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Expression and Partial Characterization of Kinesin-related Proteins in Differentiating and Adult Skeletal Muscle

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Using pan-kinesin antibodies to screen a differentiating C2C12 cell library, we identified the kinesin proteins KIF3A, KIF3B, and conventional kinesin heavy chain to be present in differentiating skeletal muscle. We compared the expression and subcellular localization characteristics of these kinesins in myogenic cells to others previously identified in muscle, neuronal, and mitotic systems (KIF1C, KIF3C, and mitotic-centromere-associated kinesin). Because members of the KIF3 subfamily of kinesin-related proteins showed altered subcellular fractionation characteristics in differentiating cells, we focused our study of kinesins in muscle on the function of kinesin-II. Kinesin-II is a motor complex comprised of dimerized KIF3A and KIF3B proteins and a tail-associated protein, KAP. The *Xenopus* homologue of KIF3B, Xklp3, is predominantly localized to the region of the Golgi apparatus, and overexpression of motorless-Xklp3 in *Xenopus* A6 cells causes mislocalization of Golgi components (Le Bot *et al.*, 1998). In C2C12 myoblasts and myotubes, KIF3B is diffuse and punctate, and not primarily associated with the Golgi. Overexpression of motorless-KIF3B does not perturb localization of Golgi components in myogenic cells, and myofibrillogenesis is normal. In adult skeletal muscle, KIF3B colocalizes with the excitation–contraction-coupling membranes. We propose that these membranes, consisting of the transverse-tubules and sarcoplasmic reticulum, are dynamic structures in which kinesin-II may function to actively assemble and maintain in myogenic cells.

INTRODUCTION

During vertebrate myogenesis, pluripotent mesodermal cells become committed to a myogenic cell fate and populate areas throughout the developing embryo. After receiving the appropriate signals, these progenitors eventually differentiate into multinucleated myofibers (reviewed in Holtzer *et al.*, 1986). In contrast to the progress made in understanding the cascade of events regulating the expression of muscle-specific genes (reviewed in Weintraub, 1993), the mechanisms that underlie the morphological rearrangements during skeletal muscle formation are less well understood.

Adult skeletal muscle consists of the highly ordered array of cytoskeletal proteins forming the contractile units of the myofibril as well as the extensive network of differentiated membrane systems that regulate muscle cell activity. The differentiation of mononucleated, presumptive myoblasts into multinucleated myotubes reflects a massive structural reorganization of cytoplasmic components. Historically, the two dynamic filament systems, microtubules and microfilaments, have been considered to be actively involved in generating the spatial organization of the cell; likewise, there is accumulating evidence suggesting that microtubules may participate in myofibril formation. During the elongation of polygonal-shaped myoblasts into bipolar, spindle-shaped myotubes a stable array of microtubules becomes longitudinally oriented in the axis of future myofibril formation (Gundersen *et al.*, 1989). Microtubule inhibitor studies indicate that microtubules are essential both for generating the initial change in cell shape as well as for proper myofibril assembly (Bischoff and Holtzer, 1968; Holtzer *et al.*, 1975; Antin *et al.*, 1981; Toyama *et al.*, 1982). Microtubule number increases during developmentally significant stages of neonatal cardiac muscle, decreasing upon maturity (reviewed in Rappaport and Samuel, 1988). Additionally, antisense inhibition of muscle-specific microtubule-associated protein-4 during differentiation has no effect on growth and cell fusion, but myofibrils lack a polarized morphology (Mangan and Olmsted, 1996). These observations imply that proper myofibril

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Abbreviations used: DHPR, dihydropyridine receptor; ER, endoplasmic reticulum; E-C, excitation-contraction; HP, *Helix pomatia*; KHC, kinesin heavy chain; KRP, kinesin-related protein; MCAK, mitotic-centromere associated kinesin; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

assembly is strongly dependent on the presence of a stable array of microtubules.

In addition to contributing to the structural integrity of eukaryotic cells, the microtubule network also provides the framework for the transport and localization of cellular components through the activity of molecular motors. The molecular motor, kinesin, was originally reported to be responsible for the transport of axoplasmic organelles along microtubules in the giant, squid axon (Brady, 1985; Vale *et al.*, 1985). A superfamily of related proteins has since been identified, shown to participate in many different cellular activities across both cell type and species (reviewed in Goldstein and Philp, 1999). In addition to transporting membrane-bound vesicles and organelles (reviewed in Hirokawa, 1998), members of this superfamily also associate with and transport nonvesicular cargoes, including chromosomes (Schaar *et al.*, 1997; Wood *et al.*, 1997; Maney *et al.*, 1998) and protein rafts for cilia/flagellar assembly (reviewed in Cole, 1999). Because kinesin proteins function in both vesicle and protein complex transport, these microtubule motors are good candidates for participating in the differentiation of the contractile and/or membrane systems of skeletal muscle.

We have used two affinity-purified, pan-kinesin peptide antisera (Sawin *et al.*, 1992) to identify kinesins that are present during differentiation of the myoblast cell line, C2C12. We compare the distribution and subcellular fractionation and extraction profiles of these kinesins in proliferating and differentiating myogenic cells to others previously identified in muscle, neuronal, and mitotic systems. In our study of the role of kinesin-related proteins (KRPs) in differentiating myotubes and adult muscle, we chose to investigate the function of kinesin-II because this heterotrimeric motor complex has been implicated in participating in both membrane and protein complex transport (Le Bot *et al.*, 1998; Cole, 1999). Kinesin-II is comprised of dimerized KIF3A and KIF3B polypeptides and a tail associated protein, KAP3. Disruption of kinesin-II motor function in C2C12 cells with the overexpression of a motorless version of KIF3B does not perturb Golgi/endoplasmic reticulum (ER) transport or myofibrillogenesis. In adult skeletal muscle, the KIF3B motor subunit of kinesin-II colocalizes with components of the excitation-contraction (E-C) coupling membranes. We propose that kinesin-II may aid in either the assembly or maintenance of the E-C membranes by participating in the transport and/or recycling of specialized membrane components.

MATERIALS AND METHODS

Cell Culture and Library Screen

C2C12 cells (Yaffe and Saxel, 1977; obtained from American Type Culture Collection, Manassas, VA), were subcloned for high differentiation by immunofluorescence analysis of acetylated-microtubule expression. Myoblasts were grown in DMEM plus 10% fetal calf serum, and triggered to differentiate upon media change to DMEM plus 10 μg/ml insulin and 5 μg/ml transferrin. C2C12 cells were grown at 37°C in 5% CO_2. Differentiation media was changed every 24–48 h. *Xenopus* A6 cells (Rafferty and Sherwin, 1969) (American Type Culture Collection) were grown at 23°C in L-15 medium plus 15% fetal calf serum.

For library production, total-RNA was purified from C2C12 myotubes differentiated for 3 d (Chomczynski and Sacchi, 1987). mRNA (10 μg) was isolated from total-RNA by using the PolyATract mRNA isolation system (Promega, Madison, WI). cDNA synthesis was performed by using the ZAP-cDNA synthesis kit and cloned into the Uni-ZAP XR vector system (Stratagene, La Jolla, CA). The expression library was amplified once as per instructions in Uni-ZAP XR cloning manual (Stratagene). Approximately 600,000 plaques were screened with affinity-purified rabbit polyclonal sera, anti-LAGSE and anti-HIPYR (Sawin *et al.*, 1992). The screening technique was tailored such that individual phage plaques could be tested for protein expression with both pan-kinesin peptide antibodies (Ginkel and Wordeman, 2000). Fifteen clones cross-reacted with both antisera and were sequenced by using [α-^{35}S]dATP (New England Nuclear, Boston, MA) and Sequenase kit 2.0 (United States Biochemical, Cleveland, OH). Sequences were submitted to a BLAST database search (National Center for Biotechnology Information, Bethesda, MD).

Transfection, Immunocytochemistry, Immunohistochemistry, and Light Microscopy

Cells grown on 12-mm glass coverslips (Carolina Biological Supply, Burlington, NC) coated with either Cell Tak (Becton Dickinson, Bedford, MA) or 1% gelatin, and placed in 24-well plates, were transfected with Superfect reagent (Qiagen, Valencia, CA) as per manufacturer's instructions. For one coverslip, 2 μg of plasmid DNA (Maxiprep; Qiagen) and a 1:3 DNA:Superfect reagent ratio was used. Cells were incubated in the DNA-Superfect solution for 3.5 h, washed 2× with phosphate-buffered saline (PBS), and 2× with normal medium. After transfection, C2C12 cells were either maintained in proliferation medium or, after 18–24 h, switched into differentiation medium. A6 cells were incubated in normal medium.

For immunocytochemistry, cells were fixed in either 4% paraformaldehyde (Ted Pella Inc., Redding, CA) for 20 min or ice-cold methanol for 10 min. Sometimes, C2C12 myotubes were extracted before methanol fixation in 0.5% Triton X-100 in microtubule-stabilizing buffer [50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.9, 5 mM $MgCl_2$, 1 mM EGTA] for 1 min. Cells were labeled by a procedure described elsewhere (Ginkel and Wordeman, 2000). For immunohistochemistry of mouse skeletal muscle, sections of hind limb from adult mice were prepared as in Ralston *et al.* (1999) and fixed in 4% paraformaldehyde for 20 min. Sections were double labeled with anti-KIF3B-T and mouse, monoclonal antibodies to dihydropyridine receptor (DHPR), ryanodine receptor (RyR), or triadin. Sections were blocked first for 30 min in Avidin solution and then biotin solution (Vector Labs, Burlingame, CA), followed by 1 h in 20% donkey serum, washing in between with PBS. Primary antibodies were applied overnight at 4°C. Biotin-conjugated anti-mouse (Jackson ImmunoResearch, West Grove, PA) was applied for 2 h. Secondary antibodies, Texas Red-conjugated streptavidin and fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit (Jackson ImmunoResearch), were applied for 2 h. Coverslips and slide sections were mounted in Vectashield containing 4,6-diamino-2-phenylindole nuclear stain (Vector Labs).

Labeled cells and sections were observed by using a Nikon FX-A photomicroscope (Melville, NY) and photographed by using Kodak Technical Pan film. Negatives were scanned by using a Nikon Coolscan II, and images were analyzed in Photoshop 5.0 (Adobe Systems, Mountain View, CA). Confocal images were acquired by using a confocal laser microscope Leica TCS-NT (Deerfield, IL) equipped with an Ar/Kr laser triple line. Collection and projection of confocal images were made with the TCS-NT software.

