4, 8 h for KIFC1 constructs or

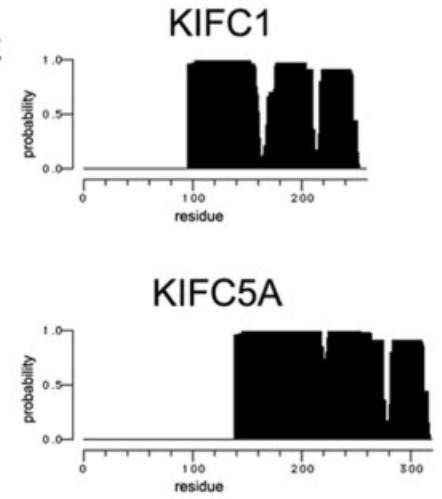
A

KIFC5A	541	ACCCTGGGGACAGAGCGGAACACCCTGGAAGGGGAGCTGGCCAGTGTACGCAGCCGAGCT
KIFC1	412	ACCCTGGGGACAGAGCGGAACACCCTGGAAGGGGAGCTGGCCAGTGTACGCAGCCGAGCT
KIFC5A	601	GAGCAGGACCAGCAGAGGCTGGAGACGCTGAGTGCCCGTGTCTTGGAGCTGGAGGAATGT
KIFC1	472	GAGCAGG-----
KIFC5A	661	CTGGGTACCAGGGAAAGGCTGCTTCAGGAGCTTCAGGGAGAGCGGCTGCAATTGCAGGAA
KIFC1	479	-----GAAAGGCTGCTTCAGGAGCTTCAGGGAGAGCGGCTGCAATTGCAGGAA
		*
KIFC5A	721	GAGCGGAGCACACTGAGCACCCAGCTGGAGGAGCAGAAGAGGAGGTTTCAGGCCACAGAA
KIFC1	527	GAGCGGA-CACACTGAGCACCCAGCTGGAGGAGCAGAAGAGGAGGTTTCAGGCCACAGAA

B

		5A	
KIFC1	1	-----	MEDALEPAKKRTRVMGA
KIFC5A	1	MDVQAQRPPLEVKRNVELKAALVKSSSRVPLSASRLKRGPDQ	MEDALEPAKKRTRVMGA
KIFC1	18	VTKVDTSRPRGPLLSTVSQTQGHTAAQKGPKKTGPRGCSAVGTVLRSQKPAPAAPAQKPG	
KIFC5A	61	VTKVDTSRPRGPLLSTVSQTQGHTAAQKGPKKTGPRGCSAVGTVLRSQKPAPAAPAQKPG	
KIFC1	78	TSTAPVVVGKRAGKRPAWDLKGQLCDLNEELKRYREKTQMLELENRGLREQLREVQEAT	
KIFC5A	121	TSTAPVVVGKRAGKRPAWDLKGQLCDLNEELKRYREKTQMLELENRGLREQLREVQEAT	
		C1	
KIFC1	138	TLGTERNTLEGELASVRSRAEQG-----KAASGASGRAAAIAG	
KIFC5A	181	TLGTERNTLEGELASVRSRAEQDQQRLETLSARVLELEBECLGTRERLLQELQGERLQLQE	
KIFC1	176	RADTLSTQLEEQKRRFQATEAALSSSQEEVVCLRQKTEAQVTLA	
KIFC5A	241	ERSTLSTQLEEQKRRFQATEAALSSSQEEVVCLRQKTEAQVTLA	

C



D

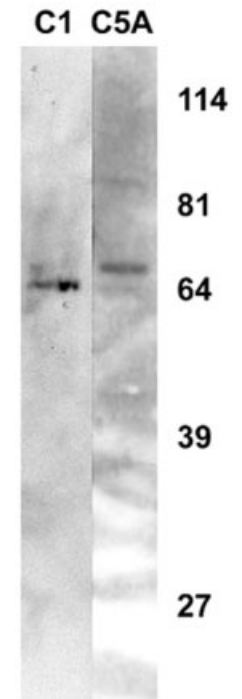


Figure 1.



16, 24, 30, 36, 48 h for KIFC5A constructs. Fluorescent images were captured using Metamorph image analysis software and pixel intensity was measured inside regions including the nucleus alone and the entire cell. These values were used to derive an average N/C (nuclear intensity/cytoplasmic intensity) ratio for each construct. Data from at least 24 cells for each transfection construct were analyzed by ANOVA. For nocodazole treatment, cells were grown to about 50–60% confluency on coverslips, then the drug was added to 33  $\mu$ M and the incubation continued for 1.5 h. For BFA, drug was added to 18  $\mu$ M followed by incubation at 37°C for 1 h. After drug treatment, cells were washed 3 times in PBS, then fixed and stained for immunofluorescence.

### Protein Transfection

The fluorescent conjugated KIFC1 peptide (GKAASGASGRAAAIAGRAD; UNC Microprotein Sequencing and Peptide Synthesis Facility, UNC-Chapel Hill, Chapel Hill, NC) was introduced into mouse NIH/3T3 cells using the Chariot reagent from Active Motif (Carlsbad, CA). Briefly, cells were grown to 40–50% confluency on coverslips in 6-well dishes. To prepare the transfer reagent, 30 ng peptide was dissolved in 100  $\mu$ l PBS and mixed with chariot reagent diluted 1:167 in water and incubated at RT for 30 min. After the media were removed and the cells rinsed with PBS, 200  $\mu$ l of the macromolecular complex was added to the cells followed by 400  $\mu$ l DMEM. Following a 1-h incubation, 1 ml of complete media was added to the cells and the incubation continued for 1 h. The cells were then fixed as described above, if desired, and viewed with a fluorescence microscope.

### Sequence Analysis

The sequence of the tail domains of KIFC1 and KIFC5A was analyzed using hydrophobic cluster analy-

sis (HCA) devised by Mornon and colleagues [Gaboriau et al., 1987]. This program was accessed at <http://smi.snv.jussieu.fr/hca/hca-form.html>. In this method, the sequence is written as an alpha helix and then cut parallel to the axis of the helix (see small arrows in Fig. 10) and unrolled. Because cutting the sequence removes adjacent sequences from one another, the sequence is duplicated for ease of analysis. Neighboring hydrophobic residues are encircled to aid visualization while residues with strand-disrupting properties are indicated with special symbols (P = star, G = filled diamond, T = box with dot, S = empty box). For our analysis, we used the tail and stalk domains of these motors from amino acids 1–318 for KIFC5A and 1–253 for KIFC1. These fragments include the putative neck regions for each motor but lack the motor domains, which are identical.

## RESULTS

### Mammalian Cells Contain at Least 2 KIFC1 Motor Isoforms

We have recently described a close relative of the KIFC1 C-terminal motor, named KIFC5A [Navolanic and Sperry, 2000]. These two motors are almost identical in their head domains. Their stalk and tail domains are more divergent but remain 64% identical at the amino acid level [Navolanic and Sperry, 2000]. KIFC5A has an N-terminal extension compared to KIFC1 while KIFC1 contains a unique sequence in its coiled-coil domain. This unique sequence in the KIFC1 coiled-coil domain is the result of a –1-nt change in reading frame 55 nt downstream of a 65-nt sequence inserted into KIFC5A (Fig. 1A and B) [Navolanic and Sperry, 2000]. This substitution results in a break in the predicted coiled-coil structure of KIFC1 compared to KIFC5A (Fig. 1C). Because the tail region of motor proteins is proposed to target them to specific cargos, we proposed that these two motors might have distinct functions in the cell despite their sequence similarity. In order to begin to address this question, we have developed isoform-specific antibodies to KIFC1 and KIFC5A.

