The C-Terminus of Tubulin Increases Cytoplasmic Dynein and Kinesin Processivity

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ABSTRACT In motor movement on microtubules, the anionic C-terminal of tubulin has been implicated as a significant factor. Our digital analyses of movements of cytoplasmic dynein- and kinesin-coated beads on microtubules have revealed dramatic changes when the C-terminal region (2–4-kDa fragment) of tubulin was cleaved by limited subtilisin digestion of assembled microtubules. For both motors, bead binding to microtubules was decreased threefold, bead run length was decreased over fourfold, and there was a dramatic 20-fold decrease in diffusional movements of cytoplasmic dynein beads on microtubules (even with low motor concentrations where the level of bead motile activity was linear with motor concentration). The velocity of active bead movements on microtubules was unchanged for cytoplasmic dynein and slightly decreased for kinesin. There was also a decrease in the frequency of bead movements without a change in velocity when the ionic strength was raised. However, with high ionic strength there was not a decrease in run length or any selective inhibition of the diffusional movement. The C-terminal region of tubulin increased motor run length (processivity) by inhibiting "detachment" but without affecting velocity. Because the major motor binding sites of microtubules are not on the C-terminal tail of tubulin (Marya et al., 1994), we suggest that the changes are the result of the compromise of a weakly attached state that is the lowest affinity step in both motors' ATPase cycles and is not rate limiting.

INTRODUCTION

Motor molecule movement on microtubules involves a highly coordinated interaction between the ATPase domains of the motor and the filament. With the definition of the crystal structure of the kinesin and ncd ATPase domains (Kull et al., 1996; Sablin et al., 1996) and the nature of the motor-microtubule complex (Hoenger and Milligan, 1997; Hirose et al., 1996), our understanding of the structural aspects of the motors has increased dramatically. Kinetic analysis of the ATPase cycle has clarified important aspects of the coordination of the heads through the comparison of one- and two-headed molecule ATPase kinetics (see review in Hackney, 1996). Motion analysis of moving beads or microtubules has also clarified the magnitude of the force generated and important aspects of the motor movement on the filament (Coppin et al., 1996, 1997; Gelles et al., 1988; Svoboda et al., 1994; Hunt et al., 1994). The interface between the kinesin motor and the filament has been defined (Woehlke et al., 1997; Tucker and Goldstein, 1997), and a role for the neck of the kinesin molecule in processive movement has been found (Romberg et al., 1998). However, the role of the microtubule surface in stabilizing the movement of the motors is poorly understood. In these studies we have focused on the role of the anionic C-terminal domain of tubulin in the motility of both kinesin- and cytoplasmic dynein-coated particles.

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Microtubules have a high negative charge on their surface and are often stabilized by cationic domains of microtubule-associated proteins (Hirose et al., 1995; Li and Joshi, 1995; Mitchison, 1993; Wade and Chretien, 1993). Tubulins have a neutral amino acid composition, except for their carboxyl-terminal 30–40 residues, which are highly acidic. Recent analyses of the microtubule structure at the atomic level have not clarified the structure of the C-terminal region, presumably because of segmental motion in that region (Nogales et al., 1998). Limited proteolysis by subtilisin cleaves a small fragment of 2–4 kDa from the C-terminal end of both α and β subunits (Serrano et al., 1984b; Sackett et al., 1985; Sackett and Wolff, 1986). The cleavage of the C-terminal domain of tubulin reduces the critical concentration for polymerization by 50-fold (Sackett et al., 1985) and promotes microtubule polymerization in the absence of microtubule-associated proteins (Serrano et al., 1984b). The binding sites of MAP2 and tau are located on the C-terminal acidic region according to limited proteolysis studies with subtilisin (Serrano et al., 1984a; Marya et al., 1994).

Microtubule-based motor proteins, cytoplasmic dynein and kinesin, do not bind to the carboxy-terminal domain, as judged by studies of the motility of subtilisin-digested microtubules on motor-coated glass (Hagiwara et al., 1994), but do bind to an adjacent region based upon structural studies. MAP2, which binds to the carboxy-terminal region, does inhibit cytoplasmic dynein and kinesin motility. However, this effect appears to be the result of a steric inhibition, because the smaller microtubule binding domain of MAP2 alone and the tau protein do not affect motility (Lopez and Sheetz, 1993), unless they are used at very high concentrations (Hagiwara et al., 1994; Heins et al., 1991; Paschal et al., 1989; Rodionov et al., 1990). Small latex particles coated with motors are sensitive detectors of motor move-

ment and have faithfully reported the run length of single motors on microtubules (processivity) (Block et al., 1990; Wang et al., 1995), the stepping distance (Coppin et al., 1996; Svoboda et al., 1993), and the tracking of motors along protofilaments (Gelles et al., 1988; Wang et al., 1995). We have now used the beads to follow the motor movements on proteolyzed microtubules and find dramatic changes in run length but not in velocity.