Production of KIF3B Fusion Protein and Antibody

The globular tail domain of KIF3B (nucleotides 1846–2310, amino acids 593–747) (Yamazaki *et al.*, 1995) was generated by polymerase chain reaction (PCR) from the pBluescript II-KIF3B clone isolated from the screen. *Nde*I and *Xho*I sites incorporated into the 5′ and 3′ PCR primers, respectively, were used to insert the fragment into the pET-21a vector (Novagen, Madison, WI). This construct was trans-

fected into BL21(DE3) cells for bacterial protein expression, and the fragment was purified on an NTA-agarose column (Qiagen, Valencia, CA) under denaturing conditions as per instruction manual (Qiagen). Protein was eluted into 1.5-ml fractions, and purest fractions were pooled. Protein was renatured by step dialysis into PBS and concentrated in dialysis tubing with carboxymethyl cellulose, sodium salt to 1 mg/ml. Rabbit polyclonal antisera against KIF3B-tail fragment was produced (Berkeley Antibody, Richmond, CA). Antibodies (anti-KIF3B-T) were affinity purified (Lane and Harlow, 1988) against the corresponding tail fragment coupled to Affigel-10 (Bio-Rad, Hercules, CA). Glycine (100 mM), pH 2.5, was used to elute the column.

Immunoblots and Immunoprecipitations

To generate C2C12 cell lysates, pellets of either proliferating C2C12 myoblasts or differentiating myotubes (3 d unless otherwise indicated) were resuspended in 50 mM Tris-HCl, pH 7.5, 4% SDS, and boiled for 10 min. To generate muscle lysates, adult mouse muscle from hind limb was frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. Muscle powder was resuspended in immunoprecipitation (IP) lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 50 mM sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% Triton X-100, phenylmethylsulfonyl fluoride, protease inhibitors) and homogenized with a polytron tissue grinder (Kinematica, Lucerne, Switzerland). Unsolubilized material in the lysates was removed by centrifugation at 10,000 rpm, 10 min, 4°C, and protein concentrations were determined by a crude A280. Total protein (100 μg) was loaded per lane. Samples were separated on 4–12% gradient gels (Novex, San Diego, CA) and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 h in NAP-*Sure*BLOCKER (Geno Technology, St. Louis, MO), and all washes were performed in Tris-buffered saline, pH 8.0. Primary and alkaline-phosphatase conjugated-secondary antibodies, diluted in Tris-buffered saline, were sequentially incubated with membranes for 2 h, at room temperature, with agitation. Membranes were developed by 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (BCIP-NBT) (Lane and Harlow, 1988).

For immunoprecipitation, a 10-cm plate of a monolayer of either transfected or nontransfected C2C12 cells was washed with PBS. Cells were lysed on the plate in 1 ml of IP lysis buffer on ice. The cell lysate was clarified by centrifugation for 10 min, 10,000 rpm, 4°C. The antibody used for immunoprecipitation (2.0 μl of anti-KIF3B-T concentrated stock, or 2.0 μl of anti-green fluorescent protein [GFP]) was incubated with 200 μl of a 10% slurry of Affi-Prep Protein-A beads (Bio-Rad) in IP lysis buffer for 1 h, 4°C, with gentle shaking. The clarified cell lysate was incubated with the antibody/Protein A solution for 4 h, 4°C, with gentle shaking. Washed beads were resuspended in 35 μl of SDS-PAGE final sample buffer, boiled for 10 min, and analyzed by immunoblot. For immunoprecipitation from adult skeletal muscle, ~250 μg from hind limb was frozen and ground as described above, resuspended in 1 ml of IP lysis buffer, and clarified. Anti-KIF3B-T was used to immunoprecipitate kinesin-II as outlined above. To immunoprecipitate kinase heavy chain (KHC), 5 μl of mouse monoclonal anti-KHC was incubated with 200 μl of a 10% slurry of anti-mouse IgG agarose (Sigma, St. Louis, MO) in IP lysis buffer. Immunoprecipitation controls were performed with Protein-A or anti-mouse agarose alone.

Antibodies and Reagents

The following antibodies were used: mouse monoclonal anti-α-tubulin (Sigma); rat monoclonal anti-tubulin (Harlan Sera-Lab, Crawley Down, United Kingdom); mouse monoclonal anti-skeletal myosin-fast (Sigma); mouse monoclonal antiacetylated-tubulin (Sigma); mouse monoclonal anti-kinesin II (K2.4; Berkeley Antibody); rabbit polyclonal anti-KIF3C, gift from Z. Yang and L.S. Goldstein (University of California, San Diego, CA); rabbit polyclonal anti-mitotic–centromere-associated kinesin (MCAK) (Wordeman and Mitchison, 1995); rabbit polyclonal anti-KIF1C, gift from R. Lammers (Max-Planck Institut für Biochemie, Martinsried, Germany); mouse monoclonal anti-KAP3 (Transduction Laboratories, Lexington, KY); mouse monoclonal anti-KHC (H2; Chemicon, Temecula, CA); rabbit polyclonal anti-GFP (Clonetech, Palo Alto, CA); mouse monoclonal anti-Na^+/K^+ ATPase α-1 (Upstate Biotechnology, Lake Placid, NY); mouse monoclonal anti-RyR and mouse monoclonal anti-triadin (Affinity Bioreagents, Golden, CO); mouse monoclonal anti-DHPR α-1 subunit (Chemicon); and FITC-, Texas Red-, and alkaline phosphatase-conjugated secondary antibodies specific for rabbit, mouse, or rat (Jackson ImmunoResearch). Texas Red-conjugated *Helix pomatia* (HP) lectin (Sigma) was resuspended in PBS and kept at 4°C. FITC-phalloidin (Molecular Probes, Eugene, OR) was resuspended in methanol and kept at −20°C.

Expression Constructs

The GFP-KIF3B-motorless deletion construct (GFP-KIF3B-ML) was made by modifying GFP-MCAK (Maney *et al.*, 1998) in pOPRSVI-CAT (Stratagene). Briefly, by using a *Nde*I site inserted at the junction of the GFP and MCAK coding regions by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene), MCAK was removed by *Nde*I-*Xho*I digestion. Before making the GFP-KIF3B-ML, a GFP-KIF3B-tail construct was made. The *Nde*I/*Xho*I fragment of KIF3B used to make the bacterial expression construct was inserted into *Nde*I-*Xho*I sites of the prepared vector. To make GFP-KIF3B-ML, the incorporated *Nde*I site was removed and replaced with an AvrII site (QuikChange site-directed mutagenesis kit; Stratagene). The fragment of KIF3B corresponding to the coiled-coil plus part of the tail domain (nucleotides 1154–1888, amino acids 364–609) was generated by PCR from the isolated pBluescript II-KIF3B. AvrII and BbsI sites, incorporated into the 5′ and 3′ PCR primers, respectively, were used to insert the fragment into the AvrII site at the GFP junction and the unique BbsI site within the tail domain. The resulting GFP-KIF3B-ML coding region was removed from pOPRSVICAT with *Not*I and inserted into pGREEN-LANTERN-1 (Life Technologies, Rockville, MD) due to increased expression in C2C12 cells.

Subcellular Fractionation

Cell fractionation was performed by using a modified version of the protocols described by Okada *et al.* (1995) and Marszalek *et al.* (1999). In brief, fifty 25-cm plates of pelleted C2C12 myoblasts or 3-d myotubes were resuspended in cell fractionation (CF) buffer (20 mM HEPES, pH 7.2, 100 mM sodium aspartate, 40 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM dithiothreitol, protease inhibitors) with a final volume of 1 ml. Resuspended cells were homogenized by using a polytron tissue grinder (Kinematica). The homogenate was spun at $3000 \times g_{avg}$ for 5 min (P1), $9000 \times g_{avg}$ for 10 min (P2), and then centrifuged in a Beckman TLA-120.1 rotor at $100{,}000 \times g_{avg}$ for 1 h (P3). Forty micrograms of total protein, as determined by using the BCA-200 protein assay kit (Pierce, Rockford, IL), from each fraction was analyzed by immunoblot. The P3 pellet was extracted with either CF buffer or CF buffer plus 1% Triton X-100 by using a dounce homogenizer and respun at $100{,}000 \times g_{avg}$ for 1 h. Pellets were resuspended in the starting volume, and equal amounts of pellet and supernatant were analyzed by immunoblot. Immunoblots were scanned with $\gamma = 1.0$ and bands were quantitated by using NIH Image. Intensity was plotted from the pixel area under each peak for that fractionation.

RESULTS

KIF3A, KIF3B, and KHC Are Expressed in the Differentiating Myogenic Cell Line C2C12

To identify KRPs expressed in differentiating skeletal muscle, we performed an antibody screen of a cDNA library

