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Characterization of Kinectin, a Kinesin-binding Protein: Primary Sequence and N-Terminal Topogenic Signal Analysis

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Kinectin is a kinesin-binding protein (Toyoshima *et al.*, 1992) that is required for kinesin-based motility (Kumar *et al.*, 1995). A kinectin cDNA clone containing a 4.7-kilobase insert was isolated from an embryonic chick brain cDNA library by immunoscreening with a panel of monoclonal antibodies. The cDNA contained an open reading frame of 1364 amino acids encoding a protein of 156 kDa. A bacterially expressed product of the full length cDNA bound purified kinesin. Transient expression in CV-1 cells gave an endoplasmic reticulum distribution that depended upon the N-terminal domain. Analysis of the predicted amino acid sequence indicated a highly hydrophobic near N-terminal stretch of 28 amino acids and a large portion (326–1248) of predicted α helical coiled coils. The 30-kDa fragment containing the N-terminal hydrophobic region was produced by cell-free in vitro translation and found to assemble with canine pancreas rough microsomes. Cleavage of the N terminus was not observed confirming its role as a potential transmembrane domain. Thus, the kinectin cDNA encodes a cytoplasmic-oriented integral membrane protein that binds kinesin and is likely to be a coiled-coil dimer.

INTRODUCTION

In eukaryotic cells, many cellular processes, including force generation for mitotic chromosomal separation, physical extension of the endoplasmic reticulum (ER), fast axonal transport, yolk granule transport from insect ovariole to the egg, are catalyzed by microtubule-based organelle transport (for review see Schroer and Sheetz (1990) and Vallee and Shpetner (1990)). Analysis of the microtubule requirement(s) in the membrane trafficking pathways in the cell have also indicated that microtubules and microtubule-based motors dramatically influence ER formation (Lee *et al.*, 1989), membrane recycling from the transitional ER back to ER (Lippincott-Schwartz *et al.*, 1990), Golgi assembly at the microtubule organizing center (MTOC) (Freed *et al.*, 1989; Ho *et al.*, 1989), early to late endosome transition during endocytosis (Greenberg *et al.*, 1993), and alignment of lysosomes along microtubules (Swanson *et al.*, 1987; Mekori *et al.*, 1989).

The microtubule-based transport machinery has currently been suggested to contain polar microtubules as tracks, motor proteins (kinesin, cytoplasmic dynein, or analogues) as engines, ATP as a fuel, accessory factors as regulators, and vesicles as cargo (Schroer *et al.*, 1988, 1991). The cargo vesicles are presumed to carry the mechanistic signals dictating the directionality of movement (Sheetz *et al.*, 1986). We have previously reported that vesicle motility and motor-membrane interaction depend on membrane protein(s) (Yu *et al.*, 1992; Burkhardt *et al.*, 1993), and these membrane anchor protein(s) might also serve as a switch to control the directionality.

An integral ER membrane protein of 160 kDa, kinectin, was shown to bind to the tail portions of kinesin (Toyoshima *et al.*, 1992). Recent findings that an anti-kinectin monoclonal antibody (mAb) can inhibit plus-end directed vesicle motility in vitro, in parallel with

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an inhibition of kinesin binding, suggest that kinectin is an essential membrane anchor for the kinesin-driven vesicle motility on microtubules (Kumar *et al.*, 1995). We report here the isolation and characterization of a full length kinectin cDNA which encodes a protein that binds to kinesin in vitro. Topogenic signal analysis of the N terminus suggests that the N terminus contains a putative transmembrane domain with the rest of the kinectin molecule in the cytoplasm, which presented us with a testable model for kinectin's functions.

MATERIALS AND METHODS

Antibody Preparation and Affinity Purification of Kinectin

The mAbs of the 160.x.1 series and 1B9 against kinectin were produced as described previously (Toyoshima *et al.*, 1992). A new anti-kinectin mAb (VSP4D), which only recognizes the native epitope of kinectin in immunoprecipitation but not the denatured kinectin on immunoblot (Kumar *et al.* 1995), was generated by procedures described previously (Yu *et al.*, 1992). Mice were injected with intact Na_2CO_3-extracted microsomes mixed with RIBI adjuvant (RIBI Immunochemicals), and hybridoma supernatants were screened for microsome binding and motility inhibiting clones. Microsome binding was assayed by enzyme-linked immunosorbent assay by incubating cell supernatant of each hybridoma clone with intact alkali-extracted microsomes. VSP4D can immunoprecipitate kinectin (Kumar *et al.*, 1995). Anti-HSTAF (human kinectin) mAbs NT-1 and CT-1 were obtained from Dr. Martin Krönke (Technical University, München, Germany). NT-1 was raised against the N terminus of human kinectin, and CT-1 was raised against the C terminus of human kinectin (Krönke, personal communication). Cell supernatants of mAbs were diluted 1:6 into 3% milk, for immunoscreening of the cDNA library.

To prepare anti-fusion protein polyclonal antibodies, 50 μg of the purified inclusion bodies (see "Bacterial Expression and Purification of Fusion Protein") were injected intraperitoneally biweekly in RIBI adjuvant for 6 wk, and the serum was tested by immunoblotting and immunostaining of chick embryo fibroblast cells at a dilution of 1:300.

For affinity purification of the kinectin, Na_2CO_3-extracted microsomes were solubilized in 1% Triton X-100, 0.5 M NaCl on ice for 1 h. A supernatant was obtained by centrifugation at 55 000 rpm for 15 min in a TL100.3 rotor (Beckman). The supernatant was diluted to 0.15 M NaCl with PMEE' (35 mM potassium 1,4-piperazinediethanesulfonic acid, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, and 0.5 mM EDTA) and incubated with 2 ml of the anti-kinectin mAb KR160.9.1 conjugated to Sepharose 4B resin (10 mg of IgG per ml) for 16 h at 4°C in a rotating mixer. After washing with 50 ml of suspension buffer, the 156-kDa protein and its associated complex were eluted with 3 ml of 0.2 M glycine, pH 2.8, containing 0.1% TX-100 in 0.3 ml of 1 M Tris-HCl, pH 8.5. The sample was then concentrated on a Centricon 30 (Amicon, Danvers, MA) 10-fold and analyzed on 0.75% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of Clones

An embryonic chick brain λgt 11 cDNA library was obtained from Dr. Don Cleveland (Johns Hopkins University, Baltimore, MD). Immunoscreening of the cDNA library was performed sequentially with each of the three mAbs (KR160.11.1, 1B9, KR160.10.1) using a modification of a standard protocol (Sambrook *et al.*, 1989). Immunoblotting was performed as described previously (Yu *et al.*, 1992). After plaque purification, the cDNA inserts were subcloned into the *Eco*RI site of the pGEM-4z vector (Promega) for further analyses.

For cell free in vitro translation and membrane translocation studies, the first 724 bp encoding the N terminus of the open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using DNA polymerase Pfu and the manufacturer's protocol (Stratagene, La Jolla, CA). PCR primers were designed with the help of a shareware "Amplify" for Mac and purchased from GENSET (La Jolla, CA). The PCR product was directionally subcloned (with *Xba*I and *Eco*RI) into a vector, pGEMBP1 (Connolly and Gilmore, 1986). "Hot Start" PCR protocol (Instruction for Ampliwax, Perkin-Elmer, Norwalk, CT) was employed in conjunction with Pfu DNA polymerase (Stratagene) and nucleotides from Perkin-Elmer. The thermocycler (Perkin-Elmer) was programmed as follows: 95°C 1′, 52°C 2′, 72°C 3′. The ends of the PCR products were sequenced to verify their identities.

For recombinant expression of the full length kinectin for kinesin binding studies, pGEMEX-1 was used as the vector (Promega). The full length kinectin was digested with *Eco*RI and *Bgl*I to release a 3.8-kb C-terminal kinectin fragment to be gel-purified. The remaining 0.9-kb N-terminal fragment with *Nhe*I site in the N terminus and *Bgl*I site at the 3′ end was generated by PCR, digested with *Nhe*I and *Bgl*I, and gel-purified. Two primers were used: primer 1 containing the 5′ end of the ORF with an in-frame *Nhe*I site, and primer 2 containing the sequence including the *Bgl*I site at position 1021. The two inserts (3.8 and 0.9 kb) were directionally subcloned into a *Nhe*I/*Eco*RI-digested and gel-purified pGEMEX-1 vector in a trimolecular ligation to generate a full length kinectin cDNA in pGEMEX-1. Similarly, the full length kinectin cDNA was subcloned into pCB6 (Dr. Evelyne Coudrier, Institute Pasteur, Paris), a eukaryotic expression vector with CMV promoter upstream of the ATG start site for transfection and expression in CV1 cells. For N-terminal truncated kinectin clone, primer 1 was replaced with a sequence at 150 bp from the 5′ end of the ORF so that the first 149 bp (approximately 50 amino acids including the putative transmembrane domain) were missing from the kinectin cDNA.

DNA Sequencing

cDNA clones subcloned into the *Eco*RI site of the pGEM-4z vector were sequenced in both directions (walking from both ends) by dideoxy-chain-termination method (Sanger *et al.*, 1977) with Sequenase 2.0 (USB, Cleveland, OH) using [^{35}S]dATP (RediVue, Amersham). Each subcloning of the full length or a portion of the kinectin cDNA was confirmed by sequencing the ends of the new clone to ensure the correct orientation.

