Kinectin-Kinesin Binding Domains and Their Effects on Organelle Motility

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Intracellular organelle motility involves motor proteins that move along microtubules or actin filaments. One of these motor proteins, kinesin, was proposed to bind to kinectin on membrane organelles during movement. Whether kinectin is the kinesin receptor on organelles with a role in organelle motility has been controversial. We have characterized the sites of interaction between human kinectin and conventional kinesin using in vivo and in vitro assays. The kinectin-binding domain on the kinesin tail partially overlaps its head-binding domain and the myosin-Va binding domain. The kinesin-binding domain on kinectin resides near the COOH terminus and enhances the microtubule-stimulated kinesin-ATPase activity, and the overexpression of the kinectin-kinesin binding domains inhibited kinesin-dependent organelle motility in vitro. These data, when combined with other studies, suggest a role for kinectin in organelle motility.

Minus- and plus-end-directed microtubule (MT)\(^1\)-based organelle motility is important for active cellular processes, especially in asymmetric cells such as neurons (for reviews, see Refs. 1 and 2). Actin-dependent organelle motility has also been proposed to be important for various short range cellular processes (3, 4). In these active transport systems, motor proteins are regulated to move membrane organelles to specific target compartments (5–10). Among the motor proteins, kinesin or its family members power the plus-end-directed MT-based motility (11, 12), while cytoplasmic dynein or its family members drive the minus-end directed motility (13–15). Myosin-V (or its relatives) is one factor shown to be responsible for the actin-based organelle motility (16–18). This myosin has recently been shown to interact with kinesin (19). Motor proteins transport organelles by anchoring to them via some type of linkage. For minus-end-directed MT-based motility, it has been proposed that cytoplasmic dynein binds to organelles via direct lipid linkage (20), via intermediates such as the dynactin complex (21), or via an Arp1-linked receptor using a classical actin-binding mechanism such as spectrin-ankyrin (22–24). For myosin-V, little information is known about its membrane anchor. In the case of kinesin, a membrane protein (kinectin) has been identified to bind kinesin to membrane organelles (25).

Kinectin is a 160-kDa integral membrane protein identified as a membrane anchor essential for kinesin-dependent organelle motility in vitro (25, 26). A shorter 120-kDa kinectin can bind the 160-kDa kinesin as a heterodimer with the amino-terminal trans-membrane domain of the 160-kDa kinectin anchoring to organelles (27). An anti-kinectin monoclonal antibody, VSP4D, inhibited cytoplasmic dynein as well as kinesin functions in both the motor-membrane binding and the organelle motility assays in vitro (26). Plus- and minus-end-directed organelle motility have also been shown to be coupled in vivo (28). Therefore, kinectin has been proposed to be involved in the regulation of the organelle motility (28). Recently, the kinesin tail, where the cargo-binding domain has been postulated has been reported to activate motor activities of the kinesin head and hereby regulate organelle motility (29–31). On the other hand, organelles that normally are driven by cytoplasmic dynein are not affected in kinesin disruption studies (32, 33). These observations, together with a lack of clearer understanding of the kinectin-motor interaction, support the hypothesis that kinectin might simply anchor kinesin to organelle membrane without an active role in organelle motility. Previous efforts to characterize the kinectin-kinesin interaction with various affinity methods have not been successful, since the interaction measured in vitro seems always weaker than expected (34, 35).\(^2\) This has led some to question whether kinectin is truly a kinesin receptor on organelles (36).

