

# Kinectin, a Major Kinesin-binding Protein on ER

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**Abstract.** Previous studies have shown that microtubule-based organelle transport requires a membrane receptor but no kinesin-binding membrane proteins have been isolated. Chick embryo brain microsomes have kinesin bound to their surface, and after detergent solubilization, a matrix with an antibody to the kinesin head domain (SUK-4) (Ingold et al., 1988) bound the solubilized kinesin and retained an equal amount of a microsome protein of 160-kD. Similarly, velocity sedimentation of solubilized membranes showed that kinesin and the 160-kD polypeptide cosedimented at 13S. After alkaline treatment to remove kinesin from the microsomes, the same 160-kD polypeptide doublet bound to a kinesin affinity resin and not to other proteins tested. Biochemical characterization localized this protein to the cytoplasmic face of brain microsomes and indicated that it was an inte-

gral membrane protein since it was resistant to alkaline washing. mAbs raised to chick 160-kD protein demonstrated that it was absent in the supernatant and concentrated in the dense microsome fraction. The dense microsome fraction also had the greatest amount of microtubule-dependent motility. With immunofluorescence, the antibodies labeled the ER in chick embryo fibroblasts (similar to the pattern of bound kinesin staining in the same cells) (Hollenbeck, P. J. 1989. *J. Cell Biol.* 108:2335-2342), astroglia, Schwann cells and dorsal root ganglion cells but staining was much less in the Golgi regions of these cells. Because this protein is a major kinesin-binding protein of motile vesicles and would be expected to bind kinesin to the organelle membrane, we have chosen the name, kinectin, for this protein.

**M**ICROTUBULE-dependent organelle transport is involved in a variety of membrane trafficking events (for review see Schroer and Sheetz, 1991a). At the molecular level both kinesin and cytoplasmic dynein have been implicated as the motors involved in powering organelle transport. In the case of kinesin, a role has been demonstrated in the movement of lysosomes (Hollenbeck, P. J., and J. A. Swanson. 1990. *Nature [Lond.]* 109:81 abstr.), melanophores (Rodionov et al., 1990), axonal vesicles (Schroer et al., 1988; Urrutia et al., 1991) and ER-like network formation (Dabora, 1989). For these movements to occur kinesin must be attached to the vesicle surface with its microtubule- (MT)<sup>1</sup> binding domain free. Mapping of the kinesin molecule has revealed that the NH<sub>2</sub>-terminal head has both the MT and ATP binding domains and a highly cross-reactive antibody, SUK-4 binds specifically to that domain (Scholey et al., 1989; Ingold et al., 1988). The COOH-terminal portion of the molecule contains the light chains of kinesin (Hirokawa et al., 1989) and is the region which logically would bind to the organelle surface. The molecules in addition to kinesin which are part of the organelle-motor

complex have yet to be defined (Schroer and Sheetz, 1991b). The hypertonic salt extraction of organelles does not block subsequent motility whereas proteolysis does (Schroer et al., 1989) suggesting that the membrane protein involved is integral. This is unlike the myosin 1 motors which bind directly to vesicle lipids (Adams and Pollard, 1989) and produce motility.

Membranous vesicles from only a few tissues have given motility when reconstituted in vitro (Dabora and Sheetz, 1988a). We have found that embryonic chick brain provides an abundant source of microsomes which are motile and will produce an ER-like network in vitro (Dabora and Sheetz, 1988b). Since brain is also the tissue which is richest in kinesin (Hollenbeck, 1989), we chose embryonic chick brain as a source of microsomes to isolate kinesin-binding membrane proteins. The kinesin-binding protein(s) responsible for vesicle motility should be on the cytoplasmic face of the vesicle where it could be labeled with an impermeant biotin reagent.

In this paper we report the identification of a 160-kD cytoplasmic membrane protein from motile brain microsomes on the basis of its interaction with kinesin. Many properties of this protein including its ER-like distribution within cells are consistent with its involvement in kinesin-dependent organelle motility.

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## Materials and Methods

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

### Heavy Microsomal Fraction

The microsome fraction from 12-d-old chick embryo brains was prepared by discontinuous sucrose gradient sedimentation. Fresh 12-d-old chick brains, typically 40 g, were homogenized in 40 ml of homogenization buffer (PMEE' [35 mM Pipes/KOH, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.5 mM EDTA] with 1 mM DTT and protease inhibitors [1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml TAME, 10 μg/ml TPCK, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor]) (Dabora and Sheetz, 1988a). The homogenate was centrifuged at 10,000 rpm for 15 min in a SA 600 rotor (Sorval Instruments, Wilmington, DE). Supernatant was layered onto a sucrose gradient (2 ml of 20% sucrose, 2 ml of 40% sucrose, and 1 ml of 60% sucrose each in homogenization buffer). After the centrifugation at 38,000 rpm for 2 h in a SW40 rotor (Beckman Instruments, Inc., Palo Alto, CA), the fraction that banded between 40 and 60% sucrose was recovered and dialyzed against PMEE' overnight. Unless noted otherwise the fraction was treated with 0.1 M sodium carbonate, pH 11, for 5 min. After washing by centrifugation through 15% sucrose in homogenization buffer at 38,000 rpm for 90 min in the SW40 rotor, the microsome membranes suspended in 8 ml of homogenization buffer were reacted with sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) (1 mg/ml in homogenization buffer) for 20 min at room temperature and quenched with 20 mM glutamine. The labeled membranes were washed by centrifugation through 15% sucrose in homogenization buffer at 38,000 rpm for 60 min. The pellet was suspended in 8 ml of homogenization buffer and treated with 0.05% Triton X-100 (Pierce Chemical Co.).

### Antibody Affinity Columns

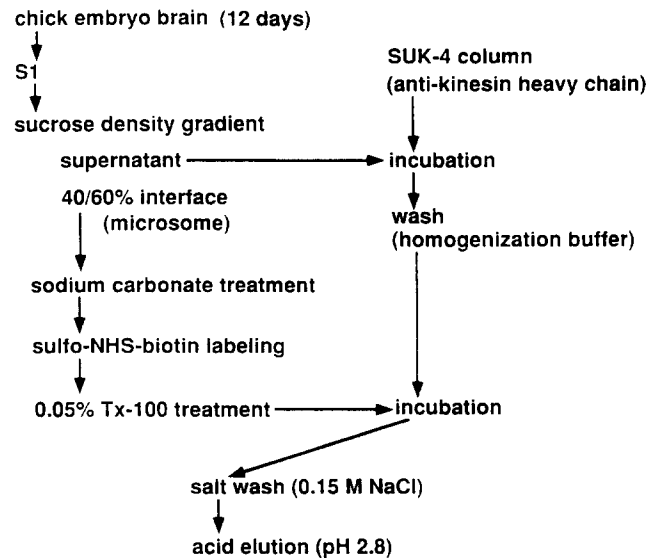
A diagram of the procedure is shown in Fig. 1. Immunoaffinity columns were made by conjugation of 5–10 mg of IgG per 1 ml of CNBr-activated Sepharose 4B. Either SUK-4 antikinesin heavy chain mAb (Scholey et al., 1989), 440.1 antidynein heavy chain mAb (Steuer et al., 1990) or nonspecific mouse IgG were conjugated to Sepharose. High speed supernatant (10 ml) from fresh embryonic chick brains was loaded on the columns (1 ml) first and then washed with 10 ml of PMEE'. Biotin-labeled microsomes in 0.05% Triton X-100 were incubated with kinesin-loaded resin for 1 h at 4°C. Resin was packed in a column and washed with PMEE' and 0.05% Triton X-100. The salt wash (0.15 M sodium chloride with PMEE' and 0.05% Triton X-100) and acid eluants (0.2 M glycine/HCl, pH 2.8, and 0.05% Triton X-100) were collected and peak fractions were analyzed by SDS-PAGE. Electrobloods were incubated with Avidin D-HRP (Vector Laboratories, Inc., Burlingame, CA) and visualized by peroxidase reaction.

### Soluble Kinesin-Membrane Protein Complex

A heavy microsomal fraction before alkaline extraction was solubilized and applied to SUK-4 immunoaffinity column. Typically 1 ml of a suspension of heavy microsomes (protein concentration 7 mg/ml) was solubilized in homogenization buffer containing 0.1% Triton X-100 and 0.5 M NaCl. After 1 h incubation at 4°C with mixing, a supernatant was obtained by centrifugation at 75,000 rpm for 30 min in a TL100.3 rotor (Beckman Instruments, Inc.). The supernatant was diluted to 0.15 M NaCl with homogenization buffer and incubated with 100 μl of SUK-4 resin (1 mg of IgG was bound) for 1 h at 4°C. After washing with 10 ml of suspension buffer, the kinesin-membrane protein complex was eluted with 100 μl of 0.2 M glycine, pH 2.8, containing 0.1% Triton X-100. Kinesin and a 160-kD protein were detected and quantified by immunoblotting with CKHC1 and KR160-11, respectively. Video densitometry was used for quantitation of kinesin and 160-kD protein. Images from the video camera (Type 67; Dage-MTI, Inc., Michigan City, IN) were processed with a microcomputer-based image analysis system (Image 1.29, developed by Wayne Rasband, National Institutes of Health (NIH) Research Service Branch, National Institute of Mental Health). Standards of purified kinesin and 160-kD protein were run on the same gel and used to define a standard curve.