Sequence Analysis

DNA sequences were assembled and analyzed using Lasergene software (DNASTAR, Madison, WI). Protein sequences from the ORF translation were analyzed for structural features such as α helices, β sheets, flexible regions, hydrophilicity, and surface probability using the Protean module of DNA-Star. A VAX/VMS program, COILS2.EXE, (EMBO, Heidelberg, Germany) was used to calculate the probability of coiled-coil formation. The kinectin sequence was analyzed using both scoring matrices, MTK and MTIK. The alignment of all kinectin homologues was performed by Megalign program in the Lasergene package using the Hein method (Hein, 1990) with a PAM250 residue weight table. A Prosite motif search was performed by MacPattern v3.0 using Prosite and Block databases (Bairoch, 1991) downloaded from EMBL databases.

Northern Blot

Fresh embryonic chick brains (4–12 d old) were homogenized in a Dounce homogenizer pretreated with diethyl pyrocarbonate (DEPC), and total RNA was purified using RNAZol RNA purification kit (Tel-Test, Friendswood, TX). Total RNA was then processed

using a PolyATtrack mRNA isolation kit (Promega, Madison, WI) to yield mRNA. mRNA (7 μg) per lane were separated on a 0.75% agarose/12% formaldehyde gel using a standard protocol (Sambrook *et al.*, 1989). The RNA was transferred to a nitrocellulose membrane (Schleicher & Schüll) in 20 × standard sodium citrate phosphate (SSCP), and the probes were P^{32}-labeled by random priming (Feinberg and Vogelstein, 1983). After hybridization, the nitrocellulose membrane was washed twice for 30 min in 0.1 × SSCP, 0.1% SDS at 60°C, and bands were visualized by autoradiography.

Bacterial Expression of the Fusion Proteins Encoded by cDNA Clones

λgt 11 phage clones were used to infect a high frequency lysogenic (hfl) *Escherichia coli* strain, BNN103 (Young and Davis, 1983), and the clones carrying the integrated phages as lysogens were isolated and screened. The lysogens were grown in 500 ml of L-broth at 32°C and, when the OD_{600} reached 0.5, were subjected to a heat shock in a preheated circulating water bath at 42°C for 15 min to induce expression. Expression was continued for an additional 2 h at 38°C, and the bacteria were harvested by centrifugation at 4000 × *g*, 4°C for 15 min.

For expression of pGEMEX-1 clones, the clones were transformed into JM109(DE3). A saturated overnight culture was diluted ~1:150, grown to OD_{600} = 0.2–0.5, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 2.5 h. The induced bacteria were pelleted, washed in phosphate-buffered saline (PBS), repelleted, and resuspended in 10 ml of suspension buffer (50 mM PBS, pH 8.2; 1 mM EDTA; 25% sucrose). Lysozyme (2 mg/ml) was added to the samples, and then protease inhibitors (10 μg/ml leupeptin; 10 μg/ml pepstatin; 1:1000 dilution of diisopropyl fluorophosphate; 5 mM benzamidine; 50 μg/ml phenylmethylsulfonyl fluoride) were added for 30 min at 4°C. $MgCl_2$ (10 mM) and 40 μg/ml DNase were added for another 30 min at 4°C before adding 20 ml of lysis detergent buffer (20 mM PBS, pH 7.4; 1% Triton X-100; 1% deoxycholate; 200 mM NaCl; 2 mM EDTA; 1 mM dithiothreitol (DTT) and fresh protease inhibitors). Each sample was extruded through a 20-gauge needle 3 × and centrifuged at 5,000 × *g* for 10 min. The supernatants were saved to check for soluble-expressed proteins, and the pellets were washed twice with lysis buffer; and two to three times with 0.4% Triton X-100, 1 mM DTT, 10 mM PBS, and 1 mM EDTA. Finally, the inclusion bodies were resuspended in 8 M urea, 10 mM PBS, 1 mM DTT, 1 M NaBr, 1 mM EDTA, 1 mM NaN_3, and 10 mM glycine and stored at −80°C for further analysis.

Dot-Blot

Solubilized fusion proteins (50 and 250 ng) in inclusion bodies (1 and 5 μg) were diluted 1:10-fold in PMEE′ buffer, kept on ice for 10 min, and filtered onto a nitrocellulose membrane which was prewetted in 10% methanol in PMEE′ and prerinsed in PMEE′ in a dot-blot apparatus (Bio-Rad). Two sets of experiments, with a five-fold difference in loading of the fusion proteins, were performed to ensure that possible differences in protein recovery were not a significant factor in our results. The nitrocellulose membrane was blocked with 3% bovine serum albumin (BSA; Sigma) in Tween-20 tris-buffered saline (TTBS) (Towbin *et al.*, 1979) for 30 min at room temperature, and incubated with 1:5 dilution of VSP4D hybridoma cell medium overnight at 4°C. For the kinesin binding assay, sucrose gradient-purified kinesin (Yu *et al.*, 1992) was dialyzed in PMEE′ for 2 h at 4°C and diluted in PMEE′ to 10 μg/ml. Incubations were performed for 1 h at 37°C. The blot was rinsed briefly three times in PMEE′, then fixed in 2% formaldehyde in PMEE′ for 15 min at 25°C, rinsed briefly with PMEE′, blocked again with 3% BSA for 30 min at room temperature, and incubated with anti-kinesin mAb SUK4 (Ingold *et al.*, 1988) as described above for VSP4D. The blot was then processed as described previously for immunoblots (Yu *et al.*, 1992). For kinesin binding to pGEMEX-1 expressed full-length kinectin, increasing concentrations of kinectin (1-, 5-, 20-μg total proteins in inclusion bodies) were used on the blot. Alkaline-washed 40/60 vesicles (Yu *et al.*, 1992) (0.05-, 0.25-, 1-μg total vesicle proteins) were used as a positive control for kinectin binding and pGEMEX-1 mock transformant in BL21(DE3) (2.4-, 12-, 48-μg bacterial proteins) were used as a negative control.

Transient Expression in CV1 Cells and Immunofluorescence Microscopy

Transfection of full length and N-terminal truncated kinectin cDNA into CV1 was performed according to the instruction accompanying LipofectAmine kit (GIBCO BRL/BRL). CV1 cells were cultured in complete CV1 media (Hamm-Alvarez *et al.*, 1993) on sterilized microscope coverslips in 12-well tissue culture plates until they were approximately 1/3 confluent. LipofectAmine (6 μl/well) was diluted into 100 μl of serum-free media (Opti-MEM-I, BRL). Purified cDNA (1.5 μg; purified by QIAGEN Midi-Prep kit) was diluted in a separate tube of 100 μl of serum-free media. The diluted DNA and LipofectAmine were mixed at 24°C for 20 min to allow complex formation. The CV1 cells were rinsed with 2 ml/well serum-free media immediately before incubation with LipofectAmine-DNA complex. The LipofectAmine-DNA complex was incubated with CV1 cells for 5 h at 37°C in 5% CO_2 tissue culture incubator. Complete CV1 media was then added to the cells for another 15 h. before changing into fresh complete CV1 media. The cells were then allowed to grow for another 5 h and rinsed with 2 ml/well of PBS. For control experiment, 100 μl of serum-free media was mixed with the diluted LipofectAmine in place of DNA.

Cells were fixed, permeabilized, and stained with KR160.4.1, rhodamine-wheat germ agglutinin (WGA) and DiOC6 (Molecular Probe) as described (Toyoshima *et al.*, 1992). The cells were imaged in a Axiophot (Zeiss) fluorescence microscope equipped with a Star-I cooled CCD camera interfaced to a Macintosh computer. An exposure time of 10 s was used to capture each image. The images were then compiled and output to a dye-sublimation color printer (Tektronix).

Cell-Free Transcription, Translation, Membrane Translocation, and Association Studies

Transcription was performed using a MegaScript T7 transcription kit (Ambion, Austin, TX). Cell-free translations were performed as described previously (Nicchitta and Blobel, 1989) using a reticulocyte lysate translation system supplemented, where indicated, with canine pancreas rough microsomes. Translation reactions (20-μl final volume) were performed for 30 min at 25°C and contained 500 ng of mRNA transcript encoding either bovine preprolactin (pPL) or a truncated kinectin (tKNT). At 30 min, reactions were supplemented with puromycin, to a final concentration of 1 mM, and the incubation was continued for an additional 10 min. Reactions were subsequently chilled on ice and processed as follows. To assess the membrane localization of the translation products, protease digestion experiments were performed by dilution to 50 μl in a physiological salt buffer (110 mM KOAc, 25 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2.5 mM $Mg(OAc)_2$ supplemented with 300 μg/ml proteinase K. Digestions were performed for 30 min on ice, and the protease was inactivated by addition of phenylmethylsulfonyl fluoride to 5 mM. The reaction products were fractionated with ammonium sulfate, and prepared for SDS-PAGE as described previously (Nicchitta and Blobel, 1989). To determine membrane association, translation reactions were diluted to 200 μl in 2.3 M sucrose/physiological salt buffer and placed into Beckman TLS-55 centrifuge tubes. Reactions were overlayed with 0.8 ml of 1.9 M sucrose, 0.6 ml of 1.5 M sucrose, and 0.4 ml of 0.25 M sucrose, in physiological salt buffer, and centrifuged for 2 h at 50,000 × rpm (4°C) in the Beckman TLS-55 rotor. Gradients were fractionated to yield four 0.5-ml fractions, and a pellet fraction. To recover the

translation products, gradient fractions were diluted with two volumes of saturated ammonium sulfate, incubated on ice for 20 min, and centrifuged for 10 min at 16,000 × g in a refrigerated microcentrifuge. The supernatants were discarded, the pellets were washed in 5% trichloroacetic acid, and the samples were prepared for SDS-PAGE as described previously (Nicchitta and Blobel, 1989).

