The Carboxyl-terminal Domain of Kinesin Heavy Chain Is Important for Membrane Binding

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Sea urchin kinesin is a plus end-directed microtubule-based motor consisting of two heavy chains and two light chains and is proposed to be responsible (a) for the transport of membranous organelles along microtubules in sea urchin mitotic spindles (Wright, B. D., Henson, J. H., Wedaman, K. P., Willy, P. J., Morris, J. N., and Scholey, J. M. (1991) J. Cell Biol. 113, 317–338) and (b) for the radial dispersion of endoplasmic reticulum and endosomal membranes in nonmitotic cultured coelomocytes (Henson, J. H., Nesbitt, D., Wright, B. D., and Scholey, J. M. (1992) J. Cell Sci. 103, 309–320). We report here that sea urchin kinesin is indeed able to bind in a concentration-dependent and saturable manner to microosomal membranes isolated from sea urchin eggs in the presence of MgATP. The kinesin light chains may not be essential for membrane binding since kinesin containing negligible amounts of light chains binds as well as kinesin containing stoichiometric amounts of light chains. Finally, we propose that kinesin binds to membranes with the carboxyl-terminal domain of the heavy chain (amino acid residues 858–1031) since the bacterially expressed and then isolated stalk-tail fragment of kinesin heavy chain, in contrast to the stalk fragment, is able (a) to bind membranes in a concentration-dependent and saturable manner and (b) to compete with native kinesin for membrane binding. Our results support the hypothesis that the carboxyl-terminal domains of the heavy chains attach kinesin molecules to their membranous cargo in mitotic and interphase sea urchin cells.

The microtubule-based motor proteins kinesin (Vale et al., 1985a, 1986b; Brady, 1985; Scholey et al., 1985) and cytoplasmic dynein (Paschal et al., 1987; Lye et al., 1987) are believed to use ATP hydrolysis to drive diverse forms of intracellular membrane transport; they are thought to mediate the anterograde and retrograde movements of axonal vesicles along microtubules and are involved in controlling the very dynamic organization of the endomembrane system consisting of endoplasmic reticulum membrane tubules, lysosomes, and Golgi membranes in a variety of cell types (for review see Brady, 1991; Schroer and Sheetz, 1991; Skoufias and Scholey, 1993).

Kinesin isolated from different sources is a tetrameric protein complex consisting of two 110–130-kDa heavy chains and usually two light chains of variable molecular weights (Bloom et al., 1988; Kuznetsov et al., 1988) Sequence and structure-function analysis has revealed that the amino-terminal ends of the two KHCs form globular motor domains that split ATP and generate motile force (Yang et al., 1989, 1990; Hirokawa et al., 1991; Scholey et al., 1989). The motor domains are linked to the carboxyl-terminal tail domain through a coiled-coil region called the stalk, which is the site of dimerization of two KHCs (Yang et al., 1989; De Cuevas et al., 1992). The stalk-tail domain of KHC is strongly conserved among kinesins from different species (Yang et al., 1989; Kostik et al., 1990; Wright et al., 1991; Navone et al., 1992), and it is thought to contain the sites of interaction with KLCs (Hirokawa et al., 1989; Curr et al., 1991; Gauger and Goldstein, 1993; Wedaman et al., 1993) and with the membranous cargo. The localization of the membrane binding sites and the relative roles of the heavy and light chains in kinesin binding to membranes, however, are not known.

We have been studying the mechanisms and functions of kinesin-driven intracellular transport in sea urchin cells. In this system, immunocytochemical studies have suggested that kinesin is associated with membranes and microtubules in the mitotic apparatus of dividing zygotes and early blastomeres; however, the distribution of kinesin-bound membranes changes during development, being dispersed into the cytoplasm of later embryonic cells and found in association with endoplasmic reticulum and endosomal membranes in terminally differentiated, nonmitotic coelomocytes maintained in culture (Wright et al., 1991; Henson et al., 1992). We are interested in the role of kinesin-driven motility in the dynamic reorganizations of intracellular membranes that occur during cell division, development, and cell motility in these sea urchin cells.

In this report we examined the ability of sea urchin kinesin to bind membranes in the manner predicted from the aforementioned immunocytochemical localization studies. Our results demonstrate that kinesin is indeed able to bind membranes in a specific and saturable manner and that the KLCs may not be essential for membrane binding. Finally, our data also suggest that the carboxyl-terminal tail domain of KHC is responsible for membrane binding.