To address these questions and understand the mechanism of the motor binding to organelles, we have characterized the interacting domains on human kinectin and three known members of the human conventional kinesin family using in vitro and in vivo assays. The kinectin-binding domains on kinesin partially overlap the head-binding domain on the kinesin tail that has been reported to regulate the kinesin motor activities (29). The kinesin-binding domain on kinectin resides in the COOH terminus, and the overexpression of the kinectin-kinesin binding domains in vivo disrupts the kinectin-dependent organelle motility. The kinectin-binding domains also overlap the reported myosin-Va binding domain on kinesin (19). Further, the kinesin-binding domain on kinectin can enhance the microtubule-stimulated kinesin-ATPase activity, supporting the notion that kinectin is the kinesin receptor on organelles not only to anchor kinesin but also to release kinesin from the inactive compact conformation. These and other studies lead us

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\(^1\) The abbreviations used are: MT, microtubule; BD, binding domain; AD, activation domain; GST, glutathione S-transferase; nKHC, neuronal kinesin heavy chain; PAGE, polyacrylamide gel electrophoresis; uKHC, ubiquitous kinesin heavy chain.

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to a better understanding of the kinecitin function in organelle motility.

MATERIALS AND METHODS

The restriction enzymes used were from Promega (Madison, WI), and all of the other reagents were purchased from Sigma unless otherwise stated.

Construction of the Kinecitin Baits—The human kinecitin gene (Gen-Bank™ accession number Z22551) was a generous gift from Dr. Martin Krejci (Christian-Albrechts University of Kiel, Germany). The kinecitin bait A (residues 46–444) and bait C (residues 987–1356) were generated by amplifying their respective region using standard polymerase chain reaction protocol (37), and their sequences were verified by sequencing. Bait B (residues 444–1049) was obtained directly by digesting the human kinecitin gene with PstI. The three cDNA fragments were directionally cloned in frame to the GAL4 DNA-binding domain (BD) of the pAS2–1 vector (CLONTECH). The details of the kinecitin bait constructs are illustrated in Fig. 1.

Yeast Two-hybrid Screening—A yeast two-hybrid system (CLON-TECH) was used according to the supplied protocol. The kinecitin baits were used to screen a human adult brain Matchmaker cDNA library fused to the GAL4 DNA-activation domain (AD) of the pACT2 vector (CLONTECH). The baits and the amplified library were sequen- ced to confirm the yeast strain X190 (CLONTECH) using the lithium- acetate method (38). The transformants were assayed for their expres- sion of his reporter gene by plating them onto the synthetic medium deficient in tryptophan, leucine, and histidine. 25 mM of 3-amino-1,2,4-triazole was included to limit the number of false positives (39). Colonies that grew successfully in the selective medium were further screened for their expression of the lacZ reporter gene by colony-ly- t assay. The cDNAs in the AD vector of the persistent positive clones were isolated. False positives were further eliminated via testing whether the clones were able to activate both reporter genes with the control bait (BD vector alone).

The primary sequences of the positive clones were determined with an ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Kit using an ABI PRISM TM 377 DNA sequencer, according to the manufac- turer’s instructions. The results were analyzed with the LASER-GENE (DNASTAR) and the BLAST 2.0 (National Library of Medicine) software.

In Vitro Binding Studies—Glutathione S-transferase (GST) fusion constructs with the kinecitin baits A, B, and C were made by subcloning the corresponding cDNA into the expression vector pGEX4T-1 (Amer- sham Pharmacia Biotech). The his tag fusion constructs of the neuronal kinecitin heavy chain (nKHC) was also made by subcloning the nKHC cDNA into an expression vector pRSET (Invitrogen). The fusion pro- teins were expressed in Escherichia coli strain BL21/pLyseS (DE3) (generous gift from Niomi Santama, University of Cyprus and Cyprus Insti- tute of Neuroscience and Genetics). Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Intact bacterial cells and bacterial cell supernatants containing the expressed fusion proteins were collected by centrifugation at 6000 × g for 10 min. Protein extracts were obtained by freezing and thawing the cell pellet and resuspended in GST purification buffer (1× phosphate-buffered saline, 50 mM Tris-HCl, pH 8.0, 0.5 mM MgCl2, 0.1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride) plus 5 mM dithiothreitol. Equal amounts of the GST fusion proteins with baits A, B, and C were immobilized onto glutathione-agarose beads by end-over-end mixing in a 1.5-ml microcentrifuge tube for 2 h at 4 °C. The beads were washed three times with GST purification buffer to remove un- bound proteins. The expressed his-nKHC fusion protein extract was allowed to interact with the GST-bait fusion protein-coated beads for 2 h at 4 °C with end-over-end mixing. Any unbound proteins were removed through extensive washings. The proteins that remained bound to the immobilized GST-baits were released by boiling in SDS gel sample buffer, analyzed by SDS-polyacrylamide gel (12%) electrophore- sise PAGE), and immobiloblotting with mouse RGS-his antibody (Qia- gen). Antibody binding was detected with goat anti-mouse secondary antibody (Bio-Rad), coupled to alkaline phosphatase according to the manufacturer’s specifications.

