Evidence for the Involvement of KIF4 in the Anterograde Transport of L1-containing Vesicles

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Abstract. In this study we present evidence about the cellular functions of KIF4. Using subcellular fractionation techniques and immunoisolation, we have now identified a type of vesicle that associates with KIF4, an NH_2-terminal globular motor domain kinesin-like protein. This vesicle is highly concentrated in growth cones and contains L1, a cell adhesion molecule implicated in axonal elongation. It lacks synaptic vesicle markers, receptors for neurotrophins, and membrane proteins involved in growth cone guidance. In cultured neurons, KIF4 and L1 predominantly localize to the axonal shaft and its growth cone. Suppression of KIF4 with anti-sense oligonucleotides results in the accumulation of L1 within the cell body and in its complete disappearance from axonal tips. In addition, KIF4 suppression prevents L1-enhanced axonal elongation. Taken collectively, our results suggest an important role for KIF4 during neuronal development, a phenomenon which may be related to the anterograde transport of L1-containing vesicles.

Key words: KIF4 • L1-glycoprotein • microtubule-based transport • axons • neuronal polarity

Introduction

During recent years a growing body of genetic, molecular biological, and immunological data has emerged suggesting that kinesin (Brady, 1985; Vale et al., 1985) and kinesin-like proteins (KLPs)[1] (Hirokawa et al., 1998; Hirokawa, 1999) are microtubule-based motor proteins specialized in the transport of membrane-bound organelles. The large number of KLPs identified in neurons (Hirokawa, 1999) can provide multiple possibilities for cargo selectivity, an event that is likely to be essential for generating the highly polarized distribution of the cytoplasmic and membrane components that distinguishes the axonal and somatodendritic domains of mature neurons. The identification of the type of cargo that each KLP translocates is therefore a key step for elucidating their functional involvement in the development and maintenance of neuronal polarity. Examples of KLPs with identified cargos include KIF1A, which transports synaptic vesicles precursors along axons (Okada et al., 1995), KIF2, which associates with nonsynaptic vesicles containing the insulin-like growth factor receptor βgc (Morfini et al., 1997), and KIFC2, which appears to transport a class of multivesicular body in dendrites (Hanlon et al., 1997; Saito et al., 1997). In neurons, the cargos transported by all other KLPs remain largely unknown.

KIF4 is a 1,231–amino acid KLP composed of an NH_2-terminal globular motor domain, a central α-helical stalk domain, and a COOH-terminal tail domain. KIF4 forms a homodimer that moves along microtubules towards the plus-end at a velocity of 0.2 μm/s (Sekine et al., 1994; Hirokawa et al., 1998; Hirokawa, 1999). KIF4 is predominantly expressed in juvenile tissues, including developing neurons, where it associates with a population of small membranous organelles localized to neurites and growth cones (Sekine et al., 1994). Based on these observations, it has been suggested that KIF4 is a unique anterograde motor that transports specific organelles involved in nerve cell morphogenesis (Sekine et al., 1994).

To test this hypothesis, in this study we have used a combination of biochemical, immunological, and antisense experiments to characterize the cargo transported by KIF4. The results obtained suggest an important role for KIF4 in neuronal morphogenesis, a phenomenon which appears to be related with the anterograde transport of a type of nonsynaptic vesicle that contains as one of its com-

ponents the cell adhesion molecule L1, a protein involved in axonal formation, fasciculation, and guidance (Walsh and Doherty, 1997; Kamiguchi et al., 1998).

Materials and Methods

Cell Culture

Dissociated cultures of hippocampal pyramidal cells from embryonic rat brain tissue were prepared as described previously (Cáceres et al., 1986; Mascotti et al., 1997). Cells were plated onto polylysine-coated glass coverslips (12 or 25 mm in diameter) at densities ranging from 5,000–15,000 cells/cm^2 and maintained with DME plus 10% horse serum for 2 h. The coverslips with the attached cells were then transferred to 60-mm petri dishes containing serum-free medium plus the N2 mixture of Bottenstein and Sato (1979). All cultures were maintained in a humidified 37°C incubator with 5% CO^2. For some experiments, an L1-Fc chimera (a generous gift of Dr. Sue Kenwrick, University of Cambridge, Cambridge, UK) consisting of the Fc region of human Ig and essentially the whole extracellular domain of human L1 (see Doherty et al., 1995) was directly added to the medium of hippocampal cell cultures at a concentration of 5 μg/ml.

Antisense Oligonucleotides

Two KIF4 antisense phosphorothioate oligonucleotides (S-modified) were used in this study. One of them, designated ASKIF4a, corresponds to the sequence ACTTGAGTGTCTGCTTC and is the inverse complement of nucleotides +2211/2227 of the sequence of mouse KIF4; antisense oligonucleotide ASKIF4b consisting of the sequence TGGTTCTGATGGCGGTTC is the inverse complement of the mouse nucleotides +4085/5002. An L1 antisense oligonucleotide was also used in some experiments. It corresponds to the sequence AGACTGTCATCACTGCCTA and is the inverse complement of nucleotides +3699/3717 of the sequence of rat L1. The oligonucleotides were purified by reverse chromatography and taken up in serum-free medium as described previously (Morfini et al., 1997). For all the experiments, the antisense oligonucleotides were preincubated with 2 μl of Lipofectin reagent (1 mg/ml; GIBCO BRL) diluted in 100 μl of serum-free medium. The resulting oligonucleotide suspension was then added to the cultured cells at concentrations ranging from 1–5 μM. Control cultures were treated with the same concentration of the corresponding sense-strand oligonucleotides. For some experiments, hippocampal pyramidal cells were treated for 1 d with an S-modified KIF2 antisense oligonucleotide (Morfini et al., 1997) in order to suppress KIF2 expression. The administration of all oligonucleotides was as follows: the oligonucleotides were added to the culture medium 4 h after plating with Lipofectin; the medium was then supplemented with additional oligonucleotide without Lipofectin every 12 h until the end of the experiment.

Primary Antibodies

The following primary antibodies were used in this study: a mAb against tyrosinated α-tubulin (clone TUB-1A2, mouse Ig; Sigma Chemical Co.) diluted 1:2,000; a mAb against β-tubulin (mouse Ig; Sigma Chemical Co.); a mAb against conventional kinesin heavy chain (uKHC) (clone H2, Pfister et al., 1989; a generous gift of Dr. Scott Brady, University of Texas, Dallas) diluted 1:50; a rabbit polyclonal antibody against α- and β-tubulin (Sigma Chemical Co.) diluted 1:100; a mAb against syntaxin (Transduction Laboratories) diluted 1:100; a mAb against a 151–amino acid peptide mapping at the NH$_2$ terminus of synaptotagmin (clone 41; Transduction Laboratories) diluted 1:100; a mAb against a 270–amino acid peptide mapping at the NH$_2$ terminus of the human L1 molecule (clone 17; Transduction Laboratories) diluted 1:100; an affinity-purified rabbit polyclonal antibody against the cytoplasmic domain of chick L1 (a generous gift of Dr. Vance Lemmon, Case Western Reserve University, Cleveland, OH; see Lemmon and McLoon, 1986) diluted 1:250; an affinity-purified rabbit polyclonal antibody against a 27.5-kD protein fragment corresponding to amino acids 170–241 of human TAX-1 (Transduction Laboratories) diluted 1:100; a mAb against the amyloid precursor protein (APP) (Boehringer Mannheim) diluted 1:50; a mAb against DCC (for deleted in colorectal carcinoma) (clone Ab-1; Oncogene Research Products) diluted 1:100; an affinity-purified goat polyclonal antibody against a peptide corresponding to an amino acid sequence mapping at the COOH terminus of the semaphorin precursor (Santa Cruz Biotechnology) diluted 1:100; and an affinity-purified rabbit polyclonal antibody against βgc (Quiroga et al.,

1995; Mascotti et al., 1997) diluted 1:100. In addition, two affinity-purified rabbit peptide antibodies against KIF4 were generated (Research Genetics). One peptide corresponds to amino acid residues 396–406 of mouse KIF4 (AKIF4), whereas the other corresponds to amino acids 123–135 of mouse KIF4 (BKIF4).

Western Blot Analysis

Equal amounts of crude brain homogenates or whole cell extracts from cultured hippocampal pyramidal cells were fractionated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes in a Tris-glycine buffer, 20% methanol. The filters were dried, washed several times with TBS (10 mM Tris, pH 7.5, 150 mM NaCl), and blocked for 1 h in TBS containing 5% BSA. The filters were incubated for 1 h at 37°C with the primary antibodies in TBS containing 5% BSA. The filters were then washed three times (10 min each) in TBS containing 0.05% Tween 20, and incubated with a secondary HRP-conjugated antibody (Promega Corporation) for 1 h at 37°C. After five washes with TBS and 0.05% Tween 20, the blots were developed using a chemiluminescence detection system.