Polyclonal sera were raised to specific peptides that include sequences unique to each motor: the N-terminal extension of KIFC5A and the peptide substitution due to the frame shift in KIFC1 (overlines, Fig. 1B). Affinity-purified antibodies to each protein recognize a unique band in an immunoblot of cell extract (Fig. 1D). The size of the reactive proteins is in good agreement with the sizes predicted from the KIFC1 and KIFC5A cDNAs. In addition, these antibodies specifically recognize KIFC1 and KIFC5A produced in transcription/translation lysates (unpublished results). The immunoblot result confirms previous predictions concerning KIFC1 and KIF5A

Fig. 1. Structure of the tail domains of KIFC1 and KIFC5A and development of tail specific antibodies. **A:** Alignment of the nucleotide sequence of a portion of the tail domains of KIFC5A and KIFC1. The 65-nt inserted sequence present in KIFC5A (insert 2) is shown. The deleted nucleotide in KIFC1 (asterisk) restores the reading frame so that the encoded amino acids are identical after that point. This discontinuity between KIFC1 and KIFC5A results in 19 amino acids unique to KIFC1 encoded between insert 2 and the frame shift (see B). **B:** Alignment of the amino acid sequence of the tails of KIFC1 and KIFC5A. The overlines indicate the peptides used to prepare specific antibodies to KIFC1 and KIFC5A. **C:** The coiled-coil content of KIFC1 and KIFC5A tail domains was predicted using the Paircoil algorithm [Berger et al., 1995] where the ordinate represents the probability of the sequence forming a coiled-coil structure and the abscissa corresponds to the linear amino acid sequence. **D:** Western blot of NIH/3T3 cell lysates with affinity-purified KIFC1 and KIFC5A antibodies. Antibodies were made to unique peptides from the tail domain of each motor (see B and Materials and Methods).

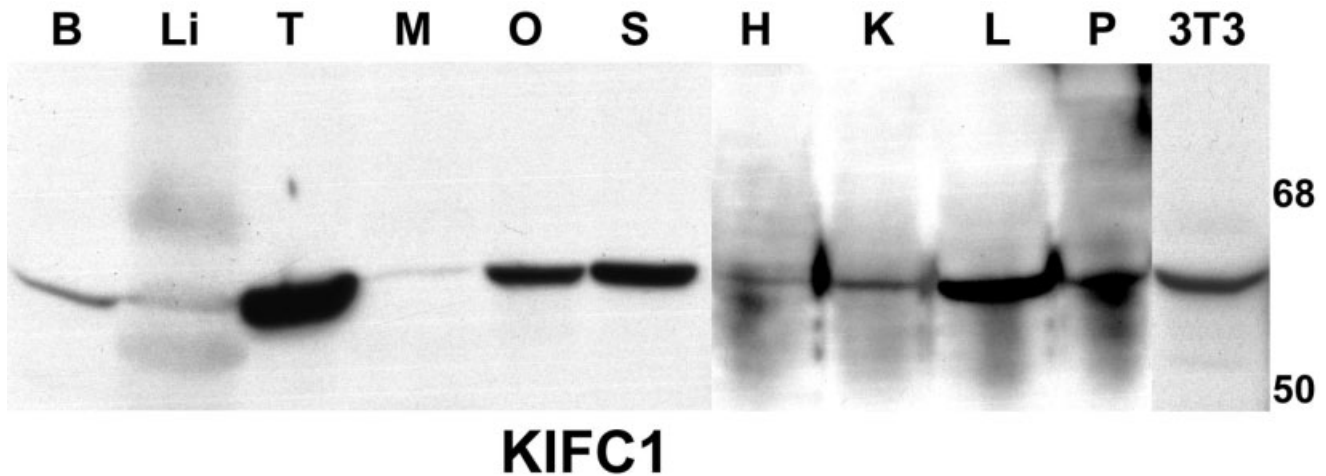


Fig. 2. Tissue distribution of KIFC1. Ten micrograms of protein from the indicated tissues was prepared and separated by electrophoresis on 10% PAGE, blotted, probed with affinity-purified KIFC1, and visualized with horseradish peroxidase-conjugated donkey secondary. B, Brain; Li, liver; T, testis; M, muscle; O, ovary; S, spleen; H, heart; K, kidney; L, lung; P, pancreas; 3T3, NIH/3T3 cells.

ORFs: that KIFC1 contains a unique sequence due to the deletion of 65 nt and the presence of a frame shift 55 nt downstream of the deletion [Navolanic and Sperry, 2000] and that KIFC5A begins at the predicted upstream start site.

#### KIFC1 and KIFC5A Motors Display Different Tissue Distribution and Subcellular Localization

Isoform-specific antibodies were used to investigate the tissue distribution of the KIFC1 and KIFC5A proteins by immunoblot. KIFC1 is a fairly abundant protein and is widely distributed, being most abundant in testis, ovaries, and spleen but also present in brain and lung (Fig. 2). Although KIFC1 is homologous to known mitotic motors, its tissue distribution is not consistent with its having a sole function in cell division. KIFC5A, on the other hand, is much less abundant compared to KIFC1 and is difficult to detect in tissue lysates. However, this motor is detectable in actively dividing cultured cells (Fig. 1D).

This differential distribution is also seen at the subcellular level using isoform-specific antibodies to localize these motors in cultured cells. KIFC1 was found associated with punctate vesicular structures in a perinuclear staining pattern (Fig. 3A). The punctate pattern of KIFC1 staining is suggestive of a vesicular localization for this motor. Indeed, treatment with detergent prior to staining resulted in abolition of KIFC1 staining in the cytoplasm (Fig. 3B); however, a fraction of KIFC1 was reproducibly found tightly associated with the nucleus (arrow, Figure 3B). KIFC1 was not found associated with the mitotic spindle (Fig. 3D). Unlike KIFC1, KIFC5A was found not on cytoplasmic vesicles but

instead on the spindle using an antibody to the N-terminus of this motor (Fig. 4A–D); this antibody did not stain interphase cells (Fig. 4E).

#### KIFC5A Contains a Nuclear Localization Signal and Microtubule Binding Site in Its Tail Domain

In order to determine the targeting sequences responsible for the differential localization of KIFC1 and KIFC5A, we undertook a deletion study of these two motors using GFP fusion proteins in order to follow their localization after transfection into mouse 3T3 cells. Figure 5A shows a schematic of KIFC5A highlighting the differences between the two motors. The black boxes numbered 1 and 2 in Figure 5A represent the two inserts of 129 and 65 nucleotides, respectively, that are unique to KIFC5A. The remaining areas of the KIFC5A diagram are almost identical with KIFC1. Below the schematic, the tail domain deletions that were fused to GFP are shown. As can be seen in Figure 5B, full-length KIFC5A (KIFC5AFL) is strongly localized to the nucleus, consistent with the presence of a putative nuclear localization sequence in insert 1 [Navolanic and Sperry, 2000]. When insert 1 alone is fused to GFP, this protein is specifically

Fig. 3. Immunolocalization of the KIFC1 motor in cultured cells. NIH/3T3 cells were fixed and stained with affinity-purified polyclonal KIFC1 antibody, visualized with a Texas Red conjugated donkey secondary and double stained with an FITC-conjugated mouse anti-tubulin antibody. Cells were treated either without (A,D) or with (B,C) 0.5% Triton prior to staining. After extraction, KIFC1 was found associated with the nucleus (B, arrow). Extracted cells exposed to secondary antibody alone are shown in C. The KIFC1 antibody does not stain the spindle in dividing cells (D). Scale bars = 10  $\mu$ m.