MATERIALS AND METHODS

Materials

Fertilized chicken eggs were from the veterinary school of North Carolina State University. Monoclonal anti-cytoplasmic dynein heavy chain antibody 440.1 was described before (Steuer et al., 1990). Taxol was purchased from Calbiochem (San Diego, CA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Methods

Preparation of motor proteins and antibody 440.1

Cytoplasmic dynein and kinesin were purified from 12-day-old chicken embryo brain as previously described (Schroer et al., 1989). Anti-cytoplasmic dynein heavy chain antibody 440.1 was raised in the laboratory (Steuer et al., 1990) and purified from ascites by sodium acetate precipitation and protein A affinity column chromatography.

Assembly of microtubules

Porcine tubulin (4 mg/ml) was purified and assembled in vitro as previously described (Wang et al., 1995).

Subtilisin digestion of microtubules

Microtubules were incubated with subtilisin at the ratio of 1:0.6 (w/w of tubulin:subtilisin) at 37°C for 1 h. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to 2 mM. The mixture was transferred into a 200-μl airfuge tube and centrifuged at 25 psi for 5 min in an airfuge (Beckman Instruments, Palo Alto, CA). The pellet was washed twice gently with microtubule stabilizing (MS) buffer (PMEE′ containing 20 μM taxol and 1 mM GTP). Then it was resuspended in MS buffer. Subtilisin was prepared as a 10 mg/ml stock in water and stored at −20°C. PMSF was dissolved in isopropanol at 100 mM in the stock solution.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and protein concentrations were determined by gel densitometry. The average number of protein molecules per bead in the motility assay was calculated accordingly (Wang et al., 1995).

Motility assay

Bead motility assays were performed as previously described (Wang et al., 1995).

To study the dependence of bead motility on the concentration of cytoplasmic dynein on beads, cytoplasmic dynein was serially diluted and mixed with a constant number of beads such that the concentration of cytoplasmic dynein on beads was serially decreased. For each concentration, 10 fields were randomly chosen and observed for 2 min for moving, diffusing, and stationary beads. A bead that bound to a microtubule was scored as stationary if it moved less than 0.2 μm from its original position during the observation period, which ranged from 2 s to 2 min. If a bead moved more than 0.2 μm in one direction, it was scored as a moving bead. If a bead moved more than 0.2 μm but changed its direction of movement, it was scored as a diffusing bead. The length of microtubules in each field was measured with the "ruler" program developed in the laboratory. The number of beads bound was calculated per unit length of 100 μm.

Data analysis

Beads and microtubules were observed by video-enhanced DIC microscopy. The criteria for defining bound beads as moving, diffusing, or stationary were as described in the previous paper (Wang et al., 1995). In brief, the moving beads progressed in one direction on a microtubule with a maximum velocity of less than 1.5 μm/s. Diffusing beads moved in both directions on microtubules and underwent frequent random changes in direction and velocity. Stationary beads did not move during the observation period. To determine the number of beads that were moving, diffusing, and stationary under different conditions, six fields of each sample were randomly scanned and scored. Each field was scored for 1 min. The total length of microtubules was determined for each field, and the numbers of beads in each category was calculated per unit length (100 μm) of microtubule. The length of microtubules and distance of bead movement were measured with the "ruler program" as previously described.

For the instantaneous velocity measurements, bead positions were tracked to nanometer precision. Bead positions were tracked with the "centroid" program developed in this laboratory (Gelles et al., 1988). The program measured the intensity of the bright part of the bead image and calculated the centroid of the image as a weighted average. Bead displacement was decomposed into displacements parallel and perpendicular to the microtubule axis. The parallel displacement was the distance moved by the bead along the microtubule. Data were filtered over five frames with a boxcar filter to decrease measurement errors. Parallel displacement between the mth frame and $(m + a)$th frame was calculated for every frame of the movement. The displacements were pooled into bins of 4 nm, and the number of displacements in each bin was counted. Displacement d was put into bin n when $4n \leq d < 4(n + 1)$ and n was an integer. To cancel the noise generated by the vibration of stationary beads, the number of displacements in bin $-n$ was subtracted from that in bin $+n$. The average displacement was divided by $a/30$ to yield the velocity.