Preparation of Microtubules from Cytosolic Fractions

For some experiments, microtubules were prepared from cytosolic fractions obtained from the cerebral cortex of 7-d-old rats by the method of Vallee (1982) in the presence or absence of AMP-PNP. The association of KIF4 with microtubules was then evaluated by Western blotting of the microtubule pellet using the anti-KIF4 antibodies.

Subcellular Fractionation and Sucrose Density Gradient Centrifugation

Multiple fractions from 3-d-old rat cerebral cortex were prepared as described previously (Morfini et al., 1997). In brief, rat cerebral cortex was gently homogenized with 10 vol of ice-cold 0.32 M sucrose, 10 mM Hepes, pH 7.4. The homogenate was centrifuged at low speed (3,000 g) for 10 min at 4°C. The supernatant was centrifuged at medium speed (9,200 g) for 15 min. The medium speed supernatant was again centrifuged at high speed (100,000 g) for 60 min to yield a cytosolic fraction (S3) and a microsomal one (P3). All the obtained fractions (supernatants and pellets) were then subjected to electrophoresis, transferred to PVDF membranes, and probed with the AKIF4 antibody. For some experiments, the microsomal fraction was further applied to either a discontinous sucrose density gradient (SDG) (0.4, 0.8, 1.2 M) or to a continuous one (0.3–1.6 or 1.0–2.0 M) at 48,000 rpm for 2 h in a Sorvall STS 60.4 rotor. Fractions were then collected from 4-ml tubes. They were then centrifuged at 100,000 g, and the resulting pellets resuspended in Laemmli buffer. The same volume from each fraction was applied to SDS-PAGE and transferred to PVDF membrane. Fractions were then analyzed by immunoblotting with antibodies against uKHC, KIF2, KIF4, synaptic vesicle markers, and growth cone membrane components.

Isolation of Growth Cone Particles (GCPs) by Subcellular Fractionation

Fetal rat brain (18 d of gestation) was fractionated according to Pfenninger et al. (1983) (see also Quiroga et al., 1995) to obtain GCPs. In brief, the low-speed supernatant (L) of fetal brain homogenate (H) was loaded on a discontinuous sucrose gradient in which the 0.75 and 1 M sucrose layers were replaced with a single 0.83-M sucrose step. This facilitated collection of the interface and increased GCP yield without decreasing purity (Paglini et al., 1998). The 0.32–0.83 M interface or A fraction was collected, diluted with 0.32 M sucrose, and pelleted to give the GCP fraction. This was resuspended in 0.32 M sucrose for experimentation. It is worth noting that this preparation (GCPs) has been extensively characterized by electron microscopic (Pfenniger et al., 1983; Li et al., 1992) and biochemical methods. These studies have revealed that GCPs contain significant amounts of c-src, tau, GAP-43 (Lohse et al., 1996), and βgc (Quiroga et al., 1995), but lack detectable amounts of high molecular weight MAP2, glial fibrillar acidic protein, and vimentin (Lohse et al., 1996).

Immunoisolation of KIF4-containing Organelles

Immunoisolation of KIF4-containing organelles was performed as described by Morfini et al. (1997) (see also Okada et al., 1995). For this ex-

periment, the AKIF4 antibody or the antibody against the cytoplasmic domain of chick L1 was covalently attached to protein A–Sepharose beads (Morfini et al., 1997). Microsomal fractions were incubated with these beads at 4°C for 6 h, and the beads were recovered by centrifugation (5,000 rpm for 120 s), and washed with 20 mM Hepes, 100 mM K-aspartate, 40 mM KCl, 5 mM EGTA, 5 mM MgCl$_2$, 2 mM Mg-ATP, 1 mM DTT, pH 7.2, supplemented with several protease inhibitors. The supernatant was spun down (75,000 rpm for 30 min) to collect the remaining organelles. Immunoblotting was then performed as described previously with antibodies against uKHC, KIF2, KIF4, L1, TAX-1, and synaptotagmin.

Immunofluorescence

Cells were fixed before detergent extraction and processed for immunofluorescence as described previously (Paglini et al., 1998). The antibody staining protocol entailed labeling with the first primary antibody, washing with PBS, staining with labeled secondary antibody (fluorescein or rhodamine conjugated), and washing similarly. The same procedure was repeated for the second primary antibody. Incubations with primary antibodies were for 1 or 3 h at room temperature, whereas incubations with secondary antibodies were performed for 1 h at 37°C. The cells were observed with an inverted microscope (Carl Zeiss Axiovert 35M) equipped with epifluorescence and differential interference contrast (DIC) optics. Fluorescent images were captured under regular fluorescence microscopy with a silicon-intensified target camera (SIT-C2400; Hamamatsu Corp.). The images were digitized directly into a Metamorph/Metafluor Image Processor (Universal Imaging Corp.). Fluorescence intensity measurements were performed pixel-by-pixel along the longitudinal axis of identified neurons (Paglini et al., 1998). Using this data, we then calculated the average fluorescence intensity within the cell body, the distal third of identified neurites (either minor processes or axons), and the central region of growth cones. Photographs were printed using Adobe Photoshop. To measure neuronal shape parameters, fixed unstained or antibody-labeled cells were randomly selected and traced from a video screen using the morphometric menu of the Metamorph as described previously (Cáceres et al., 1992; Morfini et al., 1997). Differences among groups were analyzed by the use of ANOVA and Student-Newman Keul's test.

Results

Characterization of a Peptide Antibody against KIF4

The monospecificity of the affinity-purified rabbit polyclonal antibody designated AKIF4 raised against a peptide corresponding to amino acid residues 396–406 of mouse KIF4 is shown in Fig. 1 A. This antibody recognizes a single band of ~140,000 M_r in Western blots of whole cell homogenates from the cerebral cortex of developing rats (Fig. 1 A, lanes 1 and 2). The staining generated by this antibody is completely abolished by neutralization with the corresponding purified peptide (Fig. 1 A, lane 3).

Fig. 1 B shows that in the cerebral cortex, the expression of the AKIF4 immunoreactive protein species is higher at early postnatal days, declining gradually but considerably until adulthood, where the lowest levels are detected. In addition, Western blot analysis of subcellular fractions obtained from the cortex of 3-d-old rats revealed that the 140-kD protein is relatively concentrated in the microsomal fraction compared with the cytosol or the mitochondrial fraction (Fig. 1 C). This means that a considerable amount of the protein recognized by the AKIF4 antibody is associated with small membranous organelles, but only barely associated with larger ones such as mitochondria (Fig. 1 C). Besides, microtubule binding experiments show that in the absence of ATP the 140-kD protein cosediments with taxol-stabilized microtubules obtained from the cerebral cortex; this binding occurs in the presence or absence of AMP-PNP (data not shown). Since all the
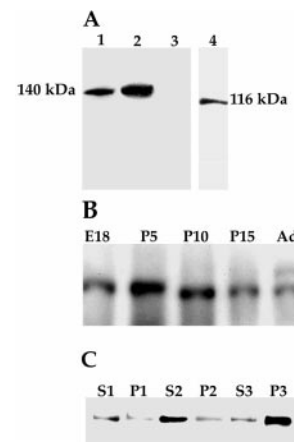

Figure 1. (A) Specificity of the affinity-purified peptide antibody against KIF4 (AKIF4) as revealed by Western blot analysis of whole tissue extracts obtained from the cerebral cortex of 3-d-old rats. The AKIF4 antibody (diluted 1:100) stains a single immunoreactive protein species with an apparent molecular weight of 140 kD (lanes 1 and 2). The staining generated by this antibody is completely abolished by neutralization with the corresponding purified peptide (lane 3). 10 μg of total protein was loaded in lane 1, and 20 μg in lanes 2 and 3. Lane 4 shows an equivalent sample to that of lane 1, but revealed with a mAb against uKHC. (B) The expression of KIF4 in the developing rat cerebral cortex as revealed by immunoblot analysis of whole tissue extracts. E18, embryonic day 18; P5–P15, postnatal days 5–15; Ad, adult. KIF4 is highly expressed in the developing cerebral cortex. The blot was reacted with AKIF4 antibody (dilution 1:100); 20 μg of total protein was loaded in each lane. (C) Western blot analysis of subcellular fractions obtained from the cerebral cortex of 3-d-old rats showing that KIF4 is enriched in the microsomal fraction (P3). S1, low-speed supernatant; P1, pellet 1; S2, medium speed supernatant; P2, pellet 2; S3, high-speed supernatant; P3, pellet 3 or microsomal fraction. The blot was reacted with AKIF4 (dilution 1:200); 20 μg of protein were loaded in each lane.

above described properties are identical to those reported previously for KIF4 (see Sekine et al., 1994), we conclude that this antibody effectively recognizes KIF4 and not a different protein having a similar molecular weight.