### Velocity Sedimentation of Solubilized Membranes

Unwashed 40/60 interface vesicles were solubilized basically as above except that the sample was incubated with 0.1% C<sub>12</sub>E<sub>8</sub> (octaethylene-



**Figure 1.** Schematic representation of protocol for affinity purification of brain microsomal kinesin binding protein(s). The procedure for identification of kinesin binding proteins on the cytoplasmic face of brain microsomes is presented. Details of the experimental procedure are described in Materials and Methods.

glycylododecyl ether; Calbiochem Corp., La Jolla, CA) and 0.5 M NaCl for 30 min at 24°C before centrifugation (50,000 rpm for 30 min, in a TL100.2 rotor at 24°C). The supernatant was then dialyzed for 2.5 h against PMEE' at 20°C with or without added kinesin. After dialysis, the sample was loaded onto a 5–20% sucrose gradient in PMEE' (11 ml linear gradient in an SW40 tube) and centrifuged (38,000 rpm for 11 h in a SW40 rotor at 20°C). Fractions (1 ml) were collected from the gradient and analyzed by immunoblot with antibodies, 9E5.KHC2 and KR160.11.1. Kinesin and cytoplasmic dynein centrifuged separately under the same conditions were used as standards.

### Chymotrypsin Digestion of Microsomal Membranes

The microsome fraction labeled with sulfo-NHS-biotin as described above was treated with 0.3 mg/ml gamma-chymotrypsin for 30 min at 37°C, blocked by 2 mM PMSF, washed, suspended in 0.05% Triton X-100 with homogenization buffer, and applied to a kinesin-saturated SUK-4 column. In addition, samples of chymotrypsin-treated membranes were analyzed by immunoblot with mAbs against the 160-kD protein.

### Purification of 160-kD Protein

Alkaline-treated microsomal membranes were solubilized in Laemmli's sample buffer and applied to a preparative SDS gel. The 160-kD region of the gel was excised and electroeluted in Laemmli's running buffer (Laemmli, 1970). After mixing with Laemmli's sample buffer, the eluate was put on small 5% acrylamide SDS gels containing 9 M urea. The 160-kD protein was separated from other proteins of similar molecular weight in the second gel and was electroeluted again.

### Monoclonal Antibodies Against 160-kD Protein

Anti-160-kD protein mAbs were made in BALB/c mice. First, chick embryonic microsome fraction was mixed with Ribi adjuvant (Ribi Immuno-Chem Res., Inc., Hamilton, MT) and injected twice. For a third boost, the eluant of the kinesin affinity column (see Fig. 3, lanes c and j) was absorbed on streptavidin agarose and injected with Ribi adjuvant. The final boost was with the eluant of the kinesin affinity column on streptavidin without adjuvant. Spleen cells were fused with myeloma cell line X63Ag8.653. Hybridomas were screened with ELISA and cloned twice. Immunoblots were incubated with hybridoma cell supernatant and visualized with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin. A second series of Balb/c mice were immunized with crude fraction of 160-kD protein and

boosted with pure 160-kD protein twice. The final boost was intraperitoneal without Ribi adjuvant. One mAb from the first series (KR160.1B9) and 10 mAbs (KR160.2-KR160.11) from the second series were obtained.

### Monoclonal Antibodies Against Chick Kinesin

SUK-4 affinity column-purified kinesin from 12-d-old chick embryo brains was injected into Balb/c mice (200  $\mu$ g per mouse) i.p. with Ribi adjuvant according to the procedure recommended by the manufacturer. Three boosts with adjuvant i.p. and a final boost i.v. without adjuvant were carried out. Hybridomas were screened with ELISA and immunoblot using immunoaffinity purified kinesin as an antigen. Three antikinesin light chain mAbs (CKLC1-CKLC3) and 12 antikinesin heavy chain antibodies (CKHC1-CKHC12) were obtained.

### In Vitro Organelle Motility Assay

The motile activity of organelles was measured in a video microscopy assay previously described (Schroer et al., 1989). In these experiments sucrose gradient-purified brain vesicles (0.2 mg/ml of vesicle protein), microtubules (0.02 mg/ml), and chick embryo fibroblast supernatant (a 1:1 dilution of S3) were combined on the coverslip and analyzed within 1–2 h. The motility is expressed as the number of different vesicle movements per minute per 22 by 28 micron video field with 20–30 MTs per field.

### Two-dimensional Gel Electrophoresis

The two-dimensional electrophoresis method of O'Farrell (O'Farrell, 1975) was modified for a smaller gel size (4  $\times$  6 cm) (Toyoshima et al., 1983).

### Cell Culture

Primary fibroblast cultures were prepared from 12-d-old chick embryos (Kelly and Schlessinger, 1978). Cells were grown to confluence in MEM (Gibco Laboratories, Grand Island, NY) with 5% FCS. The primaries were diluted 300 times to plate on glass cover slips in the same media with 10% FCS.

Primary astrocyte cultures were prepared from 12-d-old chick forebrains (Hansson, 1989). Dissected forebrains were triturated and plated in MEM with 10% FCS. The cells were trypsinized and transferred twice before plating on cover slips for immunostaining.

Dorsal root ganglia were dissected from 11- or 12-d-old chick embryos and incubated in 0.1% collagenase and 0.25% trypsin for 10 min. Triturated cells were plated in MEM with 20% FCS, additional 0.6% glucose, 25 mM Hepes, 50 ng/ml nerve growth factor, and 1% chick embryo extract. Coverslips were pretreated with collagen, poly-L-lysine, or Matrigel (Collaborative Research, Inc., Bedford, MA).

### Immunohistochemical Staining of 160-kD Protein

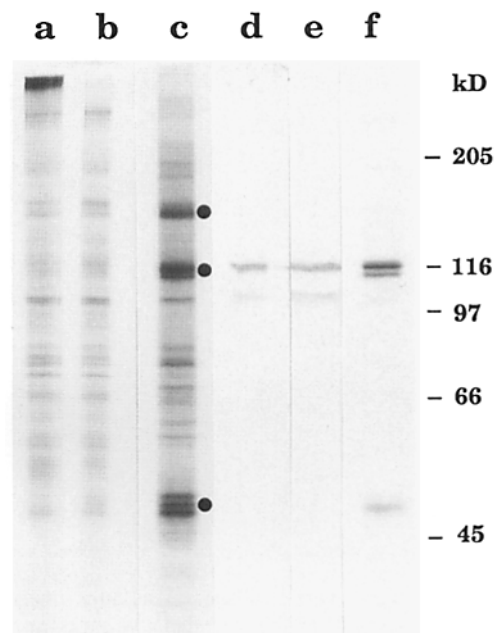
Cultured cells on glass cover slips were fixed with 3.7% formaldehyde (Polyscience, Inc., Warrington, PA) in PBS for 15 min at 37°C and permeabilized with 0.1% Brij 35 (Pierce Chemical Co.) in PBS containing 0.25% BSA and 50 mM ammonium chloride for 15 min at room temperature. Hybridoma supernatant was diluted with an equal volume of 0.25% BSA, 0.01% saponin and PBS, and incubated with cells for 1–2 h. Anti-mouse IgG and IgM-FITC (Boehringer-Mannheim Corp., Indianapolis, IN) or anti-mouse IgG-L-rhodamine (Boehringer-Mannheim Corp.) were used as secondary antibodies. Rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, Inc.) was used to visualize the Golgi complex either after or simultaneously with the secondary antibody. Both procedures gave the same results. DiOC<sub>6</sub>(3) (Molecular Probes, Inc., Eugene, OR) (5  $\mu$ g/ml) was used as an ER and mitochondrial marker.

Preparations were examined with an Axiophot microscope (Carl Zeiss Inc., Oberkochen, Germany) equipped with epifluorescence optics and a Peltier-cooled CCD camera (Star 1 from Photometrics Ltd., Tucson, AZ).

