Kinesin is a mechanoenzyme that couples adenosine triphosphate hydrolysis to the generation of force and movement along microtubules. To gain insight into the interactions of kinesin and microtubules, cross-linking, mapping, and proteolysis experiments were executed. The motor domain of kinesin was consistently cross-linked to both α- and β-tubulin subunits. Initial mapping of the cross-linked kinesin suggested that amino acids within the N- and C-terminal cyanogen bromide fragments of the motor domain formed cross-links to both α- and β-tubulin subunits. Mapping of the cross-linked tubulin suggested that cross-linking to kinesin occurred within the negatively charged, C-terminal cyanogen bromide fragments of α- and β-tubulin subunits. Treatment of microtubules with subtilisin, a protease that cleaves C-terminal fragments from α- and β-tubulin, reduced their ability to be cross-linked to kinesin motors supporting the idea that C-terminal sequences of α- and β-tubulin may interact with kinesin motors. Finally, of three synthetic peptides, a peptide consisting of the last 12 C-terminal amino acids of β-tubulin competitively interfered with the microtubule-stimulated adenosine triphosphatase activity of the kinesin motor, further suggesting that C-terminal sequences of β-tubulin may be involved in kinesin binding.

Members of the kinesin superfamily of motor proteins harness the energy liberated from ATP hydrolysis to generate a wide variety of intracellular movements (1, 2). To elucidate how kinesin generates force against the microtubule, it is important to understand how kinesin and the microtubule interact. Initial truncation studies of Drosophila kinesin heavy chain revealed that the N-terminal 340 amino acids can hydrolyze ATP and translocate microtubules (3, 4). Consistent with this observation, sequence homology among members of the kinesin superfamily is restricted to this region (2).

Recent work has focused on the interaction of the motor domain with the microtubule, which is composed of repeating dimeric subunits, each consisting of an α- and β-tubulin monomer. The binding stoichiometry of the kinesin motor to tubulin was reported to be one kinesin motor domain/tubulin dimer (5, 6). However, the issue of whether kinesin can be cross-linked only to β-subunits (6), or to both α- and β-subunits (7), remains controversial. Analysis of the atomic structure of the human kinesin motor domain led to the suggestion that loops 8 and 12 (amino acids 138–173 and 272–280) bind to microtubules, which is consistent with the observation that the C-terminal region of the 340-amino acid kinesin motor may be involved in binding to microtubules (8, 9).

In this paper, the cross-linking reaction between kinesin motors and microtubules was investigated further. Using low concentrations of cross-linker, kinesin motors can be cross-linked consistently to both α- and β-tubulin subunits. Further analysis suggests that cross-linking likely occurs within both the most N- and C-terminal CnBr fragments of the kinesin motor. Finally, to probe the sites in tubulin that interact with the kinesin motor, the sites of cross-linking on α- and β-tubulin were mapped, the behavior of subtilisin-treated tubulin was assessed, and competitive peptide inhibitors were assayed. Together, the data suggest that highly charged C-terminal regions of α- and β-tubulin interact with the kinesin motor.

EXPERIMENTAL PROCEDURES

Constructs—Unless otherwise noted, kinesin motor refers to a protein expressed from the vector pET(23b+) (Novagen); the resulting construct, pET(23b+)/K369I, encodes the first 369 amino acids of Drosophila kinesin heavy chain plus one additional isoleucine at the C terminus and is referred to as K369I (11).