RESULTS

Isolation of cDNA Clones and Confirmation of Their Identities

Immunoscreening of the embryonic chick brain cDNA library with three different anti-kinectin mAbs (KR160.11.1, 1B9, KR160.10.1) (Toyoshima *et al.*, 1992) yielded three clones (clones A, B, and C). Only one, clone A, was recognized by all three monoclonal antibodies suggesting that clone A was the most likely candidate to be a kinectin cDNA. The insert in clone A was subcloned into pGEM-4z vector for DNA sequencing and was ~4.7 kb in length which was of sufficient size to encode a full length ORF of kinectin. To verify that clone A encoded a message of reasonable size, mRNA was purified from embryonic chick brains and analyzed in a Northern blot experiment (Figure 1). The size of the message that the clone A encodes was ~5.3 kb, consistent with the observed molecular mass of purified kinectin (160 kDa, ~4 kb of message).

A fourth anti-kinectin mAb (VSP4D), that inhibited the kinesin-driven vesicle motility in vitro (Kumar *et al.*, 1995), recognized only a native epitope of kinectin in immunoprecipitation but not the denatured kinectin in immunoblot. To verify the identity of clone A as a putative kinectin cDNA, we used renatured fusion proteins from all three clones in a dot-blot experiment to react with VSP4D. Solubilized fusion proteins expressed by the clones were renatured by a 1:10-fold dilution from 8 M urea and spotted onto a nitrocellulose membrane. The renatured fusion proteins were allowed to react with the mAb VSP4D. To control for the mass effect, equal amounts of expressed proteins were loaded on the dot-blot. Two sets of samples were used such that one set (Figure 2A, set 2) had five times the amount of expressed protein as the other set (Figure 2A, set 1). It is evident in Figure 2 that fusion protein expressed by clone A was recognized by the mAb VSP4D, whereas five times the fusion protein from clone B and clone C were not. Together, we have four different anti-kinectin mAbs that reacted with fusion protein expressed from clone A.

Since kinectin was characterized as a kinesin binding ER membrane protein (Toyoshima *et al.*, 1992), we have used a blot binding assay to investigate the possible interaction between the renatured expressed fusion proteins and kinesin. As in the renaturation dot-blot experiment, we have loaded the renatured fusion proteins expressed from clones A, B, and C onto a nitrocellulose membrane. After blocking the nitrocellulose membrane with BSA, kinesin (25 nM) was in-

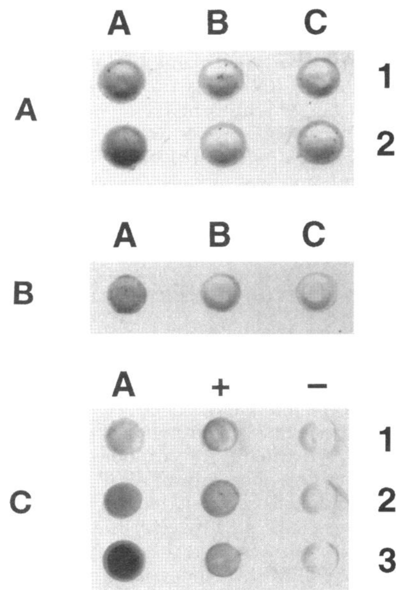

Figure 2. Renatured proteins expressed from clone A interact with kinesin and anti-kinectin mAb VSP4D. (A) Dots A, B, and C are fusion proteins of clone A, B, and C, respectively. They were probed with mAb VSP4D. Only clone A reacted with VSP4D. Row 1 has 50 ng of expressed fusion proteins, and row 2 has 250 ng of fusion proteins. (B) Fusion protein (0.5 μg) was loaded in each spot and incubated with 10 μg/ml of sucrose gradient-purified kinesin, fixed, and visualized with anti-kinesin mAb SUK4. Dots A, B, and C are fusion proteins of clone A, B, and C, respectively. Only clone A reacted with kinesin. (C) Kinesin binding to clone A (lane A), alkaline-washed chick brain microsomes (lane +) and bacterial proteins (lane −). Dots A (1–3) have 1, 5, and 20 μg of total inclusion body proteins, respectively. Dots +(1–3) have 0.05, 0.25, and 1 μg of total vesicle proteins, respectively. Dots −(1–3) have 2.4, 12, and 48 μg of total bacterial proteins, respectively. Clone A as well as native kinectin in vesicles reacted with kinesin but not bacterial proteins.

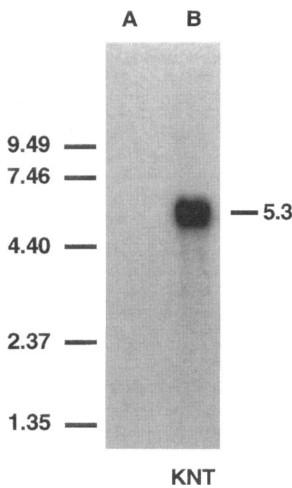

Figure 1. Northern blot. mRNA from embryonic chick brains was probed with radiolabeled cDNAs from clone A and *Eco*RI-digested pBluescript II SK^+ as negative control. Lane 1 is the negative control; lane 2 is the message (5.3 kb) encoded by clone A.

cubated with the blot and the unbound kinesin was washed away. The kinesin-fusion protein complex on the blot was fixed with formaldehyde, and the bound kinesin was visualized by immunoblotting with anti-kinesin mAb SUK4 (Ingold *et al.*, 1988). Figure 2B demonstrates that kinesin can bind to renatured fusion protein expressed from clone A but not to the ones from clones B and C. Using pGEMEX-1 (Promega)-expressed full length clone A instead of the fusion protein as described above, we have also observed kinesin binding to expressed clone A but not to endogenous bacterial proteins (Figure 2C). These data, in combination with the characteristics of the cDNA as described below, clearly indicated that clone A is a kinectin cDNA.

We have previously reported that most anti-kinectin mAbs reacted with two bands of 160 and 120 kDa on immunoblots (Toyoshima *et al.*, 1992). To further confirm that clone A is a kinectin cDNA, we have raised antisera against the fusion proteins expressed from the cDNA clones A, B, and C to investigate their ability to react with purified native kinectin. The fusion proteins purified from the clones were injected into mice, and the antisera produced were then used to probe the purified kinectin in an immunoblot. All antisera reacted with the purified kinectin on immunoblots to different degrees but only anti-clone A antiserum exhibited the same pattern as the positive control (KR160.9.1) (Figure 3). When vesicles were probed with the antisera, the major band was in the molecular weight range of kinectin (Figure 3). The antisera were also used to stain embryonic chick fibroblast cells; they stained mostly with an ER-like pattern, except that the antiserum against clone C protein stained filamentous structures in the cytoplasm (our unpublished results).

An additional question is whether or not the expression of this kinectin cDNA in CV1 cells would give the normal distribution of kinectin to the ER as seen previously. CV1 cells were chosen because of their spread morphology and because most antibodies to chicken kinectin did not label CV1 cells. When the full length kinectin cDNA was subcloned into a eukaryotic expression vector pCB6 and transfected into CV1 cells to be expressed transiently, expressed kinectin was localized mostly to ER but not Golgi (Figure 4, C–F) as expected.

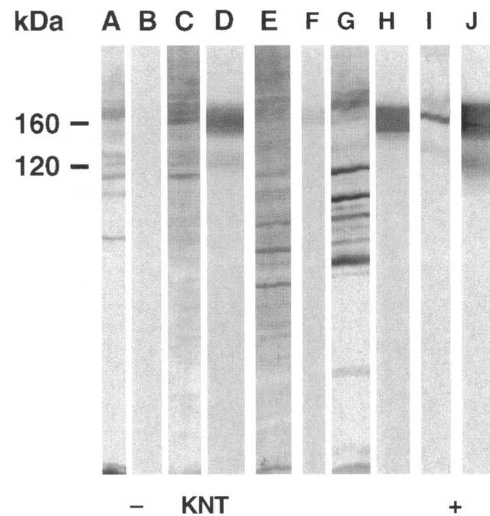

Figure 3. Anti-fusion protein antisera reacted with purified native kinectin. Lanes A, C, E, G, and I represent immunoblot of carbonate-washed vesicle proteins and lanes B, D, F, H, and J are purified kinectin reacted with various anti-fusion protein antibodies. Lanes A and B are with pre-immune serum as negative control; lanes C and D are with anti-clone A (KNT = kinectin) Ab; lanes E and F are with anti-clone B Ab; lanes G and H are with anti-clone C Ab and lanes I and J are with KR160.9.1 as positive control.

Structural Analysis of the Kinectin cDNA

The kinectin cDNA contains an ORF of 4092 bp and encodes a protein of 1364 amino acids of 156 kDa that agrees very well with the calculated relative molecular weight of the chick embryo brain kinectin (Toyoshima *et al.*, 1992). Efforts to determine the N-terminal amino acid sequence of the chick embryo brain kinectin were unsuccessful, possibly because the N-terminal residue is blocked (our unpublished results). However, several indirect lines of evidence support the assignment of the N-terminal residue in the deduced protein sequence, and the conclusion that the complete coding sequence of kinectin has been elucidated. A Kozak sequence (Kozak, 1991) is adjacent to the proposed translation initiation site at the beginning of a single ORF (there is an in-frame ochre termination triplet (TAA) which is 24–22 bp 5′ from the first AUG of the ORF). An identical start site (including a methionine followed by 5 hydrophilic residues) has been found in two human ORFs isolated independently, which appear to be human kinectin homologues (Figure 7 and see below). The ORF ends with a termination triplet UGA at position 4162–4164 and is followed by an untranslated sequence extending from position 4165 to 4668. A potential polyadenylation signal (AATAAA) is located at position 4652–4657 followed by 34 bases of Poly(A) tail starting at position 4669 (Figure 5).