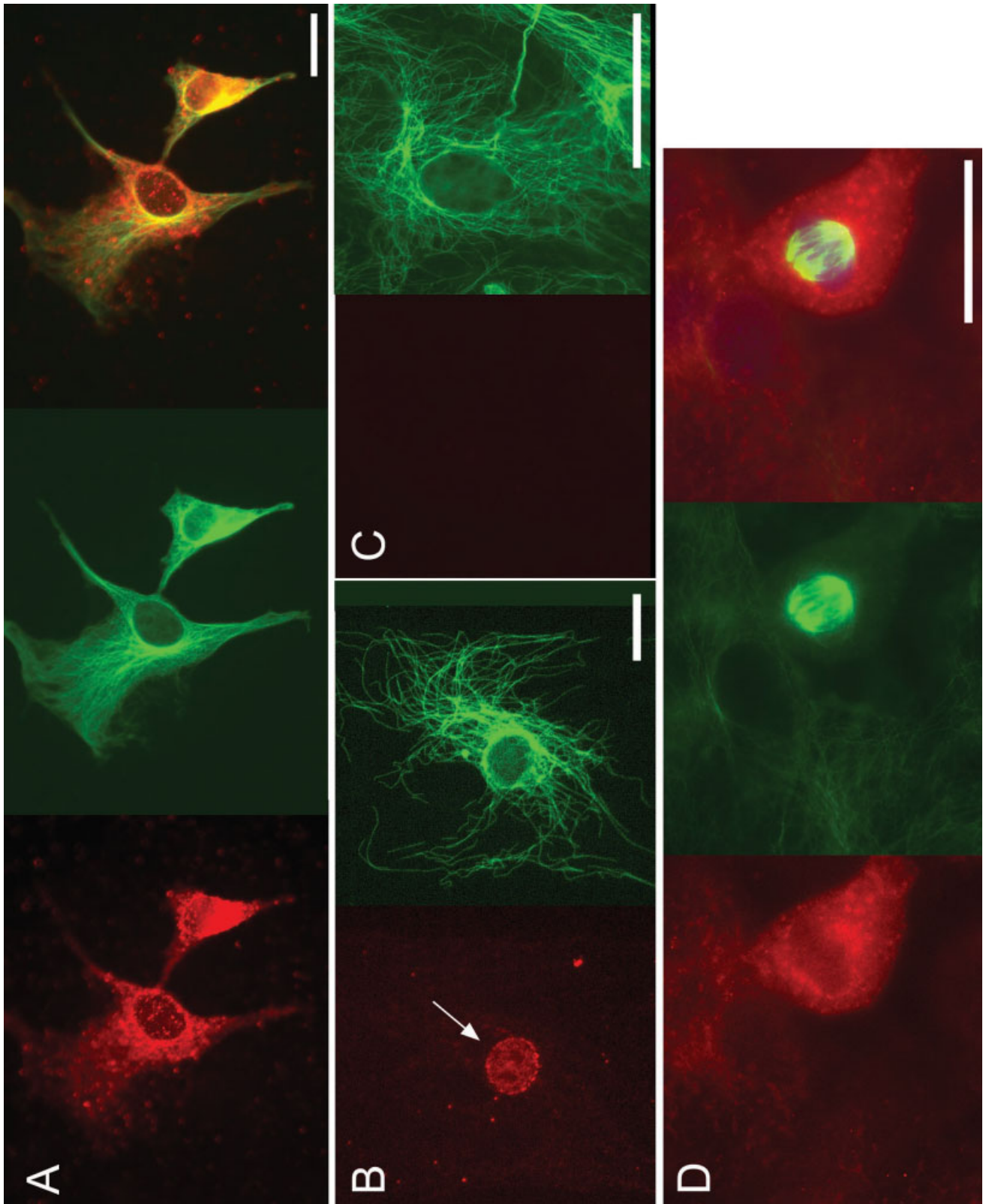


Figure 3.



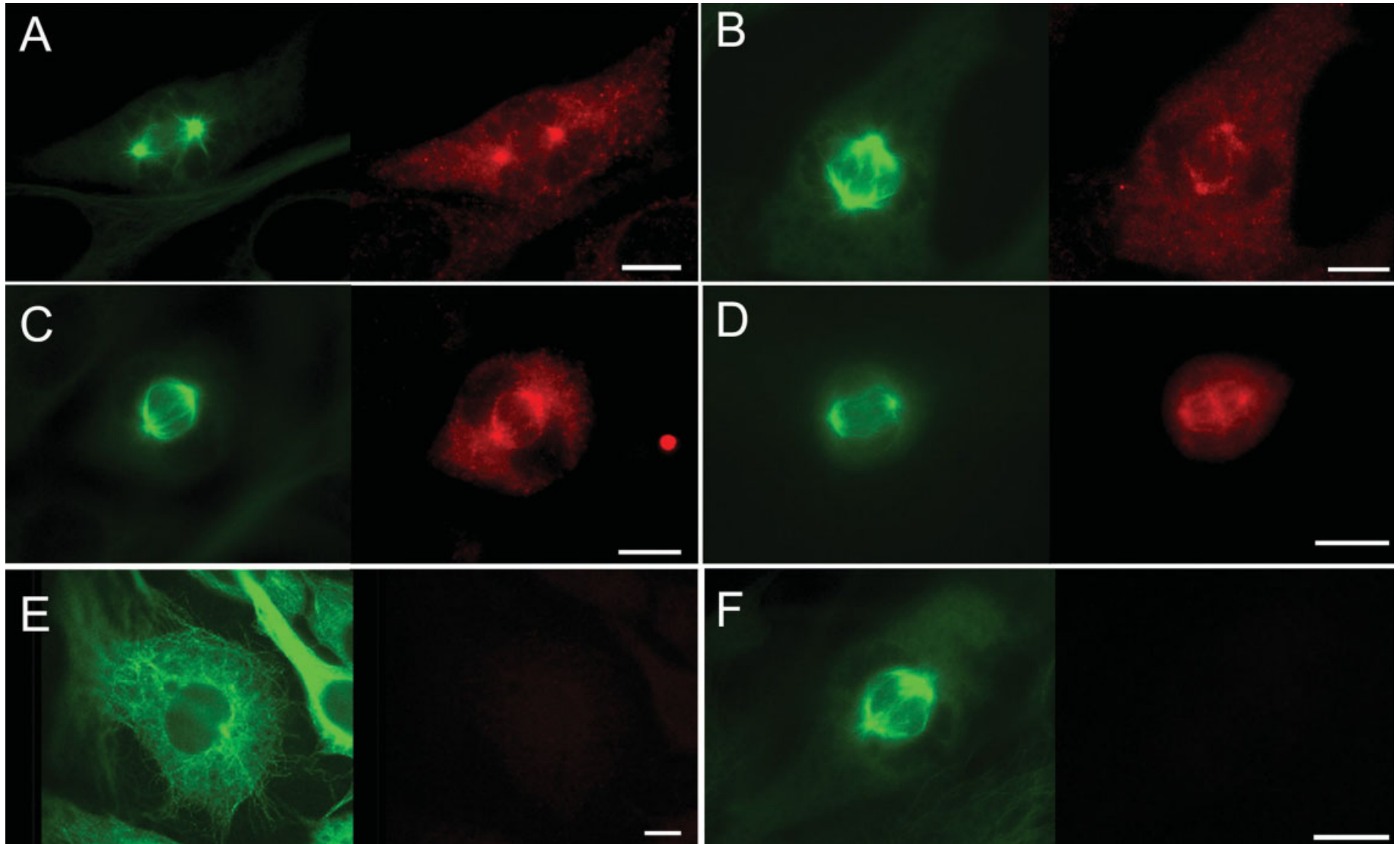


Fig. 4. Immunolocalization of the KIFC5A motor to the mitotic spindle. Fixed, cultured cells were double stained with an FITC-conjugated mouse anti-tubulin antibody and the KIFC5A affinity-purified polyclonal antibody detected with a Texas Red-conjugated donkey

secondary. Cells at different stages of mitosis are shown (A-D). An interphase cell is shown stained with the KIFC5A antibody (E) and a no primary antibody negative control staining of a dividing cell is shown in F. Scale bars = 10 μm.



found on spindle fibers (Fig. 5B, Ins 1) in dividing cells. These results indicate that this 43–amino acid sequence is sufficient for both nuclear localization and microtubule binding. This conclusion is further supported by our observation that in interphase cells expressing GFP-insert 1, this protein is found in both the nucleus and associated with cytoplasmic microtubules (arrow; Fig. 5B). In contrast, the GFP-insert 2 fusion protein is more evenly distributed throughout the cell (Fig. 5B, Ins 2). Although some nuclear localization is seen with this fusion protein, it is much reduced compared to the GFP-insert 1 fusion and the full-length KIFC5A. In addition, no spindle localization was ever observed with the insert 2 fusion.

The importance of insert 1 to KIFC5A function was demonstrated by localization of a KIFC5A fusion protein lacking the first 129 nucleotides including insert 1 (Fig. 5B, 129). The 129 deletion is no longer strongly localized to the nucleus and never appears on spindle fibers. As expected, deletion of both insert 1 and insert 2 in the GFP-669 fusion results in cytoplasmic localization of this protein (Fig. 5B, 669). The images in Figure 5B, particularly of those cells containing insert 1 constructs, are overexposed in order to show the outline of the cell. This leads to an overestimation of cytoplasmic staining relative to nuclear staining. Therefore, we quantified the ratio of nuclear staining to cytoplasmic staining for selected constructs and the results are shown in Figure 5C. The full-length KIFC5A (KIFC5AFL) is significantly concentrated in the nucleus compared with constructs lacking the insert 1 sequence.

