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The Neuron-Specific Kinesin Superfamily Protein KIF1A Is a Unique Monomeric Motor for Anterograde Axonal Transport of Synaptic Vesicle Precursors

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Summary

Axonal transport has been intensively examined as a good model for studying the mechanism of organelle transport in cells, but it is still unclear how different types of membrane organelles are transported through the nerve axon. To elucidate the function of this mechanism, we have cloned KIF1A, a novel neuron-specific kinesin superfamily motor that was discovered to be a monomeric, globular molecule and that had the fastest reported anterograde motor activity (1.2 μm/s). To identify its cargo, membranous organelles were isolated from the axon. KIF1A was associated with organelles that contained synaptic vesicle proteins such as synaptotagmin, synaptophysin, and Rab3A. However, this organelle did not contain SV2, another synaptic vesicle protein, nor did it contain presynaptic membrane proteins, such as syntaxin 1A or SNAP-25, or other known anterograde motor proteins, such as kinesin and KIF3. Thus, we suggest that the membrane proteins are sorted into different classes of transport organelles in the cell body and are transported by their specific motor proteins through the axon.

Introduction

The nerve axon has been a good model system for studying the mechanism of organelle transport in cells. By fast axonal transport, membranous organelles are bidirectionally transported through axons at 0.5–1.5 μm/s. This transport is essential for neurons, because an axon lacks protein synthesis machinery. The anterograde transport (from cell body to axon terminus) supplies materials consumed in both the axon and the synapse. Various types of membranes and membrane proteins, such as synaptic vesicles (SVs) and presynaptic plasma membranes (PMs), are transported, presumably in precursor forms. Kinesin (Brady, 1985; Brady et al., 1990; Hirokawa et al., 1991; Niclas et al., 1994; Vale et al., 1985a, 1985b) was identified as a candidate for the motor molecule of this transport. Now, it is widely accepted that kinesin is a motor for anterograde transport.

However, it is still questionable whether the complex anterograde transports of various membrane organelles are solely dependent on kinesin. First, some membrane organelles move very fast (~1.5 μm/s) in the axon. The velocity of these organelles is too fast compared with that of kinesin (~0.5 μm/s), even in the assays, to minimize the possible artifacts (Berliner et al., 1994). Secondly, kinesin is not abundantly detected in the axon. Immunofluorescent studies do detect kinesin in the axon, but staining there was weaker than in the cell body (Hollenbeck, 1989; Niclas et al., 1994; Pfister et al., 1989). Lastly, kinesin-deficient mutants of Drosophila (Gho et al., 1992) or Caenorhabditis elegans (Patel et al., 1993) do not show significant perturbation in their supplies of SVs. Hence, we consider that other motors exist, aside from kinesin, for anterograde axonal transport.

Recent molecular biological analyses revealed that many different classes of kinesin superfamily proteins (KIFs) exist, although their functions are still poorly understood (Endow, 1991; Goldstein, 1993; Hirokawa, 1993a, 1993b; Hoyt et al., 1993). The majority of KIFs characterized to date appear to be involved in force-generating events in mitosis and meiosis (Endow, 1991; Goldstein, 1993; Hoyt, 1994). On the bases of both the complexity of the axonal transports and the multiplicity of cross bridge structures between membranous organelles and microtubules (Hirokawa, 1982), we surmised that several types of KIFs would exist in neurons and that they would serve as axonal transport motors. In fact, some KIFs are supposed to participate in axonal transport of organelles (Hirokawa, 1993a, 1993b; Kondo et al., 1994; Noda et al., 1995; Pesavento et al., 1994; Sekine et al., 1994). For example, *unc-104* mutants of C. elegans show deficiencies in SV biogenesis (Hall and Hedgecock, 1991). As this locus encodes a KIF (Otsuka et al., 1991), the *unc-104* gene product (UNC-104) is hypothesized to participate in SV precursor transport. We also isolated five KIFs (KIF1 to KIF5) by systematic screening of murine brain cDNA (Aizawa et al., 1992). Among these KIFs, brain kinesin (KIF5), KIF3, and KIF1A were abundantly or specifically expressed in neurons (Aizawa et al., 1992). We hypothesized that they serve as the axonal transport motors; each carries different types of organelles, with some potential redundancy.

In this paper, we report on KIF1A. Biochemical and biophysical analyses revealed that it is a unique monomeric, globular motor protein. It was the fastest anterograde motor, a motor whose velocity agrees well with that of the fastest organelles in the axon. Furthermore, immunoisolation of axonally transported organelles from cauda equina demonstrated the existence of at least two different types of transport vesicles in the axon. One class of organelles contained various SV proteins, such as synaptophysin, synaptotagmin, and Rab3A. KIF1A was associated with these organelles, and it might serve as the motor for their transport. Other known anterograde motor proteins, such as kinesin and KIF3, were not detectable on these organelles. They would transport other organelles that are associated with another SV protein (SV2) and PM proteins, such as syntaxin 1A and SNAP-25. These results suggest that the membrane proteins are sorted into different classes of transport organelles in the cell body and are carried by their specific motor proteins through the axon.