Analysis of the deduced amino acid sequence reveals that the first 6 residues in the extreme N terminus are hydrophilic and the next 28 residues are hydrophobic (Figure 6). This N-terminal hydrophobic domain is the only domain likely to insert into the membrane (Figure 8) although there are seven potential myristylation sites at residues 135, 256, 364, 382, 618, 902, and 1114 that could be modified to interact with the membrane if those sites are exposed. The entire kinectin sequence, other than

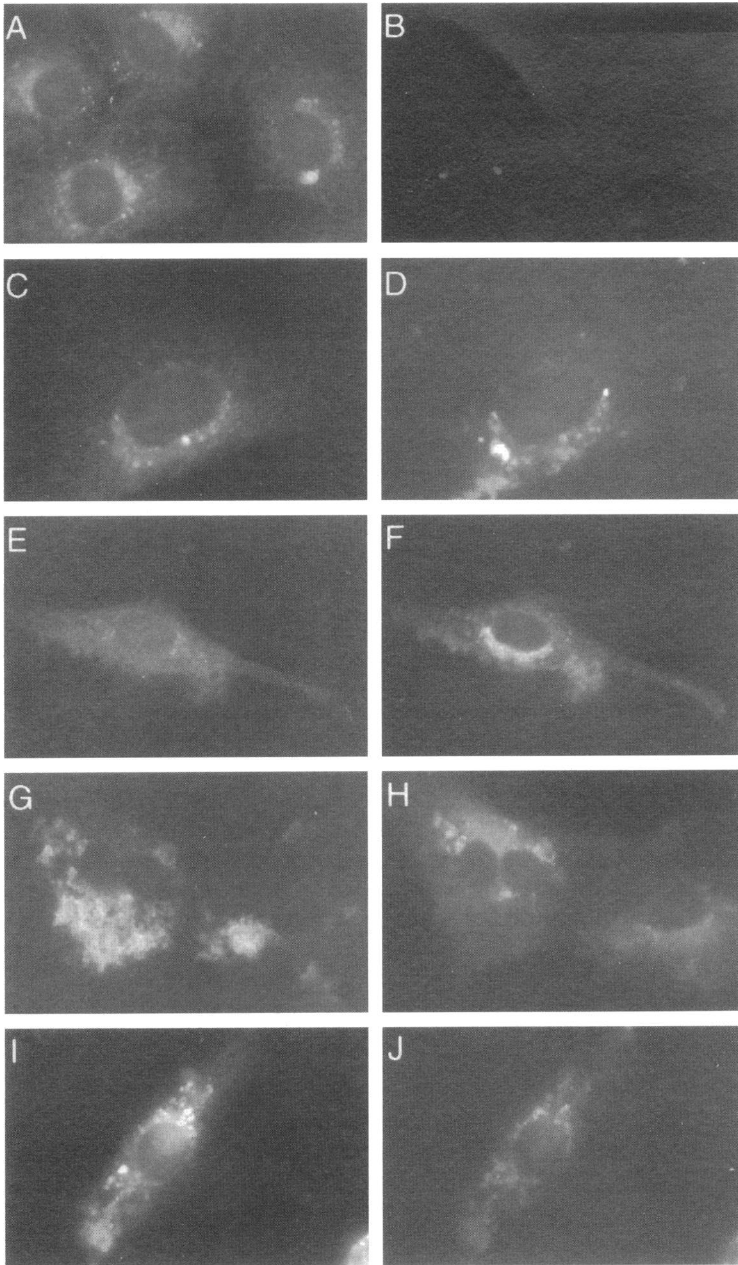

Figure 4. Immunolocalization of full-length and N-terminal truncated kinectin in CV1 cells. DiOC6 is used to stain ER to be visualized in FITC channel; WGA was used to stain Golgi to be visualized in rhodamine channel of the fluorescence microscope. Control CV1 cells that were treated with LipofectAmine but no kinectin cDNAs were stained with DiOC6 (A) and with KR160.4.1 + rhodamine-goat anti-mouse secondary Ab (B). KR160.4.1 does not recognize CV1 proteins (A and B). CV1 cells transfected with full length kinectin cDNA (C–F) were stained with DiOC6 (C), KR160.4.1 + rhodamine-secondary Ab (D), KR160.4.1 + FITC-secondary Ab (E), and WGA (F). Full-length kinectin was expressed mostly in ER (C and D) and not Golgi (E and F). CV1 cells transfected with N-terminal truncated kinectin cDNA (G–J) were stained with KR160.41 + rhodamine-secondary Ab (G), DiOC6 (H), KR160.4.1 + FITC-secondary Ab (I), and WGA (J). The truncated form of kinectin was expressed as large aggregates (G–J) not in ER (G and H) but in regions including Golgi (I and J).

the first 34 residues in the N terminus, is highly hydrophilic with all residues having high surface probability, indicating that kinectin probably is an extended molecule rather than a globular protein. Residues 326-1248 of kinectin are likely to form α helices (Figure 6) and have heptad repeats with an extremely high probability of forming coiled coils (Lupas *et al.*, 1991) (Figure 6). The residues in *a* and *d* position of the heptad repeats are mostly leucine rather than isoleucine, further implying that kinectin can form dimers (Harbury *et al.*, 1993).

When the putative amino acid sequence of the kinectin cDNA sequence was used to search GenBank and EMBL databases, two entries matched with very high scores. CG1 (accession numbers: gp|D13629 and gp|L25616) has not been characterized and another match (HSTAF or Nap156 or Human Kinectin) (accession numbers: pir|S32763 and emblu|Z22551) was a 160-kDa hypothetical protein which was characterized as an ER membrane protein that can react with anti-kinectin mAb KR160.9.1 (Krönke, personal communication). One mAb against the C terminus of the puta-

tive human kinectin (CT-1) also reacted with purified chick embryo brain kinectin and impure kinectin in carbonate-washed vesicles (Figure 7). Another anti-human kinectin mAb (NT-1) did not react with any chick protein (Figure 7). The two human ORFs were aligned with the embryonic chick brain kinectin sequence (Altschul *et al.*, 1990; Hein, 1990) (Figure 7), and they were almost identical except that the HSTAF (human kinectin) protein had a 28-residue insertion at residues 1031–1032 and a 27-residue insertion at residues 1203–1204 when compared with the CG1, implying that they were the products of alternative splicing. The chick kinectin has 71% identity and 87% similarity in the first 700 N-terminal residues when compared with both human sequences. From residue 700 to 900, the identity dropped to 63% and similarity to 83%. From residue 900 to the C terminus, the identity further dropped to 61% and similarity to 80%. These high levels of sequence homology and the antibody cross-reactivity suggest that the human ORFs are human kinectin homologues.

Cell-Free Transcription, Translation, Membrane Translocation, and Association Studies

By virtue of its resistance to extraction in alkali buffers, native kinectin is thought to be an integral membrane protein (Toyoshima *et al.*, 1992). Transient expression of a kinectin cDNA lacking the first 50 residues in the N terminus in CV1 cells resulted in an immunolocalization pattern distinctly different from the ER distribution of the native kinectin in fibroblast (Toyoshima *et al.*, 1992) and the expressed full length kinectin in CV1 cells (Figure 4). This suggested that the N terminus of kinectin was essential for its correct localization in the cells. A hydrophobicity analysis (Kyte and Doolittle, 1982) of the kinectin amino acid sequence also indicated a single hydrophobic region (residues 7–34) of sufficient length to traverse the membrane bilayer (Figure 6). However, application of the $-3,-1$ rule for signal peptide cleavage (von Heijne, 1986), suggested a putative signal peptide cleavage site between residues 39 and 40. In the absence of an additional membrane-spanning hydrophobic domain(s), the N-terminal hydrophobic domain would be expected to act as a signal for the initiation of translocation of newly synthesized kinectin across the ER membrane, yielding either a lumenally disposed or secreted translation product. To determine if the N-terminal hydrophobic domain functions as a signal sequence, a 730-bp truncated kinectin cDNA was first prepared by PCR and subcloned into pGEMBP1 (Connolly and Gilmore, 1986), a plasmid vector suitable for the in vitro transcription of the cloned DNA. After in vitro transcription, the mRNA was translated in a reticulocyte lysate translation system in the presence and absence of canine pancreas rough microsomes. As a positive control for signal peptide processing activity, parallel translation was performed with the mammalian secretory precursor, preprolactin.