#### **KIFC5A Contains a Microtubule-Binding Site in Its Tail Domain That Can Mediate MT Bundling**

The behavior of GFP-motor fusions, above, suggests that the tail of KIFC5A, unlike that of KIFC1 (see below), contains a microtubule-binding site. Indeed, insert 1 is unique to KIFC5A and was able to direct this motor to the nucleus and to microtubules. Interestingly, when the KIFC5A motor is expressed at high levels, thick bundles of microtubules form in the cytoplasm (Fig. 6). In many cases, these bundles are seen to circle the nucleus (Fig. 6A,B,D,E). This phenotype is similar to what is observed when the microtubule-bundling protein PRC1 is overexpressed in cultured cells [Mollinari et al., 2002]. Our results are consistent with the demonstration by others that motors of this kinesin subfamily mediate microtubule bundling in the spindle [Karabay and Walker, 1999; Matulienė et al., 1999; Walczak et al., 1997]. In this model, the microtubule-binding site on the head domain of these motors interacts transiently with microtubules in an ATP-regulated manner while the ATP-independent site in the C-terminal tail domain is available to bind nearby microtubule arrays. The micro-

tubule cross-linking provided by these motors stabilizes the spindle pole and serves to focus microtubules at the pole. In support of this model, constructs containing only the ATP-dependent site (KIFC5A-127, KIFC5A-669, and KIFC1) are not stably associated with microtubules under normal physiological conditions.

#### **KIFC1 Contains a Vesicle Association Sequence in Its Tail Domain**

The targeting properties of the KIFC1 tail were compared with those of KIFC5A using GFP fusion proteins in a manner similar to that described above. A schematic for KIFC1 is shown in Figure 7A with the various fusion proteins and peptide shown below. The sequence unique to KIFC1 is indicated by the black box in Figure 7A and is produced as a result of the frame shift in this protein's coding sequence with respect to KIFC5A (see Fig. 1A) [Navolanic and Sperry, 2000]. Consistent with our previous localization of KIFC1 to punctate structures in cultured cells (Fig. 3), the full-length KIFC1 fusion protein was found localized around the nucleus to punctate structures resembling membrane-bounded organelles (Fig. 7B, KIFC1FL). These structures appear larger, however, than the KIFC1 positive structures detected by immunofluorescence (see Fig. 3). The KIFC1 protein contains a unique 19-aa sequence that corresponds to the first predicted break in the coiled-coil domain of KIFC1 (Figs. 1C, 7A). This peptide is necessary for targeting of KIFC1 because when this peptide is deleted from KIFC1, it no longer localizes to vesicular structures (compare KIFC1535 to KIFC1FL; Fig. 7B). Quantification of KIFC1 targeting showed that without the unique sequence, KIFC1 showed a similar distribution compared to the KIFC5A deletion constructs (Fig. 5C).

In order to determine whether the unique sequence in KIFC1 contains information sufficient to direct KIFC1 to cytoplasmic vesicles, a peptide corresponding to this sequence was synthesized, conjugated to FITC, and introduced into cultured cells via the Chariot reagent (Active Motif; Carlsbad, CA). The KIFC1 peptide was found localized in a manner very similar to that of the GFP construct (Fig. 7B, KIFC1pep) while a peptide with the same amino acid content but a scrambled sequence was not localized in a similar manner (Figure 7B, KIFC1scrm).

#### **KIFC1 Is Enriched in a Vesicle Population Distinct From Those Containing Kinesin or Dynein**

The punctate pattern of KIFC1 staining is suggestive of a vesicular localization for this motor. Indeed, treatment with detergent prior to staining resulted in abolition of KIFC1 staining in the cytoplasm (Fig. 3B); however, a fraction of KIFC1 was reproducibly found

associated with the nucleus. In order to determine whether KIFC1 is found on vesicles containing other microtubule motors, this motor was colocalized with conventional kinesin and cytoplasmic dynein. KIFC1 does not show extensive colocalization with either the plus-end directed motor kinesin (Fig. 8A) or the minus-end motor dynein (Fig. 8B). The distribution pattern of KIFC1 is distinctive, being localized more to the perinuclear region compared to that of kinesin or dynein (compare the KIFC1 panels with the kinesin and dynein panels in Fig. 8A and B).

### KIFC1 Accumulates at the Nucleus After Treatment With Brefeldin A and Nocodazole

We next analyzed the behavior of KIFC1 vesicles after treatment with agents that disrupt vesicle trafficking including the fungal metabolite Brefeldin A, which disrupts the Golgi, and Nocodazole, which disrupts microtubules. Treatment with Brefeldin A alone caused only a slight increase in perinuclear staining (compare Fig. 9, BFA panel, with Fig. 8) with an accumulation of the KIFC1 motor in the nucleus (Fig. 9). Depolymerization of microtubules with nocodazole heightened this effect resulting in a more striking accumulation of this motor at the nucleus particularly at the nuclear rim. This behavior suggests that KIFC1 may reside in or on the nucleus when not transporting cargo. This association with the nucleus is consistent with our previous report that KIFC1 is localized to the nucleus in male germ cells [Yang and Sperry, 2003].

### The Tails of KIFC1 and KIFC5A Have Regions of Differing Secondary Structure

The tail domains of these two motors have strikingly different targeting properties. In addition, we have identified the sequences responsible for the differential localization of these motors and we predict that the linear sequence difference in the tail domain of these motors will result in important differences in their secondary structure. In order to better visualize and compare the structural differences between the tail domains of KIFC1 and KIFC5A, we used the hydrophobic cluster analysis (HCA) method of Mornon and coworkers [Gaboriaud et al., 1987]. This 2-dimensional prediction program relies on the interactions of corresponding hydrophobic residues to determine the folding of polypeptide chains and has been used to analyze the secondary structure of a number of protein families [reviewed in Callebaut et al., 1997]. HCA analysis directly reflects the dependence of proper protein folding on amino acids representing the hydrophobic core and hydrophilic surface of the protein. This technique provides easy visualization of structural domains within a protein as well as facilitating comparison between proteins. In this method, the polypeptide is

written onto an alpha helical pattern and the sequence is flattened and duplicated to make visualization of relationships between amino acid residues within the protein easier, as well as to make comparison between proteins more straightforward (see Materials and Methods). Once the sequence is displayed in this manner, residues with a strong predicted effect on secondary structure are highlighted to make pattern recognition easier. In Figure 10, clusters of contiguous hydrophobic residues (V, I, L, M, F, W, Y) are encircled; proline residues, which confer the most restraint on the polypeptide chain, are indicated with stars, and glycine residues, which confer the least restraint on chain structure, are indicated with a diamond. The small polar amino acids serine and threonine are indicated with a dotted square or a plain square, respectively, because their polarity can be masked by hydrogen bonding to residues in the main chain (Fig. 10). This combination of displaying the linear structure as a flattened  $\alpha$ -helix and labeling important amino acids as symbols allows for the easy detection and comparison of structural domains.

The most noticeable differences in the two tail domains are in the coiled-coil of KIFC1 (Fig. 10, black bar) and insert 1 of KIFC5A (Fig. 10, open bar with star). The sequence specific to KIFC1, GKAASGASGRAAA-IAGRAD, disrupts the coiled structure of the stalk domain with a glycine-rich flexible region not found in KIFC5A. Notably, this sequence contains a serine predicted to be a substrate for PKC phosphorylation (Fig. 10, red and yellow boxes). KIFC5A, on the other hand, contains an N-terminal extension rich in proline residues (Fig. 10, red stars) suggestive of a disordered structure. Insert 1 also contains clusters of hydrophobic residues and the amino acid content of insert 1 is consistent with known microtubule-binding sequences. These results, along with the localization studies described above, provide strong evidence that the sequences we have identified in KIFC1 and KIFC5A are important for motor structure and function.