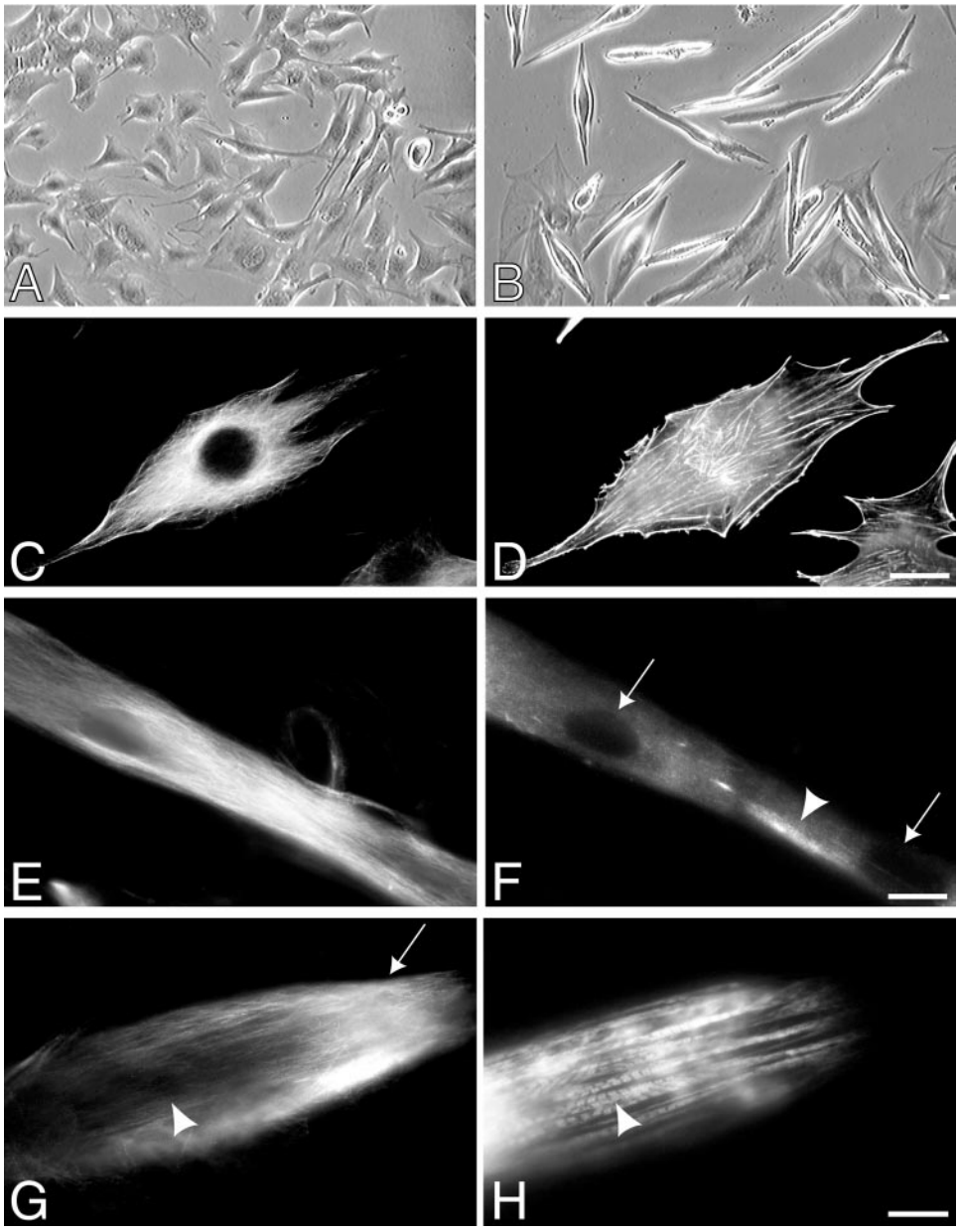

Figure 1. Phase-contrast and immunofluorescence microscopy of differentiating mouse myoblasts (C2C12). A phase-contrast image of a field of proliferating myoblasts (A) and multinucleated myotubes (B). (C and D) Immunofluorescence microscopy of a C2C12 myoblast that has been double labeled with an antibody to tubulin (C) and FITC-conjugated phalloidin (D). (E–H) Immunofluorescence microscopy of C2C12 myofibrils that have been double labeled with antibodies to tubulin (E and G) and skeletal muscle myosin (F and H). The myotube in E and F has been differentiating for 3 d and has begun to express skeletal muscle myosin (arrowhead in F). Two visible nuclei are indicated (arrows in F). The myotube in G and H has been differentiating for 6 d and contains striated myofibrils (arrowhead in H). Note regions of decreased microtubule density (arrowhead in G) versus abundant microtubules (arrow in G) at the tip of the myofibrils. Bar, 20 μm.

generated from differentiating myotubes of the mouse skeletal muscle cell line C2C12. We determined the stage of differentiation on which to focus our screen by analyzing the microtubule and myofibrillar protein content of differentiating C2C12 cells by using immunofluorescence microscopy. C2C12 myoblasts are polygonal-shaped cells (Figure 1A) containing microtubules that emanate from a microtubule-organizing center close to the nucleus and many actin stress-fibers (Figure 1, C and D). When C2C12 myoblasts are triggered to differentiate upon the depletion of growth factors from the culture medium, the myoblasts exit the cell cycle, become spindle shaped (Figure 1, B and E–H), and fuse with neighboring myoblasts (arrows in Figure 1F). During this early stage of differentiation (3 d in differentiation

media), microtubules align in the axis of future myofibril formation (Figure 1E); and the nascent myotubes begin to express myofibrillar proteins, such as skeletal muscle myosin (arrowhead in Figure 1F). Myotubes that remain in differentiation media for longer periods accumulate more mature myofibrils. Figure 1, G and H, represent a C2C12 myotube that has been differentiating for ~6 d. In comparison to the myotube shown in Figure 1, E and F, this is a more mature myotube because it is discernible by the striated pattern of skeletal muscle myosin (arrowhead in Figure 1H). Microtubule density in the region of the myotube that contains the bulk of striated myofibrils has decreased (arrowhead in Figure 1G) compared with the growing tip of the differentiating cell (arrow in Figure 1G). We decided to focus our search for KRPs that may participate in the formation of skeletal muscle on the early stage of differentiation, 3 d of C2C12 differentiation. At this stage of myogenesis, microtubules are very prominent throughout the entire myotube, and nascent myofibrils are beginning to assemble.

We generated a cDNA expression library of C2C12 myotubes that had been differentiating for 3 d. The library was screened with two affinity-purified, pan-kinesin peptide antibodies, anti-HIPYR and anti-LAGSE (Sawin *et al.*, 1992), that recognize conserved regions within the kinesin motor domain. Using this method, we identified three KRPs that are present in mouse skeletal muscle during the process of differentiation. Conventional KHC, also referred to as KIF5B (Aizawa *et al.*, 1992), was represented most frequently with nine double-positive clones. KIF3A and KIF3B (Kondo *et al.*, 1994; Yamazaki *et al.*, 1995), members of the KIF3 subfamily of KRPs (reviewed in Moore and Endow, 1996), were represented by five and one double-positive clones, respectively. DNA sequences of the overlapping clones isolated for each of these kinesin proteins were identical to the mouse sequences in the database.

Proliferating and Differentiating Myogenic Cells and Adult Skeletal Muscle Are Rich Sources of KRPs

Concurrent with our isolation of KRPs from differentiating mouse skeletal muscle, two other groups identified kinesins in myogenic tissue by using different cloning techniques. Using a yeast two-hybrid screen with the ezrin domain of the protein-tyrosine phosphatase PTPD1 as bait, the kinesin KIF1C was identified from a human skeletal muscle cDNA library (Dorner *et al.*, 1998). Additionally, the kinesins KIF3C and KIF1B were identified from a rat myogenic cell line by a PCR-based screen (Faire *et al.*, 1998). Because we are interested in the both the expression and function of KRPs both through differentiation and in adult muscle, we examined and compared the expression of those kinesins that were identified in our antibody screen, as well as the KRPs isolated from myogenic tissue by using different cloning techniques. We specifically chose to compare the expression of the following KRPs in C2C12 myoblasts, differentiating myotubes, and in adult mouse skeletal muscle (Figure 2): KIF3A, KIF3B, KIF3C, MCAK, KHC, and KIF1C.

We first examined the expression of KIF3A and KIF3B, members of the KIF3 subfamily of KRPs. Kinesin-II was originally cloned from sea urchin embryos as a novel heterotrimeric complex comprised of two homologous motors of molecular weight 85 and 95 kDa. These kinesin motors dimerize through their coiled-coil domains (Cole *et al.*, 1992, 1993; Rashid *et al.*, 1995), and a 115-kDa globular polypeptide, KAP, associates with the tail domain of the motor dimer (Wedaman *et al.*, 1996). To examine whether the entire kinesin-II motor complex is present in differentiating, mouse skeletal muscle, we first produced a polyclonal antibody to the globular tail domain of mouse KIF3B. This region is most divergent from its motor counterpart, KIF3A (Yamazaki *et al.*, 1995). The anti-KIF3B tail antibody (anti-KIF3B-T) recognizes a single band of ~95 kDa in a lysate of differentiating C2C12 cells (Figure 2A). In mouse, the kinesin-II motor complex comprises three proteins: 85-kDa KIF3A, 95-kDa KIF3B, and 100-kDa KAP3. Anti-KIF3B-T coimmunoprecipitates KIF3A, KIF3B, and the tail-associated KAP3 from differentiating C2C12 cells (Figure 2B). The anti-KIF3B-T antibody does not coimmunoprecipitate KIF3C, a third member of the KIF3 subfamily in mouse, from differentiating myotubes; however, KIF3C is present in a myotube cell lysate (Figure 2B). Anti-KIF3B-T also coimmunoprecipitates the entire kinesin-II complex from proliferating C2C12 myoblasts (our unpublished results).

We next compared the expression levels of the selected KRPs (KIF3A-C, MCAK, KHC, KIF1C) in proliferating C2C12 myoblasts and differentiating myotubes (Figure 2C), and examined their expression in adult mouse muscle (Figure 2, D and E). As a control to assess the degree of differentiation of the C2C12 myotubes in this study, the expression of skeletal muscle myosin was analyzed. Skeletal muscle myosin is undetected in proliferating myoblasts but is quickly expressed in myotubes differentiated for 3 d (Figure 2C). In our screen for motors possibly involved in skeletal muscle differentiation, we did not isolate any kinesins previously determined to have major roles during cell division. This was expected because myoblasts exit the cell cycle once triggered to differentiate. To determine whether mitotic kinesins are indeed down-regulated in differentiating cells, and how quickly this occurs, we examined the expression of MCAK by Western blot analysis in C2C12 myoblasts and myotubes (Figure 2C). MCAK is a 90-kDa protein and a member of the Kin I subfamily of KRPs (Wordeman and Mitchison, 1995; Vale and Fletterick, 1997). It is recruited to the centromeres of dividing cells and aids in chromosome separation during anaphase (Wordeman and Mitchison, 1995; Maney *et al.*, 1998). In C2C12 cells, MCAK is expressed in proliferating myoblasts but down-regulated to undetectable levels in myotubes differentiated for 3 d (Figure 2C). Additionally, MCAK was undetected in adult mouse skeletal muscle (our unpublished results). These data suggest that differentiating cells quickly down-regulate kinesin motor proteins whose function are not necessary either to the differentiation process or to aid in cell maintenance during differentiation.