As depicted in Figure 8A, in vitro translation of the truncated kinectin mRNA yielded a single translation product of M_r = 30 000 (Figure 8A, lane D). The mobility of the translation product was not altered by the cotranslational addition of rough microsomes (RM) (Figure 8A, lane E), indicating that the N-terminal hydrophobic domain was not undergoing proteolytic cleavage. The analogous experiment performed with preprolactin served as a positive control, and preprolactin was processed to mature prolactin in the presence of RM (Figure 8A, lanes A and B). To determine the membrane orientation of the translation products, protease digestion experiments were performed. As shown in Figure 8A, lane C, the addition of exogenous protease resulted in the degradation of the precursor preprolactin, whereas mature prolactin was protected. This result is consistent with translocation of the secretory precursor into the vesicle lumen. In contrast to prolactin, the truncated kinectin translation product was degraded in the presence of protease (Figure 8A, lane F), indicating exposure of the translation product to the cytoplasmic side of the vesicle membrane. On the basis of these experiments, we conclude that the N-terminal hydrophobic domain of kinectin does not serve as a signal sequence and that the bulk of the translation product remains exposed on the cytoplasmic domain of the membrane.

As noted previously, native kinectin behaves as an integral membrane protein (Toyoshima *et al.*, 1992). The hydrophobic N terminus, although apparently not acting as a canonical signal sequence, may serve a membrane anchor function through a mechanism distinct from the signal recognition particle (SRP)-mediated pathway (Walter *et al.*, 1984). An alternative assembly pathway has, in fact, been demonstrated for the α subunit of the SRP receptor (Hortsch *et al.*, 1988; Andrews *et al.*, 1989). To determine the state of membrane association of the truncated kinectin translation product, cell-free translations were performed in the presence and absence of RM, and membrane association assayed after flotation of the vesicles in a sucrose gradient. The results of these experiments are depicted in Figure 8B. In the absence of RM, both the preprolactin and kinectin translation products remained in the load zone of the gradient (Figure 8B, top, lanes A–C and F–H). In the presence of RM, preprolactin was processed to prolactin and was recovered in association with the vesicle fraction in the top fractions of the gradient (Figure 8B, bottom, lanes C–E). In the presence of RM, kinectin was also recovered in the top fractions of the gradient (Figure 8B, bottom, lanes H–J). These results indicate that the kinectin transla-

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CCCAGCCCGGCTCCTGCACTCCGGCCCCGCAGCCCGATTTTTTTTTAAATATAGCTAAGAAAAAGCACAATGGAGTTTTACGAGTCTACC 90
                                                                      M  E  F  Y  E  S  T  7
TACTTCATCATCCTCATTCCTTCTGTGGTTATTACAGTCATCTTTCTCTTCTTCTGGCTGTTCATGAAAGAAACATTATATGATGAAGTC 180
 Y  F  I  I  L  I  P  S  V  V  I  T  V  I  F  L  F  F  W  L  F  M  K  E  T  L  Y  D  E  V  37
CTTGCGAAGCAGAAAAGGGACCTAAAGTTTCCACCTACCAAGAGCGATAAAAAGAAGACAGAGAAGAAGAAGAACAAGAAGAAAGAAGCT 270
 L  A  K  Q  K  R  D  L  K  F  P  P  T  K  S  D  K  K  K  T  E  K  K  K  N  K  K  K  E  A  67
CAGAATGGGAACATCCACGAATCTGACTCTGAAAGCACACCTCGGGACTTCAAGCTCTCTGATGCCCTGGGTACAGATGAAGAGCAAGTT 360
 Q  N  G  N  I  H  E  S  D  S  E  S  T  P  R  D  F  K  L  S  D  A  L  G  T  D  E  E  Q  V  97
GCTCCTGTTCCCCTGAGTGCGACTGAGGCGTCTGCTGGCATCAGAGAACGGAAGAAGAAGGAGAAGAAGCAGAAGGCAGCTCAAGATGAT 450
 A  P  V  P  L  S  A  T  E  A  S  A  G  I  R  E  R  K  K  K  E  K  K  Q  K  A  A  Q  D  D  127
CACGTAACTAAGGAGTCGGAAGGATCCAAGTCTTCAGGCAAGAAAGTAGAACCAGTCCCTGTTACAAAACAGCCCACTCCACCATCCGAA 540
 H  V  T  K  E  S  E  G  S  K  S  S  G  K  K  V  E  P  V  P  V  T  K  Q  P  T  P  P  S  E  157
CCAGCAGCAGCTAAGAAGAAGCCTGGGCAGAAGAAGCAGAAAAATGATGATCAAGATACTAAGACTGATTCTGTTGCATCTCCTGCCAAA 630
 P  A  A  A  K  K  K  P  G  Q  K  K  Q  K  N  D  D  Q  D  T  K  T  D  S  V  A  S  P  A  K  187
AAGCAAGAACCTGTGCTGCTCCACCAGGAGGTAAAACAAGAAAATGTATCAGGAAAGAAGAAAGTCTCTGCAAAGAAGCAGAAAGTTTTG 720
 K  Q  E  P  V  L  L  H  Q  E  V  K  Q  E  N  V  S  G  K  K  K  V  S  A  K  K  Q  K  V  L  217
GTGGATGAACCTCTTATTCAGCCCACTACCTACATTCCTTTGGTGGATAACTCTGATGCTGGCGCTTTGGAAAAGAGAGAAGTGGTGGAA 810
 V  D  E  P  L  I  Q  P  T  T  Y  I  P  L  V  D  N  S  D  A  G  A  L  E  K  R  E  V  V  E  247
GTAGCAAAACAAAACATGAACGAAGGGATCCAGAAAAGCGGTGGCAAGAAAATGAAGAATGAAACTGATAAAGAGAATGCTGAAGTGAAA 900
 V  A  K  Q  N  M  N  E  G  I  Q  K  S  G  G  K  K  M  K  N  E  T  D  K  E  N  A  E  V  K  277
TTTAAAGACTTCGTAATGGCCATGAAGAATATGATCTTCACTGAAGATGAGGCCCGTTGTGTAGTGGAGGTGTTAAAGGAGAAGTCTGGT 990
 F  K  D  F  V  M  A  M  K  N  M  I  F  T  E  D  E  A  R  C  V  V  E  V  L  K  E  K  S  G  307
GCAATTCATGACGTCTTACAAAAGGCCAGCAAGGCAGAGTCGGCTGCAGCTATACATCAGCTGCAAGACAAGGAGAAACTGCTCGCTGCA 1080
 A  I  H  D  V  L  Q  K  A  S  K  A  E  S  A  A  A  I  H  Q  L  Q  D  K  E  K  L  L  A  A  337
GTGAAGGAGGAGGCAGCTGTTGCAAAGGAGCAGTGCAAGCAGTTAACTCAGGAGCTGGTTGCAGAAAAAGAGAGGAACGGCTTGCTTACA 1170
 V  K  E  E  A  A  V  A  K  E  Q  C  K  Q  L  T  Q  E  L  V  A  E  K  E  R  N  G  L  L  T  367
GCGAAAATGAGGGAACGCATCAACGCACTGGAGAAGGAGCATGGCACCTTCCAGAGTAAAATACACGTTAGCTACCAGGAATCTCAGCAG 1260
 A  K  M  R  E  R  I  N  A  L  E  K  E  H  G  T  F  Q  S  K  I  H  V  S  Y  Q  E  S  Q  Q  397
ATGAAGATAAAGTTCCAGCAACGCTGTGAGCAGATGGAGGCAGAAATAAGCCACCTGAAGCAGGAGAATACTATCCTGAGAGATGCTGTC 1350
 M  K  I  K  F  Q  Q  R  C  E  Q  M  E  A  E  I  S  H  L  K  Q  E  N  T  I  L  R  D  A  V  427
AGCACTTCTACCAATCAAATGGAGAGCAAGCAGGCAGCTGAACTGAACAAACTGCGTCAGGACTGCGCCAGGCTGGTGAATGAACTGGGA 1440
 S  T  S  T  N  Q  M  E  S  K  Q  A  A  E  L  N  K  L  R  Q  D  C  A  R  L  V  N  E  L  G  457
GAGAAGAACAGCAAGCTGCAGCAAGAGGAGCTCCAGAAGAAGAATGCGGAGCAGGCAGTGGCTCAGCTAAAGGTCCAGCAGCAGGAAGCA 1530
 E  K  N  S  K  L  Q  Q  E  E  L  Q  K  K  N  A  E  Q  A  V  A  Q  L  K  V  Q  Q  Q  E  A  487
GAGAGACGATGGGAAGAAATCCAGGTTTATCTCAGGAAAAGAACAGCAGAGCACGAAGCAGCACAACAAGATGTGCAAAATAAGCTTGTC 1620
 E  R  R  W  E  E  I  Q  V  Y  L  R  K  R  T  A  E  H  E  A  A  Q  Q  D  V  Q  N  K  L  V  517
GCCAAAGACAACGAAATCCAGAGCTTGCACAGTAAACTTACCGATATGGTGGTGTCAAAACAGCAGCTGGAGCAGAGGATGTTGCAGCTG 1710
 A  K  D  N  E  I  Q  S  L  H  S  K  L  T  D  M  V  V  S  K  Q  Q  L  E  Q  R  M  L  Q  L  547
ATTGAGAGTGAGCAAAAGAGAGCTTCTAAGGAGGACTCTATGCAATTGCGGGTGCAGGAGCTGGTAGAGCAGAACGATGCTTTGAACGCT 1800
 I  E  S  E  Q  K  R  A  S  K  E  D  S  M  Q  L  R  V  Q  E  L  V  E  Q  N  D  A  L  N  A  577
CAGCTTCAGAAGCTTCATTCACAAATGGCAGCCCAGACCTCAGCTTCAGTCCTGGCAGAAGAACTGCACAAAGTCATTGCAGAAAAGGAC 1890
 Q  L  Q  K  L  H  S  Q  M  A  A  Q  T  S  A  S  V  L  A  E  E  L  H  K  V  I  A  E  K  D  607
AAACAGCTAAAGCAGATGGAGGACTCATTAGGCAATGAACATGCCAATTTAACAAGCAAGGAAGAAGAGCTCAAGGTCTTGCAGAATATG 1980
 K  Q  L  K  Q  M  E  D  S  L  G  N  E  H  A  N  L  T  S  K  E  E  E  L  K  V  L  Q  N  M  637
AACCTGTCCCTGAAATCAGAAATACAGAAGTTACAGGCTCTGACAAATGAACAGGCTGCTGCTGCACATGAGCTAGAAAGGATGCAGAAA 2070
 N  L  S  L  K  S  E  I  Q  K  L  Q  A  L  T  N  E  Q  A  A  A  A  H  E  L  E  R  M  Q  K  667
AGCATTCACATCAAAGATGACAAAATAAGAACTCTTGAGGAGCAGCTTCGAGAAGAACTTGCTCAGACTGTGAACACAAAAGAGGAATTT 2160
 S  I  H  I  K  D  D  K  I  R  T  L  E  E  Q  L  R  E  E  L  A  Q  T  V  N  T  K  E  E  F  697
AAGATTCTCAAGGATCAAAACAAAACTTTGCAAGCTGAAGTGCAGAAGCTGCAGGCTCTCCTATCTGAGCCAGTACAGCCAACGTTTGAG 2250
 K  I  L  K  D  Q  N  K  T  L  Q  A  E  V  Q  K  L  Q  A  L  L  S  E  P  V  Q  P  T  F  E  727
GCAAACAAGGATTTGTTAGAGGAGATGGAAAGAGGCATGAGAGAGAGGGATGATAAAATCAAGACAGTTGAAGAGCTGCTTGAGGCAGGA 2340
 A  N  K  D  L  L  E  E  M  E  R  G  M  R  E  R  D  D  K  I  K  T  V  E  E  L  L  E  A  G  757
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Figure 5. Sequence analysis of the complete kinectin cDNA. Complete cDNA sequence is displayed with corresponding conceptually translated amino acid sequence underneath. The ATG at position 70–72 is the predicted N-terminal methionine residue and the potential polyadenylation signal (AATAAA) at 4652–4657 is underlined which is followed by a 34 base Poly(A) tail. The underscores surrounding the