Protein Preparation (K369I)—A 20-ml culture of BL21(DE3) containing pET(23b+)/K369I was grown for 14 h on a rotary shaker at 37 °C (12, 13). Following 1:100 dilution into LB + medium, cells were grown at 37 °C to an A of 2.0. After induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, the culture was shaken for 3 h at 37 °C. All subsequent steps were performed at 4 °C unless otherwise noted. Cells were collected by centrifugation at 4000 × g, resuspended in 5 ml of lysis buffer (50 mM imidazole, pH 7.9, 50 mM NaCl, 1 mM DTT, 0.1 mM ADP, 0.1 mM EDTA, 0.5 mM MgCl₂, 0.01% Nonidet P-40, 2 μg/ml soybean trypsin inhibitor/μl of cell pellet, and lysed with a French press set at 1000 p.s.i. Following centrifugation at 100,000 × g, the supernatant was incubated on a rocker with 2 ml of phosphocellulose resin (Whatman P-11/10 ml of supernatant. After 30 min, the solution was centrifuged at 100,000 × g, and the supernatant carefully removed. This step was repeated two times. The phosphocellulose resin was added to an Econo-Pak column (Bio-Rad, 1.5 × 12 cm) and washed with 10 column bed volumes of lysis buffer. Protein was eluted with lysis buffer containing 500 mM NaCl, dialyzed into lysis buffer, concentrated, and exchanged into 80 mM PIPES, pH 6.9, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT, 1.0 mM ADP using a Centricon-30 (Amicon). Purified protein was snap frozen with liquid N₂ in 50–μl aliquots and stored at –80 °C. Before use, each aliquot was thawed on ice and clarified by centrifugation at 100,000 × g. Protein concentration was measured by the Bradford assay (14).

Protein Preparation (Tubulin)—Bovine brain tubulin was purified by cycles of assembly/disassembly followed by phosphocellulose chromatography (15). Purified tubulin was drop frozen into liquid N₂ and stored at –80 °C. Before use, tubulin was thawed on ice and clarified by centrifugation at 100,000 × g. Protein concentration was measured by the Bradford assay (14). Microtubules were polymerized by the addition of GTP and MgCl₂, and formed polymers were pelleted by centrifugation at 10,000 × g, washed, and resuspended in 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EGTA, 10 mM imidazole, 100 μM 4[β-(N-methyl)amino]phenylacetic acid. Protein concentrations were determined spectrophotometrically using the molar absorption coefficient of 6400 M⁻¹ cm⁻¹ at 260 nm.

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The abbreviations used are: DTT, dithiothreitol; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); AMP-PNP, 5′-adenylylimidodiphosphate; EDTG, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; βME, β-mercaptoethanol; Tricine, N-tris(hydroxymethyl)methylglycine; PAGE, polyacrylamide gel electrophoresis; U-MT, uncleaved microtubule; S-MT, subtilisin-treated microtubule; MT, microtubule; K, K369I.
of 7.5% Me2SO, 1 mM GTP, 20 μM Taxol (paclitaxel) and incubation for 15 min at 37 °C (4).

Cross-linking—Kinesin motor (K369I) at 10 μM and microtubules at 10 μM were incubated together in binding buffer (80 mM PIPES, pH 6.8, 2 mM EDTA, 1 mM MgCl2, 50 mM NaCl, 1 mM DTT, 20 mM NaCl, 1 mM DTT, 20 mM NaCl) for 30 min at 25 °C. Microtubules and bound K369I were isolated by centrifugation at 50,000 × g. Pellets were resuspended in the original reaction volume with binding buffer. The cross-linking reagent EDC (Pierce) was added to 0.2 mM final concentration, reactions were incubated for 2 h at 25 °C, then terminated by the addition of 5 × sample buffer (25 mM Tris-Cl, pH 6.8, 5% SDS, 5% βME, 25% sucrose, 0.05% bromphenol blue). To study the effects of ionic strength, kinesin motors were cross-linked to microtubules under standard conditions with the concentration of NaCl adjusted from 50 to 100, 150, 200, and 250 mM NaCl.

Gel Electrophoresis—Products were diluted 1:5 with 1 × sample buffer (5 mM Tris-Cl, pH 6.8, 1% SDS, 1% βME, 5% sucrose, 0.01% bromphenol blue), and separated on 7.5% SDS gels, pH 9.1, or 10% polyacrylamide gels with a razor blade. Gel slices were dried under vacuum. Gel slices were rehydrated with 1 × sample buffer (5 mM Tris-Cl, pH 6.8, 1% SDS, 1% βME, 5% sucrose, 0.01% bromphenol blue) before loading onto a 10% Tris-Tricine gel (21). Products separated on polyacrylamide gels were electrophoretically transferred to 0.2 μm polyvinylidine difluoride (PVDF) membrane slices of 100 μM CnBr solution in 40% methanol, 10% acetic acid and destained with 40% methanol/0.1% bromphenol blue before loading onto a 10% Tris-Tricine gel (18, 21).