Biochemical Evidences for the Association of KIF4 with L1-containing Vesicles

To begin analyzing the type of cargo that KIF4 may transport, microsomal fractions from rat cerebral cortex were fractionated by isopicnic SDG centrifugation and analyzed by immunoblotting with antibodies against uKHC, KIF2, KIF4, and several membrane proteins, including synaptic and nonsynaptic vesicle constituents. This type of approach has already proven useful to identify the type of membrane-bound organelle associated with KIF1A and KIF2 (Okada et al., 1995; Morfini et al., 1997).

Approximately 70–80% of uKHC, KIF2, or KIF4 was recovered in the P3 (high-speed pellet) fraction, whereas <30% was present in the P2 (medium-speed fraction) and S3 (high-speed supernatant) fractions. When the P3 microsomal fraction was applied to a discontinuous gradient of 0.4, 0.8, and 1.2 M sucrose (see Materials and Methods), fractions with a very different molecular composition were obtained (Fig. 2, A–C). In this gradient, uKHC and KIF2 were recovered in the 0.4- and 0.8-M fractions (Fig. 2 A). In contrast, KIF4 was predominantly recovered in the 1.2-M sucrose fraction (Fig. 2 A). The distribution of KIF4 was then compared with that of synaptotagmin, a constituent of the synaptic vesicle precursor that is transported by KIF1A (Okada et al., 1995), and syntaxin, a presynaptic
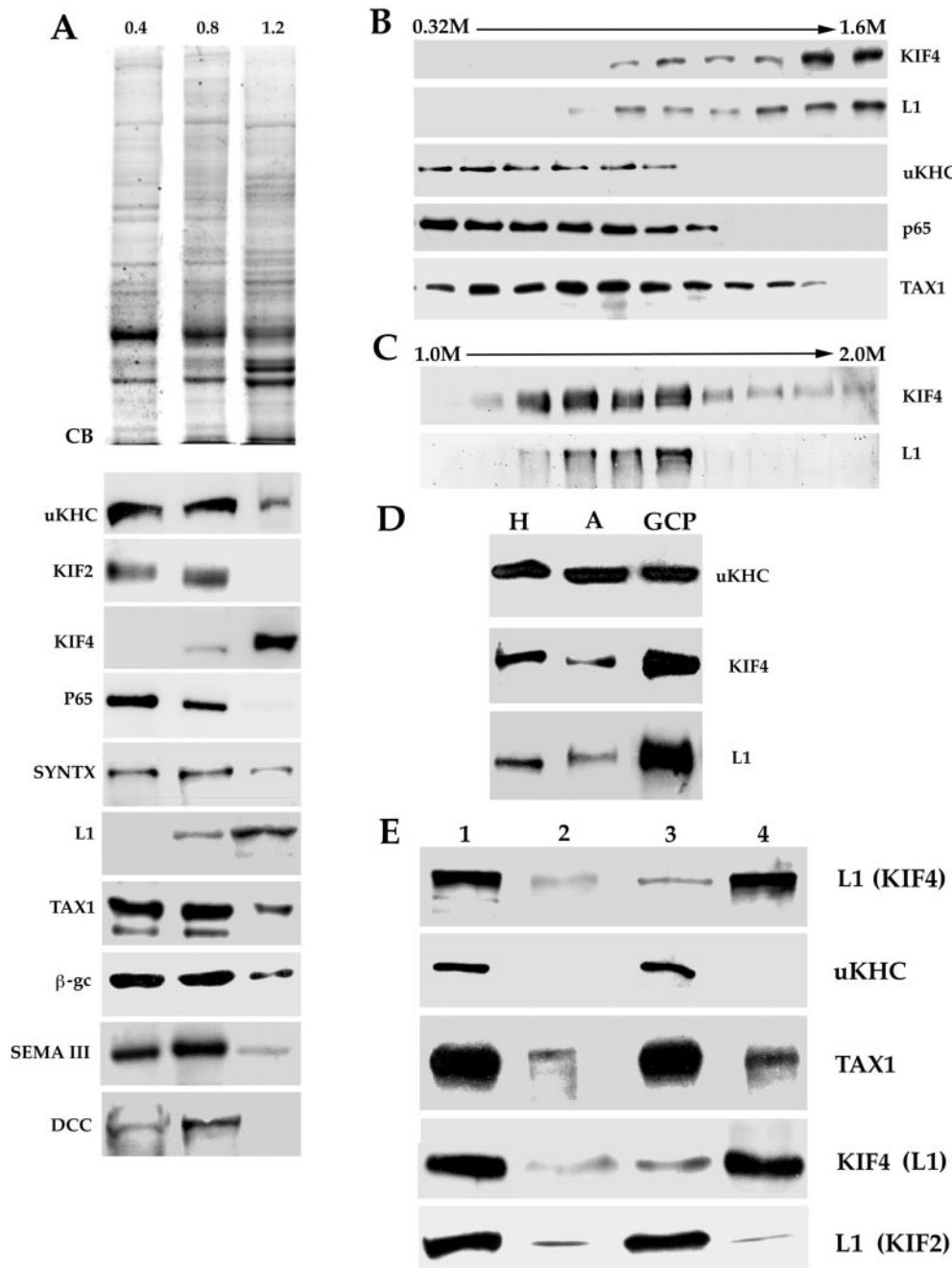
A 0.4 0.8 1.2

CB

uKHC
KIF2
KIF4
P65
SYNTX
L1
TAX1
β-gc
SEMA III
DCC

B 0.32M ──────────────→ 1.6M

KIF4
L1
uKHC
p65
TAX1

C 1.0M ──────────────→ 2.0M

KIF4
L1

D H A GCP

uKHC
KIF4
L1

E 1 2 3 4

L1 (KIF4)
uKHC
TAX1
KIF4 (L1)
L1 (KIF2)

Figure 2. (A) The binding of KIF4 to membrane vesicles. Microsome fraction from developing rat cerebral cortex was fractionated by centrifugation across a discontinuous SDG and the same volume from each fraction was applied to SDS-PAGE, stained with Coomassie blue (CB), or transferred to PVDF membranes and analyzed by immunoblotting with antibodies against uKHC, KIF2, KIF4, synaptotagmin (p65), syntaxin (SYNTX), L1, TAX-1, βgc, semaphorin III (SEMA III), and DCC. Note that KIF4 and L1 are enriched in the 1.2-M sucrose fraction. (B and C) A similar analysis to that shown in A, but for a microsome preparation separated across a continuous SDG extending from 0.32–1.6 M (B) or 1.0–2.0 M (C) sucrose. Note that KIF4 and L1 are enriched in fractions extending from 1.2–1.6 M sucrose. (D) Western blot of total brain homogenate (H), fraction A (A), and GCP reacted with antibodies against uKHC, KIF4, and L1. All antibodies were used at a concentration of 0.5 μg/ml. All fractions were obtained from E18 rat embryos as described in Materials and Methods. 20 μg of total cellular protein was loaded in each lane. Note that KIF4 and L1 are enriched in the GCP fraction. (E) L1 (KIF4): immunoisolation of L1-containing organelles with the AKIF4 antibody (dilution 1:50). Microsome fraction incubated with AKIF4 preimmune serum: remanent organelles (lane 1) and immunoprecipitated organelles (lane 2). Microsome fraction incubated with the AKIF4 antibody: remanent organelles (lane 3) and immunoprecipitated organelles (lane 4). Note that L1 is quantitatively recovered in this fraction. uKHC and TAX-1: equivalent fractions to those show for L1, but reacted with antibodies against uKHC or TAX-1. Note that both proteins are predominantly recovered in the remanent organelle fraction. KIF4 (L1): immunoisolation of KIF4-containing organelles with an antibody against the cytoplasmic domain of L1. Microsome fraction incubated with nonimmune serum: remanent organelles (lane 1) and immunoprecipitated organelles (lane 2). Microsome fraction incubated with the anti-L1 rabbit polyclonal antibody: remanent organelles (lane 3) and immunoprecipitated organelles (lane 4). Note that KIF4 is quantitatively recovered in this fraction. L1 (KIF2): failure of a rabbit polyclonal antibody against KIF2 (BKIF2, diluted 1:50) to immunoprecipitate L1-containing vesicles. Microsome fraction incubated with nonimmune serum: remanent organelles (lane 1) and immunoprecipitated organelles (lane 2). Microsome fraction incubated with the BKIF2 antibody: remanent organelles (lane 3) and immunoprecipitated organelles (lane 4).

membrane protein absent from the KIF1A cargo (Okada et al., 1995). The results obtained showed that neither synaptotagmin nor syntaxin colocalizes with KIF4; both types of synaptic vesicle proteins were enriched in the 0.4- and 0.8-M sucrose fractions, but almost absent from the heavier fraction that contains KIF4 (Fig. 2 A).