EXPERIMENTAL PROCEDURES

Purification of Sea Urchin Kinesin—Kinesin was sedimented from unfertilized sea urchin (Strongylocentrotus purpuratus) egg cytosol by AMPPNP-induced microtubule affinity binding in PMEG (100 mM potassium PIPES, pH 6.9, 5 mM EGTA, 0.5 mM EDTA, 2.5 mM threitol; TAME, N-p-tosyl-L-arginine methyl ester.)
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MgSO$_4$ (0.9 M glycerol) as described previously (Buster and Schcdley, 1991), released from microtubules in extraction buffer (PMEG with 0.2 M KCl, 10 mM taxol, 0.1 mM GTP, 10 mM MgATP), and then purified by Bio-Gel A-1.5m chromatography. Kinesin was separated into light chain-containing and light chain-depleted fractions by modifications of the sucrose density gradient centrifugation method of (Hackett, 1982). Briefly, Bacillus subtilis Gel kinesin was first concentrated with a Centricon 30 (Amicon) and then concentrated further by another round of microtube affinity binding by incubating it with 0.5 mg/ml salt-stripped taxol-stabilized sea urchin egg microtubules in the presence of 2.5 mM AMP-PNP for 30 min, at room temperature. The microtubule suspension was centrifuged at 100,000 $\times$ g in an SW 55 rotor at 4°C. SDS-PAGE of the sucrose gradient fractions revealed two peaks of kinesin, a S peak containing kinesin heavy chain and light chains and a 7 S peak containing heavy chain depleted of light chains. Membrane binding assays were performed using sucrose gradient kinesin or the Bio-Gel fractions, as were observed.

Isolation and Purification of Bacterially Expressed KHC Fragments—Two Escherichia coli bacterial strains were transferred to T7 vector containing partial KHC inserts; one 1.4-kilobase insert encodes the tail domain and a portion of the stalk domain (amino acids 579-1031), and the other (0.8 kilobase) insert encodes only a portion of the stalk domain of kinesin (amino acids 579-858) (Wright et al., 1991). To isolate the KHC fragments from these transformants, a colony from a freshly streaked plate was inoculated into Luria Broth medium containing 50 $\mu$g/ml ampicillin and 25 $\mu$g/ml chloramphenicol and was allowed to grow up to $A_{600}$ 0.6 at 37°C. The bacteria were then induced at 37°C for 3 h with 0.4 mM isopropyl-1-thio-@-galactoside. Cells were harvested, washed by centrifugation in lysis buffer (20 mM phosphate, pH 7.2, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 $\mu$g/ml pepstatin, 2 $\mu$g/ml aprotinin, 20 $\mu$g/ml benzamidine, 1 mg/ml TAME, 0.5 M NaCl) and then lysed in 5 ml of lysis buffer/g of cell pellet by sonication. The lysates were centrifuged at 12,000 $\times$ g for 30 min, and the pellets containing the inclusion bodies were solubilized in 20 M Tris, pH 8, 8 M urea, 1 mM DTT at room temperature. The insoluble material was pelleted by centrifugation at 100,000 $\times$ g in a Beckman TL100 ultracentrifuge for 90 min at 4°C. The supernatant containing the stalk-tail fragment was loaded onto a P-11 phosphocellulose column (Whatman) preequilibrated in 20 M Tris, pH 8, 8 M urea, 1 mM DTT, and the column was washed sequentially with 5 volumes of the same buffer, then with 0.1 M NaCl, and finally the fragment was released in 0.6 M NaCl. The supernatant containing the kinesin tail fragment was then loaded onto a DEAE column (Whatman) and the column was washed with 10% SDS-PAGE until the linear range of the assay (see below). SDS-PAGE and Western Blotting—Kinesin samples were analyzed with 7.5% SDS-PAGE, whereas the kinesin fragments were analyzed with 10% SDS-PAGE and then transferred to nitrocellulose. On each blot five kinesin samples of known concentration (ranging from 5 to 80 ng) were included as standards. Blots were incubated with anti-kinesin monoclonal antibodies recognizing specific epitopes on the KHC (Ingold et al., 1988; Wright et al., 1991) such as SUK4, recognizing the motor domain, and SUK2, recognizing the stalk domain. Antibody binding was visualized with goat anti-mouse antibody (Fisher Scientific), coupled to alkaline phosphatase according to the manufacturer's specifications. Care was taken to ensure that the color development for different blots was the same and within the linear range of the assay (see below).

Video Densitometry and Data Analysis—Gels and blots were quantitated by using a video densitometry system composed of an SIT168 low light video camera with a 75-mm Kern-Paillard objectives. Recorded images were digitized using the frame grabber program, NIH IMAGE 1.45, and analyzed using a Macintosh II microcomputer.

To strip the membranes of peripheral proteins, the microsomes were resuspended in 0.1 M sodium carbonate, pH 11, and incubated on ice for 10 min. The solution was neutralized with 2 volumes of PMEG and centrifuged over a 0.3 M sucrose cushion at 100,000 $\times$ g for 30 min at 4°C. The pellet was then resuspended in PMEG and stored as above.