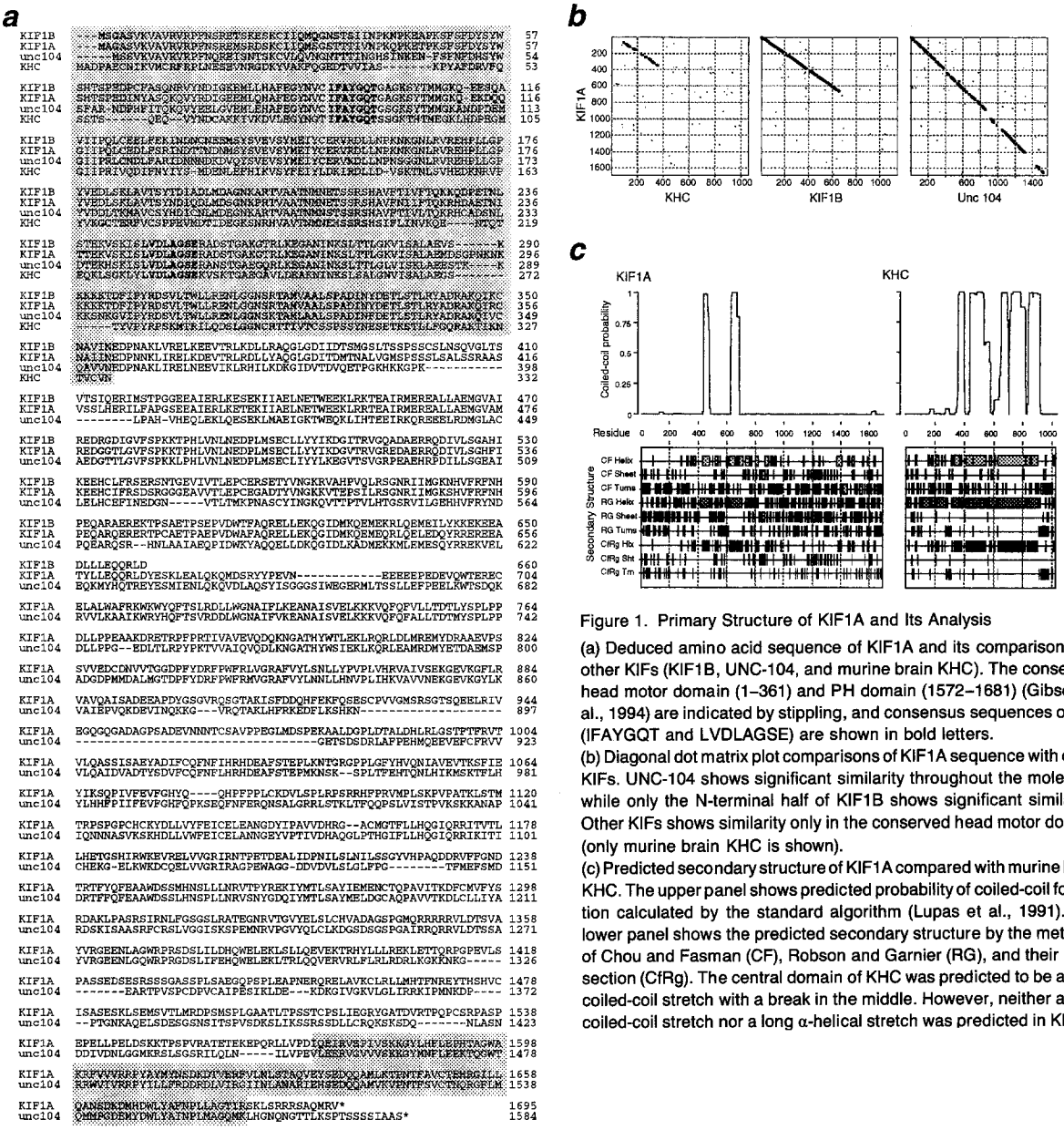

Figure 1. Primary Structure of KIF1A and Its Analysis

(a) Deduced amino acid sequence of KIF1A and its comparison with other KIFs (KIF1B, UNC-104, and murine brain KHC). The conserved head motor domain (1–361) and PH domain (1572–1681) (Gibson et al., 1994) are indicated by stippling, and consensus sequences of KIF (IFAYGQT and LVDLAGSE) are shown in bold letters.

(b) Diagonal dot matrix plot comparisons of KIF1A sequence with other KIFs. UNC-104 shows significant similarity throughout the molecule, while only the N-terminal half of KIF1B shows significant similarity. Other KIFs shows similarity only in the conserved head motor domain (only murine brain KHC is shown).

(c) Predicted secondary structure of KIF1A compared with murine brain KHC. The upper panel shows predicted probability of coiled-coil formation calculated by the standard algorithm (Lupas et al., 1991). The lower panel shows the predicted secondary structure by the methods of Chou and Fasman (CF), Robson and Garnier (RG), and their intersection (CfRg). The central domain of KHC was predicted to be a long coiled-coil stretch with a break in the middle. However, neither a long coiled-coil stretch nor a long α-helical stretch was predicted in KIF1A.

Results

Analysis of the Primary Structure of KIF1A

As reported previously (Aizawa et al., 1992), we have isolated five cDNA fragments of KIFs (KIF1 to KIF5) by polymerase chain reaction (PCR) amplification of a murine brain cDNA library. Using the KIF1 cDNA fragment as a probe, we screened the cDNA library and isolated several clones. Some clones were identical to the probe, while others were highly homologous but not identical. Therefore, we named the latter clones KIF1B (Nangaku et al., 1994) and renamed the former KIF1 clones KIF1A. Using the region unique to KIF1A as a probe, we rescreened the cDNA library and isolated several independent KIF1A cDNA clones.

Nucleotide sequence analysis of these clones revealed that the longest one contained a single 5085 nt open reading frame encoding 1695 amino acid residues (estimated M_r = 191,710; Figure 1a) with the conserved motor domain in its N-terminus. A homology search of the GenBank data base revealed that only KIFs showed significant similarity to KIF1A and that KIF1A was most similar to UNC-104 (Otsuka et al., 1991) and KIF1B.

The diagonal dot matrix comparison of KIF1A with other KIFs (Figure 1b) and multiple alignment and phylogenetic analysis of conserved motor domain (Sekine et al., 1994) confirmed that KIF1A belongs to the UNC-104-type KIF subfamily along with KIF1B and UNC-104. Among them, KIF1B was most homologous to KIF1A, with 93.2% similarity in its N-terminal half (1–650), suggesting that they share common properties in their motor activities, although their C-terminal halves showed no significant similarity. In con-

trast, UNC-104 showed 71.0% similarity throughout the whole molecule. Therefore, we consider that KIF1A is the murine homolog of UNC-104 and that KIF1B is closely related to KIF1A/UNC-104 but is not their homolog.

Regions in the C-terminal halves of KIF1A and UNC-104 were highly conserved (>80% similarity; Figure 1b), but were very different from those of KIF1B, an anterograde motor for mitochondria transport in the axon (Nangaku et al., 1994). Hence, we hypothesize that those C-terminal regions serve in specific binding to the cargo membrane. Among those regions, a pleckstrin homology (PH) domain (Gibson, et al., 1994) at the C-terminus of KIF1A/UNC-104 would be a good candidate for cargo binding or its regulation.

KIF1A is a Monomeric, Globular Molecule

All KIFs already reported are composed of three domains: a globular motor domain, a coiled-coil stalk domain, and a globular tail domain (Chandra et al., 1993; Hirokawa et al., 1989; Kondo et al., 1994; Sekine et al., 1994; Yang et al., 1989). Also, they form dimers through their stalk domain (de Cuevas et al., 1992; Hirokawa et al., 1989; Huang et al., 1994; Sekine et al., 1994; Yang et al., 1989). This stalk domain corresponds well with the region predicted to form the coiled-coil structure. Most KIFs whose sequences are already reported are predicted to have a long stretch of coiled-coil stalk and are thought to form dimers through this region. The exceptions are the UNC-104 subfamily KIFs (KIF1A, KIF1B, and UNC-104) and nod. They were not predicted to have either a long α-helical stretch or a long coiled-coil stretch. For example, KIF1A had only two short segments that were predicted to form α-helical or coiled-coil structures (Figure 1c). From this prediction, the structure of these molecules would be quite different from other KIFs: they might lack the long stalk region. There is a possibility that they might be monomeric, globular molecules.

This prediction was partially confirmed with KIF1B (Nangaku et al., 1994), which was concluded to be a monomeric, globular protein. However, some ambiguities in the determination of its molecular weight and its molecular shape still remain. Furthermore, the studies with KIF1B do not imply that KIF1A is also a monomeric, globular motor, because the C-terminal halves of the two proteins are very different. Therefore, rigorous physicochemical analyses on the molecular structure of KIF1A are important.

Such analyses require concentrated, highly purified, active KIF1A protein. Therefore, we expressed recombinant KIF1A protein by a baculovirus–insect cell expression system. The polyhistidine tag introduced at its C-terminus enabled us to purify the recombinant protein by immobilized metal affinity chromatography (Figure 2a). The fraction thus derived (lane 5 in Figure 2a) contained 0.5–1.0 mg/ml of >95% pure KIF1A (most contaminants are the degradative products). All the assays described below were performed with fresh samples to minimize denaturation by storage.