## Results

### Microsomal Membrane Preparation, Solubilization, and Binding to Antikinesin Column

Earlier studies have shown that the dense microsomes from chick brain which are isolated at a 40/60% sucrose interface

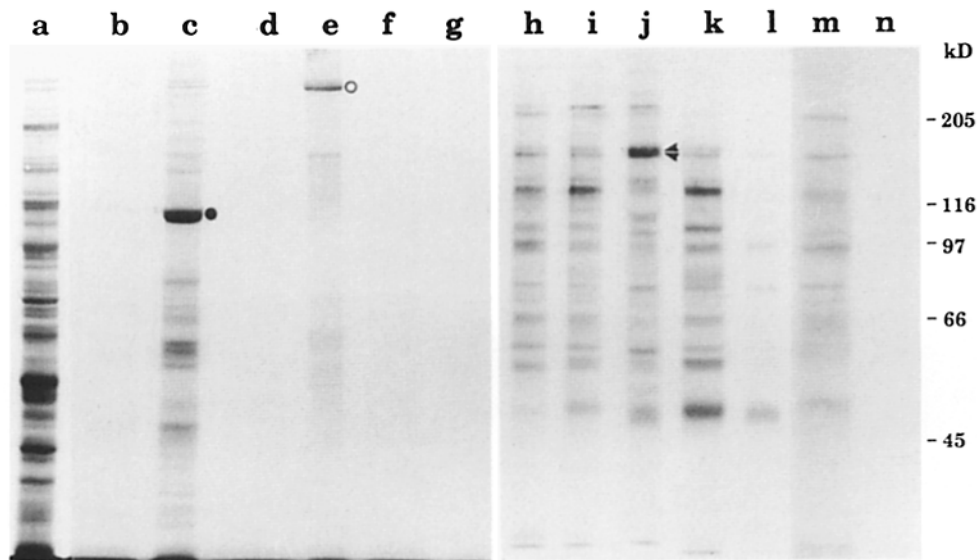


**Figure 2.** Soluble kinesin-membrane protein complex. These silver-stained gels (lanes *a–c*) and Western blots with CKHC.10 mAb (lanes *d–f*) show that kinesin is present on the heavy microsomes (lanes *a* and *d*) and that after solubilization it is coisolated with a 160-kD band. For this experiment a solubilized fraction, (lanes *b* and *e*), of native heavy microsomes, (lanes *a* and *d*), was applied to a SUK-4 (Scholey et al., 1989) antibody affinity column and eluted with acid (lanes *c* and *f*). Kinesin heavy chain in each fraction was detected with CKHC.10 mAb (lanes *d–f*) after SDS-PAGE and electroblot. The major components of the extract are noted (dots): 160-kD protein, kinesin heavy chain, and 47–50-kD component.

have the greatest motile activity of any brain vesicle fractions when reconstituted with a cytoplasmic supernatant from chick embryo fibroblasts (Schroer et al., 1989). We determined by quantitative immunoblot analysis that 40/60% interface vesicles contained bound kinesin at a level of 0.9 picomoles/mg of protein. Detergent extraction in the presence of 0.5 M NaCl resulted in the solubilization of >80% of the kinesin. After dilution of the salt to 0.15 M, ~20% of the solubilized kinesin bound to a SUK-4 antibody affinity column and eluted with an acidic solution (Fig. 2). Although there were many minor bands in the eluate which were prominent in the solubilized vesicle extract (Fig. 2, lanes *b* and *c*), the major bands were kinesin, 160-kD, and 47–50-kD proteins.

### Carbonate Extraction and Biotinylation

Because the sucrose gradient-purified vesicles were contaminated with components other than membranes, the proteins which bound to the kinesin affinity column could have been cytoskeletal proteins. There are several cytoskeletal proteins including glial fibrillary acidic protein (GFAP) which have an apparent molecular mass of 50 kD, but anti-GFAP antibodies did not react with the 47–50-kD bands (data not shown). To remove cytoskeletal proteins we extracted the membranes with 0.1 M sodium carbonate, pH 11, which removed all of the vesicle-associated kinesin and 80–85% of the total protein in the 40/60% interface fraction.



**Figure 3.** Affinity purification of microsome kinesin-binding protein after biotinylation. The Coomassie blue-stained SDS-PAGE (lanes *a-g*) and avidin-HRP stained electroblot (lanes *h-n*) of the same samples are shown for the original alkaline-treated microsome fraction (lanes *a* and *h*), the salt wash (lanes *b* and *i*) and acid eluant (lanes *c* and *j*) of the SUK-4 antibody column loaded with kinesin and biotinylated, solubilized microsomes. Similarly, solubilized microsomes were applied to a 440.1 (Steuer et al., 1990) immunoaffinity column containing cytoplasmic dynein from the supernatant and the salt wash (lanes *d* and *k*)

and acid eluant (lanes *e* and *l*) were analyzed. In addition, the salt wash (lanes *f* and *m*) and acid eluant (lanes *g* and *n*) from a control column of nonspecific mouse IgG antibody loaded with supernatant and solubilized microsomes were analyzed. Molecular mass is shown on the right side. Arrowheads indicate the 160-kD kinesin-binding microsome protein. Lanes *c* and *e* show that kinesin and cytoplasmic dynein were bound to the columns by the appropriate antibody. (*dot*) Kinesin heavy chain; (*circle*) cytoplasmic dynein heavy chain. The sample in lane *h* represents 0.0006% of the total sample to adjust the intensity with lanes *i-n*, where 1% of the total samples were applied.

Motile activity was resistant to alkaline extraction since the addition of kinesin and accessory proteins to the organelles resulted in motility on microtubules (Dabora, 1989). Further, extracted vesicles also bound the microtubule motors, kinesin, and cytoplasmic dynein and binding was saturable and protein dependent (H. Yu and M. P. Sheetz, unpublished results).

Because the kinesin binding proteins of the microsome membrane should be on the cytoplasmic face, we used the impermeable biotin derivative, sulfo-NHS-biotin to label the proteins on the cytoplasmic surface of the microsomes. The biotin tag was useful in following cytoplasmic membrane proteins during purification. We found that a distinct subset of the microsome membrane proteins were labeled with biotin (Fig. 3, lanes *a* and *h*).

#### **Binding of a 160-kD Protein to a Kinesin Affinity Column**

To assure that the membrane-binding domain of kinesin was exposed on the affinity column, a SUK-4 column which was preloaded with kinesin from a high speed supernatant of chick brain was used. Many supernatant proteins in addition to kinesin also bound to the SUK-4 column in a kinesin-dependent manner (Fig. 3, lane *c*) and we are independently characterizing them to determine if they are kinesin-binding proteins. The membranes were mildly solubilized in homogenization buffer containing 0.05% Triton X-100. When the detergent-treated membranes were incubated with the kinesin affinity resin, a set of the biotinylated proteins bound to the resin and resisted washing with 0.15 M NaCl in PMEE' but eluted with kinesin at pH 2.8 (Fig. 3, lanes *c* and *j*). The major component that was prominent only in the acid eluant was a doublet of 160-kD protein (compare Fig. 3, lanes *i* and *j*). Several minor biotinylated components in the molecular weight region >200 kD were not consistently observed (Fig.

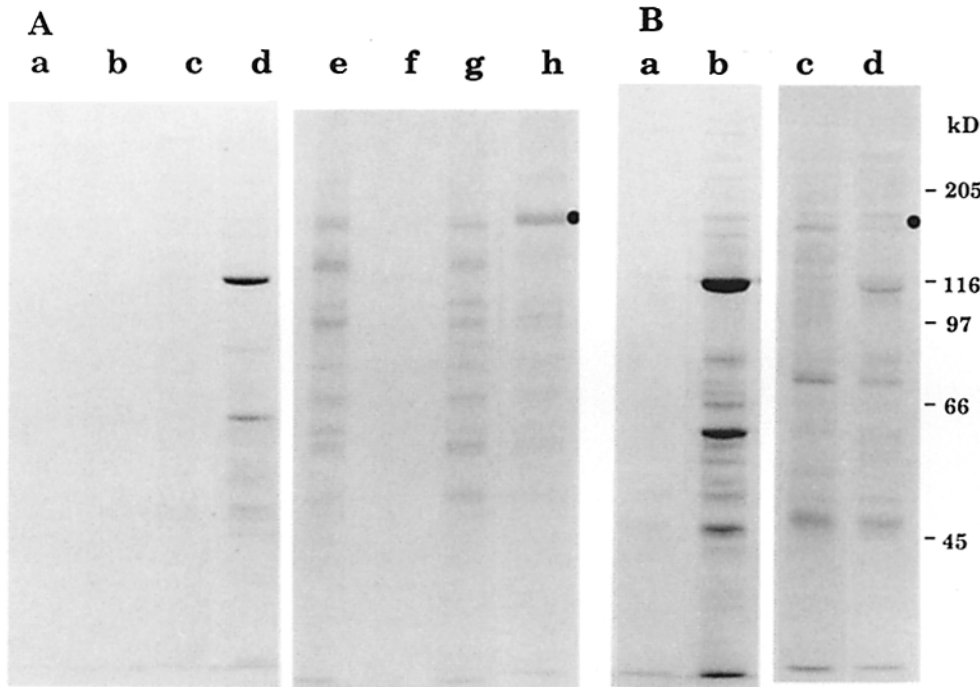
4 *A*, lane *h* and see Fig. 6 *A*, lane *g*) nor was the apparent concentration of the 105-, 75-, and 47-kD components in the acid eluant (Fig. 3, lane *j*). The other biotinylated components in the elution fraction were also prominent in the salt wash fraction and original microsome membranes and probably reflected nonspecific adsorption.