Cyanogen Bromide Digests—Proteins of interest were cut out of polyacrylamide gels with a razor blade. Gel slices were dried under vacuum before the addition of 50 μl of 100 mM CNBr solution in 70% formic acid. The reaction was incubated at 25 °C for 1 h in the dark. Slices were washed with 5 mM Tris-Cl, pH 6.8, 40% methanol, 1% SDS, 1% βME, 5% sucrose, 0.01% bromphenol blue until the dye retained a blue color. Gel slices were then washed with 100% methanol for 15 min and dried under vacuum. Gel slices were rehydrated with 1 × sample buffer (5 mM Tris-Cl, pH 6.8, 1% SDS, 1% βME, 5% sucrose, 0.01% bromphenol blue) before loading onto a 10% Tris-Tricine gel (18, 21).

Subtilisin Treatment—Paclitaxel-stabilized microtubules were digested with 1.2% (w/w) subtilisin (Sigma) at 37 °C for 12 h, which has been reported to have no observable effect on microtubule structure as judged by electron microscopy (10, 17, 22). The reaction was terminated by the addition of phenylmethylsulfonyl fluoride to 5 mM. Untreated microtubules were incubated in the same manner as microtubules treated with subtilisin. Untreated and subtilisin-treated microtubules were recovered by centrifugation through a 30% sucrose cushion at 50,000 × g and were resuspended in 80 mM PIPES, pH 6.9, 2 mM EDTA, 1 mM DTT, 20 μM paclitaxel.

ATPase Assays—The rate of ATP hydrolysis by the kinesin motor was measured by a molybdate/malachite green colometric assay for P, release (23). Typically, 10-μl aliquots of a reaction mixture containing 10 μg/ml of the kinesin motor were quenched with 90 μl of ice-cold 0.3 M HClO4 prior to addition of 100 μl of sodium molybdate/malachite green solution in 0.7 M HCl. After 20–30 min at 25 °C, the A405 of the HClO4/malachite green solution was determined using a microtiter plate reader. Rates for ATP hydrolysis were corrected for ATP hydrolysis in the malachite green/HClO4.

Peptide Synthesis—α-, β-, and scrambled peptides were synthesized on a 2-mmol scale using standard solid state methods on a Milligen 9050 peptide synthesizer. Before use, portions of the crude eluted peptides (20 mg) were purified by reverse-phase high performance liquid chromatography using a Waters DeltaPak C18 column and a gradient of 0.1% trifluoroacetic acid to 50% acetonitrile to elute peptides. Purified peptides were freeze-dried and stored under vacuum. Before use, peptides were resuspended in 80 mM PIPES, pH 6.9, 2 mM EDTA, 1 mM DTT, 20 μM paclitaxel.

Antibodies—Polyclonal rabbit antibody NK18 was made against a synthetic peptide corresponding to the N-terminal 17 amino acids of Drosophila kinesin heavy chain (MASREIPAEPSIKVVC) linked through the C-terminal cysteine to bovine serum albumin (Chiron). Polyclonal antibody PAN was generously provided by J. Scholey (University of California, Davis) (24). This antiserum resulted from pooling three separate antibodies raised against the following peptide sequences: LVDLAGSE, SSRSHSVF, and HIPYRNSKLT. Monoclonal antibody His-Tag was purchased from Sigma. Monoclonal antibody 18D6 was generously provided by D. Cleveland (University of California, San Diego) (20). Monoclonal antibodies DM1A and DM1B, which are specific for α- and β-tubulin, respectively, were purchased from Sigma.

**RESULTS**

Cross-linking Kinesin Motors to Microtubules—To study the interaction between kinesin motors and microtubules, chemical
Probing the Kinesin-Microtubule Interaction

To analyze the composition of the 97-kDa complex, products of a standard cross-linking reaction were separated on a 7.5% SDS-polyacrylamide gel adjusted from pH 8.8 to pH 9.1 to obtain optimal resolution of α- and β-tubulin subunits as well as tubulin complexes (26). Under these gel conditions, α-tubulin has a relative mobility of ~55 kDa, β-tubulin has a relative mobility of ~53 kDa and the 97-kDa complex resolves into a doublet (Fig. 3A). Probing Western blots with subunit-specific antibodies revealed that the upper band of the doublet contains α-tubulin, and the lower band contains β-tubulin (Fig. 3B); both bands react with kinesin-specific antibodies (Fig. 3C). These results support the hypothesis that kinesin motors can be cross-linked to both α- and β-tubulin subunits.