First, we performed low angle rotary-shadowing electron micrography (EM) to analyze the single molecular structure of the purified recombinant KIF1A protein. Unlike other KIFs, KIF1A was a globular molecule with a diameter of 14 nm (Figures 2b and 2c). No clearly discernable tails were observed. This observation was also confirmed by negative staining EM (data not shown).

Second, the molecular size of the recombinant KIF1A molecule was determined by several independent methods. From sucrose velocity gradient centrifugation (SVG; Figure 2g) and gel permeation chromatography (GPC; Figure 2d, DRI trace), its sedimentation coefficient and its Stokes' radius were determined as 6–7 S and 6–7 nm, respectively. The size estimated from this Stokes' radius (12–14 nm in diameter) was in good agreement with the size determined by EM. From these values, the M_r was calculated to be 220–280 kDa, suggesting that KIF1A is a monomer.

However, the resolution of the SVG is limited, and the size estimation by GPC can be biased by the shape of the molecule or its affinity to the gel matrix. Therefore, we further analyzed GPC fractions by differential light scattering (DLS). Unlike other methods, the GPC–DLS method absolutely determines the M_r of the polymer, unbiased by its shape or other properties (Wyatt, 1993). DLS itself is a very sensitive and accurate method for M_r measurement, and GPC effectively eliminates contaminating dusts and aggregates that interfere with the M_r measurement by DLS. That is, GPC is only used as a prefilter for DLS, and the M_r is measured absolutely and directly from DLS results, unbiased by the possible errors in the size estimation by GPC. As shown in Figures 2d–2f, the M_r of recombinant KIF1A was determined to be 180–220 kDa, in good agreement with the value determined by SVG–GPC. These results rigorously demonstrate that KIF1A is a monomeric protein. Furthermore, the frictional ratio was calculated as 1.1–1.4 from this M_r and the sedimentation coefficient by SVG, indicating that KIF1A is a globular protein as demonstrated by EM.

Thus, recombinant KIF1A is firmly demonstrated to be a monomeric globular protein, but what about native KIF1A? To answer this question, we analyzed the molecular structure of native KIF1A by using the recombinant protein as a marker. By both SVG and native polyacrylamide gel electrophoresis (PAGE), no difference between native and recombinant KIF1A was detected (Figures 2g and 2h). This suggests that the native KIF1A molecule is also a monomeric globular protein. Indeed, the possibility was not completely excluded that native KIF1A is a heterooligomer with very small light chains, but at least it is not associated with the large subunit and is very unlikely to contain more than one motor domain.

KIF1A Is the Fastest Anterograde Motor

Most KIFs are dimeric motors, and it is controversial whether dimerization is essential for the motor function (Hackney, 1994). Therefore, it is an important issue to characterize the motor properties of this monomeric motor, KIF1A.

For that purpose, an in vitro motility assay was performed. Purified recombinant KIF1A (0.1 μg = 3×10^{11} molecules) was absorbed on the surface of the cover glass

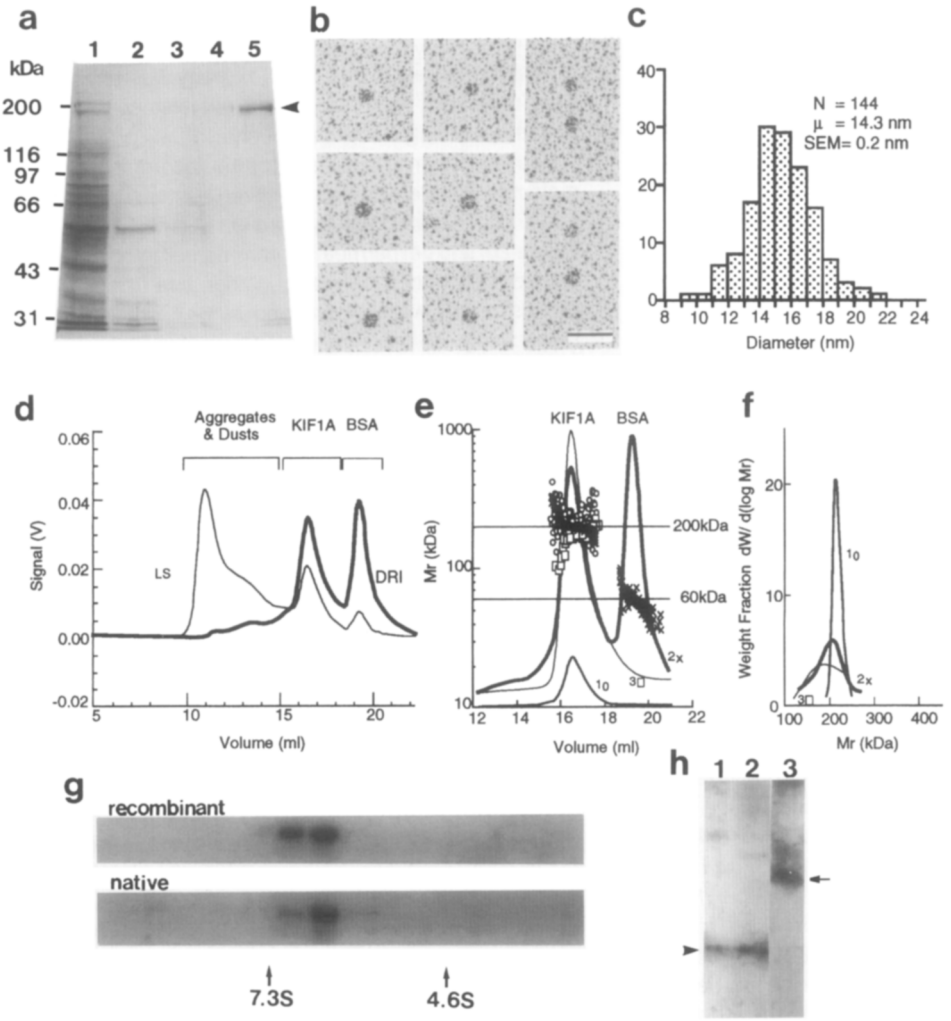

Figure 2. Physicochemical Characterization of the Molecular Structure of KIF1A

(a) Purification of recombinant KIF1A protein. Crude extract of insect cells infected with recombinant baculovirus (lane 1) was applied to immobilized Ni^{2+} column. Lanes 2, 3, and 4 are flowthrough and two wash fractions, respectively. Lane 5 shows the eluent. This fraction was used in the following assays.

(b) Low angle rotary-shadowing EM of recombinant KIF1A. Unlike other KIFs, it is a globular, tailless molecule. Scale bar, 50 nm.

(c) Size distribution of recombinant KIF1A observed by EM. The diameter of recombinant KIF1A was 14.3 nm ± 0.2 nm (mean ± SEM, N = 144).

(d–f) M_r determination by GPC–DLS. Typical light scattering chromatogram at 90° (LS) is contrasted with differential refractive index (DRI) signal (d). In this sample, bovine serum albumin (BSA) was added as an internal control. Calculated M_r of the solute in each fraction is overlaid on the DRI chromatogram (e). Results of three experiments are shown. These data are converted into differential M_r distribution (f), clearly showing that the M_r of recombinant KIF1A is ~200 kDa and that it is a monomer.