We next compared the distribution of KIF4 with that of several well-characterized nonsynaptic membrane compo-

nents. One of them, the cell adhesion molecule L1, a transmembrane protein belonging to the Ig superfamily that is highly expressed in developing neurons (Moos et al., 1988), displayed a striking codistribution with KIF4, being highly enriched in the 1.2-M sucrose fraction (Fig. 2 A). On the other hand, TAX-1, the human homologue of rat axonin-1 which is another neuronal cell adhesion molecule belonging to the Ig superfamily (Hasler et al., 1993), distributed in all fractions of the sucrose gradient with the lowest levels detected in the 1.2-M sucrose fraction (Fig. 2 A). The patterns of distribution of βgc, which is a novel variant of the β-subunit of the IGF-1 receptor, highly enriched in growth cone membranes and in vesicles that associate with KIF2 (Quiroga et al., 1995; Mascotti et al., 1997; Morfini et al., 1997), as well as of several membrane proteins involved in growth cone guidance, including semaphorin III (Messeramith et al., 1995) and the netrin receptor DCC (de la Torre et al., 1997), were all different from that of KIF4 or L1 (Fig. 2 A). The codistribution of KIF4 and L1 was further confirmed by Western blot analysis of microsomal fractions separated across a continuous SDG extending from 0.3–1.6 M sucrose (Fig. 2 B). This analysis revealed that both proteins were highly enriched in fractions extending from 1.0–1.6 M sucrose, whereas uKHC (Fig. 2 B), synaptotagmin (Fig. 2 B), syntaxin (data not shown), or TAX-1 (Fig. 2 B) were all enriched in lighter fractions. Finally, and to discard the possibility that the localization of KIF4 with L1 at the bottom of the 0.3–1.6 M sucrose gradient may represent copelleting rather than actual association at a particular density, the distribution of both proteins was analyzed across a gradient extending from 1.0–2.0 M sucrose. Fig. 2 C shows that both proteins colocalize in fractions extending from 1.2–1.6 M sucrose, but are absent from heavier ones.

Since previous immunofluorescence studies have shown that KIF4 (Sekine et al., 1994) and L1 (Kamiguchi and Lemmon, 1998) are present at neuritic tips, it became of interest to analyze their distribution in growth cone fractions isolated by subcellular fractionation according to the procedures described (Pfenninger et al., 1983; Paglini et al., 1998; see also Materials and Methods). This analysis revealed that KIF4 and L1 were not only present but also enriched in GCPs when compared with the levels detected in the total brain homogenate fraction or the A fraction that contains cytosolic soluble proteins and GCPs. In contrast, no such enrichment was detected for uKHC (Fig. 2 D).

Taken together, these observations raise the possibility of KIF4 interacting with a type of nonsynaptic vesicle that accumulates in growth cones that is distinct from those interacting with KIF2 (e.g., βgc-containing nonsynaptic vesicles), and that contains the cell adhesion molecule L1 as one of its components. Therefore, to directly test this possibility, immunoisolation of organelles from the microsomal fraction was performed with the AKIF4 antibody or with a rabbit polyclonal antibody directed against the cytoplasmic domain of L1. The remaining organelles were recovered by pelleting from the supernatant fraction. Fig. 2 E shows that with this method, the KIF4-containing organelles were collected. More importantly, in this immunoisolated organelle fraction both KIF4 and L1 were quantitatively recovered. By contrast, uKHC (Fig. 2 E), KIF2 (not shown), synaptotagmin (not shown), or TAX-1

(Fig. 2 E) were not, or were only slightly detectable in this fraction; they were quantitatively recovered in the remaining organelle fraction. In addition, they were not detected in the supernatant fraction after pelleting the remaining organelles, effectively ruling out the possibility that the lack of these proteins in the KIF4/L1 organelle-containing fraction was due to dissociation during the immunoisolation procedure. Finally, it is worth mentioning that we failed to immunoisolate L1-containing vesicles with BKIF2 (Fig. 2 E), a rabbit polyclonal antibody against KIF2 previously used to coprecipitate βgc-containing vesicles (see Morfini et al., 1997).

These results clearly and directly demonstrate that KIF4 is associated with a class of nonsynaptic membranous organelle that contains L1 as one of its components. However, they do not provide evidence about the in vivo relationship between KIF4 and the transport of L1-containing organelles, and/or the functional role of KIF4 during neuronal morphogenesis. Therefore, to obtain evidence about these aspects, we decided to examine the subcellular distribution of KIF4 as well as the consequences of its suppression on the distribution of L1 in cultured hippocampal pyramidal neurons. In this cell system, endogenous L1 and virally expressed NgCAM (the chick homologue of L1), but not several other axonal membrane proteins, become restricted to the axonal domain at early stages of morphological polarization (Jareb and Banker, 1998; Kamiguchi and Lemmon, 1998; Stowell and Craig, 1999), thus providing an excellent opportunity to test whether or not anterograde microtubule-based molecular motors such as KIF4 participate in neuronal polarization.

The Subcellular Distribution of KIF4 in Cultured Hippocampal Pyramidal Neurons

Cultured hippocampal pyramidal neurons have proven to be an excellent model system for studying growth cone formation, neurite outgrowth, the establishment of neuronal polarity, as well as the expression and function of cytoskeletal and membrane proteins involved in nerve cell morphogenesis (Craig and Banker, 1994; Bradke and Dotti, 1997, 1999; Jareb and Banker, 1998; Paglini et al., 1998; Stowell and Craig, 1999). Since protein localization can be examined with the least ambiguity in young neurons before an extensive axonal network develops (see Jareb and Banker, 1998), we examined the subcellular distribution of KIF4 48 h after cell plating, when neurons had become polarized (stage III neurons; see Craig and Banker, 1994) and the axon could be readily distinguished from the remaining minor neurites that will later develop as dendrites. Double immunofluorescence staining of these cells with the AKIF4 antibody and with a mAb against tyrosinated α-tubulin or a mAb against β-tubulin revealed that KIF4 immunolabeling is preferentially localized to the cell body and the distal third of the growing axon, including its growth cone, where the highest immunofluorescence signal is detected (Fig. 3, A–D). In contrast, minor neurites and/or their growth cones exhibit little if any staining at all. To test whether or not this distributional pattern represents a property common to other NH_2-terminal type microtubule-based motors, the subcellular localization of uKHC was also analyzed in stage III
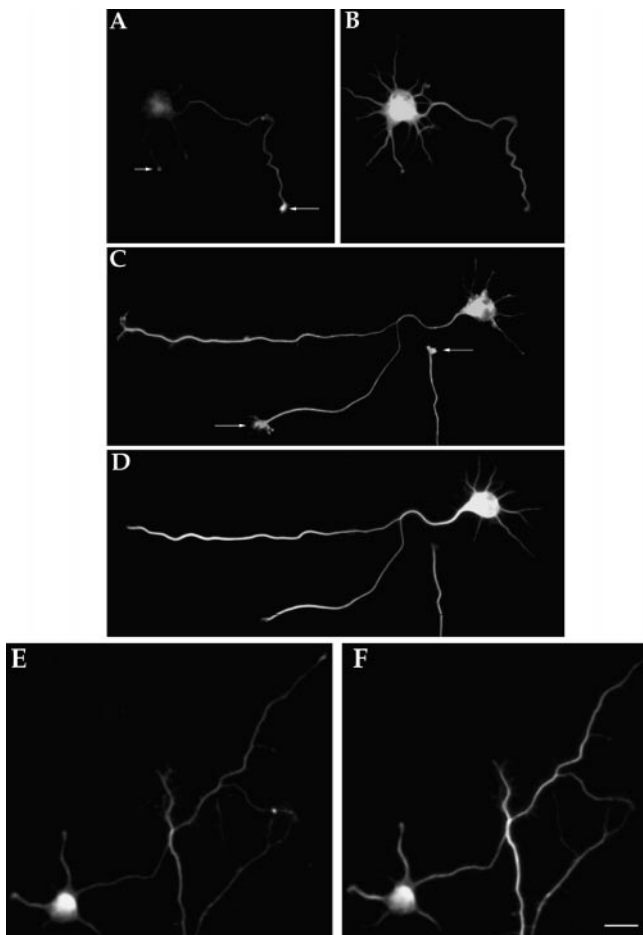
Figure 3. The subcellular distribution of KIF4 in cultured hippocampal pyramidal neurons. (A and B) Double immunofluorescence micrograph showing the distribution of KIF4 (A) and tyrosinated α-tubulin in a stage III hippocampal pyramidal cell. Note that KIF4 immunofluorescence predominantly localizes to the axon and its growth cone (long arrow). Light KIF4 immunolabeling is also detected in the growth cones (short arrow) of some minor processes. For this experiment the AKIF4 was diluted 1:100. (C and D) Double immunofluorescence micrographs showing the distribution of KIF4 and β-tubulin in a stage III hippocampal pyramidal cell. In this case the AKIF4 antibody was diluted 1:50. As shown previously, KIF4 immunolabeling preferentially localizes to axons and their growth cones (long arrows). A light immunofluorescence signal is also detected in minor processes. (E and F) Double immunofluorescence micrographs showing the distribution of uKHC (E) and total tubulin (F) in a stage III hippocampal pyramidal cell. Note that uKHC immunolabeling has a widespread distribution localizing to both axons and minor processes. Bar, 10 μm.

hippocampal pyramidal neurons. The results showed that uKHC displays a widespread distribution, localizing in axons and minor processes. In addition, this analysis revealed that uKHC immunofluorescence was not particularly prominent at the distal end of growing axons or in growth cones (Fig. 3, E and F). In a complementary series of experiments, we used quantitative fluorescence techniques to measure the relative amounts of KIF4 and uKHC in axons, minor neurites, and growth cones of stage III hippocampal pyramidal neurons. The results obtained,

which are shown in Table I, confirm our observations and clearly establish that KIF4 immunolabeling is preferentially localized to axonal processes and their growth cones.