To test whether the cross-linking of K369I to α- and β-tubulin reflects a comparable affinity of interaction, the ionic strength dependence of the reaction was assessed (Fig. 4). The data indicate that as ionic strength increases, the overall efficiency of the cross-linking reaction decreases, which suggests, as reported previously, that the binding reaction between kinesin motors and microtubules contains a significant ionic component (27). However, the relative cross-linking of kinesin motors to α-versus β-tubulin was ionic strength-independent. Comparison of the relative yields of K + α versus K + β complexes (where K is kinesin motor) revealed that the ratio of K + α complexes to K + β complexes was consistent within a given set of reactions up to 250 mM NaCl. This result argues against the interpretation that cross-links to α-tubulin are nonspecific or represent secondary sites of interaction.

Mapping Regions of the Kinesin Motor That Interact with the Microtubule—To determine which regions of the kinesin motor make contact with the microtubule, points of cross-linking were localized within the 97-kDa product. Gel slices containing the 97-kDa product (a mixture of K + α and K + β) were partially

Fig. 3. Analysis of the 97-kDa products of the cross-linking reaction. K was cross-linked to MT using 0.2 mM EDC. Products were analyzed by SDS-PAGE; however, the pH of the resolving gel was adjusted from 8.8 to 9.1 to obtain optimal resolution of α- and β-tubulin subunits as well as tubulin complexes (26). In control K + K reactions, K369I was cross-linked to K369I, and in control MT + MT reactions, microtubules were cross-linked to microtubules. K + MT indicates reactions in which K369I was cross-linked to microtubules. Products are indicated by asterisks along with their proposed composition; the symbols α and β represent α- and β-tubulin subunits, and K represents the K369I. Panel A shows the 97-kDa product resolved into a doublet. Based on their relative mobilities, the components likely resulted from cross-linking of K369I (42 kDa) to either the α-subunit (55 kDa) or the β-subunit (53 kDa). Complexes with relative mobilities slower than 97 kDa may have resulted from cross-linking the α- to the β-subunit (55 kDa + 53 kDa = 108 kDa), the α- to the α-subunit (55 kDa + 55 kDa = 110 kDa), the β- to the β-subunit (53 kDa + 53 kDa = 106 kDa) or from cross-linking K369I to the α- and the β-subunit (42 kDa + 55 kDa + 53 kDa = 150 kDa). Panel B shows the composition of the 97-kDa doublet as determined by antibody analysis. Products were analyzed by SDS-PAGE, pH 9.1, and transferred to nylon membrane. The left half of the membrane was probed with DM1A, an antibody specific for α-tubulin, and the right half was probed with DM1B, an antibody specific for β-tubulin (19). Based on the reactivity of the 97-kDa doublet, the top band is composed of K369I cross-linked to the α-subunit (K + α), and the bottom band is composed of K369I cross-linked to the β-subunit (K + β). The complex with the slowest relative mobility may result from cross-linking the α- to the β-subunit (α + β), the α- to the α-subunit (α + α), or the β- to the β-subunit (β + β). Panel C shows that both the upper and lower bands of the 97-kDa doublet react with NK18, a kinesin-specific antibody, lending further evidence that K369I can be cross-linked to both α- and β-tubulin subunits.
digested with CnBr, a chemical that cleaves proteins specifically at methionine residues (21, 28). Under these conditions, a nested set of kinesin and tubulin fragments resulted. These fragments were run on a second gel, blotted, and probed with antibodies specific for the N- or C-terminal CnBr fragments of the kinesin motor. In the hypothetical case of a kinesin motor cross-linked through its C-terminal fragment to the microtubule, when fragments are probed with an antibody specific for the N terminus of the kinesin motor, only full-length protein should shift out of register as compared with control, uncross-linked kinesin motors (Fig. 5A). If, on the other hand, the motor is probed with a C-terminal kinesin motor antibody, all products should shift out of register (Fig. 5B). When these experiments were carried out, virtually 100% of the full-length protein shifted to a slower relative mobility as compared with control, uncross-linked motor fragments when mapped from the N terminus (Fig. 6A). In addition, there are partial shifts of intermediate-sized fragments as well. Mapping with the C-terminal specific antibody generated comparable data; full-length product shifted completely, while lower fragments shifted partially (Fig. 6B). The simplest interpretation of these data is that cross-links to the microtubule occur within both the N- and C-terminal CnBr fragments of the kinesin motor. Attempts to map the K1a and K1b species separately were unsuccessful owing to low yields. Thus, the issue of whether α- and β-tubulin bind to different regions of the kinesin motor could not be resolved.