(g) SVG of recombinant (upper panel) and native (lower panel) KIF1A. Arrows show the peak position of aldolase (7.3 S) and BSA (4.6 S).

(h) Native PAGE of native (lane 1) and recombinant (lane 2) KIF1A. Kinesin (380 kDa, lane 3) is shown for comparison. Both SVG and native PAGE indicate that native KIF1A is of similar size to the recombinant KIF1A. That is, native KIF1A would be also a monomer.

($3\ cm^2 = 3 \times 10^8\ \mu m^2$) and was washed with motility buffer. Only 5%–10% of KIF1A remained on the cover glass surface, and 90%–95% of KIF1A was recovered in this wash fraction. Thus, the density of KIF1A molecule on the cover glass surface was at most 100 molecules/μm^2. On this surface, more than 90% of the microtubules moved smoothly and unidirectionally at 0.9–1.5 μm/s, more than twice as fast as conventional kinesins (Figures 3a and 3c). This speed is the fastest among those already reported for kinesins and KIFs and is the only value that corresponds well with the speed of the fastest organelles in the intact axon.

Along with all the N-terminal motor domain–type KIFs already reported, KIF1A was an anterograde, plus end–directed motor. More than 95% of Chlamydomonas axonemes moved smoothly on the KIF1A lawn toward their compact (minus) ends at 0.6–1.2 μm/s, and the rest showed no movement (Figure 3b).

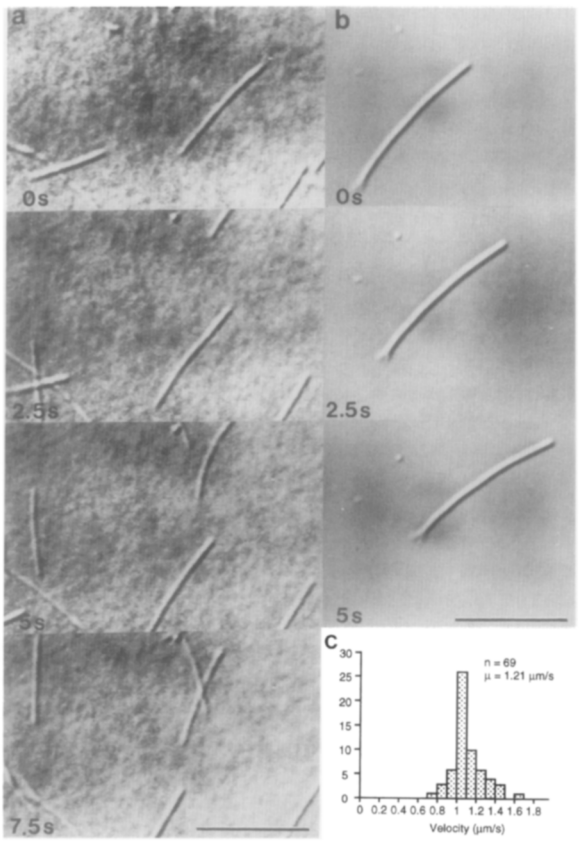

Figure 3. In Vitro Motility Assay of Recombinant KIF1A

Movement of microtubules (a) and axoneme (b) is shown. Microtubules moved smoothly at 0.5–1.5 μm/s (c), and almost all axonemes moved toward their compact (minus) ends. Consequently, KIF1A is the fastest anterograde (plus end–directed) motor. Scale bar, 10 μm.

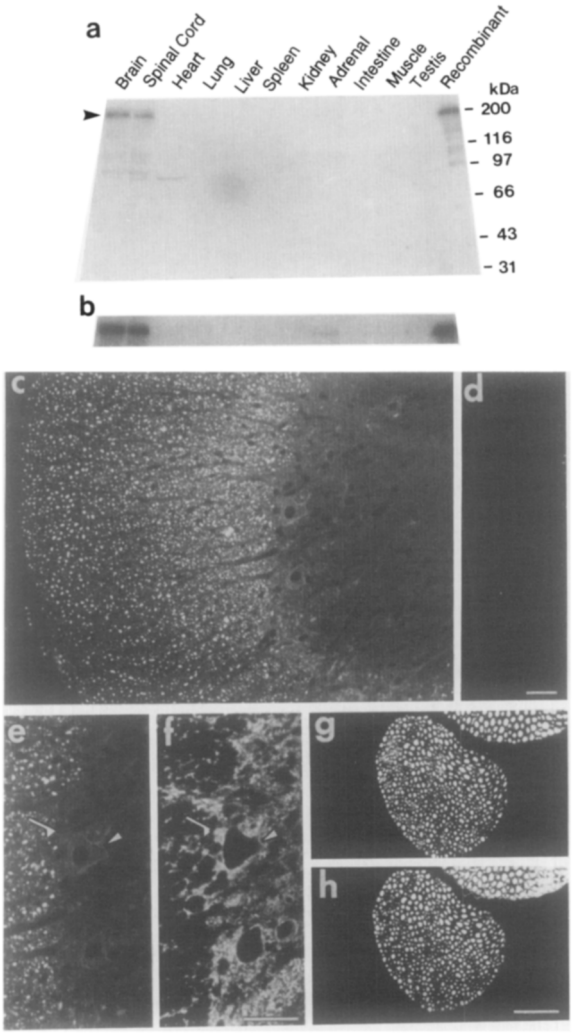

Figure 4. Localization of KIF1A

(a) Tissue distribution of KIF1A. Proteins (20 μg) extracted from adult mouse tissues were analyzed by immunoblotting with anti-KIF1A antibody. Purified recombinant KIF1A is loaded for comparison (recombinant lane).

(b) shows its longer exposure. KIF1A was abundantly expressed in brain and spinal cord and faintly in adrenal gland, confirming our previous result (Aizawa et al., 1992) that KIF1A is specifically expressed in neuronal tissues.

(c–h) Localization of KIF1A in neuron. Adult mouse spinal cord was cross-sectioned and was stained with anti-KIF1A antibody. (c) shows lower magnification view of anterior horn and ventrolateral column. All axons in the white matter were strongly stained, but only faint staining was detected in gray matter. (d) shows negative control with control antibody. (e) and (f) show higher magnification view of anterior horn. Anti-synaptophysin antibody (f) strongly stained synapses (arrow) around cell bodies of motor neurons (arrowhead), while these structures were only faintly stained by anti-KIF1A antibody (e). Only axons in the white matter were strongly stained by this antibody. (g) and (h) show cross-sectioned axons in the sciatic nerve. Axoplasm was stained with anti-phosphorylated neurofilament-H antibody (h). The staining with anti-KIF1A antibody (g) showed clear overlap with this axoplasm staining, indicating that KIF1A is abundantly expressed in all axons. Scale bars, 100 μm.

KIF1A Is Specifically Expressed in Neurons and Is Enriched in Axons

Many KIFs have been identified by homology cloning, and each single cell expresses several different classes of KIFs (Aizawa et al., 1992). If these various classes of KIFs are considered to play different roles, then what is the function of KIF1A?

KIF1A mRNA localizes specifically in neurons (Aizawa et al., 1992), while other KIFs abundant in adult neurons, such as conventional kinesin (Hollenbeck, 1989; Niclas et al., 1994) and KIF3 (Aizawa et al., 1992), are also expressed in other tissues. Thus, KIF1A is supposed to have some neuron-specific roles. To specify the KIF1A function, we raised KIF1A-specific antibody and analyzed its localization in vivo.