The expression of the motors from the KIF3 subfamily of KRPs (KIF3A, KIF3B, and KIF3C) was compared in proliferating and differentiating C2C12 cells because each has been specifically identified in myogenic cells. Western immunoblots suggest that KIF3A, KIF3B, and KIF3C are all expressed at similar levels in both proliferating C2C12 cells and myotubes differentiated for 2–3 d (Figure 2C). When examining their expression in adult mouse muscle, KIF3B and KIF3C are detected in a crude protein lysate (Figure 2D); however, KIF3A is not detected in this same lysate. To determine whether KIF3A is truly absent from adult muscle,

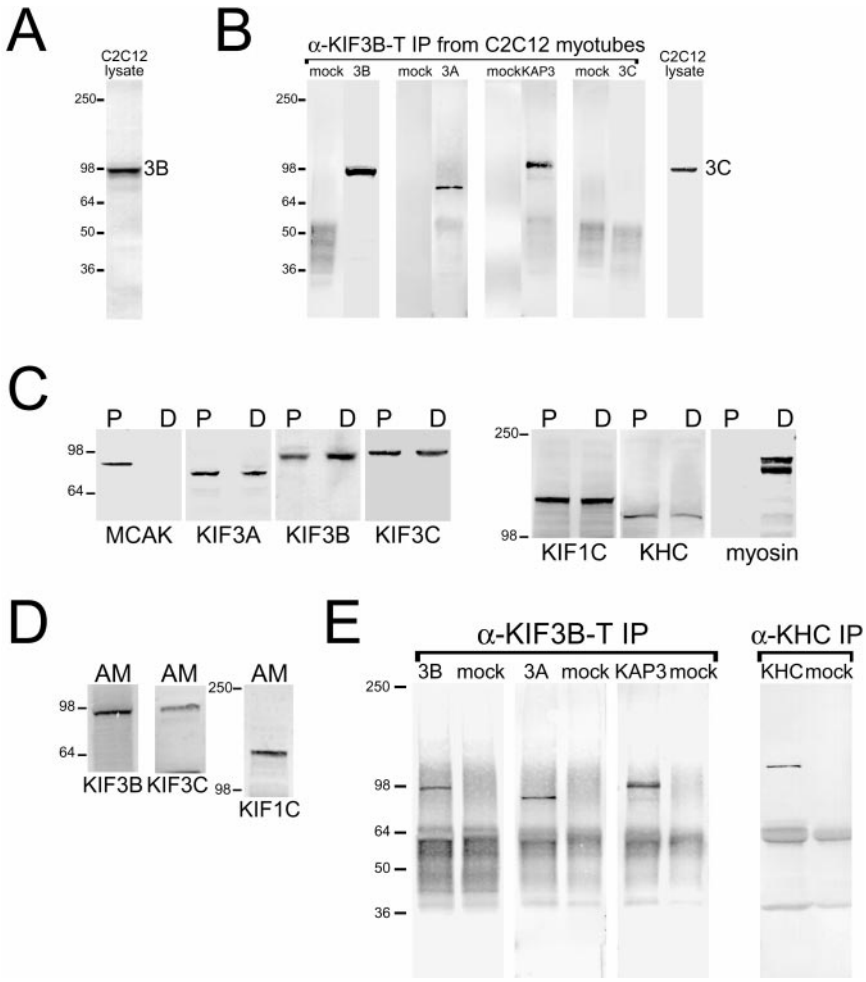

Figure 2. Expression of KRPs during C2C12 cell differentiation and in adult skeletal muscle. (A) C2C12 cell lysate labeled with α-KIF3B-T. (B) α-KIF3B-T immunoprecipitates the kinesin-II complex, consisting of KIF3A, KIF3B, and KAP3, but not KIF3C, from differentiating C2C12 myotubes. The respective IP blots are probed with α-KIF3B-T, monoclonal K2.4, MmKAP3A, and anti-MmKIF3C. Protein A beads in the absence of antibody were used in the mock lanes. (C) Comparison of the expression of KRPs in protein lysates of proliferating C2C12 myoblasts (P lanes) and 3-d differentiating C2C12 myotubes (D lanes). The expression of skeletal muscle myosin is examined as a control for the level of differentiation. (D and E) Expression of KRPs is examined in adult mouse skeletal muscle by Western blot analysis of crude muscle protein lysates (D) or immunoprecipitations by using α-KIF3B-T or α-KHC and probed on blots as indicated (E).

we immunoprecipitated the kinesin-II complex from the crude protein lysate of adult mouse skeletal muscle by using anti-KIF3B-T. KIF3A, KIF3B, and KAP3 are all coimmunoprecipitated with anti-KIF3B-T from adult muscle tissue (Figure 2E). Because the ratio of KIF3A:KIF3B:KAP3 is 1 mol:1 mol:1 mol in mouse brain (Yamazaki *et al.*, 1995), it is likely that the level of expression of KIF3A and KIF3B is also similar compared in adult muscle. This suggests that the lack of detection of KIF3A in the crude lysate is most likely due to differences in antibody affinities.

The expression of conventional KHC during myogenesis was also examined because it was identified in our screen of differentiating cells. Again, KHC is expressed in both proliferating myoblasts and differentiating myotubes at approximately equal levels (Figure 2C). Like KIF3A, KHC is not detected in the crude protein lysate of adult mouse skeletal muscle; but KHC is detected by immunoprecipitation from the crude protein lysate of adult skeletal muscle by using the monoclonal KHC antibody H2 (Pfister *et al.*, 1989) (Figure 2E). Finally, the expression of KIF1C was examined through differentiation because it was specifically isolated from adult skeletal muscle. KIF1C is a 135-kDa protein and a member of the Unc104/KIF1 subfamily of KRPs. It was previously shown to be expressed at highest levels in heart and skeletal muscle and expressed in C2C12 myoblasts (Dorner *et al.*, 1998). We found that KIF1C is expressed at approximately equal levels in proliferating C2C12 myoblasts and differentiating myotubes (Figure 2C) and is present in adult mouse skeletal muscle (Figure 2D).

These data show that myogenic cells, whether proliferating myoblasts, differentiating myotubes, or adult skeletal muscle are rich sources of KRPs. We propose that those kinesins, such as the mitotic kinesin MCAK, that are not necessary either to the cell differentiation program, or to maintain the cell during differentiation, are quickly downregulated in differentiating myotubes.

KIF3 Motors Have Altered Extraction Profiles in Differentiating C2C12 Myotubes

The subcellular fractionation characteristics and extraction profiles for some of the above-mentioned KRPs were compared in proliferating C2C12 myoblasts (Table 1) and differentiating C2C12 myotubes (Table 1). Similar studies of brain homogenates (Kondo *et al.*, 1994; Muresan *et al.*, 1998; Yang and Goldstein, 1998; Marszalek *et al.*, 1999) have demonstrated that low-speed pellets (P1) contain the nuclear and mitochondrial fractions, and medium-speed pellets (P2) con-

Table 1. Subcellular fractionation of kinesin-related proteins in undifferentiated myoblasts and differentiated myotubes

	KIF3 Family						Other KRPs				Control	
	3A PM	3A DM	3B PM	3B DM	3C PM	3C DM	K PM	K DM	1C PM	1C DM	NK PM	NK DM
P1 (3,000-g pellet)	14	14	12	13	16	25	24	14	21	24	41	45
P2 (9,000-g pellet)	12	12	12	11	15	18	17	19	23	12	40	40
P3 (100,000-g pellet)	32	29	35	25	29	27	20	28	43	24	14	10
S3 (100,000-g sup.)	42	45	41	51	40	30	39	39	13	40	5	5
P3 buffer wash	35	48[a]	43	50[a]	43	57[a]	40	42	76	80	72	72
S3 buffer wash	65	52	57	50	57	43	60	58	24	20	28	28
P3 Tx100 wash	30	51[a]	35	54[a]	41	53[a]	48	34	66	82	19	19
S3 Tx100 wash	70	49	65	46	59	47	52	66	34	18	81	81

PM, proliferating myoblasts; DM, differentiating myotubes; 3A, 3B, 3C = KIF3A-C respectively; K, kinesin heavy chain; 1C, KIF1C; NK, Na^+K^+-ATPase.

Numbers are percentages of total Western band intensity within a particular fractionation.

[a] Note shift of Kif3 family proteins from S3 to the nondetergent-extractable P3 pellet upon differentiation.

tain mostly lysosomes and synaptosomes. High-speed pellets (P3) contain mostly microsomes and organelle membranes, such as from the Golgi complex and ER. The high-speed supernatant (S3) contains soluble proteins. This differential centrifugation technique will yield similar fractional components in muscle-derived cells, with a few exceptions. Because this is not a neuronal system, synaptosomes will not be present; additionally, higher order cytoskeletal protein structures that comprise the contractile units of the myofibril will fractionate into the insoluble pool. In Table 1, the majority of the KRP protein is divided between the high-speed pellet (P3) and the remaining soluble (S3) pool. Fractionation of the integral membrane protein Na^+,K^+-ATPase was used as a fractionation control. It is found principally in the insoluble pool (P1, P2, and P3) in both myoblasts and myotubes (Table 1).

Next, we examined the interaction of the KRPs with their cargoes by extracting the high-speed pellet (P3) with either homogenization buffer alone or buffer plus 1% Triton X-100 detergent (Table 1, washes). The P3 pellet should contain the highest concentration of coupled motors, whereas motors in the S3 fraction are theoretically uncoupled from a cargo. Extracting with buffer alone examines the strength of the motor–cargo interaction; whereas extracting in the presence of detergent examines whether those motors fractionated to the insoluble, high-speed pellet (P3) are associated with membranous vesicles (Marszalek *et al.*, 1999). The extraction profile of Na^+,K^+-ATPase was analyzed as a control for the extraction experiment. In the presence of detergent, this integral membrane protein is principally extracted into the supernatant (Table 1).

In proliferating C2C12 myoblasts, all three members of the KIF3 subfamily act similarly under both extraction conditions. A proportion of each motor is extracted, relocating into the supernatant, with buffer alone. Extracting in the presence of detergent does not appreciably increase the quantity of these motors in the remaining supernatant. In proliferating myoblasts KHC and KIF1C have a similar extraction profile as the motors of the KIF3 subfamily. The lack of an appreciable increase of motor quantity in the supernatant after detergent extraction suggests that the majority of those motors present in the P3 pellet are not solely associated with membranous vesicles.

Interestingly, the extraction profiles of the KIF3 motors in differentiating myotubes are distinct when compared with both their extraction profiles in proliferating myoblasts, as well as the profiles of the other KRPs in differentiating cells. In myotubes, there is a shift in the quantity of each KIF3 motor present in the nonextractable pellet (asterisks, Table 1), indicating a higher affinity of the motors for their particular cargoes in differentiating cells. These data are not due to a hindrance to extraction by the complex myofibrillar structure because the extraction profiles of the other KRPs (KIF1C and KHC) are identical between myoblasts and myotubes. Again, there is no significant increase in extraction of the KIF3 motors in the presence of detergent. The changes in the extraction profiles of the KIF3 proteins relative to the other KRPs tested suggests that they *may* undergo some form of function switching when the myoblasts are triggered to differentiate, thus possibly participating in the differentiation program.