As a control for the possible interaction of the 160-kD protein with antibodies or other proteins, we passed the biotinylated membranes over a column containing cytoplasmic dynein from chick brain bound to a 440.1 antibody column (Steuer et al., 1990). The 160-kD protein was not concentrated in the eluate with the cytoplasmic dynein (Fig. 3, lanes *e* and *l*). The composition of the isotonic salt wash was enriched in 120-, 100-, 56-, and 49-kD biotinylated bands relative to a control nonbinding antibody (Fig. 3, lanes *k* vs *m*) but these all were major biotinylated bands in the whole membranes (Fig. 3, lane *h*) except for the 49-kD band.

#### **Lack of Reactivity of SUK-4 with 160-kD Protein**

On western blots of the brain extracts and purified microsomes there was only reactivity of SUK-4 with a 110-kD band which corresponded to kinesin. To control for interaction of the solubilized 160-kD protein with the SUK-4 antibody, the biotinylated microsomes were added to a SUK-4 affinity column without any kinesin bound to it. The flow-through from that column was then applied to a second SUK-4 column preloaded with kinesin. When the acid eluants from the two columns were compared (Fig. 4 *A*, lanes *f* and *h*), the 160-kD protein was only in the eluant of the kinesin affinity column. There was a faint band in the 160-kD region of the SUK-4 alone column (Fig. 4 *A*, lane *b*) but this protein did not react with mAbs raised to the 160-kD protein.

The proper orientation of the kinesin on the column was important for isolating the microsome kinesin binding protein, since other monoclonal antikinesin antibodies behaved



**Figure 4.** Requirement of kinesin for 160-kD protein binding (A) and sensitivity of 160-kD to protease (B). (A) These SDS-PAGE gels were stained with Coomassie blue (lanes a-d) or with avidin-HRP after electroblotting (lanes e-h) to show that the 160-kD protein did not bind to SUK-4 directly. The Biotin-labeled microsomes were applied to a kinesin-free SUK-4 column (lanes a, b, e, and f). The flow-through fraction from the column was loaded on a second SUK-4 column previously saturated with kinesin from a chick brain supernatant (lanes c, d, g, and h). The SDS-PAGE analysis of the salt wash (lanes a, c, e, and g) and acid eluant (lanes b, d, f, and h) fractions are shown. (Dot) The 160-kD protein. (B) Intact microsomes were treated

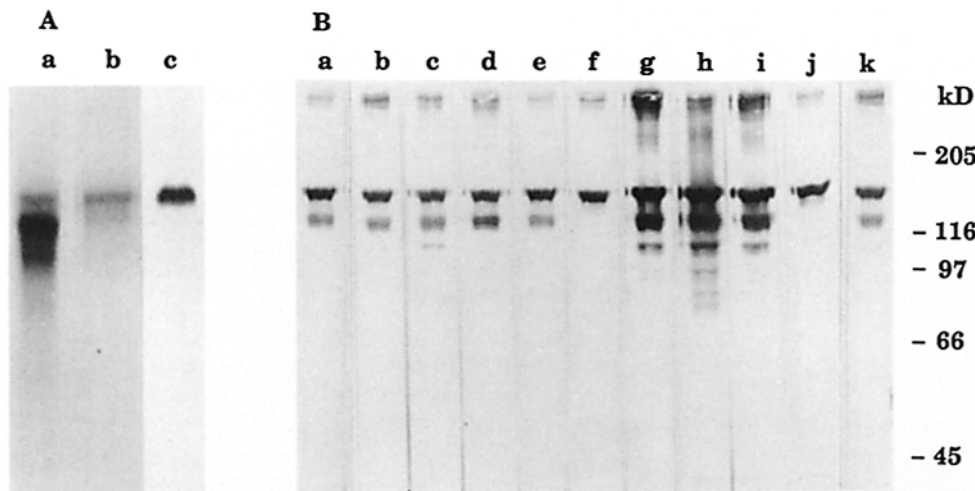
with chymotrypsin and subsequently applied to a SUK-4, kinesin affinity column. The salt wash (lanes a and c) and acid eluant (lanes b and d) are stained with Coomassie blue (lanes a and b) or avidin-HRP (lanes c and d) after SDS-PAGE and electroblot. (Dot) The position of the 160-kD protein. Most of the biotin-labeled bands were digested with extensive chymotrypsin treatment, but some of the major bands still remained in the Coomassie-stained gel.

differently. When antibody affinity columns were prepared with the antibodies which bound to the rod portion of kinesin and preloaded with kinesin, no biotinylated microsome proteins bound specifically to the column (data not shown).

#### Cytoplasmic Nature of the 160-kD Protein

Although the biotin labeling should be on the cytoplasmic face of the microsome membrane, there was a possibility that the alkaline extraction left the vesicles open to small molecules. To further test whether the kinesin binding protein was

on the cytoplasmic surface of the microsome membrane, microsomes were proteolyzed with chymotrypsin before detergent treatment and application to the kinesin column. The eluant from the column loaded with proteolyzed microsomes was greatly decreased in the 160-kD component (Fig. 4 B, lane d). Further, antibodies raised against the 160-kD protein (see below) showed that the 160-kD protein had been cleaved in proteolyzed microsomes (data not shown). Thus, both biotin labeling and protease sensitivity indicate that the 160-kD kinesin binding protein is on the cytoplasmic surface of the microsomes.



**Figure 5.** Purification of 160-kD protein and characterization of mAbs against 160-kD protein. (A) The purification of the 160-kD protein by sequential gel electrophoresis is shown after 9 M urea, 5% polyacrylamide SDS gel electrophoresis. The eluate from the 160-kD region of a preparative SDS-PAGE of alkaline-treated microsomes (lanes a and c), is compared with the eluate from the 160-kD region of a 9 M urea/SDS-PAGE (lane b) (stained with Coomassie blue). mAb KR160.1B9 (lane c) is used to identify the correct 160-kD protein. (B) These standard SDS-PAGE gels of

whole microsomes were reacted with mAbs to the 160-kD protein after electroblotting. a, KR160.1B9; b, KR160.2; c, KR160.3; d, KR160.4; e, KR160.5; f, KR160.6; g, KR160.7; h, KR160.8; i, KR160.9; j, KR160.10; k, KR160.11.

### Monoclonal Antibodies to 160-kD Protein

mAbs were raised against the 160-kD protein purified by sequential SDS and SDS-urea gel electrophoresis (Fig. 5 A). A total of one IgM (KR160.1b9) and 10 IgG (KR160.2–KR160.11) mAbs were obtained and divided into two groups according to their reactivity on immunoblots (Fig. 5 B). One group (KR160.6 and KR160.10) recognized specifically the 160-kD protein in brain microsomes but others reacted with the 160-kD and lower molecular weight bands which were probable degradation products. The band at the top of the gel represents unsolubilized 160-kD protein.

### Subcellular Distribution of 160-kD Protein and Motile Activity

The mAbs were used to determine the distribution of the 160-kD protein in the soluble and vesicle fractions from embryonic chick brain. We found that 78% of 160-kD protein in microsomes banded between 40 and 60% sucrose (Fig. 6 A, lanes a–d) and the concentration of the 160-kD protein was 2.1 picomoles/mg of 40/60% interface protein. Alkaline treatment with 0.1 M sodium carbonate, pH 11, did not solubilize the 160-kD protein and resulted in a 4.2-fold concentration of the 160-kD protein relative to microsomal protein (Fig. 6 A, lanes d and e). This is consistent with the finding that none of the 160-kD protein was in the supernatant fraction.