Western blot analysis using this antibody detected a single, or occasionally a doublet, band around 200 kDa in neuronal tissues (brain and spinal cord) and very faintly in adrenal gland (Figures 4a and 4b). Note that this antibody showed no cross-reactivity to spectrin or other PH domain–containing proteins, which guarantees the specificity of this anti-KIF1A PH domain antibody. Thus, KIF1A protein is specifically expressed in neuronal tissues.

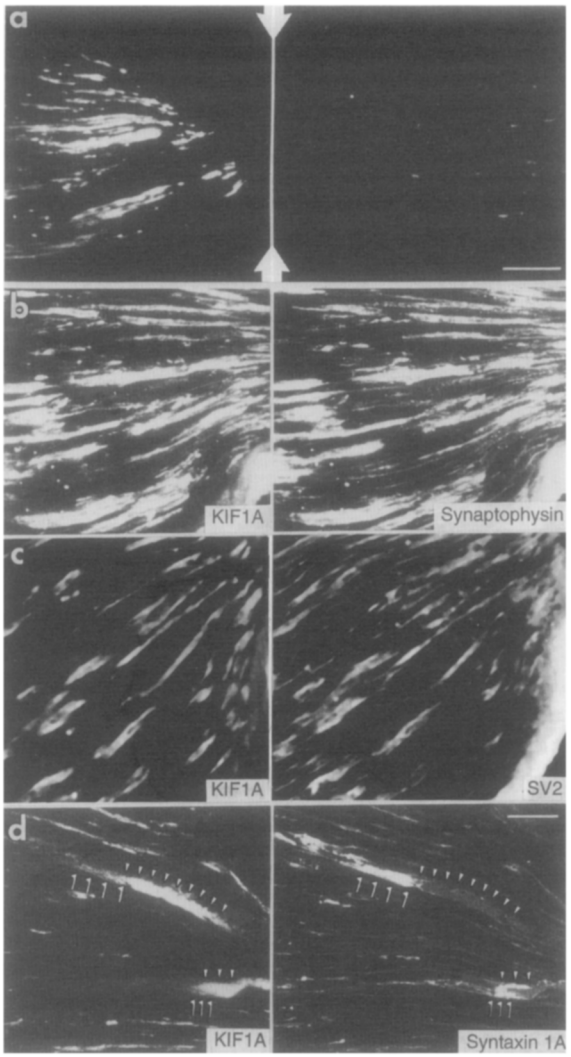

Figure 5. Association of KIF1A with Anterogradely Transported Organelles in the Axon

(a) Ligated sciatic nerve was stained with anti-KIF1A antibody. After 1 hr ligation, KIF1A significantly accumulated at the proximal region (left panel) of ligation, while almost no accumulation was observed at the distal region (right panel). Scale bar, 50 μm. Arrows show the position of ligation. In this experiment, anti-KIF1A antibody was used at higher dilution than in Figure 4c to contrast with the accumulation of KIF1A. Some axons appear to lack KIF1A staining, but this is due to bad penetration of antibody through the myelin sheath in the longitudinal sections of axons. All axons show significant accumulation when observed in the cross-sectioned sample (data not shown).

(b–d) Higher magnification optically sectioned view of the proximal region double stained with anti-synaptophysin antibody (b), anti-SV2 antibody (c), and anti-syntaxin 1A antibody (d). Orientation is the same as the left panel of (a). Right panels show anti-synaptophysin, anti-SV2, or anti-syntaxin 1A staining, and left panels show anti-KIF1A staining. Synaptophysin and SV2 apparently colocalized well with KIF1A, while syntaxin 1A (arrows) accumulated more distally than KIF1A (arrowheads) with some overlap. These results show that these membrane proteins are anterogradely transported in the same axons where KIF1A is moving anterogradely. The differential accumulation pattern of syntaxin 1A and KIF1A suggests that syntaxin 1A might not be transported by KIF1A, but it should be noted that the apparent colocalization of KIF1A with synaptophysin or SV2 does not necessarily mean that they are transported on the same organelles. Scale bar, 25 μm.

To analyze the localization of KIF1A in vivo, we stained murine neuronal tissues with anti-KIF1A antibody. As shown in Figures 4c–4h, axons were strongly stained. Perinuclear and synaptic regions were also stained, but much weaker than the axons (Figures 4e and 4f). Therefore, KIF1A is suggested to play its role in the axon, a neuron-specific structure. All the cross-sectioned axons in the white matter of spinal cord or sciatic nerve were equally strongly stained (Figures 4c–4h), suggesting that the function of KIF1A is not restricted to some specific class of axon. KIF1A is expressed in all axons and plays its role there.

KIF1A Is Associated with Some Class of Anterogradely Transported Organelles in the Axon

What then is the role of KIF1A in the axon? Its motor activity suggests that it serves as a motor for the fast anterograde axonal transport. This was confirmed by an axonal ligation experiment (Hirokawa et al., 1991; Smith, 1980). A mouse was sacrificed 1 hr after ligation of its sciatic nerves. Thus, organelles transported anterogradely or retrogradely by fast axonal transport were accumulated at the proximal or distal regions to the ligation, respectively. Stationary or slowly transported organelles do not accumulate on either side. This sample was stained with anti-KIF1A antibody. As shown in Figure 5a, gradational staining was observed on the proximal side (left panel), becoming stronger in the regions closer to the ligated portions. In contrast, staining in regions distal to the ligated parts was significantly weaker (Figure 5a, right panel). In control samples, preabsorbed antibody supplemented with preimmune immunoglobulin G (IgG) was used as the primary antibody, and only much fainter background staining occurred. This means that the KIF1A in the axon is moving fast in the anterograde direction, strongly suggesting that it serves as a fast anterograde transporter. The lack of significant accumulation at the distal regions means that none or only very small fraction of KIF1A is transported retrogradely. Considering that KIF1A is not enriched in the synapse, KIF1A may be degraded there after traveling down the axon.

Which organelles are transported by KIF1A? To answer this question, we performed double staining of accumulated organelles at the proximal end of ligation. For this purpose, antibodies to several SV proteins (SV2, synaptotagmin, and synaptophysin) and a PM protein (syntaxin 1A) were used. All of them showed clear accumulation on the proximal side (Figures 5b–5d). No, or only weak, accumulation was observed on the distal side (data not shown). Thus, the major population of these proteins is demonstrated to be transported anterogradely.