Protein Concentrations were determined by the Bio-Rad microassay using $\gamma$-globulin as a standard. Protein concentrations in the membrane preparations were determined using the Pierce BCA assay in the presence of 1% SDS. The kinesin concentration was determined by video densitometry (see below) of SDS-polyacrylamide gels stained with Coomassie Brilliant Blue using known concentrations of $\beta$-galactosidase gel samples as standards.

Kinesin-Membrane Binding Assay—Kinesin purified from sea urchin eggs or KHC fragments isolated from bacteria were mixed with 60-120 $\mu$g of vesicle protein in the presence of 1 mg/ml bovine serum albumin (BSA) and 1 mg/ml pepstatin. The vesicle mixtures were brought up to 100 $\mu$l with PMEG. The reaction mixtures were incubated at room temperature for 30 min and then loaded into polycarbonate centrifuge tubes (7 x 20 mm, Beckman) over a cushion of 100 $\mu$l of 0.3 M sucrose and centrifuged in a TL100 ultracentrifuge at 30,000 rpm for 20 min at 4°C. 100 $\mu$l from the supernatant was removed, and the top of the sucrose cushion was washed twice with 100 $\mu$l of PMEG. Finally the supernatant and the sucrose cushion were removed by suction. The membrane pellets were resuspended in 15 $\mu$l of Laemmli sample buffer with an additional 1% SDS and boiled for 5 min. For each reaction a background measurement was taken in which vesicles were omitted from the reaction.

To strip the membranes of peripheral proteins, the microsomes were resuspended in 0.1 M sodium carbonate, pH 11, and incubated on ice for 10 min. The solution was neutralized with 2 volumes of PMEG and centrifuged over a 0.3 M sucrose cushion at 100,000 $\times$ g for 30 min at 4°C. The pellet was then resuspended in PMEG and stored as above.

Microscopic Analysis of Microsomal Vesicles—Microsomal vesicles were pelleted into polycarbonate centrifuge tubes (7 x 20 mm, Beckman) and fixed with 2.5% glutaraldehyde in PMEG for 1 h at 4°C. After two washes with PMEG the membrane pellets were postfixed with 1% OsO$_4$, for 2 h at 4°C, serially dehydrated in 70, 80, 90, and 100% acetone, incubated in 100% propylene oxide for 5 min and then infiltrated with 1:1 propylene oxide/EMBED 812 mixture at 4°C, and finally 100% Epon mixture at 4°C. The membranes were finally embedded in pure Epon mixture for 3 days at 40°C and then thin sectioned. Electron micrographs were taken with a Phillips EM300 electron microscope at a 80-kV operating voltage.

Video Densitometry and Data Analysis—Gels and blots were quantitated by using a video densitometry system composed of an SIT168 low light video camera with a 75-mm Kern-Paillard objectives. Recorded images were digitized using the frame grabber program, NIH IMAGE 1.45, and analyzed using a Macintosh II microcomputer.

The concentration of kinesin from gels and the concentration of bound kinesin from immunoblots were calculated by converting the area under each peak into ng using standard curves; the standards were analyzed by linear regression of the peak areas versus known concentrations of either $\beta$-galactosidase (gels) or kinesin (immunoblots). For Coomassie Blue-stained gels the assay was linear with concentrations ranging from 0.13 to 2 $\mu$g of $\beta$-galactosidase. For immunoblots, using the monoclonal antibody SUK4, the assay was linear with concentrations of kinesin ranging from 5 to 80 ng of KHC. Similarly, for immunoblots stained with the monoclonal antibody SUK2, the assay was linear with concentrations ranging from 8 to 90 ng of isolated stalk tail KHC fragment.

The binding data were curve-fitted with Sigmaplot 4.1 software (Jandel Scientific) using the equation $Y = AX/B + X$, where $A$ is the $B_{max}$ and $B$ is the $K_d$. For each data point we subtracted the corresponding background caused by nonspecific pelleting of kinesin in the absence of membranes to obtain the amount of bound kinesin for some samples of membranes. The concentration of free kinesin was determined by subtracting bound kinesin from added kinesin. In our calculations the molecular mass of KHC was taken to be 130,000 Da.
Preparations used for membrane binding assays are shown in Fig. 1. Kinesin purified from unfertilized sea urchin eggs consisted of 130-kDa heavy chains plus 84-kDa/78-kDa light chains in approximately equimolar proportions. However, when kinesin was purified further by sucrose density centrifugation the kinesin was distributed into two peaks with sedimentation coefficients of 10 and 7 S, respectively. The 10 S peak consisted of kinesin heavy and light chains at equimolar stoichiometry (Fig. 1, +), whereas the 7 S peak consisted of KHC depleted of light chains (Fig. 1, −). The kinesin preparations were routinely tested for microtubule gliding activity prior to membrane binding assays. We also observed that this kinesin could transport the microsomal membrane vesicles used in binding experiments along microtubules (as reported previously by others, e.g. Sato-Yoshitake et al., 1992; Urrutia et al., 1991; Yu et al., 1992), but this activity has not yet been characterized extensively (data not shown).