Antisense Oligonucleotides Inhibit KIF4 Expression and Alter the Distribution of L1

Two phosphorothioate (S-modified) antisense oligonucleotides were tested for their ability to inhibit KIF4 expression. Cultured hippocampal pyramidal cells incubated for 24 h with different doses (2.5 or 5 μM) of the ASKIF4a an-
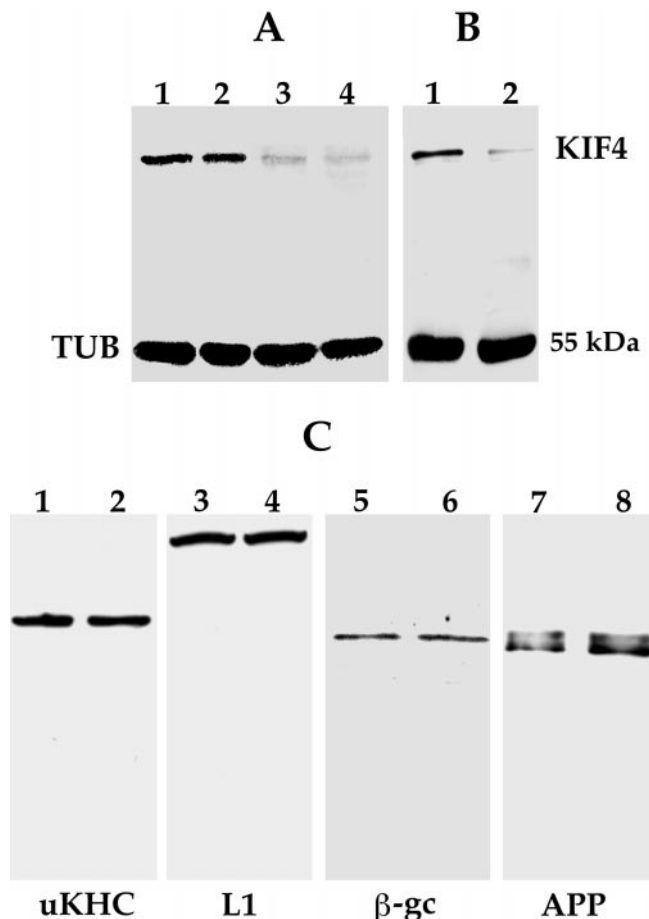

Figure 4. Antisense oligonucleotides inhibit KIF4 expression. (A) Western blot showing the effect of the ASKIF4a antisense oligonucleotides on KIF4 and β-tubulin (TUB) protein levels. Lane 1, control nontreated; lane 2, sense-treated (5 μM); lane 3, ASKF4a-treated (2.5 μM); and lane 4, ASKIF4a-treated (5 μM). The Western blot was revealed with the AKIF4 antibody (diluted 1:100) and a mAb against β-tubulin. (B) Western blot showing the effect of the ASKIF4b antisense oligonucleotide on KIF4 protein levels as revealed by the use of the BKIF4 antibody (diluted 1:100). Lane 1, sense-treated (5 μM); and lane 2, ASKIF4b-treated (5 μM). Note that the ASKIF4b antisense oligonucleotide selectively decreases KIF4 protein levels without affecting the BKIF4 immunoreactive protein band migrating at the position of tubulin (55 kD). (C) Effect of the ASKIF4a antisense oligonucleotide on uKHC, L1, βgc, or APP protein levels. Lanes 1, 3, 5, and 7, sense-treated (5 μM); lanes 2, 4, 6, and 8, ASKIF4a-treated (5 μM). For these experiments, cultures were treated for 1 d with sense or antisense oligonucleotides, starting 4 h after plating. 25 μg of total cellular protein was loaded in each lane.

Table I. Quantitative Measurements of KIF4 and uKHC Immunofluorescence in Cultured Hippocampal Pyramidal Cells

	Cell body	Axon	Axonal growth cones	Minor processes	Growth cones of minor processes
Total tubulin	230 ± 20	145 ± 20	35 ± 10	135 ± 10	35 ± 10
β-Tubulin	220 ± 25	140 ± 15	30 ± 5	135 ± 20	48 ± 10
KIF4	140 ± 20	130 ± 16	145 ± 10	30 ± 8	20 ± 8
uKHC	180 ± 15	120 ± 20	55 ± 10	120 ± 20	40 ± 10

Average fluorescence intensity measurements within the cell body, the distal third of the axon, the distal third of minor processes, and the central region of growth cones were performed as described in the Materials and Methods section. Each value represents the mean ± SEM. Pixel intensity expressed in gray values: 0 (black)/255 (white). Cells were double labeled with a rabbit polyclonal antibody against α- and β-tubulin (Total tubulin) and a mAb against uKHC, or with a mAb against β-tubulin and the AKIF4 antibody. A total of 50 cells was measured for each double immunofluorescence.

tisense oligonucleotide described in Materials and Methods showed markedly reduced reactivity to the AKIF4 antibody, as assessed by Western blotting of whole cell extracts (Fig. 4 A). In contrast, cells treated with sense oligonucleotides are comparable in their immunoreactivity to untreated control cells (Fig. 4 A). The ability of the ASKIF4b antisense oligonucleotide (5 μM) to decrease KIF4 protein levels was also evaluated using a different KIF4 antibody. This affinity-purified rabbit polyclonal antibody, designated BKIF4, raised against a peptide corresponding to amino acid residues 122–134 of mouse KIF4, recognizes a 140-kD protein band (Fig. 4 B, lane 1) and also a protein migrating at the position of tubulin. As shown in Fig. 4 B, the BKIF4 immunolabeling of the 140-kD protein band selectively decreases in whole cell extracts obtained from ASKIF4b-treated cultures (Fig. 4 B, lane 2). No such a decrease is observed with respect to the protein band migrating at the position of tubulin. Our results also show that the ASKIF4a antisense oligonucleotide does not affect tubulin expression as revealed by labeling with a mAb against β-tubulin (Fig. 4 A, lanes 1–4), or the one of uKHC (Fig. 4 B), KIF2 (data not shown), L1 (Fig. 4 B), βgc (Fig. 4 B), or the APP (Fig. 4 B). The presence of normal levels of these proteins in the KIF4-suppressed cells suggests that the effect of the antisense treatment is specific and that the regulation of the expression of other proteins, including microtubule-based motors, is independent of KIF4.

Next, we examined the distribution of L1 in control and KIF4 antisense oligonucleotide-treated hippocampal pyramidal neurons. As expected, KIF4 immunofluorescence was dramatically reduced in neurons treated with the antisense oligonucleotides (Table II). In addition, a significant alteration in the distribution of L1 was also detected (Fig. 5). Thus, whereas in >90% of nontreated or sense-treated hippocampal pyramidal neurons L1 is selectively and highly enriched in axons and their growth cones (Fig. 5, A, B, E, and F), in the KIF4 antisense-treated cells all of the labeling is present within the cell body or the inner third of the axonal shaft, being completely absent from axonal growth cones (Fig. 5, C, D, and G–J; see also Table II). In contrast, the distribution of several other membrane proteins present in growth cones, such as βgc transported by KIF2 (Morfini et al., 1997) or APP transported by KHC (Ferreira et al., 1993), was unaltered in the KIF4 antisense-treated neurons (Fig. 6, A–D; see also Table II). To determine if other KLPs may also be required for L1 transport, cells were treated with KIF2 antisense oligonucleotides (Morfini et al., 1997). As shown in Fig. 6, E and

F, KIF2 suppression does not alter the distribution of L1. However, and as described previously (Morfini et al., 1997), this treatment produces a dramatic reduction of βgc immunolabeling at the growth cone and a concomitant accumulation within the cell body (see Table II). In addition, and to obtain some evidence as to whether or not L1 may be a single major component of the KIF4 cargo, the expression and distribution of KIF4 was analyzed in neurons treated with an L1-antisense oligonucleotide. As shown in Fig. 7 A, a dose-dependent reduction in L1 protein levels was detected when cultures were exposed for 24 h to different concentrations of the L1-antisense oligonucleotide. In contrast, no such a decrease was detected in cells treated with equivalent doses of the corresponding sense-strand oligonucleotide (data not shown). The results obtained also show that L1 suppression has no effect on the levels or distribution (Fig. 7, B and C) of KIF4 as might be expected if other as-yet unidentified proteins are components of the KIF4 cargo.