Mapping the Sites on the Microtubule That Interact with the Kinesin Motor—To evaluate the regions of α- and β-tubulin that interact with the kinesin motor, partial CnBr digests of the 97-kDa cross-linked product were run on gels, blotted, and probed with tubulin-specific antibodies. If β-tubulin were linked through its C-terminal CnBr fragment to the kinesin motor, then upon probing with an antibody specific for the N terminus of β-tubulin, one would have expected that only the

![Image](http://www.jbc.org/)

**FIG. 4.** The effect of increasing ionic strength on the cross-linking reaction. K was cross-linked to MT using EDC, and the final concentration of NaCl present in the reaction was adjusted from 50 to 100, 150, 200, or 250 mM. Labeling is as in Fig. 3.

**FIG. 5.** Expectations for N- and C-terminal maps of the kinesin motor. If the kinesin motor is digested with CnBr, five fragments are expected. For the purpose of illustration, the motor is assumed to be cross-linked through its C-terminal CnBr fragment to the microtubule (C-terminal cross-link). Panel A diagrams the expected results of an N-terminal map of the kinesin motor cross-linked through its C-terminal fragment to the microtubule (X). In the left control reactions, the motor was not cross-linked to the microtubule before cleavage. On the right, the motor was cross-linked to the microtubule before cleavage. If fragments are separated by SDS-PAGE, blotted, and probed with a kinesin-specific antibody whose site of recognition (*) lies near the N terminus, then only full-length kinesin motor protein should shift to a slower relative mobility as compared with control, uncross-linked motor fragments. Cross-linked products are represented by a large box because they are unlikely to migrate as a well defined band due to partial cleavage of attached tubulin fragments, shown in gray. Panel B diagrams a C-terminal map (using an antibody whose recognition site is at the C terminus (*)) of the kinesin motor cross-linked through its C-terminal CnBr fragment to the kinesin motor. If cross-linking to the microtubule occurs exclusively within the C-terminal fragment, all fragments should shift to a slower relative mobility as compared with control, uncross-linked motor fragments on the left.
largest fragment would shift out of register as compared with control, un-cross-linked tubulin. This result was observed, suggesting that contact with the kinesin motor occurs within the most C-terminal CnBr fragment of the β-subunit (Fig. 7A). To test this result further, maps of the 97-kDa cross-linked product were generated using the antibody specific for the C terminus of β-tubulin. If cross-linking occurs within the most C-terminal CnBr fragment of β-tubulin, when fragments are probed with a β-tubulin-specific antibody whose site of recognition lies near the C terminus, all fragments should shift to slower relative mobilities as compared with control, un-cross-linked tubulin fragments. When this experiment was carried out, all fragments appeared to shift, which is consistent with the hypothesis that at least one region of contact between microtubules and the kinesin motor resides within the most C-terminal, CnBr fragment of β-tubulin (Fig. 7B).

Contacts between α-tubulin and the kinesin motor were analyzed in a similar manner except that an N-terminal α-tubulin map was not generated because an appropriate N-terminal α-tubulin antibody was not available. If cross-linking to the kinesin motor occurs through the most C-terminal CnBr fragment of α-tubulin, then when CnBr fragments from the 97-kDa product are probed with an α-tubulin-specific antibody whose site of recognition lies near the C terminus, all fragments should shift to slower relative mobilities as compared with control, un-cross-linked tubulin fragments. When this experiment was carried out, all fragments appeared to shift suggesting that at least one region of contact between microtubules and the kinesin motor resides within the most C-terminal CnBr fragment of α-tubulin (Fig. 7C).