Fig. 5. Localization of GFP-KIFC5A motor fusions after transfection into cultured cells. **A:** A schematic of the KIFC5A motor is shown with the GFP-fusion constructs indicated below. **B:** GFP-KIFC5A motor fusions were transfected into 3T3 cells and visualized 16 h after transfection. *Arrow* indicates insert 1 fusion associated with cytoplasmic microtubules. Typical transfectants of each construct are shown from 4 independent experiments. Cells transfected with GFP alone are shown at bottom right. Scale bars = 10  $\mu$ m. **C:** Quantification of pixel intensities for fusion constructs. Values are displayed as nuclear intensity/cytoplasmic intensity (N/C). The number of cells of each type analyzed is shown in the bar graph. Differences were considered significant at  $P < 0.05$ . For our samples,  $P = 0.001$ . The values for KIFC1585 (Fig. 7) are shown for comparison with KIFC5A669, whose sequence is almost identical.

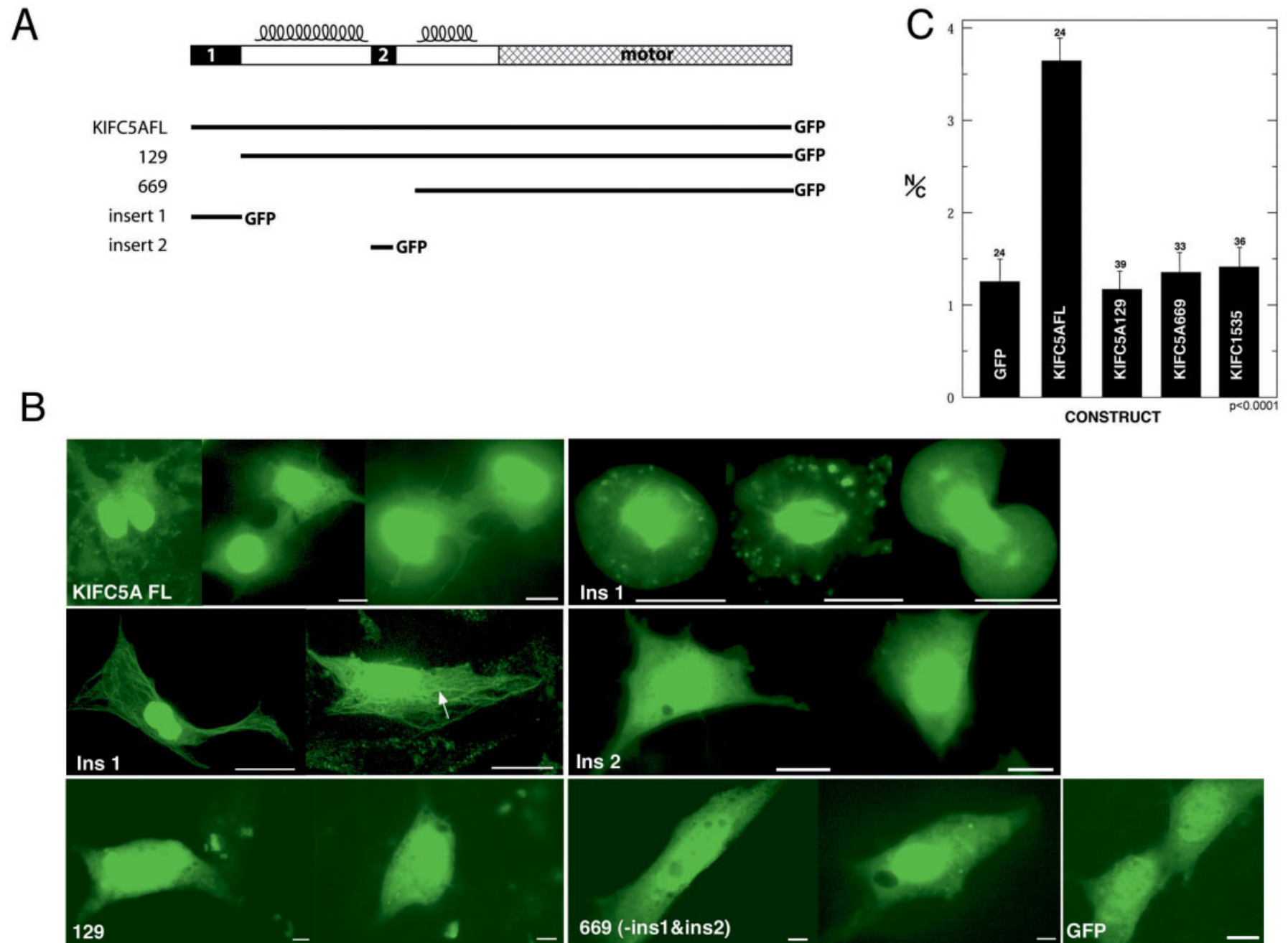


Figure 5.

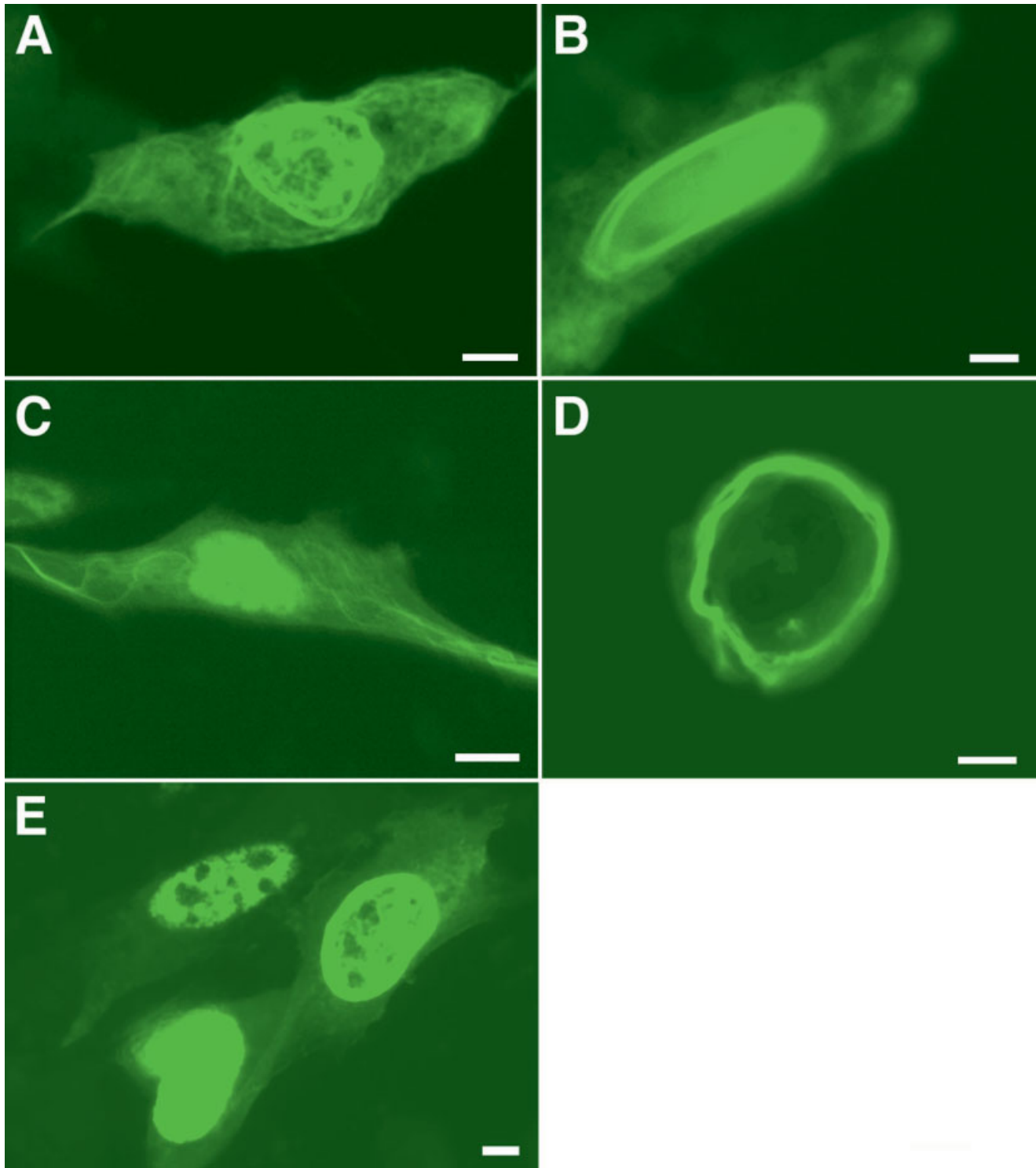


Fig. 6. KIFC5A mediates microtubule bundling. **A–E:** Full-length KIFC5A fused to GFP was transfected into NIH/3T3 cells as described in Materials and Methods and incubated for 20–24 h. Scale bars = 5  $\mu$ m (A,C,E), 2  $\mu$ m (B,D).