KIF4 Suppression Prevents L1-stimulated Axonal Elongation

Previous studies have shown that purified L1 or an L1-Fc

Table II. Quantitative Measurements of KIF4, L1, and βgc Immunofluorescence in Cultured Hippocampal Pyramidal Cells Treated with KIF4 and KIF2 Antisense Oligonucleotides

	Cell body	Axon	Growth cone
Nontreated			
KIF4	140 ± 20	130 ± 16	145 ± 10
L1	80 ± 10	95 ± 10	125 ± 12
βgc	110 ± 15	75 ± 10	85 ± 15
KIF4 sense-treated (5 μM)			
KIF4	145 ± 15	120 ± 20	145 ± 10
L1	80 ± 15	95 ± 10	115 ± 10
βgc	105 ± 15	80 ± 10	85 ± 15
ASKIF4a-treated (5 μM)			
KIF4	14 ± 4	ND*	ND*
L1	100 ± 10	10 ± 5*	ND*
βgc	105 ± 15	65 ± 10	95 ± 20
ASKIF2-treated (5 μM)			
KIF4	140 ± 20	125 ± 10	140 ± 10
L1	85 ± 10	100 ± 20	105 ± 15
βgc	145 ± 10	ND*	ND*

Average fluorescence intensity measurements within the cell body, the distal third of the axon, and the central region of axonal growth cones were performed as described in the Materials and Methods section. Each value represents the mean ± SEM. Pixel intensity expressed in gray values: 0 (black)/255 (white). A total of 30 cells was measured for each experimental condition.
*Values significantly different from those of control cells (nontreated or sense-treated). ND: not detectable.
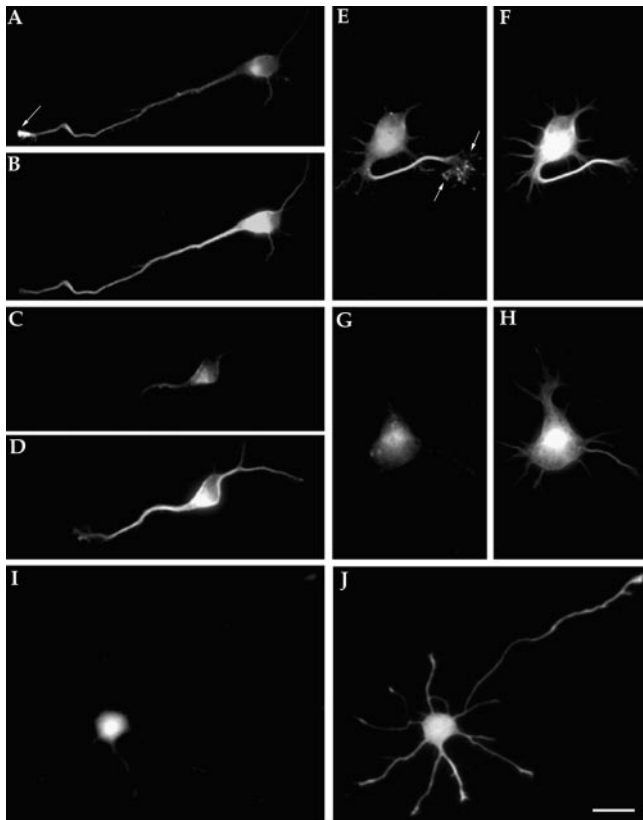
Figure 5. KIF4 suppression alters L1 subcellular localization. (A and B) Double immunofluorescence micrographs showing the distribution of L1 (A) and tyrosinated α-tubulin (B) in a stage III hippocampal pyramidal cell. Note that L1 is preferentially localized to the distal third of the axon and its growth cone (arrow). (C and D) An equivalent cell to that shown previously but from a culture treated with the ASKIF4a antisense oligonucleotide (5 μM). Note that L1 immunofluorescence has disappeared from the growth cone and the distal axon. (E and F) Double immunofluorescence micrograph showing the distribution of L1 (E) and tyrosinated α-tubulin (F) in another hippocampal pyramidal cell from a control (sense-treated) culture. The cell that is at the transition of stage II to stage III displays strong L1 immunofluorescence in the longest neurite, the presumptive axon, and its growth cone (arrows). (G and H) An equivalent cell to that shown previously, but from a culture treated with the ASKIF4a antisense oligonucleotide (5 μM). Note that L1 immunofluorescence (G) is exclusively localized to the cell body. (I and J) Double immunofluorescence micrographs showing the distribution of L1 (I) and tyrosinated α-tubulin (J) in a stage III hippocampal pyramidal cell from a culture treated with the ASKIF4b (5 μM). Note the dramatic decrease of L1 immunolabeling in the axon and its growth cone. For these experiments, cells were treated with sense or antisense oligonucleotides for 30 h, starting 4 h after plating. Bar, 10 μm.

chimera can promote as a substrate bound molecule neurite elongation in culture neurons (Lemmon et al., 1992; Doherty et al., 1995). A similar effect has been observed when using the L1-Fc chimera as a soluble molecule (Doherty et al., 1995). Since this response appears to be related with the homophilic binding of L1 in growth cones to soluble L1 (Neugebauer et al., 1988; Seilheimer and Schachner, 1988; Doherty et al., 1995), it became of inter-
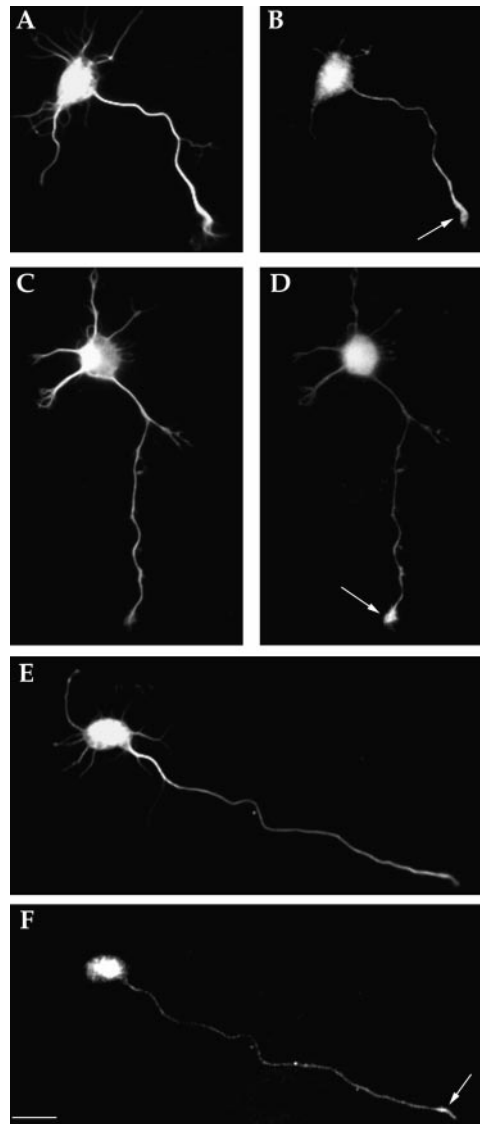

Figure 6. (A and B) Double immunofluorescence micrographs showing the distribution of tyrosinated α-tubulin and βgc in a stage III hippocampal pyramidal cell from a culture treated with the ASKIF4a antisense oligonucleotide (5 μM) for 24 h. Note that βgc immunofluorescence is higher at the distal third of the axon, including its growth cone (arrow). (C and D) Double immunofluorescence micrographs showing the distribution of tyrosinated α-tubulin and APP in a stage III hippocampal pyramidal cell from a culture treated with the ASKIF4a antisense oligonucleotide (5 μM) for 24 h. Note that within the axon APP, immunofluorescence is preferentially localized at the growth cone (arrow). (E and F) Double immunofluorescence micrograph showing the distribution of β-tubulin and L1 in a stage III hippocampal pyramidal cell from a culture treated with a KIF2 antisense oligonucleotide (5 μM). Note that L1 immunofluorescence remains high within the axonal growth cone (arrow). Cultures were treated with antisense oligonucleotides as described in the previous figure. Bar, 10 μm.