Further Evidence That the N and C Termini of α- and β-Tubulin Interact with the Kinesin Motor—To confirm whether the C termini of α- and β-tubulin interact with the kinesin motor, paclitaxel-stabilized microtubules were treated with the protease subtilisin under conditions that are reported to remove C-terminal fragments of α- and β-tubulin subunits without obviously affecting microtubule structure (10, 17, 22). These microtubules were then tested for their ability to be cross-linked to the kinesin motor using EDC in the presence of AMP-PNP (25). Cleavage of microtubules with subtilisin changes the relative mobility of microtubules by ~4 kDa (Fig. 8A); the reaction appears to be complete as there is no obvious uncleaved product (U-MT) remaining in the subtilisin-treated (S-MT) material. In reactions in which S-MT were cross-linked.

![Image](http://www.jbc.org/)

**Fig. 6. Antibody mapping of sites on the kinesin motor that interact with the microtubule.** Panel A shows an N-terminal map of K369I cross-linked to microtubules and probed with NK18, a β-tubulin-specific antibody whose site of recognition lies near the N terminus. In the K + MT lane, full-length K369I shifted completely to a slower relative mobility, marked by an arrow, when compared with the K lane. Other fragments partially shifted to slower mobilities, marked by asterisks. Panel B shows a C-terminal map of K369I cross-linked to microtubules. Fragments were probed with PAN, an antibody whose site of recognition lies within the C-terminal CnBr fragment of K369I (24). Comparable to the N-terminal map, in the K + MT lane, full-length protein shifted completely to a slower relative mobility, marked by an arrow, when compared with the K lane. Other fragments partially shifted to slower mobilities, indicated by a bracket.

**Fig. 7. Antibody mapping of sites on the microtubule that interact with the kinesin motor.** Panel A shows an N-terminal map of β-tubulin cross-linked to K369I. Control microtubules (MT) and microtubules cross-linked to K369I motor (MT + K) were partially digested, separated, and probed with 18D6, a β-tubulin-specific antibody whose site of recognition lies near the N terminus (20). In the MT + K lane, only the largest, full-length tubulin fragment shifted to a slower relative mobility as compared with the MT lane. Panel B shows a C-terminal map of β-tubulin cross-linked to K369I. Control un-cross-linked microtubules (MT) and microtubules cross-linked to K369I (MT + K) were partially digested, separated, and probed with DM1B, a β-tubulin-specific antibody whose site of recognition lies near the C terminus (19). In the MT + K lane, all fragments appeared to shift to slower relative mobilities as compared with the MT lane. Panel C shows a C-terminal map of α-tubulin cross-linked to the kinesin motor. Control microtubules and microtubules cross-linked to the kinesin motor were partially digested, separated, and probed with an α-tubulin-specific antibody, DM1A, whose site of recognition lies near the C terminus (19). In the MT + K lane, all fragments appeared to shift to slower relative mobilities as compared with the MT lane.
treating microtubules with subtilisin markedly reduces their ability to be cross-linked to kinesin motors.

The functional significance of diminishing kinesin-MT interaction by subtilisin treatment was assessed by examining MT-stimulated ATPase activity. At 50 mM salt, the $K_m$ for microtubule-stimulated ATPase activation of K369I was measured to be 1.92 $\mu M$, and the $V_{max}$ was measured to be $4.4 s^{-1}$, both of which are well within published values for comparable constructs (3, 27, 29). When subtilisin-treated microtubules were added to the kinesin motor, the $V_{max}$ remained essentially unchanged; however, the $K_m$ increased from 1.92 $\mu M$ to 9.03 $\mu M$ (Fig. 9). These data also suggest that the interaction of subtilisin-treated microtubules with the kinesin motor is impaired.