Two fragments of the sea urchin KHC were isolated from bacteria overexpressing KHC cDNA inserts, one encoding the tail and part of the stalk domain (referred to as stalk-tail) and the other encoding only part of the stalk domain, referred to as the stalk. The stalk-tail fragment has a predicted molecular mass of 51,717 Da (Fig. 1B, lane 1), whereas the stalk fragment is a 33,762 Da polypeptide (Fig. 1B, lane 2). On sucrose density gradients, the purified stalk-tail and stalk fragments sedimented as monodisperse 2.7 and 3.9 S peaks, respectively. Sucrose density gradient centrifugation did not alter the membrane binding properties of the fragments.

Characterisation of Sea Urchin Microsomes—Sea urchin microsomes were isolated from a 100,000 × g pellet sedimented from unfertilized sea urchin egg lysates. This membrane fraction has been shown previously to contain calcium storing activities (Oberdorff et al., 1988). The microsomal preparations were usually prepared in the presence of 0.5 M KCl and thus can be considered to be salt-stripped, although for some comparative experiments, we did prepare “control” membranes that were not exposed to 0.5 M salt. We further extracted peripheral proteins from the membranes with a brief alkaline wash in 0.1 M sodium carbonate at pH 11. Alkaline-washed membrane preparations, as seen by electron microscopy (Fig. 2A), were cleaner and devoid of small particles, possibly ribosomes, as compared with salt-stripped membranes. When equal amounts of vesicle protein were examined by SDS-PAGE there were no detectable differences in protein composition between the KCl-washed and alkaline-washed membrane preparations (Fig. 2B, lanes 2 and 3), although the protein composition of the control membranes was different (Fig. 2B, lane 1).

The presence of endogenous kinesin in the different membrane preparations was examined by immunoblotting using the anti-sea urchin-kinesin monoclonal antibody, SUK4; the amount of kinesin in KCl-washed and alkaline-washed membranes was negligible (Fig. 2C, lanes 2 and 3, respectively), in marked contrast to the control membranes in which kinesin was present (Fig. 2C, lane 1). A less intense band of higher molecular mass than sea urchin kinesin was occasionally detected on immunoblots probed with SUK4 (but not on those probed with SUK2) as shown in Fig. 2C, lanes 2 and 3, but only when the quantity of vesicles analyzed was much greater that the quantity analyzed routinely in the membrane binding experiments. For example the band is not visible in Fig. 3.

Kinesin Binding to Microsomal Membranes—Our aim was...
to develop a quantitative membrane binding assay that would allow us to map the membrane binding region on kinesin. To do this, we first investigated qualitatively how saturating concentrations of kinesin purified plus or minus light chains and the purified KHC fragments interact with the control, salt-washed, and alkaline-washed membranes (Fig. 3). We then optimized conditions for quantitative binding assays (Fig. 4) which were subsequently used for the mapping experiments (see Figs. 5–7).

The basic assay involved incubating the kinesin preparations with the membrane preparations in the presence of 0.1 mM MgATP to prevent the kinesin motor domain from interacting with the tubulin present in the microsomes, even after salt and alkaline washes (not shown). The mixtures were centrifuged to pellet the membranes which were then examined for kinesin content by quantitative immunoblotting. The results reported were obtained using membranes from unfertilized eggs at interphase, but we did not observe any significant differences when we used membranes from mitotic zygotes at first metaphase.

**Fig. 3.** Membrane binding by native kinesin and KHC fragments. Panel A, native kinesin (173 nM) isolated by Bio-Gel chromatography, 10 S kinesin purified by sucrose density centrifugation with stoichiometric amounts of light chains (+LC, 230 nM), and 7 S kinesin depleted of light chains (−LC, 139 nM) were incubated in the absence (lanes 1) and presence of 79 μg/ml control (lanes 2) and KCl-washed (lanes 3) and alkaline-washed (lanes 4) membranes. Panel B, similarly the bacterially expressed and then isolated KHC fragments, stalk (S, 210 nM) and stalk-tail (ST, 207 nM), were analyzed for their ability to cosediment with membranes. After centrifugation the kinesin content of the membrane pellets was determined by immunoblotting using the monoclonal antibodies SUK4 to the motor domain (panel A) and SUK2 to the stalk (panel B). The numbers on the left side of both panels refer to the molecular masses in kDa of the corresponding polypeptides.

**Fig. 4.** Quantitative analysis of kinesin binding to membranes. Panel A, influence of increasing amounts of vesicle protein on the binding of kinesin to KCl-washed (circles) and alkaline-washed (squares) membranes.
Fig. 3 shows that native kinesin prepared with or without light chains, and also the KHC stalk-tail fragment, bound to the three different types of membrane preparation and coprecipitated with them. The precipitation of the kinesin polypeptides was membrane-dependent since they did not pellet in the absence of membranes. In contrast, the stalk fragment did not bind and cosediment with any of the membrane preparations.