In Vivo Binding Studies—The kinecitin baits, A, B, and C, were cloned into the plasmid pEGFP-C1 (Invitrogen). The kinecitin baits, A, B, and C, were expressed in E. coli in the presence of truncated kinectin fragments at the final concentrations of 0.1 or 0.2 mg/ml was measured at 37 °C as described previously (40, 41). The final concentrations of taxol-stabilized micro- tubules, kinecitin, and kinecitin fragments in ATPase assay were 1.0 mg/ml, 15 μg/ml, and 0.1 or 0.2 mg/ml, respectively.

In Vivo Kinetin-dependent Organellar Motility Assay—The kinecitin- binding domain on kinecitin (KNT™, residues 1188–1288) and a larger fragment containing the domain (K1, residues 987–1356) were subcloned into the pEGFP-C vector for optimal expression in mammalian cells (CLONTECH). KNT™ (residues 1049–1146) that showed no interaction with the nKHC bait in yeast two-hybrid screening and the pEGFP-C vector alone were used as controls. In addition, the kinecitin- binding domain on uKHC (uKHC™, residues 833–900) that interacts with kinecitin and a control domain that does not bind kinecitin (ukHC™, residues 735–801) were also cloned into the pEGFP-C vector. COS7 cells were cultured and transfected as described above. Transfected cells were identified by GFP expression. Lysosome-specific staining was obtained by incubating cells with 50 μM Lysotracker DND-99 (Molecu- lar Probes, Inc., Eugene, OR) in normal Ringer’s solution for 30 min. Lysosomal redistribution upon acidification as described previously (44) was assayed. Cells were subsequently fixed with 4% paraformaldehyde, mounted in FluorSave (Calbiochem), and imaged using a Carl Zeiss LSM 510 laser-scanning confocal microscope. A total of 90 cells were observed for each clone for their lysosome redistribution. The results were tabulated based on the mean percentage of cells in each of the four categories, namely clustered around cell center, clustered centrally with radiating tubules, dispersed throughout cell, or dispersed with peripheral organelle clusters (44, 45).

RESULTS

Neuronal Kinecitin Heavy Chain Interacts with Kinecitin—We performed a yeast two-hybrid assay to screen for proteins that interact with kinecitin. Three overlapping kinecitin fragments, A, B, and C, were constructed as baits (Fig. 1). Most of the positive clones identified were those that interact with the kinecitin bait C. A total of 14 positive clones were identified from the screening of approximately 1.8 × 106 transformants.
Among them, two were nKHC cDNAs (GenBank™ accession number U06698). The two clones (2.5 and 1.1 kilobases) code for two nKHC fragments with residues 284–1032 and 601–957, respectively. Both nKHC fragments interacted with the kinesin bait C but not with the control baits (A or B or BD vector alone). Bait C encodes the kinectin fragment (residues 987–1356) that does not contain the predicted leucine zipper motifs alone. Bait C interacted with the kinesin bait C, but not with bait A nor B, is consistent with the interactions observed in the yeast two-hybrid assay.