All these markers accumulated in the same axon in which KIF1A showed accumulation, but did not necessarily colocalize perfectly with KIF1A. For example, synaptophysin (Figure 5b), SV2 (Figure 5c), and other SV proteins appeared to colocalize with KIF1A, but syntaxin 1A accumulated at more proximal regions than KIF1A, with some overlap (Figure 5d). This suggests that KIF1A is not associ-

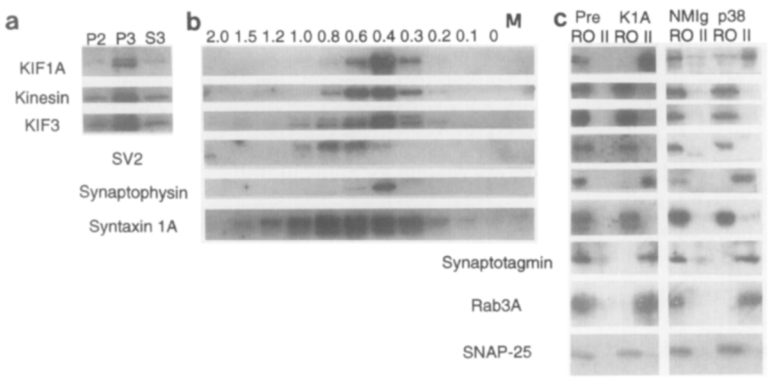

Figure 6. Analysis of Axonally Transported Organelles

(a) Fractionation by differential centrifugation. KIF1A, kinesin, and KIF3 were mostly (>80%) recovered in the P3 fraction, and only a very small fraction (<5%) was recovered in the P2 or S3 fraction. This means that most of these motors are associated with small membranous organelles, but not or only barely associated with larger membranous organelles such as the mitochondria recovered in the P2 fraction. (b) Fractionation by isopicnic SDG. KIF1A, kinesin, and KIF3 were recovered in 0.3–0.6 M sucrose fractions. Synaptophysin was also recovered in these fractions, while SV2 was recovered in denser fractions (0.4–1.0 M). Syntaxin 1A was recovered as a broad peak. This suggests that synaptophysin, SV2, and syntaxin 1A are transported in different cargoes. (c) Immunoisolation with anti-KIF1A (K1A) or anti-synaptophysin (p38) antibody. With anti-KIF1A or anti-synaptophysin antibody, KIF1A, synaptotagmin, and synaptophysin were quantitatively recovered in immunoisolated (II) fraction. This immunoisolated fraction abundantly contained Rab3A, while kinesin, KIF3, syntaxin 1A, SNAP-25, and SV2 remained on the remaining organelles (RO). These proteins were not recovered in II fraction with preimmune (Pre) or nonspecific mouse IgG (NMIg).

ated with syntaxin 1A–containing organelles. They might be transported by a slower motor than KIF1A.

KIF1A Is Associated with Synaptophysin-Containing Membrane Organelles in the Axon

From the results of the ligation experiments, it was surmised that KIF1A might transport SV-precursor rather than PM-precursor organelles. However, the resolution of light microscopy is limited. It is impossible to conclude that synaptophysin, SV2, and KIF1A are transported on the same membrane organelles. There is a possibility that they are on different organelles that are transported at similar velocities and, thus, accumulate similarly. To clarify this point, we performed biochemical analyses of axonally transported organelles collected from adult rat cauda equina.

First, they were roughly fractionated by differential centrifugation (Figure 6a). Nearly 90% of KIF1A was recovered in the P3 (high speed pellet) fraction, and about 80% of both kinesin and KIF3 was also recovered in this fraction. Only <10% was detected in the P2 (medium speed pellet) and S3 (high speed supernatant) fractions.

This P3 fraction was further fractionated by isopicnic sucrose density gradient centrifugation (SDG). Organelles were pelleted from these fractions and were analyzed by immunoblotting. None of the proteins probed here were detected in the supernatant, confirming that they were not dissociated by this fractionation. Most organelles were collected in a single broad peak (Figure 6b). Syntaxin 1A (HPC-1 antigen), a presynaptic PM integral protein (Bennett, et al., 1992), was recovered in the broad peak (0.2–1.5 M sucrose fractions), while SV2, a SV membrane integral protein (Buckley and Kelly, 1985), was concentrated there in denser fractions (0.4–1.0 M). Synaptophysin, another SV membrane integral protein (Jahn et al., 1985; Wiedenmann and Franke, 1985), was in lighter fractions (0.3–0.6 M) in which KIF1A was also recovered. These results suggest that there exist at least two different classes of organelles that contain SV membrane integral proteins. One contains SV2, and the other contains synaptophysin. The former is unlikely to be transported by KIF1A. KIF1A is a good candidate for the motor of the synaptophysin-containing organelles; however, from this fractionation, definite conclusions cannot be drawn on the motor for these organelles, because the synaptophysin-enriched fractions abundantly contained other KIFs (kinesin and KIF3). It was unclear whether these KIFs are all on the same organelle or whether they are on different organelles with similar density. The relation between syntaxin 1A–containing organelles and the two classes of organelles containing SV membrane proteins was also unclear.

To clarify these points, immunoisolation of organelles from the S2 fraction was performed (Figure 6c). KIF1A-containing organelles were isolated by beads conjugated with anti-KIF1A antibody (Figure 6c, lanes K1A). The remaining organelles were recovered by pelleting from the supernatant fraction. With this method, the KIF1A-containing organelles were quantitatively collected. In this immunoisolated organelle fraction, synaptophysin and synaptotagmin were quantitatively recovered. Rab3A was also abundant in this fraction; about half of Rab3A was coimmunoisolated with KIF1A, and the rest remained in the supernatant soluble fraction (data not shown). In contrast, kinesin, KIF3, SV2, syntaxin 1A, and SNAP-25 either were not, or were only barely, detectable in this fraction. These proteins were quantitatively recovered in the remaining organelle fraction. They were not detected in the supernatant fraction after pelleting these remaining organelles, effectively ruling out the possibility that the lack of these proteins in the KIF1A-organelle fraction is due to the dissociation during the immunoisolation process. Control fractions isolated with beads conjugated with preimmune IgG contained only trace amount of proteins (Figure 6c, lanes Pre).

These results clearly and directly demonstrate that KIF1A is associated with a class of membrane organelles that contains SV proteins, such as synaptotagmin, synaptophysin, and Rab3A. These SV proteins are on the same

membrane organelles, because immunoisolation with anti-synaptophysin antibody quantitatively recovered these proteins (Figure 6c, lanes p38). These organelles, putative SV precursors, will be anterogradely transported by KIF1A. Interestingly, these KIF1A-organelles do not contain SV2. SV2 was not recovered on the organelles immunoisolated with anti-KIF1A beads or anti-synaptophysin beads, though synaptic vesicles immunoisolated from synaptosomal membranes with anti-synaptophysin beads abundantly contain SV2 (data not shown). Also, the KIF1A organelles were not associated with PM proteins (syntaxin 1A and SNAP-25). They may be transported on the same or different organelles by motors other than KIF1A.

Discussion

We have cloned KIF1A, a novel neuron-specific kinesin-type motor. We analyzed its molecular structure and its motor activity in vitro. Unexpectedly, KIF1A was discovered to be a monomeric, globular protein. It showed the fastest reported anterograde motor activity. These features of this molecule seems suitable for the study of mechanochemical transduction. Furthermore, we examined its function in vivo by using immunocytochemistry of axons after ligation and immunoisolation of axonally transported organelles. The ligation experiments strongly suggested that KIF1A really works as an anterograde motor for fast membrane organelle transport, and the immunoisolation enabled clear identification of KIF1A cargo. KIF1A was abundantly expressed in all axons, in which it associated with putative SV precursor organelles. These organelles do not contain SV2, syntaxin 1A, SNAP-25, or other KIFs (kinesin and KIF3). This result not only suggests the cargo of KIF1A, but also provides evidence for the sorting and transport mechanism of membrane proteins in the axon. These issues are discussed below.