To optimize conditions for quantitatively assaying membrane binding (Fig. 4), we used varying amounts of native kinesin and both salt-stripped and alkaline-stripped membranes. Even though exogenously added kinesin bound satisfactorily to control membranes (e.g. Fig. 4B, inset), we did not use these microsomes routinely because of the complicating effects of their endogenous kinesin content (Fig. 2C). Generally, the affinity of kinesin for KCl versus alkaline-washed membranes was constant (below), but we did note some differences, albeit variable in extent, in the maximal amount of kinesin that bound to the two types of membranes and between different batches of membrane prepared the same way; as reported by Yu et al. (1992), alkaline-washed membranes usually had a higher capacity for kinesin than the KCl-washed membranes, as shown in the example displayed in Fig. 4, although in some experiments the differences were far less pronounced (see Fig. 3).

Increasing amounts of kinesin precipitated as the amount of KCl-washed and alkaline-washed microsomes was increased. However, alkaline-washed membranes were able to bind more kinesin per mg of membrane protein compared with the salt-stripped membranes (Fig. 4A). The binding reaction was examined quantitatively to determine the $B_{\text{max}}$ and $K_0$, where the $B_{\text{max}}$ is defined as the maximum amount of kinesin bound to 1 mg of microsomal protein and the $K_0$ as the dissociation constant of the kinesin-membrane complex. As shown in Fig. 4, $B$, kinesin binds saturably to KCl-washed membranes with a $K_0$ of 19 ± 6 nM and a $B_{\text{max}}$ of 76 ± 10 pmol/mg of microsomal protein, and to alkaline-washed microsomes with a $K_0$ of 22 ± 7 nM and a $B_{\text{max}}$ of 445 ± 38 pmol/mg of microsomal protein. We also analyzed the membrane binding data according to the Hill method (Fig. 4C) and the Scatchard method (inset in Fig. 4C); we interpreted the data in terms of simple linear plots, but there is too much scatter to rule out the existence of multiple components in the binding data.

As noted above, although the measured $K_0$ is highly consistent, the maximal binding capacity is somewhat variable, even for different batches of membranes prepared identically (see "Discussion"). Double-reciprocal analysis of kinesin binding to alkaline-washed membranes (which appeared "cleaner" by electron microscopy) yielded a regression line that fitted the experimental data more closely than the data obtained with KCl-washed membranes (Fig. 4D). Therefore, to map the membrane binding site on the kinesin molecule, we standardized our experiments by working with alkaline-washed membranes and by always performing direct comparisons between kinesin prepared with or without light chains and the KHC fragments in a single experiment, allowing reliable determination of the relative $B_{\text{max}}$. In addition, we worked routinely below 100 nM added kinesin; in this concentration range, curve fitting and quantitation were the most reliable and reproducible obtained. Finally, we repeated the experiments multiple times, and below we present data that are typical and reproducible.

Role of the Light Chains in Kinesin Binding to Membranes—We examined the role of KLCs in membrane binding by comparing the membrane binding abilities of 10 and 7 S kinesin (Fig. 5). The 10 S kinesin bound to alkaline-washed microsomal membranes saturably, with a $K_0$ of 14 ± 6 nM and a $B_{\text{max}}$ of 48 ± 7 pmol/mg of microsomal protein, whereas the 7 S kinesin bound with a $K_0$ of 11 ± 5 nM and a $B_{\text{max}}$ of 26 ± 4 pmol/mg of microsomal protein. Saturable binding was also observed for both kinesin preparations with KCl-washed and control membranes (data not shown). The similar affinities of the 10 and 7 S kinesin for membranes suggest that KLCs are not essential for kinesin binding to membranes. However, the results do not exclude regulatory or targeting roles for the light chains in the kinesin-membrane binding reaction as the differences in the $B_{\text{max}}$ values may suggest. The results obviously suggest that the KHC contains a membrane binding domain.

The KHC Membrane Binding Domain.—To identify the membrane binding domain of the KHC we tested the ability of KHC fragments expressed in, and purified from, bacteria transformed with corresponding cDNAs, to inhibit native kinesin binding to microsomal membranes. The stalk fragment of KHC did not appear to inhibit kinesin binding to membranes, whereas the stalk-tail fragment did (compare panels A and B in Fig. 6). Furthermore, the stalk-tail fragment inhibits kinesin binding to membranes by competing for the same binding sites with native kinesin as shown by Dixon plots which conform to the pattern expected for competitive inhibition since they intersect above the inhibitor axis (Fig. 6, panel C). From the slope of the line which results by
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![Graph A](image1.png)
![Graph B](image2.png)
![Graph C](image3.png)