The in vitro binding study was also extended to cytosolic kinesin for further assessment of the kinectin-kinesin interaction. Mouse brain cytosol was extracted and incubated with the immobilized GST-bait (kinectin) fusion proteins. The cytosolic proteins remained bound to the immobilized GST-baits after extensive washings were analyzed by immunoblotting with anti-FLAG antibody (Fig. 3B). The association of the nKHC in vitro with the kinesin bait C, but not with bait A nor B, is consistent with the interactions observed in the yeast two-hybrid assay.

The interaction of cytosolic kinesin with the kinectin bait C. A, SDS-PAGE analysis of the proteins immobilized on glutathione-agarose beads indicates that the GST-bait C fusion protein is the only protein on the beads. B, equal amount of the his-nKHC, his-uKHC, his-KIAA0531 protein extracts was incubated with the bead-immobilized GST-bait fusion proteins. After extensive washings, bound proteins were eluted and fractionated by SDS-PAGE and analyzed in an immunoblot with anti-his antibody. nKHC specifically binds to bait C, but not bait A or bait B. MW, molecular weight marker; TP, total protein; FT, flow-through; W, wash; E, eluate.

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To corroborate the yeast two-hybrid data, we performed an in vitro binding study. GST fusion constructs with the kinesin baits A, B, and C were made, and equal amounts of the individual fusion-protein extracts were immobilized onto glutathione-agarose beads. The beads were washed extensively to remove unbound proteins such that only the kinesin baits remained bound to the beads (Fig. 2A). An nKHC fusion construct with a his tag was expressed in E. coli, and the cell extract was allowed to interact with the immobilized GST-bait fusion proteins. The proteins remained bound to the immobilized GST-baits after extensive washings were analyzed by immunoblotting with anti-his antibody (Fig. 2B). The association of the nKHC in vitro with the kinesin bait C, but not with bait A nor B, is consistent with the interactions observed in the yeast two-hybrid assay.

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Figure 4. The kinectin-binding domain on nKHC. The nKHC (residues 601–957) clone was truncated by both amino- and COOH-terminal deletions. The interaction of the truncated clones with the kinectin bait C was tested by the yeast two-hybrid assay. The solid line indicates the cDNA clones whose gene products activated the his and lacZ reporter genes in >90% of the transformants, whereas the discontinuous line indicates the clones that activated the reporter genes in <10% of the transformants.

FLAG-tagged nKHC with a GST-tagged kinectin bait C into COS7 cells. Immunoprecipitates of the co-transfected COS7 cell extracts via the GST-tagged kinectin bait C on a glutathione-agarose column were analyzed by immunoblotting with anti-FLAG and anti-human kinectin antibodies. The FLAG-nKHC co-precipitates with the GST-bait C.

Characterization of the Kinectin-binding Domain on Neuronal Kinesin Heavy Chain—To identify the minimally sufficient domain of nKHC that interacts with kinectin, we have constructed a series of truncated fragments of nKHC (Fig. 4) and assayed for their interaction with the kinectin bait C using the yeast two-hybrid assay. Clones N1, N2, N3, N4, N5, N6, N9, N10, and N11 could interact with bait C as indicated by the activation of the his and lacZ reporter genes, whereas N7, N8, N12, and N13 could not. A minimally sufficient kinectin-binding domain on nKHC, clone nKHCN10, that encodes a 54-amino acid fragment (residues 836–890), was deduced from these results, and its interaction with the kinectin bait C was experimentally confirmed using the yeast two-hybrid assay.

Interaction Sites of Three Human Conventional Kinesin Heavy Chains with Kinectin—From our yeast two-hybrid screening for kinectin-binding proteins, we have identified another member of the human conventional kinesin heavy chains (KIAA0531) as a kinectin-binding protein. KIAA0531 (GenBank accession number AB011103) was reported as one of the 100 large human proteins whose genes were sequenced. The COOH terminus of human kinectin is an elongated molecule with multiple domains (47–49). The amino terminus has a trans-membrane domain in the 160-kDa form but not the 120-kDa form of kinectin (27). There are up to six variable domains in the middle to tail (COOH terminus) of kinectin (49). Multiple modifications (glycosylation, myristoylation, and phosphorylation) sites in or near these domains were postulated (48, 49) based on sequence data as well as demonstrated experimentally (50). To understand whether and how these modifications modulate kinesin binding to kinectin and involvement in organelle motility, it is important to characterize the kinesin-binding domain on kinectin.