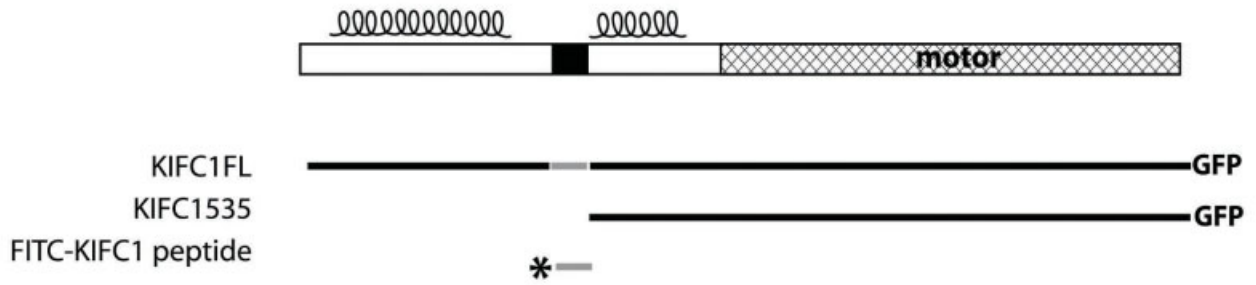
## DISCUSSION

In this report, we describe the structure and localization of two highly related kinesin motors, KIFC1 and

KIFC5A [Navolanic and Sperry, 2000; Noda et al., 1995]. These motors are encoded by 2 members of a family of 4 genes located on chromosomes 17, 9, and 10



A



B

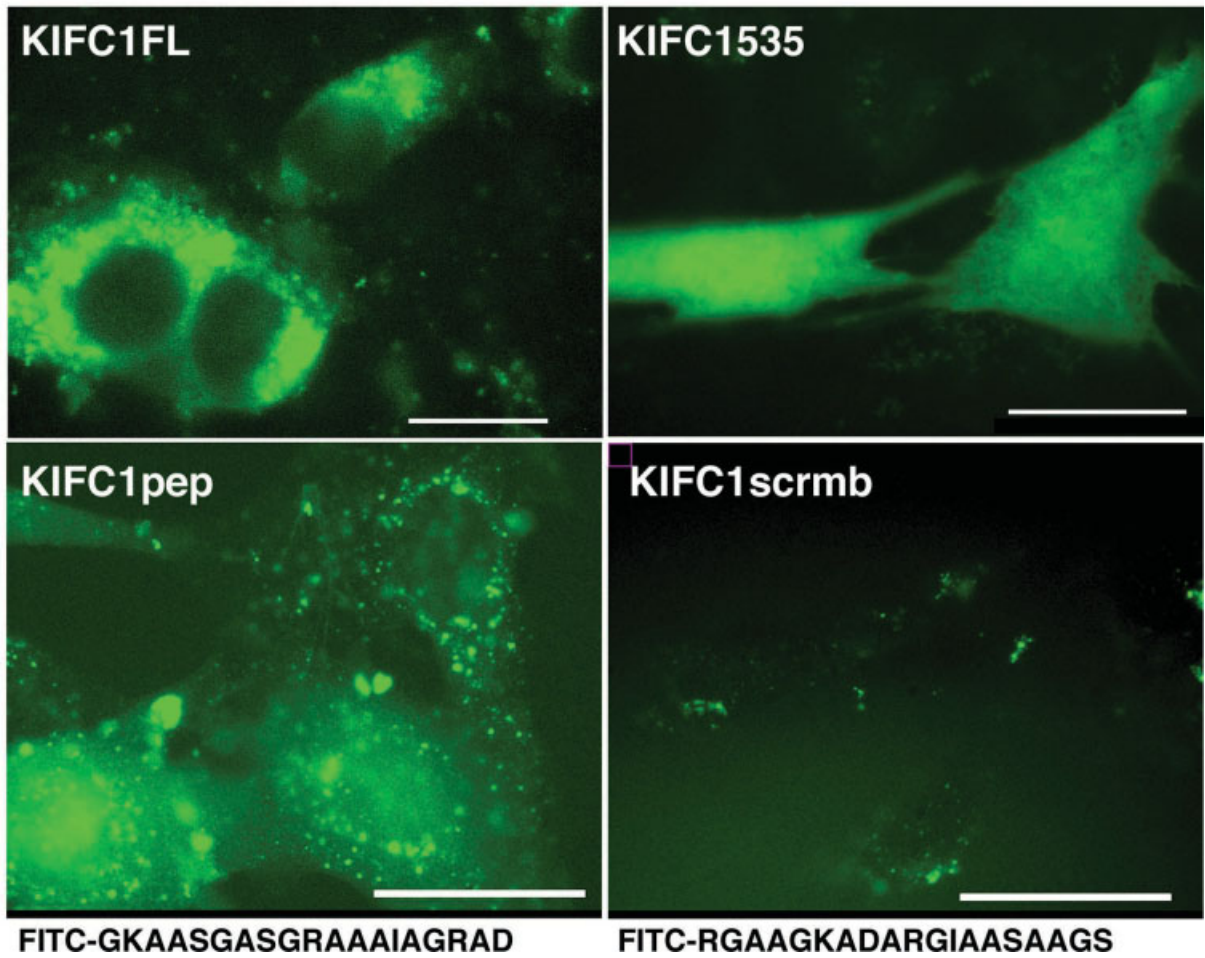


Fig. 7. Localization of GFP-KIFC1 motor fusions after transfection into cultured cells. **A:** Schematic of the KIFC1 motor is shown with the GFP-motor fusions shown below and the unique sequence indicated by a black box. **B:** Fluorescent localization of full-length (KIFC1FL), deletion mutant (KIFC1535), and peptides (KIFC1pep, 19 aa unique sequence; KIFC1scrmb, 19 aa scrambled sequence). Typical transfectants of each construct are shown from at least 3 independent experiments. Scale bars = 10  $\mu$ m

