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

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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 ovariolo 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; Burkhart et al., 1993), and these membrane anchor protein(m) might also serve as a switch to control the directionality.

An integral ER membrane protein of 160 kDa, kinesin, was shown to bind to the tail portions of kinesin (Toyoshima et al., 1992). Recent findings that an anti-kinecin 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 kinesin 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 kinesin 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 kinesin molecule in the cytoplasm, which presented us with a testable model for kinesin’s functions.

MATERIALS AND METHODS

Antibody Preparation and Affinity Purification of Kinectin

The mAbs of the 160.x series and 1B9 against kinesin were produced as described previously (Toyoshima et al., 1992). A new anti-kinesin mAb (VSP4D), which only recognizes the native epitope of kinesin in immunoprecipitation but not the denatured kinesin on immunoblot (Kumar et al., 1995), was generated by procedures described previously (Yu et al., 1992). Mice were injected with intact Na2CO3-extracted microsomes mixed with RIBI adjuvant (RIBI Immunocchemicals), and hybridoma supernatants were screened for mouse monoclonal antibodies that inhibit kinesin's motility.

Microsome binding was assayed by enzyme-linked immunosorbent assay by incubating cell supernatant of each hybridoma clone with intact alkaline-extracted microsomes. VSP4D can immunoprecipitate kinesin (Kumar et al., 1995). Anti-HSTAF (human kinesin) 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 kinesin, and CT-1 was raised against the C terminus of human kinesin (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 kinesin, Na2CO3-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 MgCl2, 5 mM EGTA, and 0.5 mM EDTA) and incubated with 2 ml of the anti-kinesin 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 150-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 Aqt 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 EcoRI 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 XhoI and EcoRI) into a vector, pGEMBP1 (Connolly and Gilmore, 1986). "Hot Start" PCR protocol (Instruction for AmpliWax, PerkinElmer, Norwalk, CT) was employed in conjunction with Pfu DNA polymerase (Stratagene) and nucleotides from PerkinElmer. 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 kinesin for kinesin binding studies, pGEMEX-1 was used as the vector (Promega). The full length kinesin was digested with EcoRI and BglI to release a 3.8-kb C-terminal kinesin fragment to be gel-purified. The remaining 0.9-kb N-terminal fragment with NheI site in the N terminus and BglI site at the 3’ end was generated by PCR, digested with NheI and BglI, and gel-purified. Two primers were used: primer 1 containing the 5’ end of the ORF with an in-frame NheI site, and primer 2 containing the sequence including the BglI site at position 1021. The two inserts (3.8 and 0.9 kb) were directionally subcloned into a NheI/EcoRI-digested and gel-purified pGEMEX-1 vector in a trimolecular ligation to generate a full length kinesin cDNA in pGEMEX-1. Similarly, the full length kinesin cDNA was subcloned into pCB6 (Dr. Evelyne Cordier, 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 kinesin 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 kinesin cDNA.

DNA Sequencing
cDNA clones subcloned into the EcoRI 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 [35S]dATP (RediVue, Amersham). Each subcloning of the full length or a portion of the kinesin 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 Protoman module of DNA-Star. A VAX/VMS program, COILS2.EXE, (EMBO, Heidelberg, Germany) was used to calculate the probability of coiled-coil formation. The kinesin sequence was analyzed using both scoring matrices, MTK and MTIK. The alignment of all kinesin homologues was performed by Megalign program in the Lasergene package using the Hein method (Hein, 1990) with a PAM250 residue weight table. A Prosise motif search was performed by MacPattern v3.0 using Prosise 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 RNASEl RNA purification kit (Tel-Test, Friendswood, TX). Total RNA was then processed.
using a PolyATract mRNA isolation kit (Promega, Madison, WI) to yield mRNA. mRNA (5 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 & Schuell) in 20 × standard sodium citrate phosphate (SSCP), and the probes were P³²-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**

Agt 11 phage clones were used to infect a high frequency lysogenic (hiI) *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 OD₆₅₀ 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 OD₆₅₀ = 0.2−0.5, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. The induced bacteria were pelleted, washed in phosphate-buffered saline (PBS), resuspended, 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. MgCl₂ (10 mM) and 40 μg/ml of DNA 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 5000 × 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 EDTA, 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 M NaNO₃, 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 fivefold 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. Incubation (same) was 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 kinecin, increasing concentrations of kinecin (1-, 5-, 20-μg total proteins in inclusion bodies) were used on the blot. Alkaline-washed 40/60 well plates (Costar) were incubated with 0.2-0.5 μg of kinecin or 0 μg of kinecin (only buffer), and 200 μl of PBS was added as a positive control for kinecin 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 kinecin 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 cover slips 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% CO₂ 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 experiments, 100 μl of serum-free media was mixed with the diluted LipofectAmine in place of DNA.

Cells were fixed, permeabilized, and stained with KR160.1.1, rhodamine-wheat germ agglutinin (WGA) and DIOC6 (Molecular Probe) as described (Toyoshima et al., 1992). The cells were imaged in a Axiphot (Zeiss) fluorescence microscope equipped with a Star-1 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 kinecin (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 protease 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). The 250 μg/ml membrane association and 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 x 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 (Nichitta 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 1](https://example.com/fig1.png)

**Figure 1.** Northern blot. mRNA from embryonic chick brains was probed with radiolabeled cDNAs from clone A and EcoRI-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.

![Figure 2](https://example.com/fig2.png)

**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.
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 kinctin cDNA.

We have previously reported that most anti-kinctin mAbs reacted with two bands of 160 and 120 kDa on immunoblots (Toyoshima et al., 1992). To further confirm that clone A is a kinctin 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 kinctin. The fusion proteins purified from the clones were injected into mice, and the antisera produced were then used to probe the purified kinctin in an immunoblot. All antisera reacted with the purified kinctin 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 antiserum, the major band was in the molecular weight range of kinctin (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 kinctin cDNA in CV1 cells would give the normal distribution of kinctin to the ER as seen previously. CV1 cells were chosen because of their spread morphology and because most antibodies to chicken kinctin did not label CV1 cells. When the full length kinctin cDNA was subcloned into a eukaryotic expression vector pCB6 and transfected into CV1 cells to be expressed transiently, expressed kinctin was localized mostly to ER but not Golgi (Figure 4, C–F) as expected.

Structural Analysis of the Kinctin cDNA

The kinctin 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 kinctin (Toyoshima et al., 1992). Efforts to determine the N-terminal amino acid sequence of the chick embryo brain kinctin 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 kinctin 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 kinctin 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 kinctin sequence, other than...
Figure 4. Immunolocalization of full-length and N-terminal truncated kinectin in CVI 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 CVI 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 CVI proteins (A and B). CVI 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). CVI 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).

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: pjr|S2763 and embl|Z2551) was a 160-kDa hypothesetical 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-

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).
tive human kinectin (CT-1) also reacted with purified chick embryo brain kinectin and impure kinectin in carbonate-washed vesicles (Figure 7). Another antihuman 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 Mr = 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-
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).

Figure 5. Sequence analysis of the complete kinectin DNA. 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 (AAATAAA) at 4652–4657 is underlined which is followed by a 34 base Poly(A) tail. The underscores surrounding the
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 kinetin (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 kinetin, 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 kinetin 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 to the structural features of Kinectin.

Figure 5 cont. Translation initiation site represents the most recent Kozak consensus sequence. The underscore at position -24 to -22 represents the in-frame ochre termination codon UAA. The BankGen accession number is U15671.
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).
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 luminal-associated proteins (Kumar et al., 1995) which is consistent with the hypothesis that kinectin may bind luminal 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...
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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REFERENCES