KIF1A Might Serve as a Model Motor Molecule for the Study of Mechanochemical Transduction

To elucidate the function of the mechanism for mechanochemical transduction in the motor molecule, measurement of a single motor activity is very informative. Kinesin has been precisely analyzed as such a model molecule (Hunt et al., 1994; Malik et al., 1994; Svoboda and Block, 1994; Svoboda et al., 1993). However, there remain ambiguities in the interpretation of the results because kinesin has two heads. The simplest way to overcome this problem is to use a single-headed kinesin; however, despite attempts by several research groups, active recombinant single-headed kinesin constructs have not yet been reported. K339-SP (Stewart et al., 1993) might be a candidate, but nothing has been reported on its molecular structure. KIF1A is a monomeric kinesin-type motor, and it has a highly active motor activity. Under the conditions reported here, each microtubule is estimated to be associated with at most ten KIF1A molecules. Several preliminary experiments support this estimation, and we consider that it would be possible to make precise measurements of the motor activity by a single KIF1A monomer. Such trials are now underway. Furthermore, a fairly large amount of active KIF1A can be purified by the baculovirus–insect cell expression system. This allows us to perform intensive sophisticated biochemical, physicochemical, and structural analyses. Therefore, we consider that KIF1A will serve as the ideal model motor protein to solve the mysteries in the mechanochemical transduction.

Axonally Transported Organelles Are the Better Material for the Analysis of Motor-Cargo Association

Along with its biophysical characterization, we also studied the in vivo function of KIF1A. For those analyses, we used axonally transported organelles collected from cauda equina. In the past, biochemical studies on the subcellular localization of motor proteins, organelles derived from the homogenate of total brain, or cultured cells were analyzed. They contain vast amounts of membranes such as PM, ER, Golgi, or SV. The transport organelles may constitute only a small fraction. Furthermore, as we have demonstrated in the previous studies (Sato et al., 1992; Okada et al., 1995), there exists a decommission mechanism at the axon terminus that dissociates kinesin from its cargo. We consider that these factors caused the discrepancies in the previous studies on the subcellular localization of kinesin. In fact, from the analyses with organelles derived from total brain homogenate, we could only obtain variable results.

Therefore, we used cauda equina organelles. In this way, we can collect transport organelles free from the cell body or the synaptic organelles, because cauda equina does not contain cell bodies or synapses, but instead contains only axons. Furthermore, we screened for a suitable buffer to maximize the recovery of motor proteins in a cargo-bound form. In conventional buffers for subcellular fractionation (0.3 M sucrose buffered with Tris or HEPES), motor proteins gradually dissociated from membranes, and only variable results were obtained. Using IM-D buffer (see Experimental Procedures for the buffer composition), motor proteins remained on organelles even after 24 hr incubation followed by ten times washing. We also noticed that motor proteins, especially KIF1A, were very susceptible to proteolysis; hence, it was essential to supplement the buffer with protease inhibitors. With these improvements, we could reproducibly recover more than 80% of motor proteins in a cargo-bound form. This preparation will serve for many biochemical assays on motor–cargo association.

Immunoisolation Is Necessary for the Fractionation of Axonally Transported Organelles

In our previous paper (Kondo et al., 1994), we tried to identify the KIF3 cargo by SDG fractionation, but as shown in Figure 6b, almost all axonally transported organelles were recovered in the same or adjacent fractions, and they were not well separated. This is not surprising, because anterogradely transported organelles are of similar size and shape (Hirokawa et al., 1991). Thus, these organelles would have similar density and would be unable to fractionate by SDG or SVG.

Therefore, we performed immunoisolation to isolate the

KIF1A cargo from other organelles. Interestingly, this KIF1A cargo contained synaptophysin, synaptotagmin, and Rab3A. SV2, syntaxin 1A, SNAP-25, kinesin, or KIF3 were not detectable on the KIF1A organelles. The lack of coimmunoisolation of these proteins could have many different causes, although most of them are not consistent with the results reported here.

First, there is a possibility that these proteins dissociated from the KIF1A cargo during the immunoisolation process. This is unlikely because the proteins were quantitatively recovered in an organelle fraction pelleted from the flowthrough fraction after immunoisolation. None of them were detectable in the supernatant fraction.

It is also unlikely that the bulk of these proteins are expressed in axons other than those expressing KIF1A and synaptophysin, because KIF1A was equally strongly detected in all axons (Figures 4c–4h), and the proteins undetectable on KIF1A organelles accumulated in the same axons in which KIF1A accumulated (Figure 5d).

Furthermore, this ligation experiment effectively confirmed that all the marker proteins we probed here are anterogradely transported by fast axonal transport. That is, the lack of the coimmunoisolation of SV2 or PM proteins would not be caused by the possibility that the bulk of these proteins are in stationary form or in retrogradely transported form and that the anterogradely transported population is too small to detect by immunoblotting with the antibody we used here.

Thus, it is interpretable from the results of our immunoisolation experiment that KIF1A is associated with a class of organelles that contains SV proteins, such as synaptotagmin, synaptophysin, and Rab3A. SV2, syntaxin 1A, and SNAP-25 are not associated with these KIF1A organelles. They may be transported on another class(es) of organelles through the axon.

The KIF1A organelles were not associated with kinesin or KIF3. Most of these motor proteins were recovered in almost the same fractions with KIF1A organelles in SDG or SVG experiments, suggesting that these motors are also associated with some class(es) of membrane organelles of similar size and density with KIF1A organelles. In fact, immunoisolation with anti-KIF3 antibody isolated membranous organelles (H. Y. et al., unpublished data). The characterization of organelles immunoisolated with anti-kinesin antibody is now underway.

A Model for the Sorting and Transport Mechanism of Membrane Proteins in the Axon and Synapse

The results derived from the analyses of axonally transported organelles suggest a model for the sorting and transport mechanism of membrane proteins in the axon and synapse. Our results show at least two different classes of anterogradely transporting organelles in the axon. SV proteins such as synaptotagmin, synaptophysin, and Rab3A are transported on the same organelles. Interestingly, SV2, another SV protein, is on different organelles. This differential behavior of SV2 is consistent with some previous results describing differential localization of SV2 and synaptophysin in the axon (Mundigl et al., 1993; Schmidle et al., 1991). This differential behavior might reflect the functional difference of these SV proteins: the proteins on KIF1A organelles are all believed to be involved in membrane fusion, while SV2 is a putative vesicular transporter.

Syntaxin 1A and SNAP-25, presynaptic PM proteins, are also on different organelles. From the results of SDG fractionation, syntaxin 1A and SV2 would be on different organelles, but we cannot definitely conclude whether these SV2 organelles are the same or different from syntaxin 1A and SNAP-25 organelles. At least, our results definitely rule out the possibility that membrane proteins in the synaptic region are transported on the same organelles to the synapse where they are sorted to their specific destinations. Membrane proteins are already sorted in the cell body to their specific cargo organelles. These organelles will be transported by their specific motors to their destinations. It should be noted that this idea does not contradict with the widely believed idea that exocytotic–endocytotic cycling at the synapse is essential for the synaptic vesicle biogenesis (Bauerfeind and Huttner, 1993; Matteoli et al., 1992; Régnier-Vigouroux et al., 1991). Rather, we consider that this membrane cycling at the synapse redistributes SV2 and other SV proteins to the same SV.