est to test whether or not KIF4 suppression prevents L1-stimulated axonal extension. For such a purpose, control and ASKIF4a-treated 1-d-old hippocampal pyramidal cells were cultured for an additional 24-h period in the presence
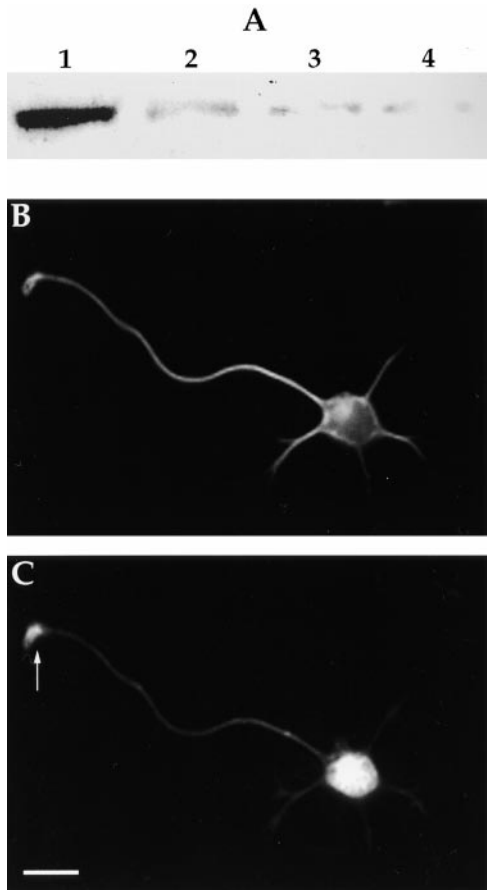
Figure 7. (A) Western blot showing the effect of an L1-antisense oligonucleotide on L1 protein levels. Lane 1, sense-treated (5 μM); lane 2, antisense-treated (1 μM); lane 3, antisense-treated (2.5 μM); and lane 4, antisense-treated (5 μM). The Western blot was revealed with the rabbit polyclonal antibody against the cytoplasmic domain of L1. For this experiment cultures were treated for 1 d with sense or antisense oligonucleotides, starting 4 h after plating. 25 μg of total cellular protein was loaded in each lane. The levels of L1 in sense-treated cultures were equivalent to those of nontreated cells. (B and C) Double immunofluorescence micrograph showing the distribution of tyrosinated α-tubulin (B) and KIF4 (C) in a stage III hippocampal pyramidal cell from a culture treated with the L1 antisense oligonucleotide (5 μM). Note that KIF4 immunofluorescence remains high within the axonal growth cone. Bar, 10 μm.

Table III. The Inhibitory Effect of KIF4 Antisense Oligonucleotides on L1-stimulated Axonal Elongation Is Reversible

	Hours after release from antisense treatment		
	0	12	24
Sense-treated (5 μM)			
Axonal length	250 ± 25	320 ± 30	420 ± 40
KIF4	145 ± 20		140 ± 25
L1	120 ± 10		125 ± 20
Sense-treated (5 μM) + L1-Fc (5 μg/ml)			
Axonal length	450 ± 45	580 ± 60	700 ± 40
KIF4	145 ± 20		145 ± 20
L1	120 ± 10		125 ± 20
ASKIF4a-treated (5 μM) + L1-Fc (5 μg/ml)			
Axonal length	260 ± 30*	380 ± 40	525 ± 25*
KIF4	ND*	40 ± 15*	110 ± 15
L1	ND*	20 ± 10*	95 ± 10

For this experiment the cells were treated for 48 h with the oligonucleotides starting at the time of plating. After 1 d in vitro, 5 μg/ml of the L1-Fc chimera was added to the culture medium. An additional pulse was given 24 h later. Cells were fixed 72 h after plating. Each value represents the mean ± SEM. Axonal length is expressed in μm. KIF4 and L1 immunofluorescence were measured within the central region of axonal growth cones as described in Table 1. Values are expressed in pixels. ND: not detectable.
*Values significantly different from those of the corresponding control group.

dition of the antisense oligonucleotides, being highly coincident with the reduction in KIF4 protein levels. When, after 48 h in the presence of the antisense oligonucleotide, the cells are released from antisense inhibition by changing the medium, axonal extension resumed at a rate equivalent to that observed under control conditions. This phenomenon is accompanied by reexpression of KIF4 and appearance of L1 at axonal tips (Table III).

Discussion

KIF4 and the Subcellular Distribution of L1

These results confirm and extend previous observations by Sekine et al. (1994) suggesting that KIF4 is an anterograde microtubule-based motor involved in the transport of a class of nonsynaptic membrane organelle abundant in developing neurons. The new information presented here suggests that at least one of the components of the KIF4 cargo is the L1 glycoprotein, a cell adhesion molecule present in axonal growth cones (Van den Pol and Kim, 1993; Jareb and Banker, 1998; Kamiguchi and Lemmon, 1998; this study) and implicated in promoting axonal elongation (Neugebauer et al., 1988; Seilheimer and Schachner, 1988; Doherty et al., 1995). Thus, one striking finding in KIF4-suppressed hippocampal pyramidal neurons is the altered distribution of L1. In untreated cells, or cells treated with sense oligonucleotides, L1 is found selectively concentrated within axonal shafts and their growth cones. In antisense-treated cells, on the other hand, L1 is confined to the cell body or the most proximal third of the axon.

Several observations suggest that this effect is the result of a specific and selective blockade of KIF4 expression by the antisense treatment. First, sequence analysis of the regions of the KIF4 mRNAs selected for designing the antisense oligonucleotides reveals no significant homology
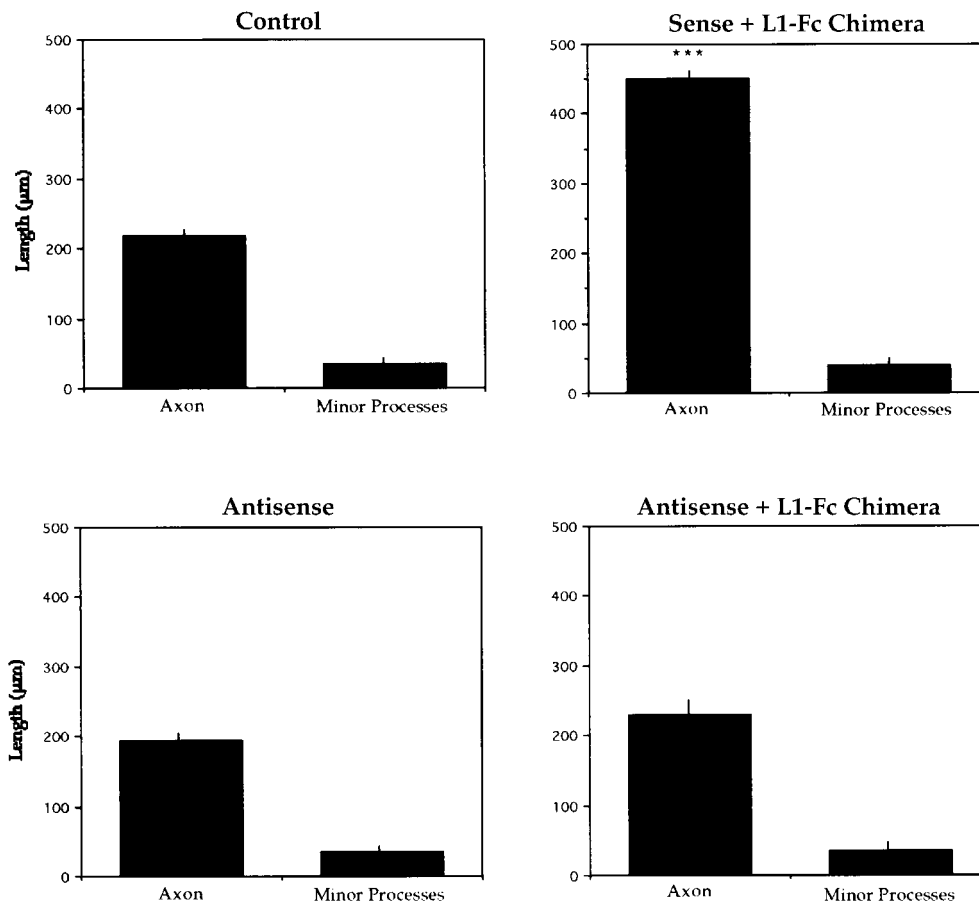
of an L1-Fc chimera. The neurite outgrowth response was then evaluated by morphometry of fixed cultures (Doherty et al., 1995; see also Materials and Methods). The results obtained show that the addition of the L1-Fc chimera to the tissue culture medium at a concentration of 5 μg/ml selectively increases axonal length in control and sense-treated neurons (Fig. 8). On the other hand, KIF4 suppression completely inhibits L1-stimulated axonal elongation without affecting either the length (Fig. 8) or the number of minor processes. This effect appears to be dependent on the presence of L1, since KIF4 suppression has no effect on minor neurite formation and/or axonal outgrowth in neurons growing on polylysine alone (Fig. 8). Inhibition of L1-stimulated axonal elongation in KIF4 antisense-treated neurons becomes evident 24 h after the ad-