KIF3B Is Punctate and Diffuse in C2C12 Cells, but Nonextractable KIF3B Colocalizes with the Myofibril in C2C12 Myotubes

The subcellular localization of the kinesin-II complex in myogenic cells was determined by analyzing the distribution of the 95-kDa motor subunit of kinesin-II, KIF3B, by immunofluorescence microscopy with the anti-KIF3B-T antibody. In C2C12 myoblasts, KIF3B is punctate and diffusely distributed throughout the cell. It is more densely concentrated in the region surrounding the nucleus (Figure 3A) where microtubule density is greatest (Figure 3B). When C2C12 cells are triggered to differentiate, individual myoblasts are within different stages of the cell cycle. Therefore, at any given time, the level of maturity for individual, differentiating myotubes is slightly different. The myotubes in Figure 3, C and D, have been differentiating for 3 d. They have an elongated shape, and a subset is expressing and beginning to assemble skeletal muscle myosin into sarcomeres (arrows, Figure 3D). Those myotubes expressing skel-

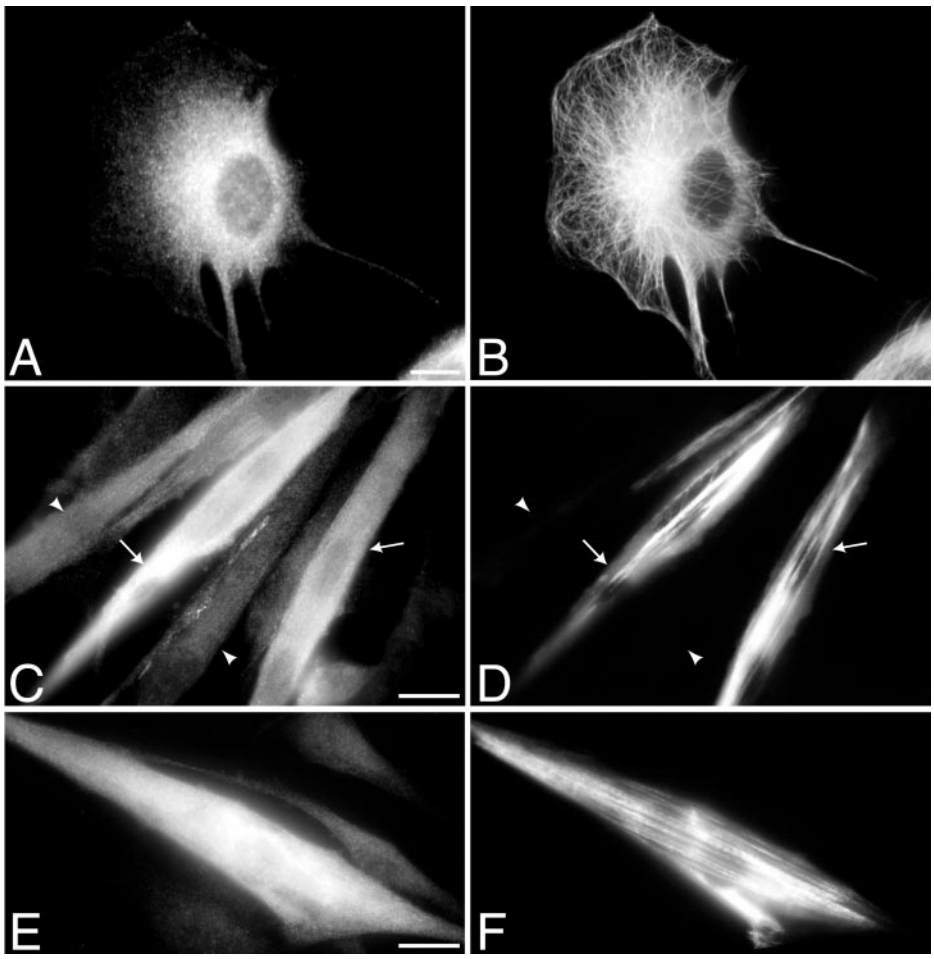

Figure 3. Cellular localization of KIF3B in proliferating C2C12 myoblasts and differentiating C2C12 myotubes. (A and B) C2C12 myoblast labeled with α-KIF3B-T (A) and an antibody to tubulin (B). (C–F) C2C12 myotubes labeled with α-KIF3B-T (C and E) and an antibody to skeletal muscle myosin (D and F). Myotubes, 3 d (C and D); myotubes, 6 d (E and F). C2C12 myotubes differentiate at different rates; therefore, a subset is expressing and assembling skeletal muscle myosin (arrows in C and D), while others are not (arrowheads in C and D). Expression of KIF3B is brighter in cells containing skeletal muscle myosin. Bar, 20 μm.

etal muscle myosin display brighter KIF3B labeling (arrows, Figure 3C) compared with KIF3B labeling in the neighboring myotubes that are less mature (arrowheads, Figure 3, C and D). The myotubes in Figure 3C that are not expressing skeletal muscle myosin, and contain less KIF3B labeling, are similarly elongated. Therefore, we believe that there is an increase of KIF3B expression in more highly differentiating myotubes. When comparing the expression of KIF3B in proliferating myoblasts with myotubes that had been differentiating for only 2–3 d by immunoblot analysis, there was not an appreciable difference (Figure 2C). However, these myotube populations are heterogenous in their extent of differentiation, making the absolute quantitation on blots less reliable than single-cell microscopic analysis. The expression and localization of KIF3B in C2C12 myoblasts and myotubes mimicked KIF3B labeling of primary myogenic cultures isolated from mouse embryos (our unpublished results).

KIF3B labeling in differentiating C2C12 myotubes is abundant, fairly punctate, dense, and diffuse (Figure 3, C–F). KIF3B is not obviously localized to a particular region of either the myoblast or myotube. To visualize the insoluble and nonextractable pool of KIF3B that is present in differentiating myotubes, we detergent extracted the cells in 0.5% Triton X-100 in microtubule-stabilizing buffer before fixation for immunocytochemistry. Pre-extracted myotubes, double labeled for KIF3B and skeletal muscle myosin by immunocytochemistry, were visualized by immunofluorescence, confocal microscopy (Figure 4, A–C). Under these conditions, the labeling of soluble KIF3B is significantly dimmer compared with labeling in nonextracted myotubes. The nonextractable KIF3B component (Figure 4B) primarily colocalizes with aggregates of skeletal muscle myosin (Figure 4A, merge in Figure 4C). KIF3B labeling in myoblasts lysed similarly under microtubule-stabilizing conditions is also significantly decreased and is not localized to a specific region of the cytoskeleton (our unpublished results).

KIF3B Is Not Predominantly Associated with the Golgi Apparatus in C2C12 Cells

In the *Xenopus* A6 cell line, Le Bot *et al.* (1998) showed that the *Xenopus* homologue of KIF3B, Xklp3, specifically localizes to the Golgi apparatus, overlapping with labeling of lectin from HP (Le Bot *et al.*, 1998). HP lectin binds to *N*-acetyl-galactosamine residues that are generated in the first step of O-linked glycosylation (Roth, 1984; Pavelka and Ellinger, 1985) occurring in the Golgi apparatus (Le Bot *et al.*, 1998). Using epifluorescence microscopy, we did not see any obvious Golgi localization of KIF3B in C2C12 myoblasts or myotubes (Figure 3). In *Xenopus* cells, anti-KIF3B-T cross-

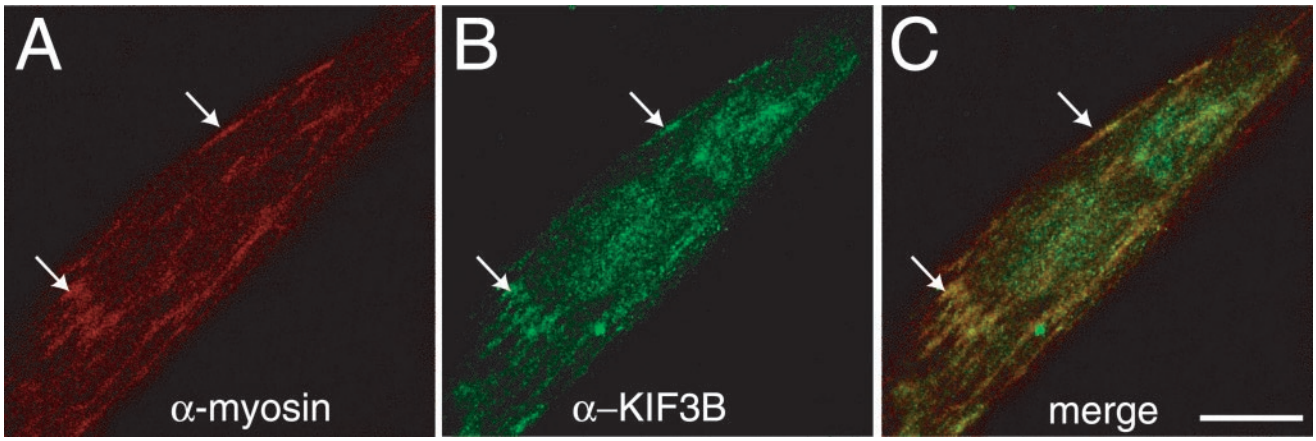

Figure 4. Nonextractable KIF3B colocalizes with skeletal muscle myosin in differentiating C2C12 cells. (A and B) Confocal image of C2C12 myotube that has been double labeled with an antibody to skeletal muscle myosin (A, red) and α-KIF3B-T (B, green). Before methanol fixation, the myotube was extracted with 0.5% Triton X-100 in microtubule-stabilizing buffer for 1 min. Merge of images is shown in C. Patches of skeletal muscle myosin, being assembled into myofibrils (arrows in A) colocalize with nonextractable accumulations of KIF3B (arrows in B). Yellow in C demonstrates colocalization of the two markers under these conditions. Bar, 20 μm.

reacts with Xklp3, the KIF3B homologue in *Xenopus*, and labels a bright, punctate region surrounding the nucleus. This staining is much brighter than the diffuse labeling in the surrounding cytoplasm (Figure 5A, green) and overlaps with Golgi-localized HP lectin staining (Figure 5A, red). In Figure 5B, a C2C12 myoblast has been double labeled with anti-KIF3B-T (Figure 5B, green) and Texas Red-HP lectin (Figure 5B, red). In general there is no significant colocalization between KIF3B and the Golgi marker (Figure 5B, merged). However, close examination reveals small regions of HP lectin labeling that may colocalize with aggregates stained for KIF3B in the C2C12 myoblast (arrows in Figure 5B).

Kinesin-II Is Not Necessary for Either Golgi/ER Transport or Myofibrillogenesis

Because a small subset of KIF3B appears to colocalize with the Golgi apparatus in C2C12 cells, we examined the effect of a motorless version of KIF3B on membrane transport in C2C12 myoblasts and differentiating myotubes. Motorless-KIF3B protein will dimerize with endogenous KIF3A to produce a single-headed kinesin-II complex. A similar technique has successfully been used to disrupt motor function of kinesin-II in *Xenopus* cells (Le Bot *et al.*, 1998; Tuma *et al.*, 1998), MCAK (Maney *et al.*, 1998), and KIF1C (Dorner *et al.*, 1998). Le Bot *et al.* (1998) showed that HP lectin no longer labeled the Golgi apparatus in *Xenopus* cells expressing a motorless version of XKlp3, however, the structure of the Golgi apparatus remained intact. They propose that O-glycosylating enzymes are mislocalized in cells transfected with motorless-Xklp3, indicating that Xklp3 is involved in the global transport of components recycling between the ER and Golgi apparatus (Le Bot *et al.*, 1998). We generated a similar deletion construct of mouse KIF3B, deleting the motor domain, and replacing it with GFP (pGFP-KIF3B-ML).