FIG. 6. Kinesin binding to membranes in the presence of the stalk and stalk-tail fragment of KHC. Panel A, kinesin at 223 nM (squares) and 92 nM (circles) was incubated with alkaline-washed microsomal vesicles (60 μg/ml) for 30 min at room temperature in the presence of increasing concentrations of the bacterially expressed stalk domain. Panel B, kinesin at 69 nM (circles) and 28 nM (squares) was incubated with alkaline-washed microsomal vesicles (50 μg/ml) in the presence of increasing concentrations of the bacterially expressed stalk-tail domain. Panel C, data from panel B in the form of a Dixon plot.

replotting the slopes of the two Dixon plots in Fig. 6C against the reciprocal of kinesin concentration and by using the $K_d$ and $B_{max}$ values for kinesin binding to alkaline-washed membranes we obtained an estimate for the $K_i$ value for stalk-tail of approximately 35 nM; additional Dixon plots are needed to check the accuracy of this value.

When we tested directly the ability of the stalk-tail fragment to bind to membranes, we observed that it binds to alkaline-washed microsomal membranes saturably (Fig. 7) with a $K_d$ of $9 \pm 4$ nM and a $B_{max}$ of $102 \pm 11$ pmol/mg of vesicle protein. The stalk-tail fragment was also able to bind to control and KCl-washed membranes in a similar saturable manner (data not shown). In contrast, membrane binding by the stalk fragment was not observed (e.g. see Fig. 3B). These results strongly suggest that the carboxyl-terminal tail domain of the KHC is responsible for the ability of the kinesin heterotetramer to bind to membranes.

The sequence of the sea urchin KHC obtained by cDNA cloning (Wright et al., 1991) predicts that the carboxyl-terminal (tail) domain of KHC is highly basic with a pI of 11.53. Moreover, membrane binding by kinesin (Fig. 8) and the stalk-tail fragment is highly salt-sensitive (data not shown), suggesting that ionic interactions between the basic tail of kinesin and acidic receptor molecules on the membranes (protein and/or phospholipids) may be important in the binding reaction.

To test the specificity of the presumptive electrostatic interaction, we tested the effect of commercially available proteins with different pI values on kinesin binding to membranes, in addition to the 1 mg/ml of bovine serum albumin that was always present in our binding reactions. As shown in Table I there was no inhibition of kinesin binding to membranes by most nonspecific proteins, even those with high pI values. Histone 1 type IIIS (lysine-rich) was an exception though, showing a concentration-dependent inhibition of kinesin binding to membranes. However, a different fraction of histones, histone 3 type IIIS (arginine-rich fraction), did not significantly inhibit kinesin binding to membranes.

![Graph D](image4.png)

FIG. 7. Binding of the KHC stalk-tail fragment to membranes. The bacterially expressed stalk-tail fragment was incubated with alkaline-washed membranes (80 μg/ml). The amount of stalk-tail bound to membranes was determined from quantitative immunoblotting using the SUK2 monoclonal antibody recognizing specifically the stalk domain of KHC as described under “Experimental Procedures.”

![Graph E](image5.png)

FIG. 8. Influence of salt on kinesin binding to membranes. Kinesin (138 nM) was incubated for 30 min with alkaline-washed membranes (40 μg/ml) at room temperature in the presence of increasing salt concentrations. The percent inhibition of kinesin binding to membranes was calculated on the basis of a control reaction in which kinesin and membranes were incubated in PMEG in the absence of any added NaCl.
**TABLE I**

Control showing effects of "irrelevant" proteins on kinesin binding to membranes

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI</th>
<th>% inhibition*</th>
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<tr>
<td>γ-Globulin</td>
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<td>11</td>
</tr>
<tr>
<td>RNase</td>
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<td>8</td>
</tr>
<tr>
<td>Cytochrome c</td>
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<td>0</td>
</tr>
<tr>
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<td>75</td>
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<tr>
<td>Histone 3</td>
<td>&gt;10</td>
<td>10</td>
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*The % inhibition of kinesin binding to membranes was calculated on the basis of a control reaction in which kinesin and membranes were incubated alone.

**DISCUSSION**

We employed a membrane cosedimentation assay to test the ability of sea urchin kinesin to bind microsomal membranes isolated from the same source. Our results show that all preparations of kinesin that contain the KHC carboxy-terminal domain bind in a specific and a saturable manner to three types of microsomal vesicles with a $K_d$ in the range of 10–20 nM. Chick brain and rat brain kinesins have been shown to bind brain microsomes and synaptic vesicles, respectively, with similar affinities (Yu et al., 1992; Sato-Yoshitake et al., 1992). In addition, bovine brain cytoplasmic dynein binds synaptic vesicles with a similar affinity as well (Lacy and Haimo, 1992).