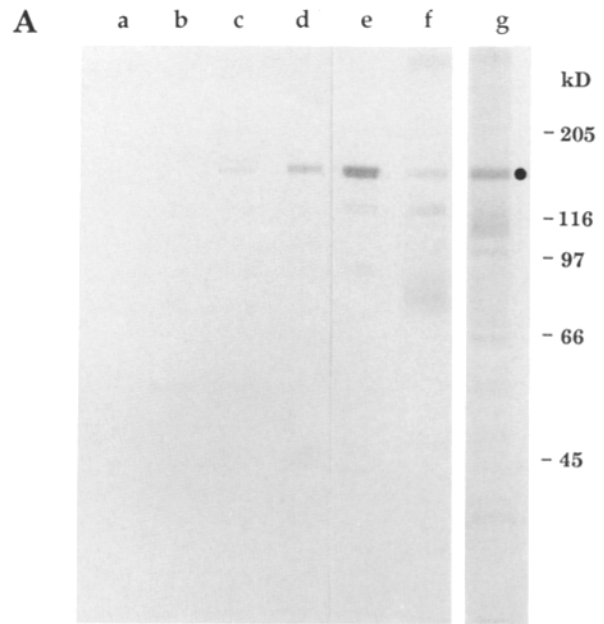
There was a dramatic difference in the motility of the three vesicle fractions (Fig. 6 B) when assayed for organelle motile activity at constant vesicle protein concentration. Motility was dependent upon added chick embryo fibroblast supernatant and was not seen with casein alone. ~85% of the total motile activity was found in the 40/60% interface fraction (Fig. 6 B). This correlated with the fraction of the 160-kD protein found in the 40/60% interface fraction.

### Cosedimentation of Soluble 160-kD Protein and Kinesin

Because the 40/60% sucrose interface microsomes contained kinesin as well as the 160-kD polypeptide, we tested whether or not they would cosediment after solubilization. Starting with unfrozen brain, we found that both kinesin and the 160-kD polypeptide sedimented together with a broad range of sedimentation coefficients which peaked in a fraction corresponding to 13S (Fig. 7). With material from frozen brains they did not cosediment and the 160-kD polypeptide had an apparent sedimentation coefficient of 5S while kinesin sedimented at 9S as in the absence of membrane proteins. In separate experiments the 160-kD polypeptide from frozen brains did not bind to kinesin, SUK-4 affinity columns either. Thus, we find that kinesin and the 160-kD polypeptide cosediment under conditions where the complex will bind to a SUK-4 antibody column.

### Quantitation of the Soluble Kinesin–160-kD Complex

When the solubilized 40/60% interface microsome proteins were incubated with a SUK-4 antibody column, a 160-kD protein was the major band that was brought down with kinesin (Fig. 2). The antikinectin antibodies reacted with the 160-kD band and a quantitative immunoblot was used to determine the ratio of the 160-kD protein to kinesin in the



### B Organelle Motile Activity (movements/min/field)

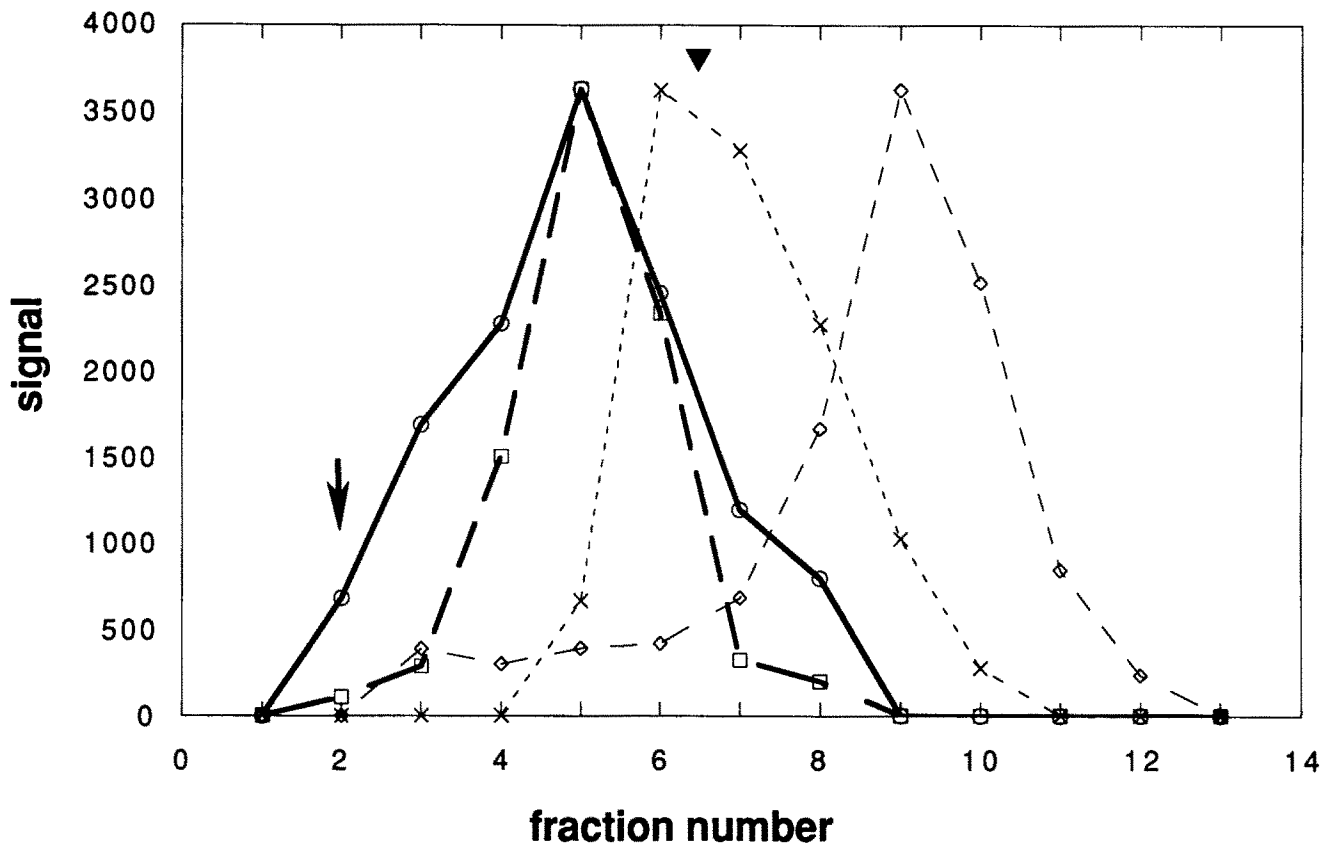
0/20	0
20/40	$0.6 \pm 0.5$
40/60	$4.6 \pm 0.7$

Figure 6. Distribution of 160-kD protein in supernatant and membrane fractions and correlation with motility. (A) These gels compare the amount of 160-kD protein present in 10  $\mu$ g of protein from the supernatant (lane a), microsomal fractions between 0–20% (lane b), 20–40% (lane c), and 40–60% sucrose (lane d) from a discontinuous sucrose gradient of embryonic chick brains and alkaline-treated 40–60% sucrose fraction (lane e) using KR160.1 to stain for the 160-kD protein. To establish the specificity of the antibody, the acid eluant of the kinesin affinity column (lane f) stained with KR160.1B9 was compared with the 160-kD visualized with avidin-HRP (lane g, ●). (B) The amount of motility in each membrane fraction was quantitated in an *in vitro* reconstitution assay (see Schroer et al., 1989). The same amount of vesicle protein was used in each assay as well as a standard concentration of microtubules and chick embryo fibroblast supernatant.

eluted fraction. The molar ratio of 160-kD protein (calculated as monomer) and kinesin (heterotetramer of 350 kD) in the acid eluate was estimated to be 1.0. The association of the 160-kD protein with kinesin was maintained under conditions where the concentration of the two proteins was <1 nM.

### Two-dimensional Gel Identification of the 160-kD Protein

There are many proteins in the molecular weight range of the 160-kD protein, including a component which copurifies with cytoplasmic dynein (Collins and Vallee, 1989). The dynein-associated 150-kD component has been identified as an accessory protein involved in cytoplasmic dynein-dependent organelle motility called dynactin (Schroer and Sheetz, 1991a). In addition, dynactin is present on the microsome surface and is not extracted by alkaline treatment (data not shown). We therefore compared dynactin and the 160-kD



**Figure 7.** Cosedimentation of solubilized 160-kD and kinesin. The plots of the normalized immunostaining intensity of kinesin and 160-kD protein versus gradient fraction show the distribution of both kinesin and 160-kD protein after velocity sedimentation in a 5–20% sucrose gradient. Cosedimentation of kinesin (□) and 160-kD protein (○) at 13S is seen in extracts of 40/60% sucrose interface microsomes prepared from fresh brain. In corresponding extracts from frozen brain, kinesin (×) sedimented with its normal sedimentation coefficient (9S) and 160-kD protein (◇) at 5S. In these experiments, the 40/60% microsomes were solubilized and dialyzed with added kinesin (1 μg of kinesin with 1 mg of solubilized microsome protein). The dialyzed material was centrifuged and analyzed as described in Materials and Methods. The positions of cytoplasmic dynein (long arrow) and kinesin (solid inverted triangle) under such conditions are noted.

kinesin binding protein by two-dimensional gel electrophoresis. The 160-kD kinesin binding protein has an isoelectric point of pH 5.0–5.5 for both components of the doublet (Fig. 8a, b, d, and e). Cytoplasmic dynein when run on the same gel system has a 150-kD subunit which is also a doublet but has an isoelectric point of pH 5.5–6.0 (Fig. 8f). mAbs raised against dynein (Steuer et al., 1990) reacted with the microsomes but only weakly with the eluant of the kinesin affinity column (data not shown). Further, coelectrophoresis showed that the lower band of the biotinylated 160-kD protein is apparently higher in molecular weight than that of dynein. A mAb against the 160-kD kinesin binding proteins did not react with the dynein associated 150-kD protein (data not shown). Thus, although these proteins are similar in molecular weight and both bind to membranes and are associated with microtubule motors, they are distinct proteins.