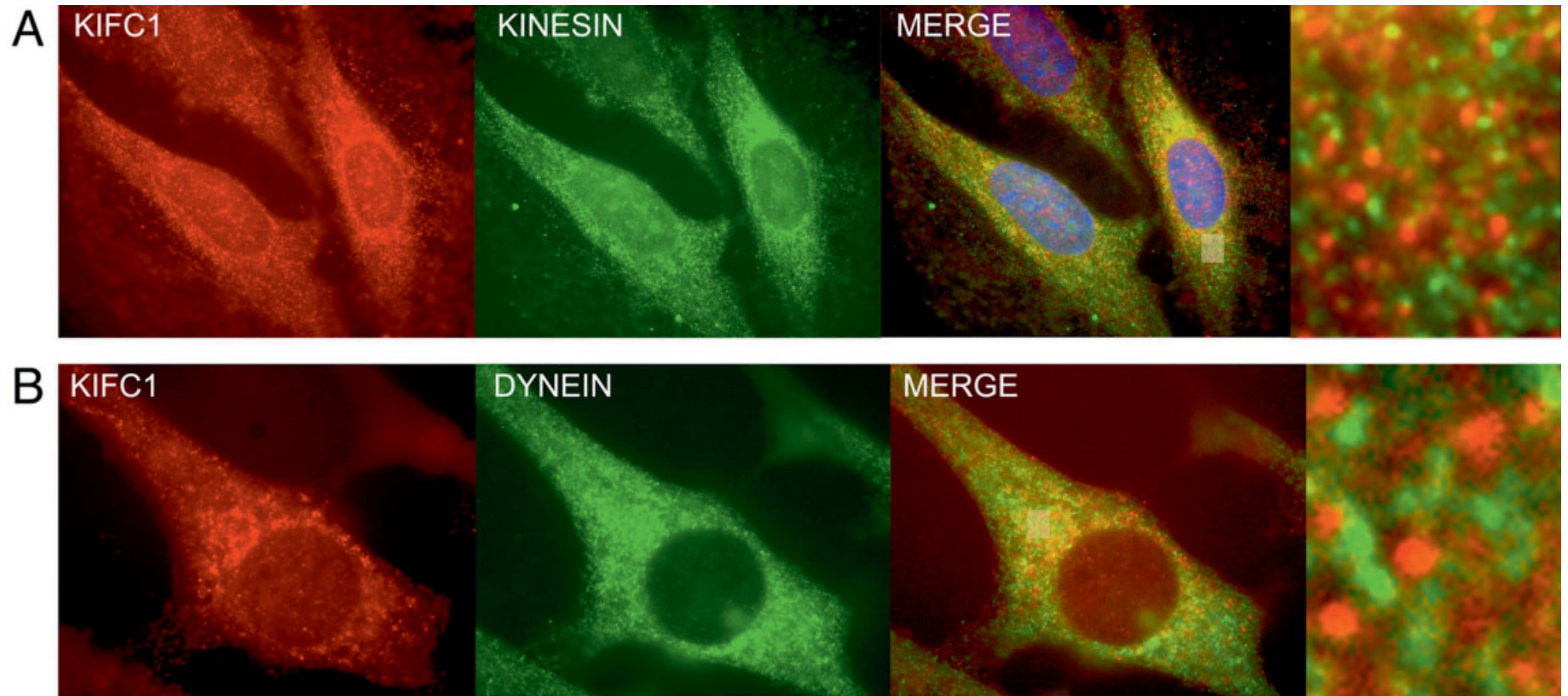


Fig. 8. Dual staining of KIFC1 with kinesin and dynein. **A:** Cells were fixed and stained for KIFC1 with the affinity-purified polyclonal antibody, visualized with a Texas Red-conjugated secondary; kinesin was stained with the H2 antibody and visualized with a FITC conjugated secondary antibody. **B:** Cells were labeled with the KIFC1 antibody as in A and with an antibody to the cytoplasmic dynein IC74 intermediate chain detected with an FITC-conjugated secondary. For both experiments, an area in the cytoplasm was enlarged (**right**) to determine colocalization of these motors.

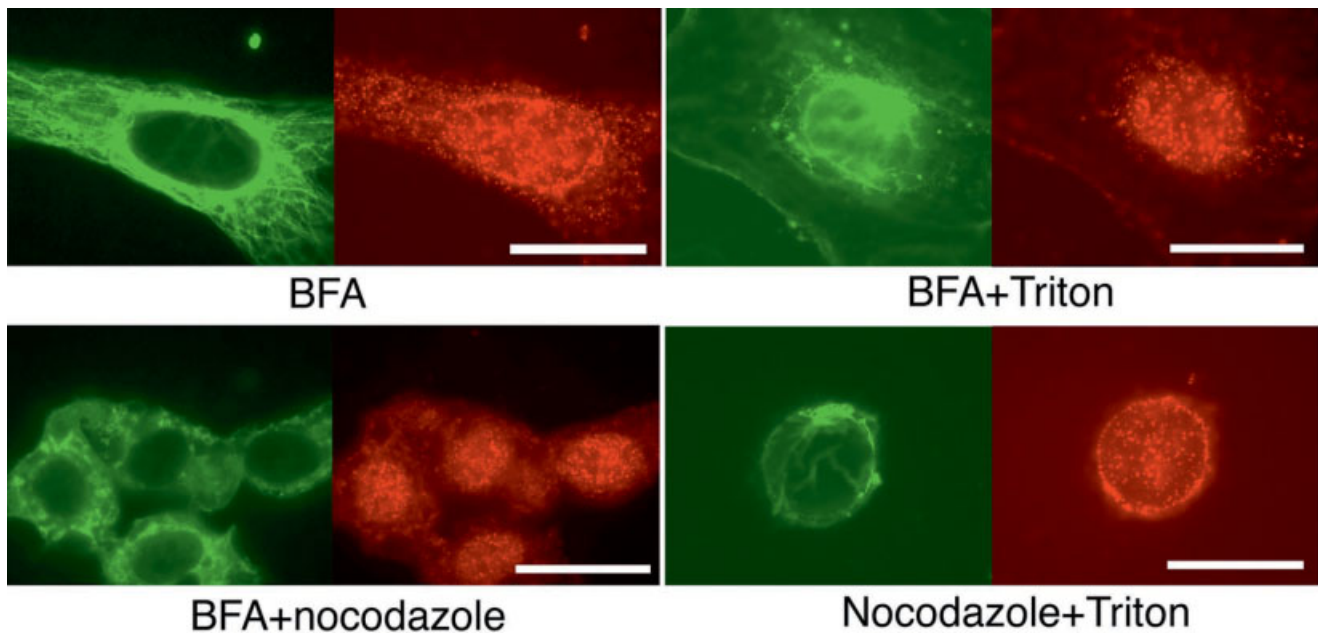


Fig. 9. KIFC1 redistribution with Brefeldin A and Nocodazole treatment. Cells were treated with either BFA alone, BFA with Triton X-100, BFA with nocodazole, or nocodazole plus Triton. Cells were double stained for tubulin (FITC) and KIFC1 (Texas Red). Scale bars = 10  $\mu$ m.

(Nancy Jenkins, unpublished results). KIFC1 and KIFC5A are identical in their head domains but differ in their tail domains by the presence of 2 sequences unique to KIFC5A and 1 sequence found only in KIFC1. Because KIFC1 and KIFC5A are so closely related in structure, they provide a unique opportunity to examine the structural requirements for motor targeting.

We produced antibodies specific for each motor protein in order to localize them in cells and tissues. Western analysis showed that KIFC1 is recognized by an antibody raised to the KIFC1-specific sequence, while KIFC5A is recognized by an antibody to the unique N-terminus of this motor. These results represent an important finding and confirm previous predictions concerning KIFC1 and KIF5A ORFs: that KIFC1 contains a unique sequence due to deletion of 65 nt and the presence of a frame shift 46 nt downstream of the deletion [Navolanic and Sperry, 2000] and that KIFC5A begins at the predicted upstream start site. The reactivity of our KIFC1 antibody to the sequence predicted due to the frame shift in KIFC1 relative to KIFC5A supports the idea that these two motors result from different genes and not alternative splicing.

KIFC1 and KIFC5A are further differentiated by their expression and localization patterns. KIFC1 is found in many tissues, being most abundant in the testis, while KIFC5A is not detectable in tissue lysates but is found in rapidly dividing tissue culture cells. KIFC5A is localized to the mitotic spindle, more concentrated at the

poles, and is a relative of the minus-end motor HSET [Mountain et al., 1999]. Like HSET, KIFC5A likely participates in stabilization of the spindle pole. KIFC1, on the other hand, behaves like a vesicle motor. We have recently shown that KIFC1 is associated with Golgi vesicles and the acrosome in developing spermatids [Yang and Sperry, 2003]. Because formation of the acrosome is a major biosynthetic activity of the spermatid, the abundance of KIFC1 in the testis is consistent with a role for this motor in formation of this organelle.

Consistent with our previous identification of a putative nuclear localization signal in the KIFC5A tail, the KIFC5A-GFP fusion is strongly localized to the nucleus [Navolanic and Sperry, 2000]. Further deletion analysis narrowed down the responsible sequence to insert 1 of KIFC5A. This sequence of 43 amino acids also contains a microtubule-binding site that targets the motor to cytoplasmic and spindle microtubules. Although there is no strict consensus for microtubule-binding sites, the KIFC5A insert 1 is rich in lysine, arginine, and valine as are the microtubule-binding sites of MAP2 and Tau. Its amino acid makeup is consistent with a role for insert 1 in microtubule binding. In addition, this microtubule-binding site can mediate microtubule bundling in combination with the microtubule-binding site of the head domain. Overexpression of KIFC5A constructs containing insert 1, but not those lacking this sequence, results in formation of thick bundles of microtubules within transfected cells.