Using a yeast two-hybrid assay, we have established above that uKHC interacts with the kinectin bait C (residues 987–1356). To identify the minimally sufficient domain of kinectin that interacts with uKHC, we have constructed a series of truncated kinectin fragments (Fig. 1) and assayed for their interaction with an uKHC-bait (residues 602–963) that contains the kinectin-binding domain (residues 833–900). Among the 14 clones, 10 were positive as indicated by the activation of the his and lacZ reporter genes in the yeast two-hybrid assay (Fig. 1A). Clones K1, K5, K6, K7, K8, K9, K12, K13, and K14 could interact with the uKHC-bait, whereas K2, K3, K4, K10, and K11 could not. The minimally sufficient kinesin-binding domain KNT (residues 1188–1288) on kinectin was deduced from these results, and its interaction with the uKHC-bait was confirmed experimentally with the yeast two-hybrid assay.

The COOH Terminus of Human Kinectin Interacts with Kinectin—Kinectin is an elongated molecule with multiple domains (47–49). The amino terminus has a trans-membrane domain in the 160-kDa form but not the 120-kDa form of kinectin (27). There are up to six variable domains in the middle to tail (COOH terminus) of kinectin (49). Multiple modifications (glycosylation, myristoylation, and phosphorylation) sites in or near these domains were postulated (48, 49) based on sequence data as well as demonstrated experimentally (50). To understand whether and how these modifications modulate kinesin binding to kinectin and involvement in organelle motility, it is important to characterize the kinesin-binding domain on kinectin.

The Kinesin-binding Domain on Kinectin Enhances the Microtubule-stimulated Kinesin-ATPase Activity—Since the COOH terminus of kinectin can bind to the kinesin tail where the head and tail of kinesin interact with each other to maintain the inactive compact conformation, we hypothesize that kinectin is the postulated cargo receptor for kinesin activation. When purified KNT (kinectin-binding domain, residues 1188–1288) and KNT (negative control, residues 1049–1146) were added to an assay measuring the microtubule-stimulated kinesin-ATPase activity, KNT significantly (74 ± 10%) enhanced the kinesin-ATPase activities whereas KNT did not (Fig. 5).

The Sites of Kinectin-Kinesin Interaction Are Important for Kinesin-dependent Organelle Motility—To further investigate the physiological relevance of our characterization of the sites of kinectin-kinesin interaction, we have overexpressed kinectin and uKHC fragments in COS7 cells and analyzed their effects
on the disruption of the kinesin-dependent organelle motility. In our studies, cDNAs encoding the kinesin fragments K1 (residues 987–1356), KNT$^+$ (kinesin-binding domain, residues 1188–1288), and KNT$^-$ (residues 1049–1146) (Fig. 1) were cloned into the pEGFP-C vector. cDNAs encoding the uKHC fragments uKHC$^+$ (kinectin-binding domain, residues 833–900) and uKHC$^-$ (residues 735–801) were also cloned into the pEGFP-C vector. The cDNAs in the vector and the pEGFP-C vector alone were introduced into COS7 cells. The cells were subjected to brief acetate treatment to change the medium pH from 7.2 to 6.9, which has been reported to cause redistribution of lysosomes to the cell periphery (44). Such redistribution is kinesin-dependent, since mutation of the kinesin motor domain inhibited the redistribution (45).