Analysis of the alkaline washed microsomes revealed that the 160-kD bands were only weakly labeled with biotin (Fig. 8d). We estimate that the fraction bound to the kinesin affinity column represents a >200-fold purification of the 160-kD protein.

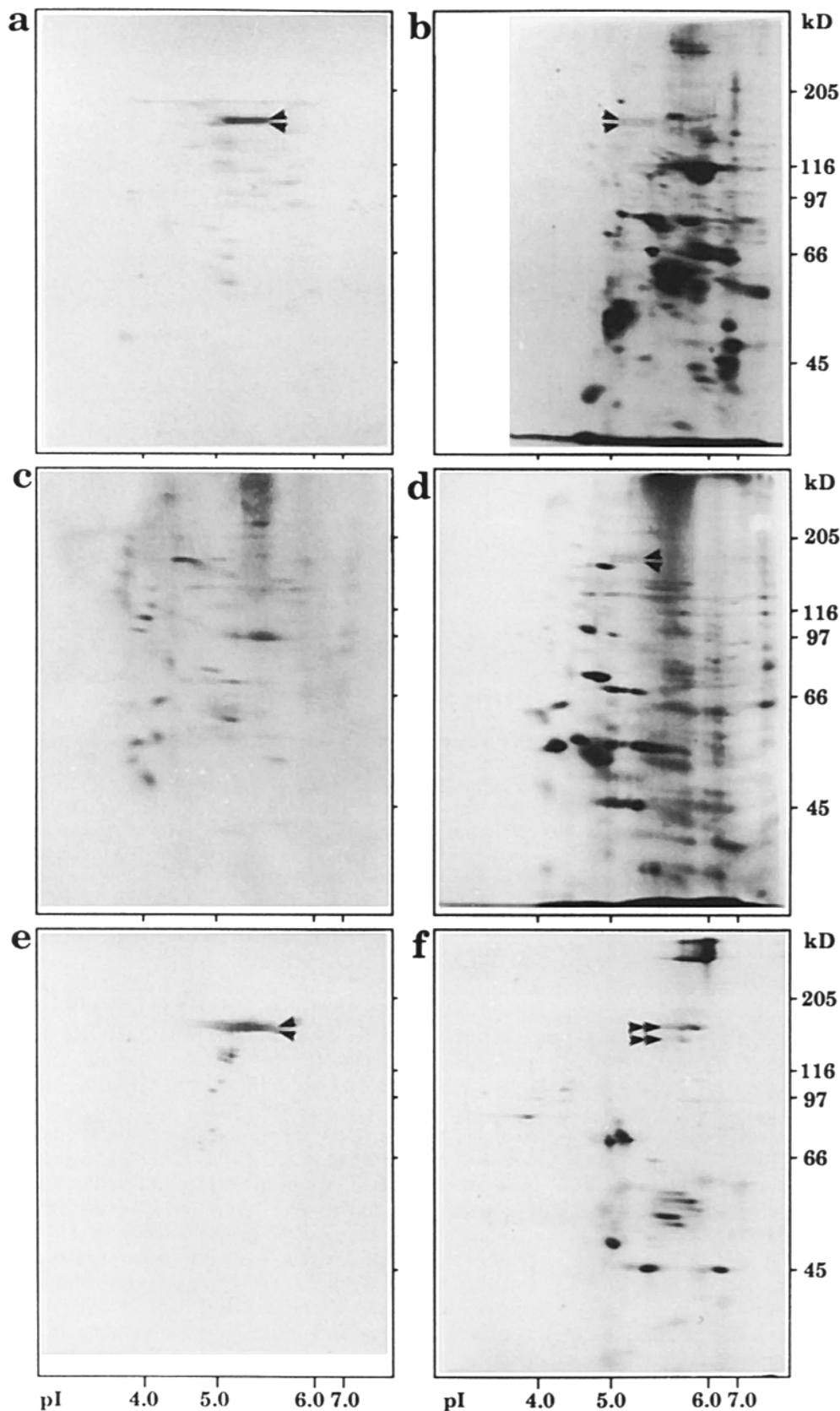
#### **160-kD Protein Concentrated in the ER and not Golgi Complex**

Immunostaining of chick embryo fibroblasts with mAbs

against the 160-kD protein showed an ER-like network pattern (Fig. 9). Eight mAbs (KR160.2, .3, .4, .6, .7, .9, .10, and .11) stained fibroblasts with the same pattern that was tubular-granular, intense in the perinuclear region, and faint in the periphery (Fig. 9a). The staining pattern was unaltered by different detergent treatments used for permeabilization; 0.1% and 1% Brij 35, 0.1% and 1% saponin, 0.1 or 1% Triton X-100.

Similar staining patterns were found in astroglia, dorsal root ganglia, and Schwann cell bodies (Fig. 9, c, e and g). The most intense staining was in neuronal cell bodies which is consistent with the isolation of the 160-kD protein from brain. Staining in neuronal processes was considerably less intense but punctate in nature (data not shown) and is currently under further study to determine if it correlates with transport vesicles.

An area weak in anti-160-kD protein staining in the perinuclear region was strongly stained with rhodamine-conjugated wheat germ agglutinin (WGA) that is a Golgi complex marker (Virtanen et al., 1980) (Fig. 9, b, d, f, and h). Peripheral vesicles stained with WGA are possible endosomes and lysosomes (Virtanen et al., 1980), and did not react strongly with anti-160-kD protein mAbs (Fig. 9, a, b). The astrocytes and neuronal cells had a wider area of Golgi staining which was weak in anti-160-kD mAb staining.