This model will be tested by further intensive analyses using the experimental paradigm reported here coupled with gene disruption and other techniques. Such an approach will hopefully solve many important questions on membrane dynamics in the neuron.

Experimental Procedures

Cloning of KIF1A and Its Analysis

KIF1A cDNA was cloned and sequenced by standard methods as previously described (Aizawa et al., 1992; Kondo et al., 1994; Nangaku et al., 1994; Sekine et al., 1994). KIF sequences were aligned by a program package Clustal V (Higgins et al., 1992). Diagonal dot matrix plot analysis and secondary structure prediction were performed with MacVector DNA analysis software package (International Biotechnologies, Incorporated). Coiled-coil probability was calculated by the standard algorithm (Lupas et al., 1991).

Purification of Recombinant KIF1A Protein from Insect Cells

Recombinant baculovirus for the expression of KIF1A was obtained as described (Nangaku et al., 1994; Sekine et al., 1994). A polyhistidine tag was introduced at the C-terminus of the molecule. Synthetic oligonucleotide (GATCTGGATCCGCGCAGATGCGGGTCGGGGGTTCT-CATCATCATCATCATCATATGCGGGTCTGAATTC) was inserted at the BglII site (5185), resulting in an additional 12-residue (GGSHHH-HHHMRV) insert at the C-terminus. After infection (66 hr), IPLB-Sf21AE cells were homogenized in 100 mM PIPES (pH 7.2) supplemented with protease inhibitors. The clarified supernatant was applied to an immobilized Ni^{2+} column (chelating Sepharose FF, Pharmacia). The column was washed first with 100 mM PIPES and then with 30 mM imidazole (pH 6.9). Recombinant KIF1A was eluted with 500 mM imidazole (pH 6.6) and was diluted 10-fold with 10 mM imidazole (pH 6.6).

Physicochemical Analyses on the Molecular Structure of KIF1A

Low angle rotary-shadowing EM was performed as described (Hirokawa et al., 1989; Kondo et al., 1994; Nangaku et al., 1994; Sekine et al., 1994). For GPC and GPC–DLS, a Shodex GPC–DLS system (Shoko Company Limited) was used. Separation was achieved with KW804 and KW803 Shodex HPLC columns in imidazole buffer containing 0.01% Triton X-100. DRI and DLS were measured with Shodex RI-71 and DAWN DSP-F (Wyatt Technology), respectively. M_r was

calculated by DAWN software assuming the dn/dc of KIF1A as 0.18. SVG and native PAGE were performed as described (Nangaku et al., 1994). KIF1A was detected by immunoblotting with anti-KIF1A antibody.

In Vitro Motility Assay

Recombinant KIF1A was diluted with motility buffer (50 mM imidazole, 1 mM EGTA, 1 mM $MgCl_2$, 0.1% Triton X-100 [pH 6.6]) to the final concentration of 10 μg/ml. This solution was introduced into the motility chamber, and 5%–10% of KIF1A was absorbed to the cover glass surface. The surface was washed with motility buffer, and taxol-stabilized microtubules or Chlamydomonas axonemes were introduced. Their movement was observed as described (Sekine et al., 1994; Okada et al., 1995) in the presence of 5 mM Mg-ATP.

Antibody Production

Anti-KIF1A antibody was raised by injecting a PH domain (indicated in Figure 1a) polypeptide into a rabbit (number 60). The antigen was expressed as a GST–fusion protein using baculo-GEX vector (Davies et al., 1993). Polypeptide in the C-terminal half of KIF1A (717–937) was also expressed as a GST–fusion protein using pGEX2T vector (Pharmacia) in Escherichia coli (JM109) and was injected into two other rabbits (numbers 91 and 92). Antibodies were affinity purified with recombinant KIF1A protein expressed by insect cells. To obtain the control antibody, these affinity-purified antibodies were passed through this recombinant KIF1A protein column, and the flowthrough fractions were supplemented with IgG fractions purified from preimmune sera. All three antibodies (60, 91, and 92) gave the same results in immunoblotting, immunohistochemistry, and immunoisolation. Therefore, only results derived by using the number 60 antibody (anti-KIF1A PH domain antibody) are presented.

Immunoblotting and immunohistochemistry were performed as described previously (Sekine et al., 1994; Okada et al., 1995).

Nerve Ligation Experiment

Ligation of sciatic nerves and immunofluorescent staining were performed as described (Hirokawa et al., 1991). Samples were observed with a Olympus BMAX microscope or a Bio-Rad MRC-1000 confocal laser scanning microscope.

Isolation of Axonally Transported Organelles from Cauda Equina

Axonally transported organelles were collected by the method described previously (Okada et al., 1995) with slight modifications. In brief, the cauda equina was dissected from two adult rats and was cut into <10 μm fragments with fine scissors in 1 ml of internal medium (IM-D: 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 protease inhibitors (10 μM phenylmethylsulfonylfloride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml tosylarginylmethylester, and 0.25 mM benzamidine). After a 10 min incubation on ice, the debris was removed by centrifugation (1000 rpm for 5 min). Then, large organelles (P2 fraction) were spun down (10,000 rpm for 10 min). From the supernatant (S2 fraction), small organelles (P3 fraction) were pelleted by ultracentrifugation (75,000 rpm for 30 min), and the remaining supernatant was the soluble fraction (S3). To avoid aggregate formation by pelleting, this S2 fraction was used for SDG or immunoisolation.

SDG Fractionation of Axonally Transported Organelles

For SDG, 0–2.0 M continuous or discontinuous sucrose density gradient formed in IM-D was used. Better separation was achieved with discontinuous gradient, and its result is shown. S2 fraction was layered on the gradient and was spun at 40,000 rpm for 24 hr. Fractions collected from the bottom were diluted with IM-D, and organelles were collected from each fractions by ultracentrifugation (75,000 rpm for 30 min).

Immunoisolation of KIF1A-Containing Organelles

For immunoisolation, anti-KIF1A antibody, anti-synaptophysin antibody, or control antibody was covalently attached to beads. Antibody was absorbed on protein A–Sepharose FF (Pharmacia) and was covalently cross-linked by dimethylpimelimidate. S2 fraction was incubated with these beads at 4°C for 4–12 hr, and the beads were recovered by centrifugation (5000 rpm for 10 s), and washed with IM-D (1 ml, three times). The supernatant was spun down (75,000 rpm for 30 min) to collect the remaining organelles. Immunoblotting was performed as described (Sekine et al., 1994; Okada et al., 1995) using affinity-purified antibodies against KIF1A and KIF3, and monoclonal antibodies against conventional kinesin (H2) (Pfister et al., 1989), synaptotagmin (CI41.1) (Brose et al., 1992), synaptophysin (SY38 [Wiedenmann and Franke, 1985] or 171B5 [Obata et al., 1986]), Rab3A (Mizoguchi et al., 1989), syntaxin 1A (HPC-1) (Barnstable et al., 1985), SNAP-25 (Oyler et al., 1989), and SV2 (Buckley and Kelly, 1985).

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