Coimmunoprecipitating motorless-KIF3B from transfected C2C12 cells by using an antibody against GFP demonstrated that motorless-KIF3B dimerizes with endogenous, wild-type KIF3A (Figure 6A). Furthermore, overexpression of motorless-KIF3B in C2C12 cells induces a dramatic increase in endogenous KIF3A expression (Figure 6B). The transfection and subsequent overexpression of either motorless- or full-length KIF3A does not induce the expression of KIF3B in transfected cells (our unpublished results). The overexpression of control GFP also does not induce the expression of either endogenous KIF3A of KIF3B (our unpublished results). Therefore, the transcription/translation of endogenous KIF3A appears to be regulated by KIF3B expression.

We first transfected our motorless-KIF3B construct into *Xenopus* A6 cells and examined HP lectin labeling of the Golgi apparatus. In 60–70% of transfected A6 cells that are overexpressing motorless-KIF3B for at least 48 h, Golgi labeling with HP lectin is either abnormal or absent. This is compared with the control transfection of a GFP construct where only 10% of transfected cells display abnormal HP lectin labeling (quantification, Figure 6C). In myoblasts transfected with motorless-KIF3B, HP lectin properly labels the juxta-nuclear Golgi apparatus (quantification, Figure 6C; Figure 6Da,b). In differentiating myotubes, the Golgi apparatus normally reorganizes, to form a ring surrounding the nucleus (Ralston, 1993). In differentiating C2C12 cells expressing motorless-KIF3B (Figure 6Dc,d), HP lectin still labels the Golgi apparatus; and proper Golgi reorganization in the differentiating cell is preserved. Transfection of a motorless version of KIF3A, and cotransfection of motorless-KIF3A and motorless-KIF3B, produced the same results (our unpublished results).

The above-mentioned data suggest that kinesin-II does not function in maintaining Golgi/ER transport in either proliferating myoblasts or differentiating myotubes. We next sought to determine whether kinesin-II participates in myo-

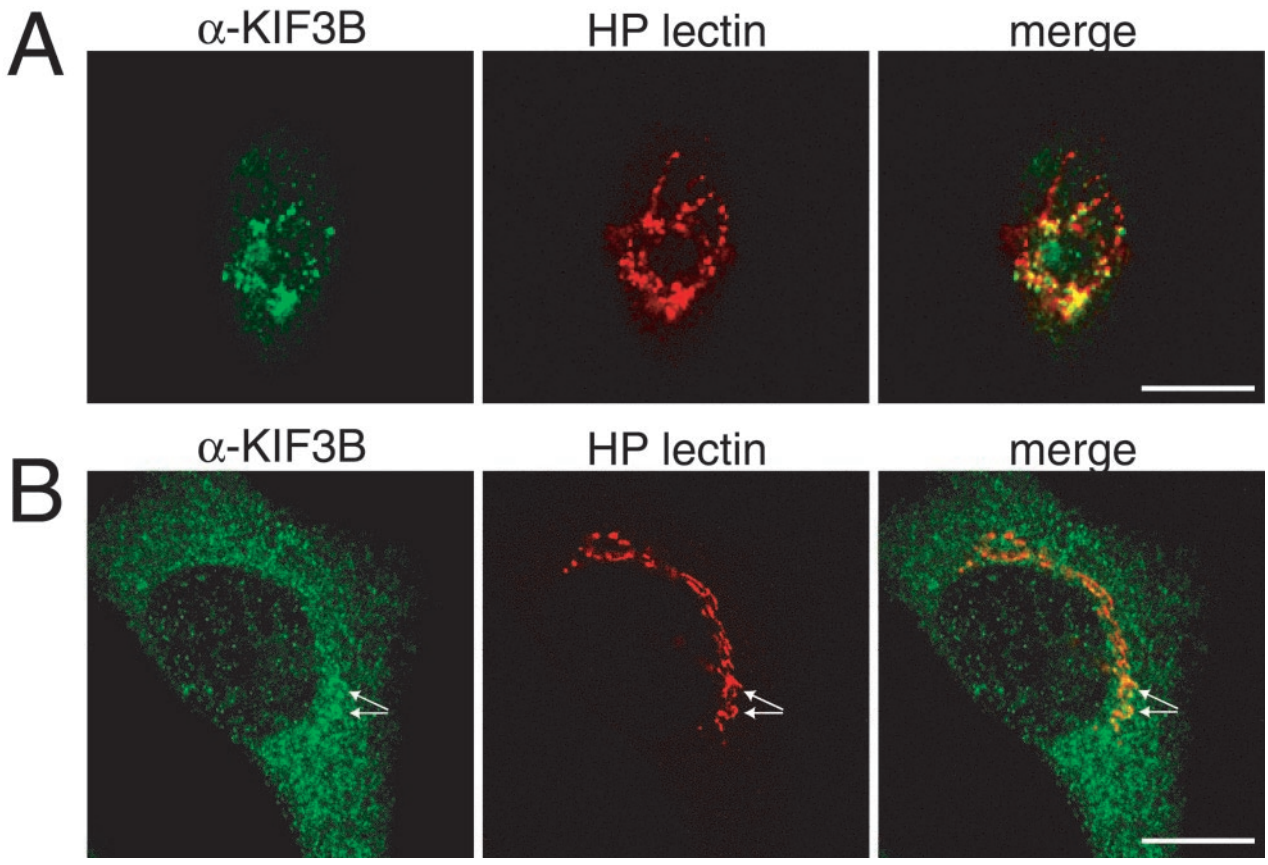

Figure 5. KIF3B primarily colocalizes with a Golgi marker in *Xenopus* A6 cells. In C2C12 cells, only a small subset of KIF3B colocalizes with the same Golgi marker. (A and B) Confocal images of a *Xenopus* A6 cell (A) and C2C12 myoblast (B) labeled with α-KIF3B-T (green) and Texas Red-conjugated HP lectin (red), with merged images to the right. HP lectin labels the Golgi apparatus (red) in both cell types. In A6 cells, labeling of our anti-KIF3B-T antibody overlaps with HP lectin (A, yellow in merged image). In C2C12 myoblasts (B), KIF3B is diffuse and punctate throughout the cytoplasm (B, green). KIF3B does not significantly colocalize with HP lectin staining in these cells; however, there are minor aggregates of KIF3B that appear to colocalize with regions of HP lectin staining (arrows in B). Bar, 20 μm.

fibrillogenesis, assembly of the contractile units of the myofibril. To test this possibility, C2C12 myoblasts that had been transiently transfected with motorless-KIF3B were triggered to differentiate. After differentiating from between 3 d and 1 wk, the expression of skeletal muscle myosin was examined by immunofluorescence microscopy (Figure 7). Myotubes with abundant motorless-KIF3B expression (Figure 7, A and C) assemble skeletal muscle myosin properly (Figure 7, B and D). In addition to examining the distribution and organization of skeletal muscle myosin in differentiating myotubes containing motorless-KIF3B, we also analyzed the localization of a battery of myofibrillar and cytoskeletal proteins, including the following: actin, microtubules, acetylated microtubules, α-actinin, sarcoplasmic α-actinin, vinculin, γ-tubulin, and nonmuscle myosin (our unpublished results). Motorless-KIF3B did not disrupt the organization of any of these proteins in transfected myoblasts or myotubes, suggesting that kinesin-II is not necessary for proper sarcomere assembly and cytoskeletal reorganization in these cells. The transfection of motorless-KIF3A, and cotransfection of both motorless-KIF3A and motorless-KIF3B, produced the same results (our unpublished results).

KIF3B Colocalizes with the E-C Coupling Membranes in Adult Skeletal Muscle

In differentiating C2C12 myotubes that are lightly extracted with Triton X-100 before fixation, KIF3B colocalizes with the developing myofibril (Figure 4); however, disruption of kinesin-II function does not inhibit myofibrillogenesis. To determine whether the association of KIF3B with the myofibril persists through differentiation, we examined the localization of KIF3B in adult mouse skeletal muscle. In adult muscle, KIF3B does not associate with the contractile apparatus of the muscle cell; but instead, it localizes to the membrane compartment. KIF3B colocalizes with three components of the E-C coupling membranes in skeletal muscle: DHPR, RyR, and triadin. In mammalian skeletal muscle, there are two triad structures per individual sarcomere. In a cross-section of adult mouse skeletal muscle (Figure 8B), KIF3B (green) and DHPR (red) colocalize within the interfibrillar space between the contractile machinery of the muscle cell. In a longitudinal section, KIF3B labeling overlaps exactly with triadin (Figure 8A). It is difficult to resolve individual triads at the A-I junction within each sarcomere in this tissue