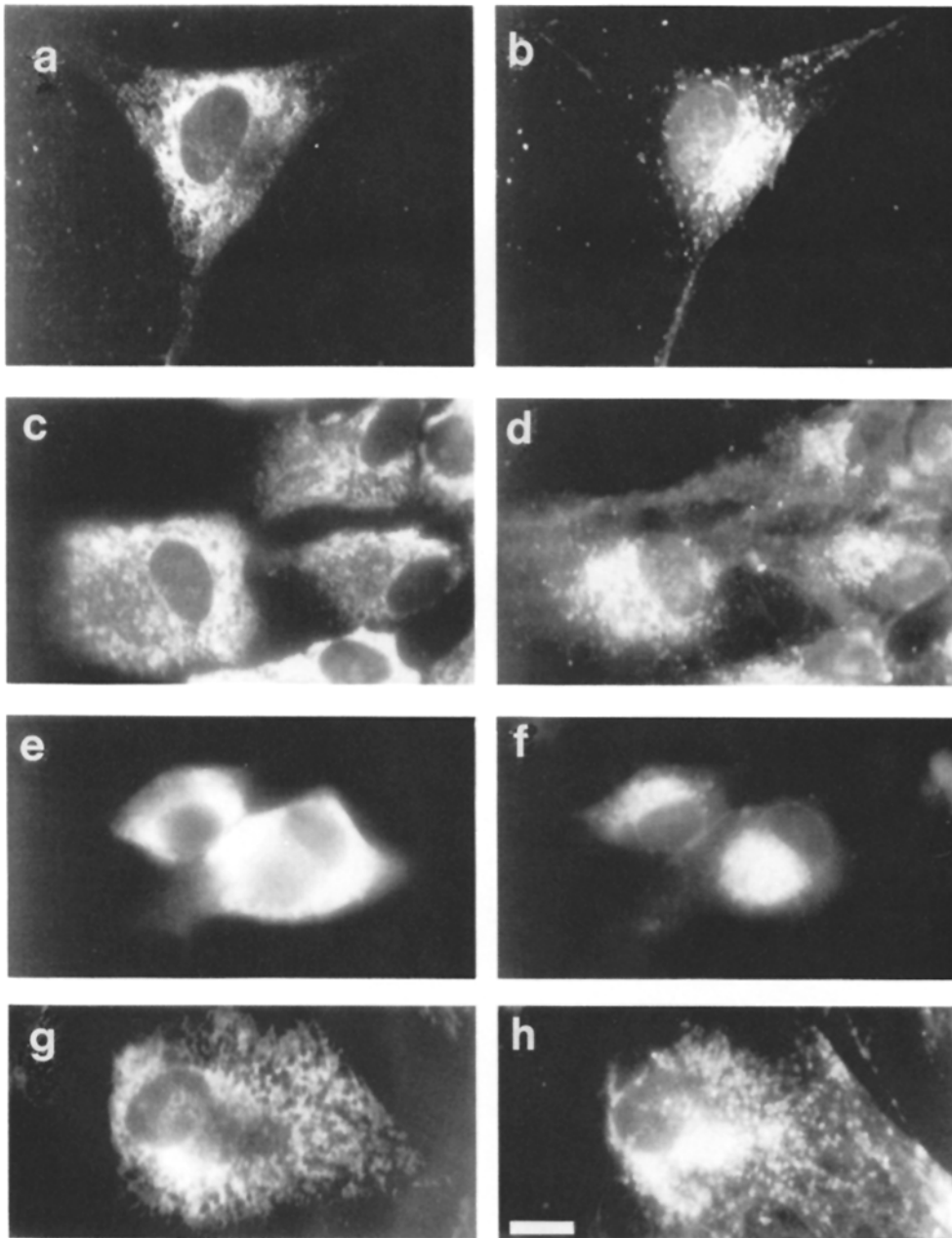


**Figure 8.** Two-dimensional gel electrophoretic analysis. These two-dimensional gels show that the 160-kD protein was the major biotinylated microsomal protein which bound to the kinesin affinity column. The kinesin affinity column acid eluant (*a* and *b*), alkaline-treated microsomal fraction (*c* and *d*), purified 160-kD protein, and microtubule affinity purified dynein (*e* and *f*) were stained with silver (*b*, *d*, and *f*), avidin-HRP after electroblot (*a* and *c*), or KR160.1B9 mAb to the 160-kD protein (*e*). The same samples in Fig. 3 (lanes *g*, *c*, *h*, and *a*) were run as *a*, *b*, *c*, and *d*, respectively. The amount of protein loaded on *d* was twice that on *c*. Molecular mass and isoelectric point are shown on the right and bottom, respectively. (*arrowheads*) The kinesin-binding 160-kD protein. (*double arrowheads*) The cytoplasmic dynein associated 150-kD protein.

The reticular distribution of the 160-kD protein suggested that it was primarily in the ER. To confirm this DiOC<sub>6</sub>(3) was used as ER marker (Terasaki et al., 1984). Anti-160-kD protein mAbs were visualized with a rhodamine-conjugated

anti-mouse mAb and recorded with CCD camera and VCR, and then fibroblasts were stained with DiOC<sub>6</sub>(3). Only the peripheral region was available for matching because of overlap in the central region. DiOC<sub>6</sub>(3) staining of ER (Fig. 10





**Figure 9.** Distribution of 160-kD protein in cells. These fluorescence micrographs show the distribution of 160-kD protein antibody, KR160.4 (*a*, *c*, *e*, and *g*) and WGA binding (*b*, *d*, *f*, and *h*) in fibroblasts (*a*, *b*), astroglia (*c*, *d*), neurons in a dorsal root ganglion (*e*, *f*) and Schwann cells (*g*, *h*). Bar, 10  $\mu$ m.

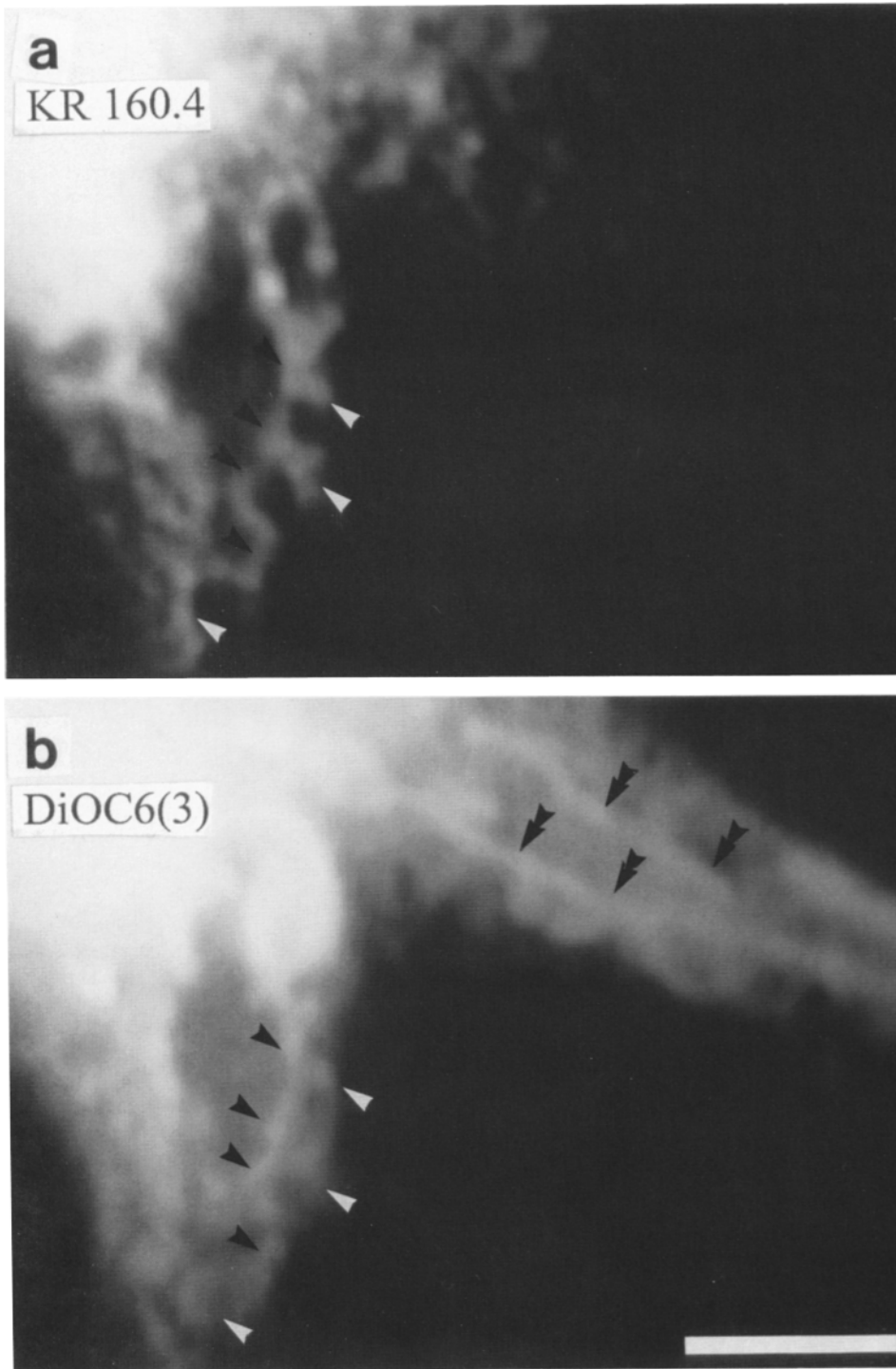
*b*) corresponded with the anti-160-kD protein staining (Fig. 10 *a*). However, the DiOC<sub>6</sub>(3) staining was smooth while the antibody showed an uneven pattern. mAbs did not stain mitochondria that stained brightly with DiOC<sub>6</sub>(3) (Terasaki et al., 1984) (Fig. 9 *b*). When the same cultures were stained with antikinesin mAbs, a diffuse staining pattern was observed (data not shown) which was similar to that reported previously (Hollenbeck, 1989).

### Discussion

We have identified a 160-kD protein which copurifies with kinesin in solubilized microsomes and binds to kinesin affinity columns. Because the SUK-4 antibody binds the motor domain of kinesin (Ingold et al., 1988; Scholey et al., 1989), kinesin on the affinity resin would be in the proper configuration with the tail portion of the molecule exposed.

No binding of the 160-kD protein was observed when kinesin was bound by other mAbs directed against the rod portion of the molecule or by SUK-4 in the absence of kinesin. Velocity sedimentation of the solubilized 40/60% sucrose microsomes shows a cosedimentation of kinesin and the 160-kD protein. We suggest, therefore, that kinesin associates with the 160-kD protein.