tion product can associate with RER-derived membranes. The mechanism of association remains, however, to be determined. Additional studies to assess the degree of membrane integration, through alkali extraction, proved equivocal, as the kinectin translation product formed large aggregates when synthesized in the absence of RM (our unpublished results).

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CTCATACAGATGGCTAATAAAGAGGAGGAGCTGAAGGTACTGCGAACAGAAAATTCATCCTTGAGAAAAGAACTTCAAAGTCTGCAAATT 2430
 L  I  Q  M  A  N  K  E  E  E  L  K  V  L  R  T  E  N  S  S  L  R  K  E  L  Q  S  L  Q  I  787
CAGCTATCAGAGCAGGTTTCCTTCCAATCACTGGTAGACGAACTCCAGAAAGTGATCCATGAGAAGGATGGCAAAATAAAATCAGTGGAA 2520
 Q  L  S  E  Q  V  S  F  Q  S  L  V  D  E  L  Q  K  V  I  H  E  K  D  G  K  I  K  S  V  E  817
GAACTCCTACAAGCAGAAATCCTTAAAGTAGCAAACAAGGAGAAGACTGTTCAGGCTTTGACACAGAAAATAGAGGCTCTGAAAGAAGAA 2610
 E  L  L  Q  A  E  I  L  K  V  A  N  K  E  K  T  V  Q  A  L  T  Q  K  I  E  A  L  K  E  E  847
GTAGGAAATTCCCAGCTTGAGATGGAGAAACAGGTGTCAATTACATCCCAAGTCAAAGAACTTCAGACTCTGTTGAAGGGGAAGGAAAAT 2700
 V  G  N  S  Q  L  E  M  E  K  Q  V  S  I  T  S  Q  V  K  E  L  Q  T  L  L  K  G  K  E  N  877
CAGGTGAAAACCATGGAGGCCTTACTGGAAGAGAAGGAGAAAGAGATTGTTCAGAAAGGAGAACGGCTGAAGGGTCAGCAAGATACAGTT 2790
 Q  V  K  T  M  E  A  L  L  E  E  K  E  K  E  I  V  Q  K  G  E  R  L  K  G  Q  Q  D  T  V  907
GCACAATTAACCAGTAAAGTTCAGGAGTTAGAGCAGCAGAACCTGCAACAGCTCCAACAGGTACCTGCTGCATCCCAAGTCCAGGACCTG 2880
 A  Q  L  T  S  K  V  Q  E  L  E  Q  Q  N  L  Q  Q  L  Q  Q  V  P  A  A  S  Q  V  Q  D  L  937
GAGAGCCGGTTGAGAGGGGAGGAAGAACAAATCAGTAAGTTGAAAGCTGTATTGGAAGAAAAGGAGAGAGAAATTGCAAGCCAAGTTAAA 2970
 E  S  R  L  R  G  E  E  E  Q  I  S  K  L  K  A  V  L  E  E  K  E  R  E  I  A  S  Q  V  K  967
CAGCTGCAGACTATGCAGAGTGAAAATGAATCTTTTAAAGTCCAAATTCAAGAACTAAAGCAGGAGAACTGCAAGCAGGCGTCTCTGGCT 3060
 Q  L  Q  T  M  Q  S  E  N  E  S  F  K  V  Q  I  Q  E  L  K  Q  E  N  C  K  Q  A  S  L  A  997
GTCCAAAGTGAGGAGCTCCTGCAAGTGGTTGCAGGGAAAGAGAAGGAAATTGCCAGTCTCCAAAATGAGCTCGCCTGCCAGAGAAATGCT 3150
 V  Q  S  E  E  L  L  Q  V  V  A  G  K  E  K  E  I  A  S  L  Q  N  E  L  A  C  Q  R  N  A  1027
TTTGAGCAACAGAGGAAAAAGAATAACGACCTTCGGGAGAAAAACTGGAAAGCAATGGAGGCATTGGCATCAACTGAAAAATTGCTGCAG 3240
 F  E  Q  Q  R  K  K  N  N  D  L  R  E  K  N  W  K  A  M  E  A  L  A  S  T  E  K  L  L  Q  1057
GACAAAGTGAACAAGACTGCCAAGGAGAAGCAGCAGCACGTGGAAGCTGCCGAGGTGGAGACACGAGAGCTGCTCCAGAAGCTGTTCCCC 3330
 D  K  V  N  K  T  A  K  E  K  Q  Q  H  V  E  A  A  E  V  E  T  R  E  L  L  Q  K  L  F  P  1087
AACGTCTCTCTTCCTGCCAACGTGAGCCATAGCGAATGGATATGTGGATTTGAAAAGATGGCTAAAGAGTATTTGAGAGGGGCTTCAGGA 3420
 N  V  S  L  P  A  N  V  S  H  S  E  W  I  C  G  F  E  K  M  A  K  E  Y  L  R  G  A  S  G  1117
TCTGAAGATATCAAGGTTATGGAGCAGAAGTTGAAGGAGGCTGAGGAGCTGCACATACTGTTGCAACTGGAATGTGAGAAATACAAATCA 3510
 S  E  D  I  K  V  M  E  Q  K  L  K  E  A  E  E  L  H  I  L  L  Q  L  E  C  E  K  Y  K  S  1147
GTTCTGGCGGAGACGGAGGGGATTTTACAGAGGCTACAGAGGAGCGTGGAAGAGGAAGAAAGCAAATGGAAGATAAAAGTTGAAGAATCA 3600
 V  L  A  E  T  E  G  I  L  Q  R  L  Q  R  S  V  E  E  E  E  S  K  W  K  I  K  V  E  E  S  1177
CAGAAGGAACTCAAACAGATGCGCTCATCGGTCGCCTCTTTGGAGCACGAGGTAGAGAGGTTAAAAGAAGAGATCAAAGAAGTTGAAACT 3690
 Q  K  E  L  K  Q  M  R  S  S  V  A  S  L  E  H  E  V  E  R  L  K  E  E  I  K  E  V  E  T  1207
CTTAAAAAAGAGAGAGAACATTTGGAAAGTGAACTAGAAAAGGCAGAAATAGAGCGATCTACTTATGTTTCAGAAGTCAGAGAGCTCAAA 3780
 L  K  K  E  R  E  H  L  E  S  E  L  E  K  A  E  I  E  R  S  T  Y  V  S  E  V  R  E  L  K  1237
GATCTGTTGACTGAATTGCAGAAAAAACTTGATGATTCATATTCTGAAGCAGTAAGACAGAATGAAGAGTTAAATTTGCTGAAAATGAAA 3870
 D  L  L  T  E  L  Q  K  K  L  D  D  S  Y  S  E  A  V  R  Q  N  E  E  L  N  L  L  K  M  K  1267
TTAAGTGAAACACTGTCAAAACTCAAAGTTGATCAAAACGAGAGGCAGAAGGTAGCTGGTGACTTGCCCAAGGCCCAGGAATCCTTGGCA 3960
 L  S  E  T  L  S  K  L  K  V  D  Q  N  E  R  Q  K  V  A  G  D  L  P  K  A  Q  E  S  L  A  1297
TCTCTTGAGAGAGAAATTGGAAAAGTTGTTGGGGATGCCAATGTGATTGAAAACAGCGACGTTCGCACAGAGTCGGAATTGACTGATAAA 4050
 S  L  E  R  E  I  G  K  V  V  G  D  A  N  V  I  E  N  S  D  V  R  T  E  S  E  L  T  D  K  1327
AGACTAAATGTGGCGGTGAATCTTAACCAGGATGTAGGTCATCTCAAGAAGCTGCTCGTGTCAGTAAGCCAAATGCTGTCAAAGGGACGA 4140
 R  L  N  V  A  V  N  L  N  Q  D  V  G  H  L  K  K  L  L  V  S  V  S  Q  M  L  S  K  G  R  1357
GAACATTACCAGCTAGTAGAATGAAGTCACGGGGGAATCGCTGATCTGAGGATAGCAGAAGGCATTGTATTATATTTGCCAAATTAAAG 4230
 E  H  Y  Q  L  V  E  .                                                                     1365
CCTTATTTATGTTTTCACCCTTTCTACTTCGTCAGAGACACTGAACAGAGTTTTGTCTTTTCTAATCCTTGTTAGACTACTGATTTAAAG 4320
GAAAAAAAACCCGACTCTGTAGACACCTCCAGAGTTTAGTTTTATAATAACAAGAAAACCTGTTTGGATAATTAGACCTTTACATTCCTG 4410
GAGAGACAGTAAACACATAATCTTTCGTCGTCTTTTGCTCAGTAAAACTTGTTCAGAAAACTCCAACGTGGCAAAGTCACCAATGGGATA 4500
TAACATTTTAATACTGATGTTGTACACTGTTTTACTTAAATACATTTTTGGGATTAGCTGCCTCTGACTTCAACCTCAAGTAAACACTCC 4590
TTTTTTTTTTGTTCTTTTTTTTTTTGTTGCTAATGTAATCAGTTTTTGTAATGGTGTCAGCAAATAAAGGGATGCTATTAAAAAAAAAAA 4680
AAAAAAAAAAAAAAAAAAAAAAAAGGAATT 4709
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Figure 5 cont. translation initiation site represent the most recent Kozak consensus sequence. The underscore at position −24 to −22 represents the in-frame ochre termination codon UAA. The GenBank accession number is U15617.