In contrast to the consistent value of the $K_a$, the $B_{max}$ varied among batches of the three types of membranes (control, KCl-washed, and alkaline-washed) even though the isolation protocols were followed in a consistent manner. Usually the variation was 2- or 3-fold, but differences as great as 10-fold have been observed. The polypeptide composition of the membrane preparations are complex (Fig 2), and it seems likely that the variations in $B_{max}$ could be caused simply by variations in the relative amounts of irrelevant proteins in the preparations; such proteins would not interfere with kinesin-membrane interactions (Table I), but variations in their concentration in different batches of membranes would lead to variations in the measured amount of kinesin bound per mg of membrane protein.

Generally, the maximum capacity of membranes for kinesin was increased by alkaline washing. In the extreme, alkaline-washed membranes had five to six times higher binding capacity for kinesin than salt-stripped membranes as suggested previously for chick brain microsomal membranes (Yu et al., 1992); but here again we saw some variation. Our electron microscopic data suggest that the variable increase in $B_{max}$ following alkaline treatment could result entirely from variations in the amount of “particulate” proteinaceous material associated with KCl-washed membranes and its subsequent removal by alkaline treatment. However, we cannot rule out the possibility that the higher capacity of alkaline-washed membranes for kinesin might be because of the exposure of a higher number of specific binding sites for kinesin per mg of membrane protein after removal of peripheral membrane proteins, and the observed variability in the measured $B_{max}$ may be caused in part by the partial inactivation of such binding sites, for example by denaturation or by base-catalyzed dephosphorylation of a kinesin receptor.

In general, the determination of the maximal capacity of the isolated membranes for kinesin in vitro is a complex issue whose clarification will require further analysis. For the present purposes, the relative $B_{max}$ values for different kinesin preparations are reliable and reproducible, but uncertainty in the actual $B_{max}$ values precludes any accurate estimate of the number of receptors present in the membranes.

Under our conditions of assay, kinesin depleted of light chains retained its ability to bind microsomal membranes with an affinity similar to kinesin containing stoichiometric amounts of light chains, suggesting that light chains are not essential for membrane binding. Indeed, we have identified the carboxyl-terminal domain of the KHC (amino acids 858–1031) as the site of interaction of the heterotetrameric kinesin molecule with membranes, based on the ability of the stalk-tail fragment of KHC expressed and isolated from bacteria to compete specifically with native kinesin for membrane binding in contrast to the stalk domain, which did not. The fact that the stalk-tail domain, in contrast to the stalk, was able to bind to membranes in a saturable manner supports our conclusion and eliminates the possibility that the stalk-tail fragment inhibits native kinesin binding to membranes by competing with the native kinesin for the light chains.

These results give a more complete picture of our understanding of the domain organization of the kinesin molecule as summarized in Fig. 9. Kinesin, consisting of two heavy chains and two light chains, is able to bind to membranes with its carboxy-terminal domain and to use its amino-terminal motor domains to generate force for movement along the microtubules in an appropriate direction. From electron micrographic studies it has been inferred that KLCs are situated at the carboxyl-terminal part of the heavy chain (Hirokawa et al., 1989). Recent studies suggest that the light chains may bind to the stalk domain of KHC by forming α-helical coiled-coils as shown in Fig. 9 (Cyr et al., 1991; Gauger and Goldstein, 1993; Wedaman et al., 1993). The physiological role of the light chains is unknown; in addition it is not known if a fraction of kinesin is depleted of light chains during the preparative procedure or if light chain-depleted KHC exists normally in cells and has a physiological role. Possible regulatory and targeting roles for the light chains in the kinesin-membrane binding reaction have not been eliminated by our results; indeed antibodies to the light chains have been shown to reduce chick brain kinesin binding to membranes although this inhibition may be simply a result of steric hindrance (Yu et al., 1992). Recently it has been shown that kinesin phosphorylated by cAMP-dependent protein kinase binds synaptic vesicles significantly less than unphosphorylated kinesin (Sato-Yoshitake et al., 1992). This observation provides us with a very attractive hypothesis as to how light chains may regulate kinesin binding to membranes, although it is not yet

![Fig. 9. Schematic model of the sea urchin kinesin molecule summarizing conclusions from this and previous studies (Scholey et al., 1989; Johnson et al., 1990; Wright et al., 1991; Wedaman et al., 1993). The amino-terminal motor domains are linked to the coiled-coil stalk (De Cuevas et al., 1992). The KLCs are speculative presented as forming coiled-coils with the heavy chains, although the precise mechanism of binding between KLC and the indicated region of KHC has not been determined (for a detailed discussion see Gauger and Goldstein, 1990). The carboxy-terminal domains of the heavy chains are proposed to be membrane binding domains, and the role of the light chains is unknown.](image-url)
known if indeed kinesin is phosphorylated in vivo by the same kinase.