Since treatment of microtubules with subtilisin affects both $\alpha$- and $\beta$-tubulin, it was important to know whether the modification of $\alpha$-, $\beta$-, or both tubulin subunits resulted in the observed decrease in microtubule-stimulated ATPase activity. To address this question, peptides $\alpha$ (SVEGEGEEGE) and $\beta$ (GEFEEEDEEDEE), which correspond to the portions of tubulin (30–32), were synthesized and tested for their ability to inhibit microtubule-stimulated ATPase activity. Addition of 1 or 5 mM $\alpha$-peptide had little effect on the $K_m$ for ATPase activation of the kinesin motor (Fig. 10A). In contrast, the addition of 1 or 5 mM $\beta$-peptide increased the $K_m$ from 1.89 $\mu M$ to 3.16 $\mu M$ or 4.15 $\mu M$, respectively (Fig. 10B). The addition of a control scrambled peptide (EGEVEESEGEE) had little effect on the $K_m$ (Fig. 10C). In all cases, $V_{max}$ remained unchanged. The increase in $K_m$ upon the addition of the $\beta$-peptide, but not the $\alpha$- or the scrambled peptide, suggests that the $\beta$-peptide competitively interferes with the binding of microtubules to the kinesin motor.
**Fig. 10. Competition of synthetic peptides with microtubules for activation of the kinesin ATPase.** The ability of synthetic peptides to compete with microtubules for binding to K369I was assayed. Reported values result from averaging the data from three separate trials. In the control, no peptide reactions, reciprocal microtubule-stimulated ATPase activity of K369I was plotted against reciprocal concentrations of microtubules. Panel A shows the addition of the α-peptide, panel B shows the addition of 1 or 5 mM β-peptide to the reaction, and panel C shows the addition of 1 or 5 mM scrambled peptide.

**DISCUSSION**

The Kinesin Motor Can Be Cross-linked to Both α- and β-Tubulin Subunits—The data presented here demonstrate that the kinesin motor domain can be reproducibly cross-linked to both α- and β-tubulin subunits of microtubules. These results are in agreement with a recently published study (7), but are in conflict with an earlier report (6). Both previous studies carried out cross-linking reactions with a 10-fold higher concentration of EDC (2.0 mM) than the concentration used in these studies (0.2 mM). If 1 or 3 mM EDC is used in the cross-linking reactions, the resolution between K + α and K + β complexes is lower than if 0.2 mM EDC is used; therefore, distinguishing between K + α and K + β complexes might be more difficult as the concentration of EDC increases (Fig. 1). An alternative explanation for these discrepancies could be that one study (6) used squid kinesin, while the other study (7), and the present study, used *Drosophila* kinesin. It is possible that squid kinesin may bind α-tubulin, but may not present the EDC-reactive groups in the necessary orientation or proximity to be cross-linked to α-tubulin (7). Nevertheless, the comparable ionic strength dependence, and the consistency of cross-linking to α- and β-tubulin observed here, suggests that the cross-links to both subunits are specific, and therefore that both are likely to reflect relevant biological interactions.

The view that kinesin motors make multiple contacts with the microtubule is consistent with other studies of the interactions of kinesin and microtubules. For example, the motor is highly processive and releases the microtubule for only a small fraction of its 50-ns cycle time (25). One way to maintain such a strong hold on the microtubule might be through the use of multiple contacts with α- and β-tubulin monomers as the motor moves over the microtubule lattice. Data indicating an 8-nm step in combination with the data suggesting that kinesin could only be cross-linked to β-tubulin led to a model in which the kinesin motor steps from β-subunit to β-subunit (6, 33). Speculatively, a more complex model may be invoked to explain contacts with both α- and β-tubulin. One possibility is that kinesin takes steps smaller than 8 nm. Indeed, recently improved methodology suggests that the motor might take 5- and 3-nm steps as well as 8-nm steps (34).

The N and C Termini of K369I Interact with the Microtubule—The initial mapping data suggest that both the N- and C-terminal CnBr fragments of K369I interact with the microtubule, which supports models in which the kinesin motor has more than one region capable of interacting with the microtubule, or alternatively, possesses a single, discontinuous microtubule binding site. Analysis of the atomic structure of the kinesin and NCD motors (8, 44) led to the suggestion that two regions, one corresponding to residues 145-180 and a second corresponding to residues 278-287 of K369I, might interact with the microtubule. The latter region is contained within the cross-linked C-terminal CnBr fragment, but the former region is not included within the cross-linked N-terminal CnBr fragment. Although further work will be required to resolve the inconsistencies, both lines of investigation agree that the region defined by the C-terminal CnBr fragment of K369I interacts with the microtubule.