When the lysosome redistribution upon acidification was quantified, the controls (untransfected cells or cells transfected with KNT$^-$ or uKHC$^-$ in pEGFP-C vector or pEGFP-C vector alone) showed the typical lysosome redistribution, with 90, 90, 84, and 86% of the lysosomes spreading throughout the cytoplasm, respectively, or near the cell periphery (Fig. 6 and Table I). In cells overexpressing K1, KNT$^+$, and uKHC$^+$ in pEGFP-C vector, there were only 19, 33, and 30% of the lysosomes redistributing throughout the cytoplasm or near the cell periphery upon acidification. The inhibition of the lysosome redistribution upon acidification was slightly more pronounced in K1-transfected than in KNT$^-$- or uKHC$^-$-transfected COS7 cells. Nonetheless, overexpression of the kinectin and uKHC fragments that contain the interaction sites inhibited the lysosome redistribution, confirming that the characterized sites of kinectin-kinesin interaction are important for the kinesin-dependent organelle motility.

DISCUSSION

In our search for kinectin-associated proteins using yeast two-hybrid, direct binding, and co-immunoprecipitation assays, we have identified members of the conventional kinesin family as kinectin-associated proteins. This provided independent evidence to the original experiments with the kinesin affinity approach (25) to confirm the interaction between kinectin and kinesin. This helps to settle a controversy on the role of kinectin as the kinesin receptor (36). We have also constructed a series of deletion mutants of kinectin and kinesin and characterized the minimally sufficient sites for the kinectin-kinesin interaction. The kinectin-binding domain on kinesin resides near the COOH terminus within a region called the "coiled-coil tail," which is adjacent to the globular tail at the extreme COOH terminus (51). This coiled-coil tail has been postulated as the cargo-binding domain on kinesin (34). In other words, kinectin binds to the region where cargoes bind. This is consistent with the proposed role of kinectin as the membrane receptor for kinesin binding to membrane cargoes.

The resolution of the fine mapping of the kinectin-binding domain on nKHC was restricted by the qualitative nature of the yeast two-hybrid assay. Further deletion at the single amino acid level gave a gradient of results with respect to whether the two reporter genes were activated. Therefore, the minimally sufficient kinectin-binding domain boundaries were deduced from N6, N7, N11, and N12 (Fig. 4). The N6 and N11 showed positive in >90% of the transfected yeast cells in activating the two reporter genes, whereas N7 and N12 showed positive in <10% of the transfected cells. A more sensitive assay is needed to more precisely determine the boundaries of the binding domain.

The kinectin-binding domain on kinesin is highly conserved for all three members of the human conventional kinesin family. This is the only highly conserved region on kinesin other than the motor domain on the kinesin head (51). Therefore, it is not surprising that all three members of the human conventional kinesin family interact with kinectin in our assays. Whether the slight variants near the edge of the characterized kinectin-binding domain on kinesin contribute to variations in the interaction between the different kinesins and kinectin has to be defined when the precise boundaries of the kinectin-binding domain are defined at the single amino acid level with a more quantitative assay.

The characterized kinectin-binding domain on kinesin partially overlaps two other important domains (Fig. 7). One is the myosin-Va binding domain (19) that overlaps 23 residues on the amino edge of the kinesin-binding domain on kinesin (Fig. 7). The interaction of myosin-Va and kinesin has been postulated as a key link between the microtubule- and actin-based organelle motility (19). The other important domain is the kinesin head-binding domain on the kinesin tail that overlaps 12 residues on the COOH edge of the kinectin-binding domain on kinesin (Fig. 7). The kinesin tail has been shown to interact with the kinesin globular head to inhibit the motor activities of the head (29). It was reported that deletion or mutation of the kinesin tail constitutively activated the kinesin motor activities (29, 30). The implication was that the cargo binding to the kinesin tail could prevent the kinesin head-tail interaction and thereby derepress the kinesin motor activities (30). Since kinectin is the cargo anchor for kinesin and the kinectin-binding domain on the kinesin tail overlaps its head-binding domain and the myosin-Va binding domain (Fig. 7), we suggest that kinectin plays a bigger role in the organelle motility than simply anchoring kinesin. Indeed, we have observed that the kinesin-binding domain on kinectin can significantly enhance the microtubule-stimulated kinesin-ATPase activity (Fig. 5). We have also observed that the overexpression of the sites of kinectin-kinesin interaction in COS7 cells disrupted the lysosome redistribution upon acidification (Fig. 6 and Table I), which is a well-established kinesin-dependent organelle motility assay in vivo (44, 45).