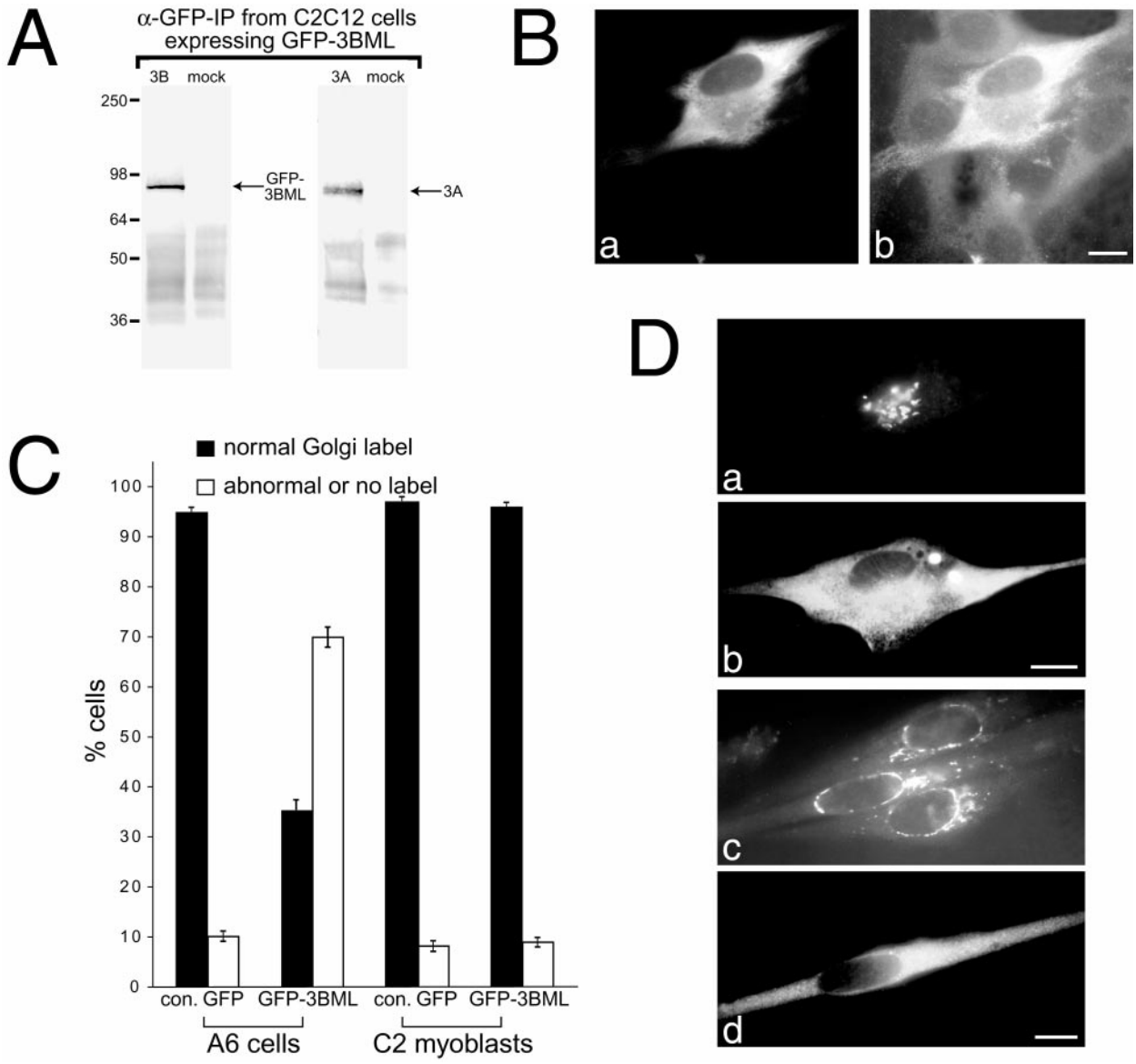

Figure 6. C2C12 myoblasts and myotubes transfected with a KIF3B motorless deletion construct contain normal HP lectin labeling. (A) Motorless-KIF3B dimerizes with endogenous KIF3A in C2C12 cells as demonstrated by the immunoprecipitation of overexpressed GFP-KIF3B-ML from transfected C2C12 myoblasts by using a polyclonal anti-GFP antibody. Anti-GFP coimmunoprecipitates both GPF-KIF3B-ML, as determined by staining with anti-KIF3B-T (to left), and endogenous KIF3A, as determined by staining with the monoclonal K2.4 (to right). Controls were done using Protein A beads alone (mock lanes). (B) Overexpression of motorless-KIF3B induces an increase of endogenous KIF3A expression in C2C12 cells. C2C12 cell transiently transfected with GFP-KIF3B-ML (a) are labeled with the monoclonal K2.4 that recognizes KIF3A (b). Note the increase in KIF3A labeling in the transfected cell compared with the neighboring, nontransfected cells. (C) Quantification of HP lectin labeling of the Golgi apparatus in *Xenopus* A6 cells and C2C12 myoblasts transiently transfected with motorless-KIF3B (GFP-3BML) and control GFP (con. GFP). Cells (100) were counted for each condition. Bars correspond to the number of cells in each category, and SDs are shown (n = 4 for each condition). Black bars (■) represent normal HP lectin labeling, whereas white bars (□) represent abnormal labeling or lack of staining altogether. Motorless-KIF3B-transfected A6 cells (60–70%) show abnormal labeling of the Golgi apparatus. This is not seen in motorless-KIF3B-transfected C2C12 myoblasts. (D) Immunofluorescence of motorless-KIF3B in transfected C2C12 cells. GFP fluorescence is seen in a transfected myoblast (b) and transfected myotube (d). The transfected cells are double labeled with Texas Red-conjugated HP lectin (a and c). In the myoblast, HP lectin correctly labels the Golgi apparatus in cells expressing GFP-KIF3B-ML. In differentiating myotubes, the Golgi apparatus becomes redistributed to a ring around the nucleus (c). HP lectin still recognizes the reorganized Golgi apparatus in differentiating cells containing GFP-KIF3B-ML. Bar, 20 μm.

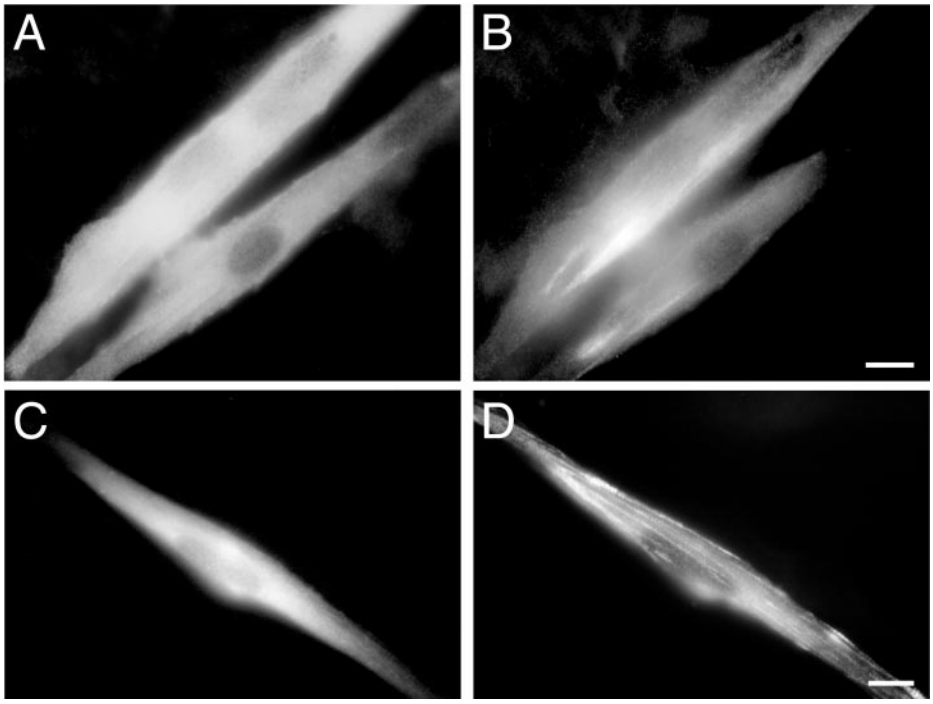

Figure 7. Motorless-KIF3B does not disrupt myofibrillogenesis is differentiating C2C12 myotubes. C2C12 myoblasts were transfected with a motorless-KIF3B deletion construct (GFP-KIF3B-ML). After recovering from the transfection procedure, myoblasts were triggered to differentiate for 3 d. Myotubes were immunofluorescently labeled with an antibody to skeletal muscle myosin (B and D). GFP fluorescence of GFP-KIF3B-ML is seen in A and C. Skeletal muscle myosin expression and assembly into developing myofibrils is not perturbed in C2C12 myotubes expressing motorless-KIF3B. Bar, 20 μm.

section; however, it is clear that KIF3B and the triad marker colocalize. In Figure 8C, a longitudinal section of skeletal muscle labeled with anti-KIF3B-T (a) and an antibody against RyR (b) has been enlarged, and the Z-lines are marked by arrows. Although individual triads are sometimes visible with RyR labeling, they are harder to visualize with KIF3B, most likely due to difficulties with the antibody. However, labeling on either side of the Z-line, at the A-I junction, is visible with both RyR and KIF3B labeling (Figure 8C).

DISCUSSION

This study is the most comprehensive analysis of KRPs in muscle tissue, as well as the first to begin to investigate the function of these motors during differentiation. We have shown that muscle tissue is a rich source of KRPs; and our partial characterization of KRPs during skeletal muscle development and in adult tissue provides a start in elucidating the function of microtubule motors during this process and in this tissue.

A few studies in the literature that have documented the expression of individual kinesins in myogenic cells. KHC was previously shown to be expressed in adult skeletal muscle (Hollenbeck, 1989; Rahkila *et al.*, 1997) where it colocalizes with a variety of ER and pre-Golgi markers (Rahkila *et al.*, 1997). One known function of KHC is to transport recycling membranes between the ER and Golgi apparatus (Lippincott-Schwartz *et al.*, 1995). KIF1C, member of the Unc104/KIF1 subfamily of KRPs, is most strongly expressed in adult heart and skeletal muscle (Dorner *et al.*, 1998). Although the function of KIF1C has not been specifically analyzed in muscle tissue, this KRP exclusively labels the Golgi apparatus in NIH3T3 cells (Dorner *et al.*, 1998). The overexpression of a motorless version of KIF1C in NIH3T3 cells blocks the brefeldin A-induced flow of Golgi membranes into the ER; and thus KIF1C is thought to participate in retrograde membrane trafficking from the Golgi to ER in that system (Dorner *et al.*, 1998). It is likely that both KHC and KIF1C act to maintain membrane recycling during myogenesis, as well as in adult muscle. Finally, Faire *et al.* (1998) identified KIF3C and KIF1B from myogenic rat cells by using a PCR-based cloning strategy; however, the function of these motors has not been analyzed in muscle tissue.