The C Termini of α- and β-Tubulin Interact with the Kinesin Motor—The data presented here suggest that the C termini of α- and β-tubulins may both interact with the kinesin motor domain. Some reports indicated that these regions may also interact with microtubule-associated proteins including kinesin, tau, dynein, and MAP2 (10, 17, 35–37), although other reports contradicted these findings (22, 38, 39). The work reported here is the first attempt to map directly the sites of interaction between microtubules and the kinesin motor; these results point to the C termini of α- and/or β-tubulin as sites where binding may occur. Furthermore, treatment of paclitaxel-stabilized microtubules with the protease subtilisin, which is reported to remove C-terminal fragments from the α- and β-tubulin subunits (30), increased the $K_m$ for ATPase activation of the kinesin motor while the $V_{max}$ remained unchanged. One interpretation is that subtilisin removes one of the kinesin binding sites on α- or β-tubulin, but that additional sites exist. Perhaps the site removed by subtilisin is a relatively low affinity site whose absence can be overcome by the addition of a greater concentration of microtubules.

Consistent with the idea that the C termini of tubulins interact with K369I is the finding that addition of a C-terminal peptide corresponding to the last 12 amino acids of pig β-tubulin can increase the $K_m$ for microtubule-stimulated ATPase activity of kinesin from 1.89 μM up to 4.15 μM. These data suggest that the β-peptide competes with the binding of microtubules to the kinesin motor and may include some portion of a kinesin binding site. The lack of interference by the α-peptide is not clear given the cross-linking results, but
perhaps the α-peptide does not assume a correct conformation. Future work should include the analysis of longer C-terminal tubulin peptides, since it is possible that the peptides used in the current experiments may be too short to encompass fully a C-terminal site of interaction with the kinesin motor. In fact, earlier reports indicate that the sites of subtilisin cleavage are about 20–25 amino acids more N-terminal than the ones chosen for the peptide design (40, 41).

A functional role for the C termini of tubulins has also been suggested previously based on sequence alignments of these regions (17). Although the C termini of tubulins display high phylogenetic variability, the sequences EGEE and EEGEE are well conserved (42, 43). This conservation of sequence and negative charge is consistent with the idea that the C termini of α- and β-tubulin bind to the kinesin motor. The finding that negatively charged amino acids on tubulin make contact with kinesin motors, suggests that positively charged residues on the motor will be important for microtubule binding. Indeed, many conserved positively charged residues are found in the cross-linked N- and C-terminal Cnb fragments.

A Model for the Kinesin-Microtubule Interaction—Combining data contained in this report with what is known about tubulin and kinesin structure, a simple model can be put forth to describe the kinesin-microtubule interaction. Cross-linking data suggest that the C-terminal domain of the tubulin β-subunit interacts with the N-terminal domain of the tubulin α-subunit (45). In addition, a 6-Å resolution structure of tubulin suggests that the monomers are similarly oriented within the filament (46). Therefore, the distance between the C termini of α- and β-tubulin could be as much as 4 nm depending on the flexibility and mobility of the C termini relative to the bulk of the subunits. Accordingly, the motor domain could make contact with the C termini of both α- and β-tubulin, especially if these domains are somewhat flexible.

Given the finding that the highly charged C terminus of β-tubulin is likely to interact with the kinesin motor, it is tempting to speculate that kinesin binds to its polymeric substrate and generates force using a similar mechanism as myosin. The first step of the myosin-actin interaction is thought to be mediated by ionic interactions that generate weak binding between the two proteins. Subsequent step(s) are thought to be mostly hydrophobic in nature and are proposed to strengthen overall binding before the power stroke occurs (47). One possibility is that the ionic interactions characterized in this report are the counterpart to the initial ionic interactions reported for the myosin-actin system. Other sites of interaction may be important for the final, stereospecific positioning of the motor on the microtubule before the power stroke occurs. It is likely that future studies of how the kinesin motor binds and releases microtubules will address these questions and expand the understanding of how these molecular machines operate.

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