If kinectin-kinesin interaction is important in organelle motility, then it is likely that the interaction is regulated. Phosphorylation has been proposed to be a way to modulate or-
sequences were boxed dotted line head (residues 889–955; thin line) were aligned, and the overlapping sequences were boxed.

The regulation of the kinectin-kinesin interaction can also occur on kinectin, since it is an extended molecule with multiple domains (49). Kinectin is also highly phosphorylated, and phosphorylation states can affect organelle motility (50). We have characterized the kinesin-binding domain on kinectin using the same in vitro and in vivo assays as for the kinectin-binding domains on kinesin. The kinesin-binding domain resides near the COOH terminus of kinectin within a region near the COOH-edge of the putative coiled-coils. It covers the last two heptad repeats (17 and 18) with a break of 26 amino acids that can render some potential flexibility in modulating the kinectin-kinesin interaction. These heptads were found throughout most of the kinectin molecule (residues 327–1362) and are essential features for forming α-helical coiled-coil structures (47, 48). There are two potential phosphorylation sites for tyrosine kinase and casein kinase 2 and a potential N-glycosylation site in the kinesin-binding domain that may be involved in such a modulation. It is interesting that the kinesin-binding domain on kinectin also resides in the region where the COOH terminus of kinesin within a region near the COOH-edge of the putative coiled-coils. It covers the last two heptad repeats (17 and 18) with a break of 26 amino acids that can render some potential flexibility in modulating the kinectin-kinesin interaction. These heptads were found throughout most of the kinectin molecule (residues 327–1362) and are essential features for forming α-helical coiled-coil structures (47, 48). There are two potential phosphorylation sites for tyrosine kinase and casein kinase 2 and a potential N-glycosylation site in the kinesin-binding domain that may be involved in such a modulation. 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action seems to be weaker (~50× lower affinity) compared with the measured kinesin-organelle interaction (34, 35). Such a discrepancy might be due to the lack of post-translational modifications on the bacterially expressed kinectin. Alternatively, regulatory modifications such as phosphorylation or association with other regulatory proteins might play a role in modulating the interaction. Since kinectin-kinesin interaction seems to be playing an important role in organelle motility, it is likely that such an interaction is tightly regulated such that the high affinity interaction only occurs in specific conformation or modification. Since the yeast two-hybrid assay is known to detect weak or transient interactions between proteins (54), it is not surprising that we have been able to use this assay to characterize the sites of kinectin-kinesin interaction. Our characterization of the kinectin-kinesin interaction is consistent with other studies leading to our current understanding of the kinesin-dependent organelle motility.