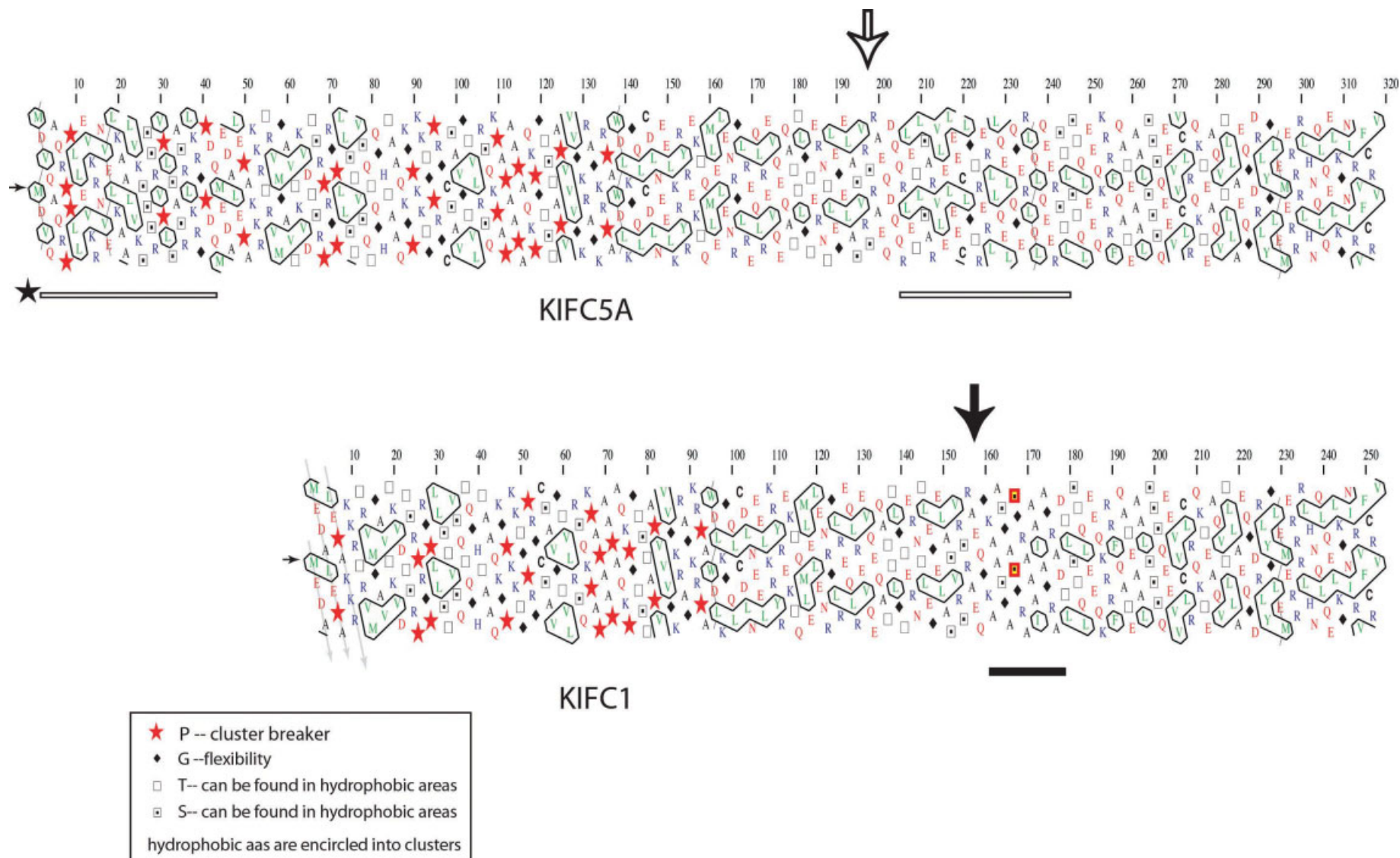


Fig. 10. Sequence analysis of KIFC1 and KIFC5A tail/stalk domains. The motor tail/stalk domains of KIFC5A (amino acids 1–318) and KIFC1 (amino acids 1–254) were analyzed by hydrophobic cluster analysis as described in Materials and Methods [Gaboriaud et al., 1987]. The following symbols are used to represent structurally important amino acids: P = star, G = filled diamond, T = empty box, S = box with dot. Hydrophobic amino acids are encircled into clusters. The unique sequence(s) of each motor is indicated with an *open bar*

with *star* (KIFC5A insert 1), *open bar* (KIFC5A insert 2), and *filled bar* (KIFC1). The putative PKC phosphorylation site in the unique sequence of KIFC1 is indicated by a yellow dotted box bounded in red. *Arrows* above the sequences indicate the break in homology between these two motors. *Arrows* to the left of the panel indicate the axis through which the sequence was duplicated.



Our prediction that the tail domains of the two motors contain functionally distinct targeting sequences was further supported by our analysis of KIFC1. KIFC1 contains a unique sequence of 19 amino acids in its stalk domain that effectively breaks the coiled-coil structure of this part of the motor (see Fig. 10). This sequence is necessary and sufficient to direct the KIFC1 motor to vesicles. Double immunofluorescence localization determined that KIFC1 is found on a vesicle population distinct from that containing the motors dynein or kinesin. Disruption of vesicle trafficking by either Brefeldin A or Nocodazole resulted in accumulation of KIFC1 in the nucleus and at the nuclear membrane. This behavior is of particular interest given our previous finding that this motor accumulates in the nucleus of round spermatids during male germ cell development [Yang and Sperry, 2003]. KIFC1 first appears in the nucleus of round spermatids; however, once the acrosome begins to form, this motor exits the nucleus to become associated with the acrosome. KIFC1 continues to be found on the acrosome during the elongation of this organelle over the spermatid nucleus. Interestingly, KIFC1 was found to interact with the nuclear transport factor importin  $\beta$  and this interaction was increased at developmental times before KIFC1 was found on the surface of the spermatid nucleus. We proposed that KIFC1 might be regulated by nuclear sequestration prior to its release to participate in acrosome motility perhaps by interaction with importin  $\beta$ .

Our proposal is particularly pertinent given a recent report that the nuclear import factors importin  $\alpha/\beta$  directly inhibit the function of the XCTK2 tail domain through a mechanism involving Ran GTPase [Ems-McClung et al., 2003]. XCTK2 is a C-terminal motor structurally related to the motors described here and, like these motors, contains an NLS in its tail domain. These authors [2003] show that the nucleotide-bound state of Ran regulates the inhibitory binding of the importin  $\alpha/\beta$  complex to the NLS, thus blocking the microtubule-binding activity of the XCTK2 tail domain. We propose that the importin  $\alpha/\beta$  complex binds to the tails of these motors in a similar fashion, thereby inhibiting their particular functions: microtubule binding for KIFC5A and binding to a specific subset of cellular membranes such as the acrosome for KIFC1.

## CONCLUSIONS

KIFC1 and KIFC5A represent two highly homologous motor proteins with divergent sequences in their cargo-binding domains. The results presented here support our initial hypothesis that the unique sequences of each motor directs them to distinct cellular targets: KIFC5A is directed to cytoplasmic microtubules and to the mitotic spindle and KIFC1 is found on membrane-

bounded organelles. Recently, numerous investigators have been able to assign individual kinesin-related motor proteins to their specific cargos including several receptor types, DNA, signaling molecules, different organelle subtypes, and others [Levesque and Compton, 2001; Nakagawa et al., 2000; Nangaku et al., 1994; Verhey et al., 2001; Xu et al., 2002]. Although the cargo-binding site(s) has been mapped for some of these motors, few of these sites have been defined to such a small protein sequence as for KIFC5A (43 amino acids) and KIFC1 (19 amino acids). Indeed, we have established that the 43-amino acid insert 1 of KIFC5A contains two functionalities: nuclear localization and microtubule binding. Further studies will determine whether these functions can be separated or are functionally interdependent and will identify the binding partners for the KIFC1 and KIFC5A motors.

## ACKNOWLEDGMENTS

The authors thank Sarah James and Yong Zou for their expert technical assistance. We are grateful to Jason Kait of Southern Micro Instruments for programming the quantitation software. This work is supported in part by grant GM60628 from the NIH (to A.O.S.).

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