RESULTS

Subtilisin digestion of microtubules

To remove the C-terminus, in vitro assembled microtubules were incubated with subtilisin at a 1:0.6 ratio (w/w) at 37°C. At 10-min intervals, aliquots were mixed with 2 mM PMSF to stop the reaction, and samples were run on 10% SDS-PAGE to monitor the size change of tubulin (Fig. 1). At time 0, the α and β tubulins were seen in one wide band. After 10 min, two faster running bands were seen; the lowest was previously identified as the cleaved product of the β subunit, and the upper band was the product of the α subunit (Sackett et al., 1985; Bhattacharyya et al., 1985). This result was consistent with previous reports that limited subtilisin digestion of tubulin cleaved a small fragment of 2–4 kDa from the C-terminal end (Serrano et al., 1984b;

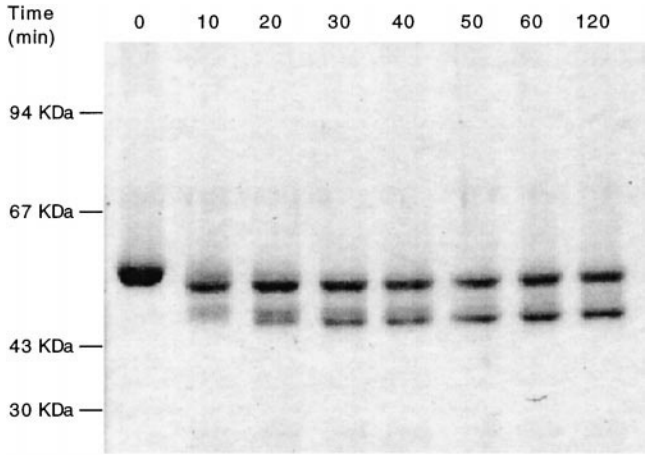

FIGURE 1 Time course of subtilisin digestion of microtubules. Microtubules assembled in vitro with porcine tubulin (4 mg/ml) were incubated with 2.4 mg/ml subtilisin (final concentration) at 37°C. Aliquots have been taken out at the time indicated at the top of the gel. Enzymatic reaction was stopped by the addition of PMSF to 2 mM. Samples were run on 10% SDS-PAGE. The numbers at the left of the gel indicate the molecular mass.

Sackett et al., 1985). The subtilisin-digested microtubules used in all assays were digested for 1 h, and the reaction was stopped by PMSF.

After subtilisin cleavage, the C-terminal piece came off of the assembled microtubules (White et al., 1987; Serrano et al., 1984b). Digested microtubules were centrifuged to separate them from the C-terminal small fragments and subtilisin, and over 80% of the original microtubule protein was recovered. After 1 h of digestion, the average length of the treated microtubules was about one-half that of the original. The control microtubules had an average length of 9.5 μm (n = 244), whereas subtilisin-digested microtubules were ~5.5 μm long on average (n = 476). No further decrease in size was observed, after the digestion was terminated. Previous studies showed that subtilisin-treated tubulin polymerized at a critical concentration 50-fold lower than that of intact tubulin (Serrano et al., 1984b; Sackett et al., 1985). Therefore, it is unlikely that the shortening was due to increased critical concentration for assembly. Because previous analysis of subtilisin-digested microtubules revealed that some of the tubes reorganized into protofilament bundles and sheets (Serrano et al., 1984b; Bhattacharyya et al., 1985; White et al., 1987), we looked for evidence of altered microtubules in the video images. Changes in microtubule organization or aggregation of microtubules should appear as differences in the contrast of the microtubules, as viewed by video-enhanced DIC microscopy. When we checked the contrast of microtubules at the same angle relative to the DIC shear axis, we found no differences in contrast between the normal and subtilisin-digested microtubules.

Inhibition of motility of motor proteins

We scored the number of moving beads as a function of cytoplasmic dynein concentration (see Materials and Methods). In parallel, the number of moving and stationary beads was also scored. In the presence of 1 mM ATP, the number of diffusing beads increased linearly with cytoplasmic dynein concentration on the beads (Fig. 2 *a*), as did the number of moving beads (Fig. 2 *b*) and the number of stationary beads (Fig. 2 *c*). The proportions of moving, diffusing, and stationary beads remained constant. The results indicated that a component(s) in the cytoplasmic dynein fraction was mediating bead diffusion on microtubules. In separate studies we have removed dynactin from the dynein fraction by antibody absorption and there was no change in the dynein motility. The linear dependence of motile activity of the beads implies that the motility is not dependent upon cooperativity in motor binding, i.e., single motor molecules appear to initiate these motile activities.