DISCUSSION

Here we have reported the isolation and characterization of a complete cDNA (clone A) of kinectin. The protein product expressed from this cDNA can be recognized by all four classes of anti-kinectin mAbs (Toyoshima *et al.*, 1992), one of which can recognize the functional domain of kinectin (Kumar *et al.*, 1995). A β-galactosidase fusion protein (Figure 2B) as well as the *E. coli*-expressed full length protein from clone A (Figure 2C) bound kinesin, whereas the fusion proteins from clones B and C (Figure 2B) and the endogenous bacterial proteins (Figure 2C) did not. This kinesin binding capability agrees well with the proposed role of kinectin in vesicle motility. The size of the mRNA encoded by clone A is ~5.3 kb. Assuming ~1 kb of 3′ untranslated sequences (Figure 5), 4 kb of ORF corresponds

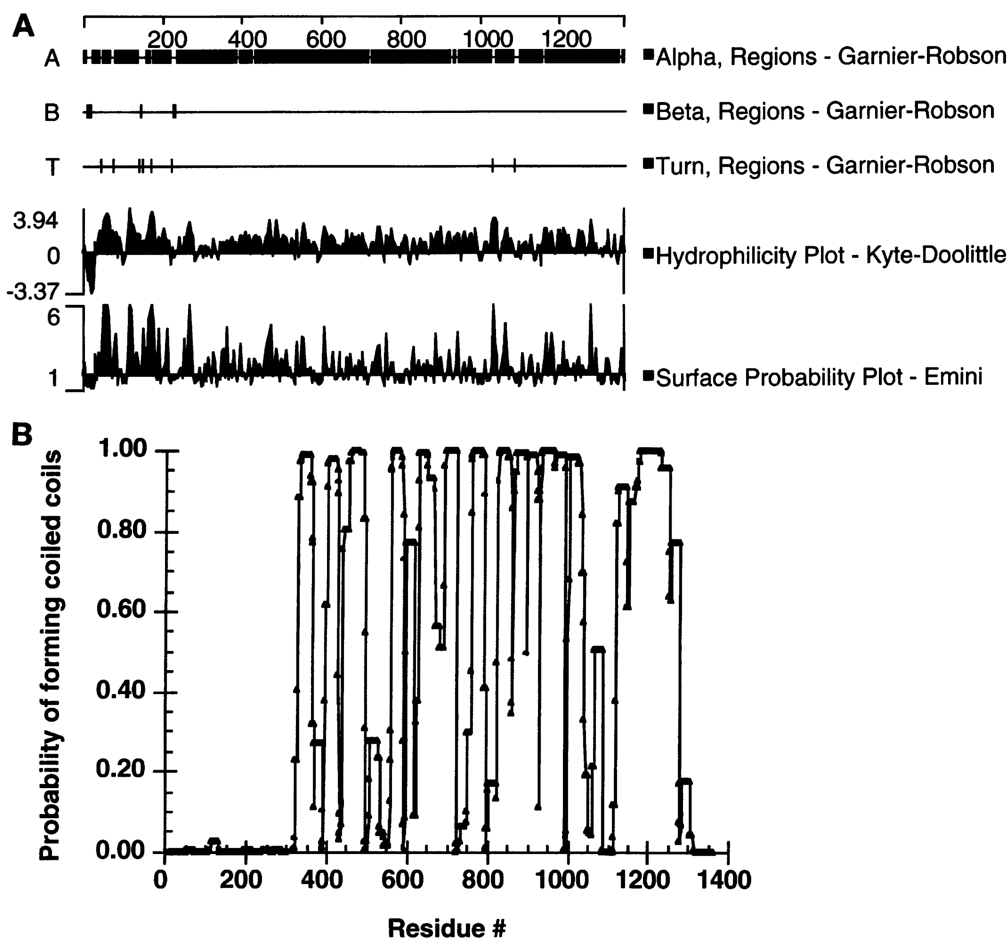

Figure 6. Predicted structural elements based on primary sequence. (A) Various plots of the putative structural elements/regions based on the conceptual translation of the kinectin cDNA. (Top to Bottom) Kinectin is mostly α-helical with a few β-sheets and turns (Biou *et al.*, 1988). Other than the N terminus, the entire molecule is hydrophilic (Kyte and Doolittle, 1982) and has high surface probability (Emini *et al.*, 1985) suggesting that kinectin might be an extended molecule rather than a globular protein. The N terminus contains the only hydrophobic region as a putative transmembrane domain. (B) Putative coiled coils. Calculated probability of coiled coil forming region by comparing with other coiled coils in various databases (Lupas *et al.*, 1991) illustrates that the region of residues ~400–1300 has high probability of forming coiled coils.

to 156 kDa of protein which is consistent with the measured kinectin molecular mass of ~160 kDa. The pI calculated based on the conceptual translation of the cDNA sequence is 5.78 which agrees very well with the measured pI in the denatured state (~5.3) given the fact that native kinectin has post-translational modifications such as phosphorylation (Hollenbeck, personal communication). Also, the antisera raised against the fusion protein reacted with purified kinectin (Figure 3) and stained ER in fibroblast cells (our unpublished results) and CV1 cells (Figure 4, C–F). Two human proteins CG1 and HSTAF (human kinectin) matched very well with the kinectin sequence (Figure 7B). Antibodies against kinectin and HSTAF (human kinectin) cross-reacted with each other (Figure 7A (Krönke, personal communication)). Immunostaining of cells and immunoblots by anti-HSTAF mAbs agreed very well with the characteristics of chick kinectin (Krönke, personal communication; Toyoshima *et al.*, 1992). Since HSTAF shares homology with kinectin cDNA at the primary sequence level, the fact that an anti-HSTAF mAb reacted with native kinectin indicated that CG1 and HSTAF are the human homologues of kinectin and clone A is a complete cDNA for chick embryonic brain kinectin.

The structural analysis indicated that the 28 hydrophobic residues in the N terminus constituted the only hydrophobic membrane domain of kinectin. This domain was indeed not cleaved like the signal peptide of secretory proteins, and the N-terminal fragment was associated with microsomal membranes (Figure 8).