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REFERENCES
1. Hirokawa, N., Noda, Y., and Okada, Y. (1998) Curr. Opin. Cell Biol. 10, 60–73
2. Hirokawa, N. (1998) Science 279, 519–526
3. Langford, G. M. (1995) Curr. Opin. Cell Biol. 7, 82–88
4. Bi, G. Q., Morris, R. L., Liao, G., Aberton, J. M., Scholey, J. M., and Steinhardt, R. A. (1997) J. Cell Biol. 138, 999–1008
5. Aizawa, H., Sekine, Y., Takemura, R., Zhang, Z., Nangaku, M., and Hirokawa, N. (1992) J. Cell Biol. 119, 1287–1296
6. Kondo, S., Sato-Yoshitake, R., Noda, Y., Aizawa, H., Nakata, T., Matsuura, Y., and Hirokawa, N. (1994) J. Cell Biol. 125, 1095–1107
7. Nangaku, M., Sato-Yoshitake, R., Okada, Y., Noda, Y., Takemura, R., Yamazaki, H., and Hirokawa, N. (1994) Cell 79, 1209–1220
8. Sekine, Y., Okada, Y., Noda, Y., Kondo, S., Aizawa, H., Takemura, R., and Hirokawa, N. (1994) J. Cell Biol. 127, 187–201
9. Noda, Y., Sato-Yoshitake, R., Kondo, S., Nangaku, M., and Hirokawa, N. (1995) J. Cell Biol. 129, 157–167
10. Okada, Y., Yamazaki, H., Sekine-Aizawa, Y., and Hirokawa, N. (1995) Cell 81, 769–780
11. Vale, R. D., Schnapp, B. J., Mitchison, T., Steuer, E., Reese, T. S., and Sheetz, M. P. (1985) Cell 44, 623–632
12. Porter, M. E., Scholey, J. M., Stemple, D. L., Vigers, G. P., Vale, R. D., Sheetz, M. P. and McIntosh, J. R. (1987) J. Biol. Chem. 262, 2794–2802
13. Vale, R. D., Wall, J. S., Paschal, B. M., and Shpetner, H. S. (1988) Nature 332, 561–563
14. Schnapp, B. J., and Reese, T. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1548–1552
15. Schroer, T. A., Steuer, E. R., and Sheetz, M. P. (1989) Cell 56, 937–946
16. Wu, X., Bowers, B., Rao, K., Wei, Q., and Hammer, J. A. R. (1998) J. Cell Biol. 143, 1899–1818
17. Tabb, J. S., Molyneaux, B. J., Cohen, D. L., Kuznetsov, S. A., and Langford, G. M. (1998) J. Cell Sci. 111, 3221–3234
18. Rogers, S. L., and Gelfand, V. I. (1998) Curr. Biol. 8, 161–164
19. Huang, J. D., Brady, S. T., Richards, B. W., Stenoloen, D., Resau, J. H., Copeland, N. G., and Jenkins, N. A. (1998) Nature 397, 267–270
20. Lacey, M. L., and Haimo, L. T. (1994) Cell Motil. Cytoskeleton 29, 205–212
21. Vaughan, K. T., and Valleé, R. B. (1995) J. Cell Biol. 131, 1507–1516
22. Beck, K. A., and Nelson, W. J. (1998) Biochim. Biophys. Acta 1404, 153–160
23. Burkhart, J. K. (1998) Biochim. Biophys. Acta 1404, 113–126
24. De Matteis, M. A., and Morrow, J. S. (1998) Curr. Opin. Cell Biol. 10, 542–549
25. Toyoshima, I., Yu, H., Steuer, E. R., and Sheetz, M. P. (1992) J. Cell Biol. 118, 1121–1131
26. Kumar, J., Yu, H., and Sheetz, M. P. (1995) Science 267, 1834–1837
27. Kumar, J., Erickson, H. P., and Sheetz, M. P. (1998) J. Biol. Chem. 273, 31738–31743
28. Sheetz, M. P., and Yu, H. (1996) Semin. Cell Dev. Biol. 7, 329–334
29. Coy, D. L., Hanecyk, W. O., Wagenbach, M., and Howard, J. (1999) Nat. Cell Biol. 1, 288–292
30. Friedman, D. S., and Vale, R. D. (1999) Nat. Cell Biol. 1, 293–297
31. Kirchner, J., Seiler, S., Fuchs, S., and Schliwa, M. (1999) EMBO J. 18, 4404–4413
32. Burkhart, J. K., Echeverri, C. J., Nilsson, T., and Valve, R. B. (1997) J. Cell Biol. 139, 469–484
33. Kraemer, J., Schnitz, F., and Drenckhahn, D. (1999) Eur J Cell Biol. 78, 265–277
34. Schroer, T. A., Steuer, E. R., and Sheetz, M. P. (1989) J. Cell Biol. 113, 809–822
35. Tabb, J. S., Molyneaux, B. J., Mitchison, T., Steuer, E., Reese, T. S., and Sheetz, M. P. (1997) J. Cell Biol. 133, 1087–1107