Early immunocytochemical experiments localized kinesin to the mitotic apparatus in sea urchin zygotes and early blastomeres, suggesting a role for kinesin in cell division (Schley et al., 1985). Further immunocytochemical studies revealed that kinesin is associated with membranous elements that concentrate in the mitotic apparatus of dividing early but not late sea urchin embryos (Wright et al., 1991). In addition, recent studies have suggested that kinesin is associated primarily with endoplasmic reticulum-derived membranes in sea urchin coelomocytes and, in a subset of these cells with endosomes, but not with mitochondria, lysosomes or Golgi membranes (Henson et al., 1992). These observations led to our working hypothesis that kinesin is responsible for the association of membranous organelles with microtubules in the mitotic apparatus and is involved in intracellular membrane trafficking in interphase cells. Certainly, our in vitro kinesin-membrane binding data support the above hypothesis, and it will now be interesting to further fractionate membrane preparations from interphase cells such as coelomocytes and test the hypothesis that kinesin associates with only a subset of membranous organelles. A plausible hypothesis is that KHC may be targeted to a particular class of organelles depending on the identity of the light chains associated with the heavy chains. A number of light chain isotypes have been identified in sea urchin as well as rat, making the above hypothesis particularly attractive (Wedaman et al., 1993; Cyr et al., 1991).

Further work will be required to learn the precise relationship between our "in vitro" experiments and the mechanism of membrane attachment in cells. Kinesin is usually isolated from cytosol (Vale et al., 1985a, 1985b; Brady, 1985; Schley et al., 1985), but a fraction of kinesin remains membrane-bound upon gentle cell lysis (Hollenbeck, 1989). One explanation is that kinesin in cells partitions itself between soluble and membrane-bound forms in accordance with the dissociation constants measured by ourselves and others in vitro. However, in certain cases kinesin can be found stably associated with isolated membrane-bound organelles, sometimes being resistant to extraction by elevated ionic strength or chaotropic agents (Leopold et al., 1992; Schnapp et al., 1992). We detected appreciable amounts of kinesin present in membranes which were prepared in the absence of any salt in the lysis buffer. However, we did not detect any kinesin stably associated with sea urchin membranes prepared in the presence of salt. The fact that sea urchin kinesin binding to membranes is salt-sensitive, as shown in this report, may explain the above discrepancy. Nevertheless it seems plausible that the interaction of kinesin with membranes can be stabilized under certain physiological circumstances, perhaps by the carboxyl-terminal domain. The above hypothesis implies that the attachment of a lipid anchor, for example, could be involved.

Our binding experiments do suggest that the kinesin carboxyl-terminal domain of KHC is important for membrane binding. The highly basic nature of this domain provides us with a suggestion that ionic interactions between the tail and the membrane receptor (protein or phospholipid) are involved. Certainly the salt sensitivity of the membrane binding reaction of native kinesin and that of the stalk-tail fragment supports the presence of such interactions. Similar results have been obtained with rat brain kinesin binding to synaptic vesicles (Sato-Yoshitake et al., 1992). The kinesin binding to membranes appears to be specific though, since a number of unrelated highly basic proteins do not inhibit kinesin binding to membranes. Strangely enough, only histone 1 (lysine-rich) but not histone 3 (arginine-rich) was able to inhibit kinesin binding.

The identity of the receptor for kinesin in membranes is obviously of great interest. In vitro binding studies from other groups have shown that kinesin binding to membranes is protease-sensitive, suggesting that the membrane binding site is a protein (Yu et al., 1992). A 160-kDa endoplasmic reticulum integral membrane protein, kinectin, has been isolated recently from chick brains and proposed to be the kinesin receptor (Toyoshima et al., 1992). Could histone 1 inhibit kinesin binding to membranes by blocking the kinesin docking site on a membrane receptor? Interestingly, histone 1 has been shown to interact with microtubules (Erickson and Voter, 1976) and to be associated with microtubules in sea urchin axonemes as a stabilizing factor (Müllinger et al., 1992). These results coupled with our observation that tubulin is present in our control and carbonate-stripped membrane preparations (data not shown) suggest that the kinesin carboxyl-terminal domain may bind to membrane-embedded tubulin, and this reaction might be inhibited by histone 1. Tubulin is a well known amphipotential protein that associates with membranes, although the physiological significance of this association is not known (Klausner et al., 1981; Kumar et al., 1981). If indeed tubulin can play a role as a kinesin receptor in membranes, then the tail domain of kinesin should be able to interact with tubulin. Over-expression and subsequent immunolocalization of the human kinesin stalk-tail domain in transformed cells revealed that the stalk-tail localizes not only to the membranes but also decorates microtubules (Navone et al., 1992). These results are in agreement with our in vitro observation that the stalk-tail binds in a salt-sensitive, nucleotide-independent manner to taxol-stabilized microtubules and causes unassembled tubulin (even when complexed with colchicine) to precipitate. These observations taken together suggest that the membrane-associated tubulin may be involved in the kinesin binding to membranes. This hypothesis can be tested by reconstituting kinesin binding to artificial membranes such as liposomes that have been exposed to tubulin.

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