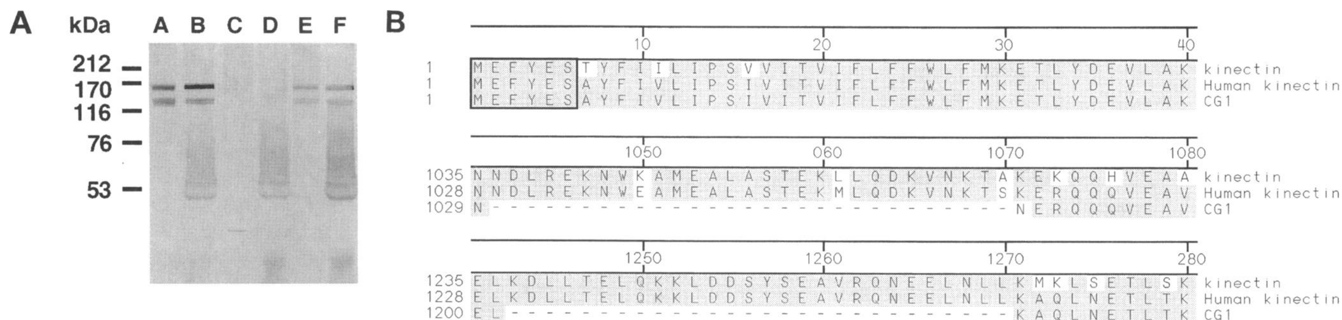

Figure 7. Chick and human kinectin compared: (A) Anti-HSTAF (human kinectin) mAbs react with purified kinectin. Protein A-bound KR160.9.1 affinity column was used to purify kinectin. Lanes A, C, and E are carbonate-washed vesicles reacted with various anti-HSTAF mAbs. Lanes B, D, and F are affinity-purified kinectin reacted with anti-HSTAF mAbs, and the 60-kDa bands represent the IgG. Lanes A and B are probed by KR160.9.1 as positive control. Lanes C and D are probed by anti-N-terminal mAb (NT-1) which did not cross-react with any proteins in chick. Lane E and F are probed by anti-C-terminal mAb (CT-1), which recognized both the 160- and 120-kDa bands in vesicles and purified kinectin like the positive control in lanes A and B. (B) Feature comparison of kinectin with human homologues. The J. Hein method (Hein, 1990) with PAM250 weight table was used to align kinectin, HSTAF (human kinectin), and CG1. The results are displayed by aligning identical and similar residues. Gaps are represented as "--." The top sequence is kinectin; the middle one is HSTAF (human kinectin); and the lower one is CG1. Three interesting regions were displayed here. Top, the N-terminal region of the protein: the boxed hexa-peptides were the N-terminal hydrophilic domain possibly residing in the lumen of vesicles (see DISCUSSION) and were identical between chick and human. Middle and bottom, the two regions of human kinectin where alternative splicing might have occurred.

Similar behavior has been observed with the SRP receptor α subunit which is assembled onto the membrane post-translationally (Hortsch *et al.*, 1988; Andrews *et al.*, 1989). The fact that the hydrophobic N terminus was uncleaved even in the cleavage-competent microsomes and was associated with the membrane indicates that the 28 hydrophobic residues constitute the membrane insertion domain for kinectin. This is consistent with the observation that the deletion of the N-terminal hydrophobic domain of the human kinectin abolished the ER distribution in CV1 (Figure 4) and in human cells (Krönke, personal communication). The vulnerability of the kinectin molecule to protease digestion in the presence of microsomes (Figure 8) agrees well with the proposed cytoplasmic orientation of kinectin. The 6 residues in the extreme N terminus may protrude into the lumen of microsomes. Immunoprecipitation of the kinectin complex has indeed identified some lumenal-associated proteins (Kumar *et al.*, 1995) which is consistent with the hypothesis that kinectin may bind lumenal factors.

Residues ~400–1300 have extremely high probabilities of forming coiled coils (Figure 6) mostly with leucine at position *d* of the heptad repeats implying that kinectin is likely to dimerize (Harbury *et al.*, 1993). Whether they form homo-dimers among themselves or there exists a β-chain to form hetero-dimers remains unclear. This possibility of dimerization, together with the seven potential myristylation sites in the middle region of kinectin, allowed us to propose a model of how kinectin might be positioned on the membrane and its likely modes of interaction with kinesin (Figure 9).

Many membrane anchors may exist for kinesin just as there are many variants of kinesin molecules (Vale and Goldstein, 1990; Endow, 1991). Even the canonical kinesin light chain has multiple isoforms and they exhibit heterogeneity in the C terminus (Cyr *et al.*, 1991; Gauger and Goldstein, 1993; Wedaman *et al.*, 1993). It has been proposed that specific kinesin isoforms can bind to specific vesicle populations. This suggests that membrane anchors might also exhibit specificity for different vesicles, which seems to be supported by the observation that kinectin may have alternatively spliced multiple isoforms (Figure 7). Within the first 28-residue variant region of the human kinectin homologues, there is one potential phosphorylation site for CK2 and PKC kinases.In the second 27-residue variant, there is one CK2 phosphorylation site. It is hard to predict how these variants will affect the conformation of kinectin near or at the kinesin binding domain just based on sequence information. Further direct binding studies, mutagenesis to find the kinesin binding site, and crystallization of kinectin domains will eventually provide us with clearer pictures of the detailed roles of kinectin in kinesin driven microtubule-based transport machinery.

From the many previous studies of organelle motility, there are suggestions that the membrane binding site for kinesin is a critical point for the regulation of both the direction and level of organelle motility. In vivo observations of the modulation of

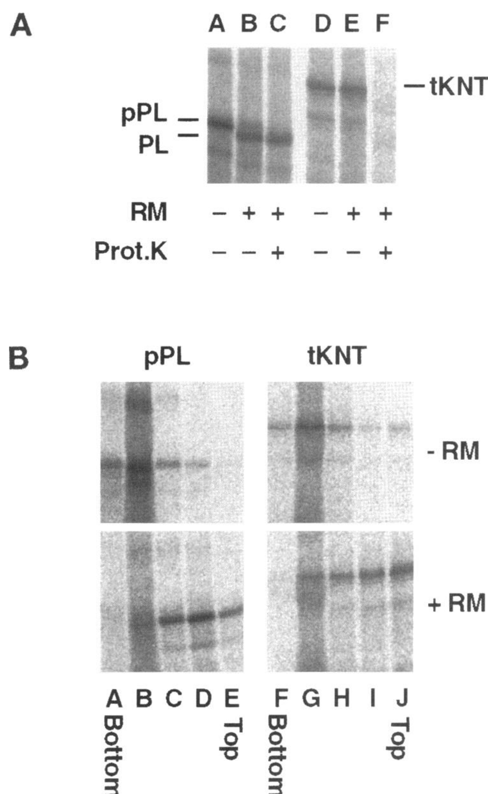

Figure 8. In vitro translation, translocation, and membrane association of kinectin. (A) Preprolactin (pPL) translocates to the lumen of canine pancreas microsomes (RM) and is cleaved to generate prolactin (PL). The translation product (pPL) without RM (lane A) and with RM (lane B) is shown. pPL is cleaved to generated prolactin (PL). Lane C is lane B-treated with protease (prot.K). N-terminal fragment of kinectin (tKNT) does not translocate to the lumen of RM cotranslationally, and no cleavage is observed. Lanes D–F are translation product (tKNT) without RM (lane D) and with RM (lane E). Lane F is lane E-treated with protease. (B) Sucrose gradient flotation analysis of tKNT association with RM. Left, pPL without RM (top left) and with RM (bottom left). Right panels are tKNT without RM (top right) and with RM (bottom right). Lanes A–E and F–J are 200-μl fractions from bottom to top of the sucrose gradient as indicated. pPL floats to the top of the gradient in the lumen of RM; tKNT also floats with RM indicating that the N-terminal fragment of kinectin could associate with RM.

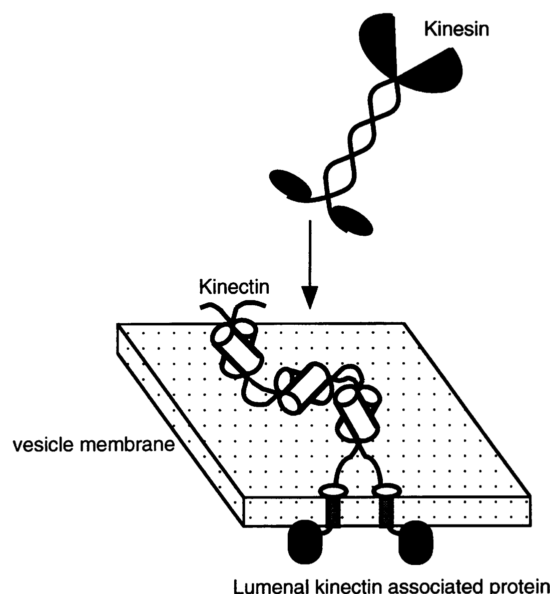

Figure 9. Schematic drawings of kinectin structure and proposed mode of interaction with kinesin. This diagram is exaggerating the kinectin and kinesin to illustrate their predicted structures and the number of coils (3) are used for illustration purpose. Other components were represented by simple shapes to be included in the diagram to illustrate the environment in which kinesin and kinectin are likely to interact. Two kinectin monomers form coiled coils and then interact with kinesin tails. N terminus penetrates through membrane and may interact with lumenal proteins.

volume of membrane traffic have shown that, over a 40-fold range in the level of microtubule-dependent organelle transport, there is a strict coordination of the inward and outward organelle movements in CV1 cells (Hamm-Alvarez *et al.*, 1993). This suggests that both motors are regulated coordinately along with all of the processing steps in between. For example, in the endocytic pathway the late endosomes move to the Golgi region where they are processed and a portion of the membrane is carried outward. Motor depletion studies have suggested that kinesin and cytoplasmic dynein seem to be involved in the same complex or have shared factors (Schroer and Sheetz, 1991). Previous competition data between motors for membrane binding (Yu *et al.*, 1992) have suggested that kinesin and cytoplasmic dynein anchors are functionally different but physically linked or in proximity to each other, implying that the anchors play an active role in regulating vesicle transport more than mere docking sites. The structural analysis of kinectin domains (membrane and kinesin binding) will also provide us with insight into their potential modes of function. Does kinectin only bind kinesin or does it also bind cytoplasmic dynein and communicate with the lumen of vesicles? Whether and how such a regulation takes place can be investigated through further understanding of the kinectin molecule and the eventual identification and characterization of the proteins that interact with kinectin.

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