The concentration of kinesin on the affinity column was high (typically 0.2 mg/ml) and a low affinity interaction would possibly retain the 160-kD protein. It was, therefore, important to find a more native interaction. Because the microsomes from the 40/60% sucrose interface do have a significant amount of kinesin bound to them (an amount which corresponds to  $\sim 1$  kinesin molecule per vesicle of 100 nm [this is calculated assuming 6 pmoles of kinesin per milligram of membrane protein] [i.e., protein which resists carbonate solubilization] and  $2 \times 10^{-16}$  gm of protein per 100



*Figure 10.* 160-kD protein localizes to ER. These micrographs compare the structures stained with KR160.4 and DiOC<sub>6</sub>(3). Arrowheads and double arrowheads show ER and mitochondria, respectively. Bar, 5  $\mu$ m.

nm vesicle), we were able to look for a complex in the solubilized microsome proteins. The fact that equimolar amounts of the 160-kD protein and kinesin bound to the SUK-4 resin and they cosedimented further indicates that the interaction is strong between them. We cannot rule out the possibility that the interaction between kinesin and the 160-kD protein is indirect through another protein or requires an additional component. These components are not trapped in a large aggregate since they have a sedimentation coefficient of 13S. Although other proteins bind to the resin as well, none of

them are present in equimolar amounts except for the 50-kD bands which are not present in the alkaline extracted microsome experiments.

In the unextracted vesicles there is a twofold molar excess of the 160-kD protein over kinesin. This could indicate that the 160-kD protein forms an oligomer (Hurtley and Helenius, 1989), that a part of the 160-kD protein is inactive for kinesin binding or that kinesin binding is reversible. We would not expect all of the motor binding sites to be active at any time, because only a few motors are needed for organelle

motility (Howard et al., 1989) and only a small fraction of the vesicles move at any given time (Dabora and Sheetz, 1988a). Another possibility is that the 160-kD protein binds to other motors such as cytoplasmic dynein (indeed kinesin and cytoplasmic dynein can compete for binding to some sites on brain microsomes [H. Yu, I. Toyoshima, and M. Sheetz, unpublished results]). The antibodies to cytoplasmic dynein have not been mapped to know whether or not they would interfere with dynein binding to microsome sites, which would prevent the 160-kD protein from binding to cytoplasmic dynein on the affinity resin. Studies of the binding of kinesin to chick brain microsomes do show that the number of specific binding sites in ATP is equal to the number of 160-kD protein molecules (H. Yu and Y. Toyoshima, unpublished results).

From the distribution of the 160-kD protein staining within cells it is clear that it is on the ER. This is consistent with the antibody distribution data which shows it is not soluble in cytoplasm or sodium carbonate extracts of microsomes but is strongly bound to the cytoplasmic surface of the dense microsomes. Fluorescence microscopy of the kinesin distribution in chick embryo fibroblasts has shown that it is distributed along the ER just as the 160-kD protein (Hollenbeck, 1989). Similarly, a membrane system in the spindle which may resemble the ER is labeled in sea urchin eggs with an antikinesin antibody (Wright et al., 1991). Other studies have shown that the spreading of ER on microtubules in vitro is dependent upon kinesin as well as cytoplasmic dynein (Dabora, 1989) and microtubule-dependent movement of ER to the periphery is seen in vivo (Lee et al., 1989). There is no particular concentration of the 160-kD protein staining in the periphery of the ER and it is depleted in the Golgi region. It is unclear whether 160-kD protein is present in transport vesicles within axons or other compartments which come after the Golgi complex in the processing of exported proteins. Thus, the 160-kD protein is primarily an ER protein and the kinesin in permeabilized cells colocalizes with the ER (Hollenbeck, 1989; Wright et al., 1991). Because the 160-kD protein will bind kinesin to the surface of cytoplasmic vesicles, we have chosen to name it kinectin (from kinesin and the Latin verb *necto*, which means to bind to or connect).

Since many microtubule-dependent transport events occur simultaneously within cells and are seemingly regulated by different cues (Schroer and Sheetz, 1991a), we believe that it is unlikely that only one kinectin is present as a motor-binding protein on membranous organelles. We suggest that it represents a class of proteins which reside on the cytoplasmic face of intracellular organelles and are involved in organelle trafficking through the regulation of motor function. Studies are underway to further define the role of this kinesin binding membrane protein in cell function and in reconstitution studies to determine if this protein will support vesicle motility or serves some other function.

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## References

- Adams, R. J., and T. D. Pollard. 1989. Binding of myosin I to membrane lipids. *Nature (Lond.)* 340:565-568.
- Collins, C. A., and R. B. Vallee. 1989. Preparation of microtubules from rat liver and testis: cytoplasmic dynein is a major microtubule-associated protein. *Cell Motil. Cytoskeleton*. 491-500.
- Dabora, S. L. 1989. Microtubule-based organelle motility: role in spatial organization of organelles and membrane sorting. Ph.D. thesis. University of Connecticut, Storrs, Connecticut. 1-215.
- Dabora, S. L., and M. P. Sheetz. 1988a. Culture cells extracts support organelle movement on microtubules in vitro. *Cell Motil. Cytoskeleton*. 10: 482-495.
- Dabora, S. L., and M. P. Sheetz. 1988b. The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell*. 54:27-35.
- Hansson, E. 1989. Co-existence between receptors, carriers and second messengers on astrocytes grown in primary cultures. *Neurochem. Res.* 14: 811-819.
- Hirokawa, N., K. K. Pfister, H. Yorifuji, M. C. Wagner, S. T. Brady, and G. S. Bloom. 1989. Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. *Cell*. 56: 867-878.
- Hollenbeck, P. J. 1989. The distribution, abundance and subcellular localization of kinesin. *J. Cell Biol.* 108:2335-2342.
- Howard, J., A. J. Hudspeth, and R. D. Vale. 1989. Movement of microtubules by single kinesin molecules. *Nature (Lond.)* 342:154-158.
- Hurtley, S. H., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277-307.
- Ingold, A. L., S. A. Cohn, and J. M. Scholey. 1988. Inhibition of kinesin-driven microtubule motility by monoclonal antibodies to kinesin heavy chains. *J. Cell Biol.* 107:2857-2667.
- Kelly, B., and M. J. Schlessinger. 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell*. 15:1277-1286.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lee, C., M. Ferguson, and L. B. Chen. 1989. Construction of the endoplasmic reticulum. *J. Cell Biol.* 109:2045-2055.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis. *J. Biol. Chem.* 250:4007-4021.
- Rodionov, V. I., F. K. Gyoeva, A. S. Kashina, S. A. Kuznetsov, and V. I. Gelfand. 1990. Microtubule-associated proteins and microtubule-based translocators have different binding sites on tubulin molecule. *J. Biol. Chem.* 265:5702-5707.
- Scholey, J. M., J. Heuser, J. T. Yang, and L. S. B. Goldstein. 1989. Identification of globular mechanochemical heads of kinesin. *Nature (Lond.)* 338: 355-357.
- Schroer, T. A., and M. P. Sheetz. 1991a. Functions of Microtubule-based Motors. *Annu. Rev. Physiol.* 53:629-652.
- Schroer, T. A., and M. P. Sheetz. 1991b. Two activators of microtubule-based vesicle transport. *J. Cell Biol.* 115:1309-1318.
- Schroer, T. A., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1988. The role of kinesin and other soluble factors in organelle movement along microtubules. *J. Cell Biol.* 107:1785-1792.
- Schroer, T. A., E. R. Steuer, and M. P. Sheetz. 1989. Cytoplasmic dynein is a minus-end directed motor for membranous organelles. *Cell*. 56:937-946.
- Steuer, E. R., T. A. Schroer, L. Wordeman, and M. P. Sheetz. 1990. Cytoplasmic dynein localizes to mitotic spindles and kinetochores. *Nature (Lond.)* 345:266-268.
- Terasaki, M., J. Song, J. R. Wong, M. J. Weiss, and L. B. Chen. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. *Cell*. 38:101-108.
- Toyoshima, I., K. Tanaka, N. Fukuhara, N. Kumamoto, and T. Miyatake. 1983. Dissection and electrophoretic protein analysis of single muscle fibers from histologically identified freeze-dried sections. Application to muscle fibers with rimmed vacuoles. *Biomed. Res. (Aligarh)*. 4:456-486.
- Urrutia, R., M. A. McNiven, J. P. Albanesi, D. B. Murphy, and B. Kachar. 1991. Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. *Proc. Natl. Acad. Sci. USA*. 88:6701-6705.
- Virtanen, I., P. Ekblom, and P. Laurila. 1980. Subcellular compartment of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* 85: 429-434.
- Wright, B. D., J. H. Henson, K. P. Wedaman, P. J. Willy, J. N. Morand, and J. M. Scholey. 1991. Subcellular localization and sequence of sea urchin kinesin heavy chain: evidence for its association with membranes in the mitotic apparatus and interphase cytoplasm. *J. Cell Biol.* 113:817-833.