An expression library screen of differentiating myotubes isolated three previously identified KRPs (KHC, KIF3A, KIF3B) that are expressed during skeletal muscle development. As would be expected from a postmitotic system, we did not isolate any mitotic KRPs from our screen of differentiating muscle. We compared the characteristics of these KRPs to others that have been previously identified (MCAK, KIF1C, KIF3C). The mitotic kinesin MCAK is expressed in C2C12 myoblasts but undetected in differentiating myotubes. Excluding MCAK, each member of the kinesin superfamily that was examined in this study is expressed in both proliferating and differentiating C2C12 cells, and present in adult muscle. Thus, kinesin proteins that do not participate in either myogenesis or maintenance of the cell during differentiation and in adult muscle are quickly down-regulated once differentiation is triggered. KHC and KIF1C have been identified in muscle (Hollenbeck, 1989; Dorner *et al.*, 1998), but the expression of the three members of the KIF3 subfamily was previously shown to be negligible in skeletal muscle when examined by multitissue immunoblots or Northern blots (Kondo *et al.*, 1994; Yamazaki *et al.*, 1995; Yang and Goldstein, 1998). We suggest that the high concentration of contractile proteins that is expressed in muscle

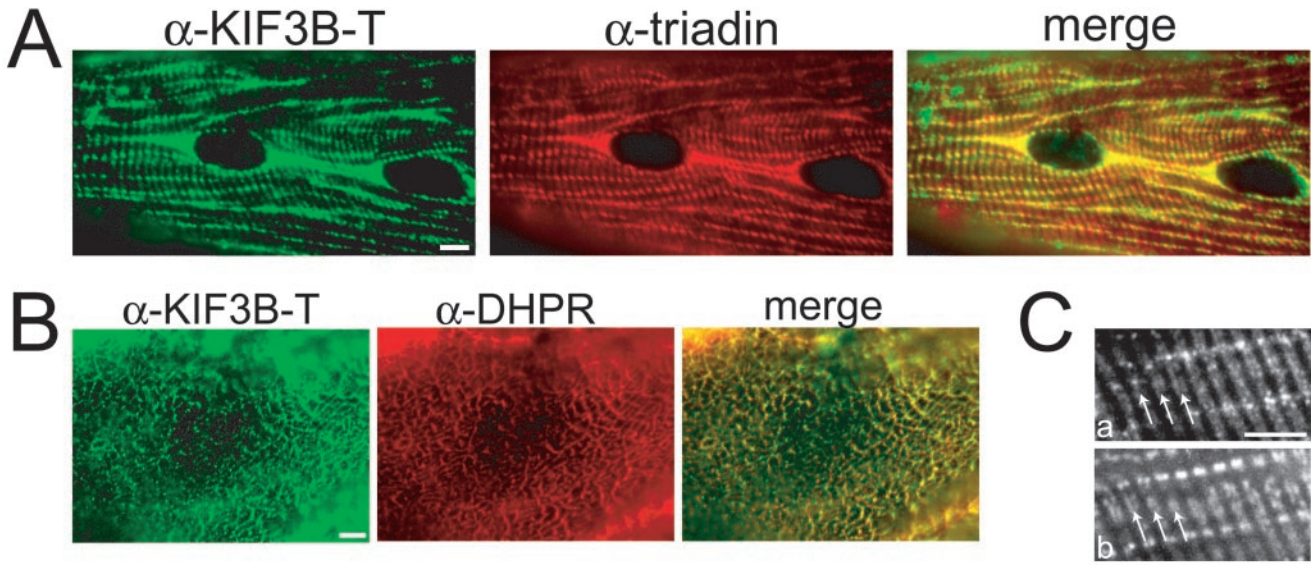

Figure 8. KIF3B colocalizes with components of the E-C coupling membranes in adult mouse skeletal muscle. (A) Longitudinal section of adult mouse skeletal muscle double labeled with α-KIF3B-T (green) and a monoclonal antibody to triadin (red). (B) Transverse section of adult mouse skeletal muscle double labeled with α-KIF3B-T (green) and a monoclonal antibody to the α-1 subunit of DHPR (red). In A and B, images were colorized in Adobe Photoshop so that areas of overlap in the merged images appear yellow (merge). In A, KIF3B and triadin colocalize in linear points transversely oriented along the surface of the muscle cell. In B, KIF3B and DHPR colocalize within the interfibrillar spaces between the contractile apparatus of the muscle cell. (C) Longitudinal section of adult mouse skeletal muscle double labeled with α-KIF3B-T (a) and a monoclonal antibody to RyR (b). The Z-lines are marked by arrows. KIF3B and RyR are located at either side of the Z-line at the A-I junction. Bar, 20 μm.

prevents the accurate representation of KRPs in this system compared with equal proportions of other tissues.

Because the KIF3 motors were abundant and exhibited an altered cargo extraction profile in differentiating myotubes, we focused our research on the KIF3 motors, especially the kinesin-II complex. In mouse, kinesin-II is a heterotrimeric complex comprised of dimerized KIF3A and KIF3B proteins, and a tail-associated protein, KAP. Kinesin-II has been identified in many different organisms and implicated in a variety of biological processes (reviewed in Goldstein and Philp, 1999). There is precedence for kinesin-II-mediated transport of both membranous vesicles and protein complexes. Le Bot *et al.* (1998) elegantly demonstrated that the *Xenopus* homologue of KIF3B, Xklp3, localizes predominantly to the Golgi apparatus and functions in the transport of vesicles between the Golgi/ER systems. There is also abundant evidence that kinesin-II is responsible for the transport of protein complexes within flagella and cilia of many organisms (reviewed in Cole, 1999). Kinesin-II function is particularly intriguing with respect to muscle differentiation because of the massive reorganization and development of both the proteinaceous myofibril and the specialized membrane components that occur during this process.

We have shown that overexpression of KIF3B will induce the overexpression of KIF3A in myogenic cells. This is not surprising for two proteins that dimerize to form a functional complex, as demonstrated by α- and β-tubulin expression (Ben-Ze'ev *et al.*, 1979; Cleveland *et al.*, 1981). Because KIF3A can dimerize with both KIF3B and KIF3C, it is logical that the expression of KIF3A is dependent on the expression of either of its motor partners. Because the expression of KIF3A is linked to motorless-KIF3B expression, and motorless-KIF3B is shown to dimerize with wild-type KIF3A, we are confident that a single-headed, kinesin-II complex is overexpressed in cells transfected with motorless-KIF3B.

Motorless-KIF3B dimerizes with endogenous KIF3A in transfected C2C12 cells, producing a single-headed motor complex. *Xenopus* cells overexpressing a motorless version of Xklp3, the KIF3B homologue, lack HP lectin staining of the Golgi apparatus and show abnormal localization of newly synthesized Golgi proteins within the ER and cytoplasm (Le Bot *et al.*, 1998). Le Bot *et al.* (1998) suggest that disrupting kinesin-II function perturbs the global flux between the ER/Golgi membrane systems. Interestingly, in C2C12 cells, myotubes, and mouse myoblast primary cultures (our unpublished results), KIF3B is not predominantly localized to the Golgi apparatus as seen in *Xenopus* cells. We examined the effect of motorless-KIF3B on ER/Golgi transport in myogenic cells due to the colocalization of a small subset of KIF3B with HP lectin staining in C2C12 myoblasts. Motorless-KIF3B does not alter the labeling of Golgi membranes with HP lectin in C2C12 myoblasts or myotubes; although, 60–70% of *Xenopus* cells overexpressing our murine motorless-KIF3B construct exhibit abnormal Golgi labeling. The motors KIF1C, predominantly found in skeletal and heart muscle, and KHC have already been shown to participate in ER/Golgi recycling (Dorner *et al.*, 1998). It is unknown whether KIF1C is expressed in amphibians; and it remains possible that, in higher vertebrates, KIF1C replaces the kinesin-II-mediated ER/Golgi function seen in *Xenopus* fibroblasts. Therefore, we propose that, in our myogenic-derived mammalian cells, kinesin-II has a different function.

Because motorless-KIF3B did not reveal an ER/Golgi membrane defect in C2C12 cells, we examined its effect on myofibril assembly. There is significant evidence demonstrating that microtubules are intimately involved in the proper assembly of the myofibril (Antin *et al*., 1981; Toyama *et al*., 1982; Gundersen *et al*., 1989). In the presence of motorless-KIF3B, myofibrillogenesis proceeded normally at the level of detection of our differentiation system. A recent model for kinesin-II-dependent intraflagellar transport in *Chlamydomonas* proposes that multiple kinesin-II motors may be responsible for the transport of large intraflagellar transport particles within the flagellum (Cole *et al*., 1998). Similarly, large complexes of myofibrillar proteins could be transported during myofibril formation. Hancock and Howard (1998) showed that single-headed kinesin proteins are capable of processive motility at high motor concentrations. Our evidence supports the notion that kinesin-II does not have a role in myofibrillogenesis; however, it cannot be ruled out that multiple single-headed kinesin-II complexes may be bound to large aggregates of myofibrillar proteins, permitting sufficient motility of the protein complexes. Under these conditions, only the time scale of myofibrillogenesis may be affected. In a heterogeneous culture system, the time difference may be minimal and difficult to detect.

However, thus far our evidence indicates that kinesin-II is not involved in either the assembly of contractile units or the trafficking of ER/Golgi components in myogenic cells. One other potential function for kinesin-II during myogenesis is to aid in the differentiation of the complex membrane systems, the sarcoplasmic reticulum (SR) and T-tubules. SR and transverse tubule (T-tubule) formation occurs concurrently with the organization of the contractile units (reviewed in Engel and Franzini-Armstrong, 1994). Immunofluorescence and ultrastructural studies have shown that the SR maintains a close association with the Z-line during early differentiation (Walker *et al*., 1969; Flucher *et al*., 1992); and it is speculated that this interaction may be mediated by microtubules (Flucher *et al*., 1993). We have shown that at early stages of myofibrillogenesis in C2C12 cells, microtubules are still prominent. Therefore, kinesin-II may be transporting components of the SR to sites of preliminary association with the nascent myofibril. KIF3B colocalizes with three components of the E-C coupling membranes in skeletal muscle: DHPR, RyR, and triadin. DHPRs are located within the T-tubule membrane and considered to be the voltage sensor of the E-C coupling system in skeletal muscle. The RyR is a Ca^{2+} release channel located at the terminal cisternae of the SR and juxtaposed to the DHPR in the neighboring T-tubule membrane. Triadin is a 95-kDa protein that colocalizes with both receptors. These three proteins are part of a structure called the triad that is located, in mammalian skeletal muscle, at the overlapped region of myosin thick filaments and actin microfilaments, the A-I junction. KIF3B localization overlaps with protein components of the E-C membrane network in adult skeletal muscle, opening the possibility that kinesin-II may be involved in the differentiation and maintenance of E-C coupling membranes.

The newest model for the formation/differentiation of triads proposed by Flucher and Franzini-Armstrong (1996) separates the process into three steps: 1) docking of SR to T-tubule/surface membranes, 2) accumulation of RyRs in ordered arrays at the membrane junction, and 3) accumulation of DHPRs in the junctional domain. Kinesin-II may be responsible for either the initial targeting/docking of the two membranes systems and/or the addition of RyRs and DHPRs to the junctional areas. Alternatively, instead of participating in the development of the specialized membrane systems, kinesin-II may transport components of the SR and T-tubules for structural maintenance during development and in adult muscle. In this model, the SR and T-tubules would represent very dynamic structures. Transport pathways from the Golgi apparatus to both the SR and T-tubules in adult skeletal muscle have been identified (Rahkila *et al*., 1996; Roy and Marette, 1996; Thomas *et al*., 1989). Furthermore, microtubule localization in adult muscle supports this model (Rahkila *et al*., 1997). Because only the initial stages of SR and T-tubule formation are established in our current system of differentiation (Parton *et al*., 1997), it is difficult to assess a true defect in membrane differentiation by using C2C12 cells. To further analyze this possibility, it will be necessary either to investigate the effect of kinesin-II inhibition in muscle of the whole animal or to develop a more mature, in vitro differentiation system.

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