Figure 8. KIF4 suppression prevents L1-stimulated axonal elongation. Graphs showing the effect of the antisense oligonucleotide ASKIF4a (5 μM) on axonal elongation in neurons cultured in the presence or absence of the L1-Fc chimera. For this experiment the L1-Fc chimera (5 μg/ml) was added to the tissue culture medium of sense- or ASKIF4a-treated 1-d-old hippocampal cell cultures. 24 h later, the cells were fixed and the length of axons and minor neurites quantified using the morphometric menu of the Metamorph. Groups: Control, nontreated neurons cultured on polylysine alone for 2 d; Sense + L1-Fc chimera, neurons cultured for 2 d with a sense oligonucleotide plus the L1-Fc chimera; Antisense, neurons cultured on polylysine alone for 2 d in the presence of the ASKIF4a oligonucleotide; and Antisense + L1-Fc chimera, neurons cultured for 2 d with the ASKIF4a oligonucleotide plus the L1-Fc chimera. Note that the L1-Fc chimera selectively stimulates axonal elongation in sense-treated neurons. A similar effect was observed when the L1-Fc chimera was added to nontreated cultures (data not shown). In contrast, the ASKIF4a oligonucleotide completely prevents this response. However, the ASKIF4a oligonucleotide has no effect on axonal elongation in neurons, growing on polylysine alone. Each value represents the mean ± SEM. A total of 100 cells was analyzed for each experimental condition. Asterisks indicate values significantly different from those of control or ASKIF4a-treated cultures.

with any other reported sequence, including other members of the kinesin superfamily. In addition, none of the S-modified antisense oligonucleotides used in this study contains four contiguous guanosines residues, which are believed to increase oligomer affinity to proteins and hence generate nonspecific antisense inhibitory effects (Morfini et al., 1997). Second, the antisense oligonucleotide treatment significantly reduces KIF4 protein levels without altering the levels of several other proteins, including tubulin, uKHC, L1, TAX-1, βgc, etc. Third, the effects of the antisense oligonucleotides are dose-dependent and not observed when the cells are treated with equivalent doses of the corresponding sense oligonucleotides. In addition, they are also observed when the antisense oligonucleotides are used at very low concentrations (2.5 μM), a phenomenon that also contributes to rule out the possibility of nonspecific binding of the oligonucleotides to proteins. Fourth, the antisense treatment does not cause irreversible damage to the cells; hippocampal pyramidal cells recover the normal distribution of L1 after a change to medium free of the antisense oligonucleotide. Fifth, KIF4 suppression selectively alters L1 distribution without affecting the subcellular localization of other growth cone

components such as APP or βgc, which are transported to the neurite terminus by other microtubule-based anterograde motor (Ferreira et al., 1993; Morfini et al., 1997). Conversely, the distribution of L1 is not modified by KIF2 antisense oligonucleotides, a treatment that significantly disrupts the growth cone localization of βgc (Morfini et al., 1997).

Several additional lines of evidence also support the idea that KIF4 may serve as a plus-end motor involved in the anterograde transport of L1-containing vesicles. First, analysis of subcellular fractions obtained by SDG centrifugation of microsomal fractions revealed a striking colocalization of KIF4 with L1. Second, and more importantly, immunoisolation experiments allow us to isolate the KIF4 cargo from other organelles and determine that it contains L1 and a small amount of TAX-1. On the other hand, it lacks uKHC, KIF2, βgc, and synaptic vesicles markers such as synaptotagmin. Third, both KIF4 and L1 are predominantly expressed in developing brain tissue and highly enriched in growth cone membrane preparations obtained from fetal brain tissue. Finally, immunofluorescence studies show that in stage III hippocampal pyramidal neurons, both proteins predominantly localize to ax-

onal shafts and their growth cones (Van den Pol and Kim, 1993; Jareb and Banker, 1998; Kamiguchi and Lemmon, 1998; this study). In this regard, it is important to note that a small amount of both proteins exists in minor neurites of stage III hippocampal pyramidal neurons. Although the reasons for this are not clear at present, it is possible that they reflect the potential of all minor neurites to become an axon (Dotti and Banker, 1987; Esch et al., 1999) and/or that the sorting machinery is still not fully developed in young neurons (Bradke and Dotti, 1997).

On the other hand, it is unlikely that the preferential localization of KIF4 to axons is the result of bulk cytoplasmic flow. Such a mechanism has been proposed recently for explaining a higher amount and transport of membrane-bound organelles to the axon during the initial establishment of polarity (Bradke and Dotti, 1997). The predominant axonal localization of KIF4, as opposed to the widespread distribution of uKHC, argues against bulk flow being a major single determinant of its subcellular localization. Several additional mechanisms could account for the axonal enrichment of KIF4 in developing neurons. As recently discussed for KIF21B, a plus-end–directed motor enriched in dendrites (Marszalek et al., 1999), this may include differences in protein stability between axons and minor processes, as well as interactions between KLPs and structural microtubule-associated proteins that may differentially affect attachment/detachment of motors with microtubules (Ebneth et al., 1998; Trinczek et al., 1999). The axonal localization of KIF4 may provide an excellent opportunity to examine these possibilities.

KIF4 and the Development of Neuronal Polarity

KIF4-suppressed neurons that grow on polylysine alone extend axons that are almost identical to those of control neurons in terms of their morphological appearance and length (nontreated or sense-treated). Although these observations indicate that KIF4 as well as the components of its cargo are not essentially required for axonal development, they do not preclude a role for KIF4 in the generation of neuronal polarity. In fact, several of our observations suggest quite the opposite. For example, the preferential axonal localization of KIF4 and the consequences of its suppression on the distribution of L1, an early and well-established marker of axonal differentiation (Van den Pol and Kim, 1993; Jareb and Banker, 1998; this study), raise the possibility of KIF4 having a specific and important contribution to the generation of polarity. Thus, by transporting L1-containing vesicles to the axon, KIF4 contributes to the establishment of a clear distinction between the axonal membrane and that of the remaining minor processes. Moreover, by mediating the transport of its cargo to the axonal growth cone, KIF4 is likely to play an equally critical role in regulating the outgrowth response of axons to growth-promoting substances, such as cell adhesion molecules. In favor of this possibility, we have shown here that KIF4-suppressed neurons are not capable of exhibiting an enhanced axonal outgrowth response when exposed to a soluble chimeric form of the L1 glycoprotein. The most likely explanation for such an inhibitory effect is a lack of homophilic binding of L1 within the axonal growth cone to soluble L1 (Neu-

gebauer et al., 1988; Seilheimer and Schachner, 1988; Doherty et al., 1995) due to a blockade of its anterograde transport. Obviously, we cannot discard the possibility that other as-yet unknown components of the KIF4 cargo are also required for this response.

In any case, our results suggest a highly specialized role for KIF4 during neuronal polarization. In this regard, KIF4 seems to be different from other anterograde microtubule-based motors mediating the transport of membranous organelles in developing neurons. For example, uKHC appears to be involved not only in the transport of axonal proteins such as synaptophysin and GAP43 (Ferreira et al., 1992), but also in the trafficking of APP, a protein that first appears axonally and later localizes to the somatodendritic domain (Ferreira et al., 1993; Tienari et al., 1996). In addition, and consistent with its widespread subcellular localization and association with different classes of membranous organelles (Ferreira et al., 1992; Feiguin et al., 1994; Nakata and Hirokawa, 1995; Tanaka et al., 1998), suppression of uKHC in cultured hippocampal pyramidal neurons has a general inhibitory effect on neurite outgrowth (Ferreira et al., 1992). Other KLPs appear to have more unique functions, but in mature neurons. Thus, KIF1A, a microtubule-based motor that transports the synaptic vesicle precursor to the axon, does not appear to be essentially required for its transport during the initial establishment of neuronal polarity (Yonekawa et al., 1998). In cultured hippocampal pyramidal neurons, KIF1A expression increases 8 d after plating, well after synapse formation and the consequent accumulation of synaptic vesicle proteins. In addition, neither axonal nor dendritic formation is impaired in cultured hippocampal pyramidal neurons from KIF1A knockout mice. Interestingly, after 8 d in culture, at which time the level of KIF1A expression in wild-type mice increases, neuronal cell death begins in the KIF1A mutants (Yonekawa et al., 1998). Future studies leading to the identification of the cargo transported by other KLPs will certainly allow us a better understanding of the transport events involved in the generation and maintenance of neuronal polarity.

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