On subtilisin-digested microtubules, the three different types of bead-microtubule interactions, directed movement, diffusion, and stationary binding, were found to be decreased. At both low and high ratios of motor molecules to beads, the number of beads bound to subtilisin-digested microtubules was lower than the number bound to control microtubules (data shown for a high ratio of motors to beads in Fig. 3, *a* and *b*). Overall the binding of actively moving kinesin and cytoplasmic dynein-coated beads to microtubules decreased by approximately threefold. Actively moving beads were in equilibrium with beads in the solution phase, inasmuch as they continually bound, moved, and released from the microtubules. Because the cycle of binding, movement, and release was completed normally in the observation period, the threefold decrease in actively moving beads represents a threefold decrease in the on rate of motor binding to the microtubule as a result of subtilisin digestion, and this is at a concentration of motor where binding is linearly dependent upon motor concentration on the bead.

For the stationary beads, there was also a decrease in cytoplasmic dynein-bead binding to ~33% of control levels. With kinesin, the level of stationary binding did not change significantly. It is difficult to interpret the stationary binding data because the basis of the interaction is only poorly understood. Normal microtubule binding is nucleotide dependent, and this nucleotide-independent binding may be through other portions of the molecule or through denatured proteins on the bead surface.

The most dramatic change in bead binding was a 20-fold decrease in the number of randomly diffusing dynein beads. The random movement of the beads characterized the diffusive binding. By definition, diffusing beads moved more than 0.5 μm, with many reversals of direction. No diffusing kinesin beads were observed, which was consistent with

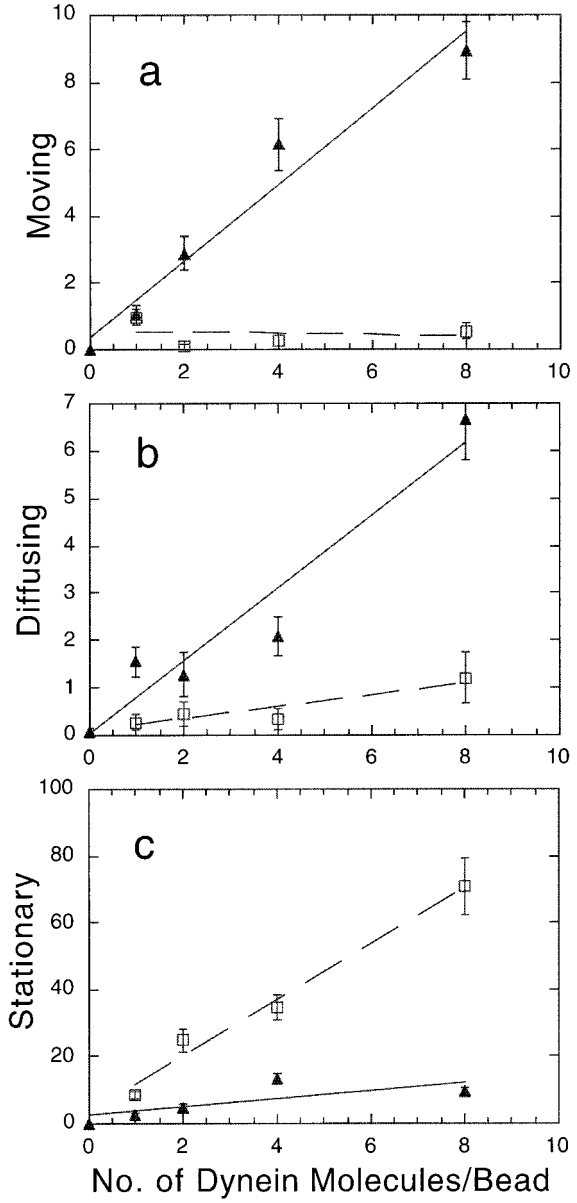

FIGURE 2 Effect of dynein concentration on bead motility. Bead motility assays were performed in the presence of 1 mM ATP (▲) or 5 mM AMP-PNP (□). The numbers of moving (*a*), diffusing (*b*), or stationary (*c*) beads were scored at different motor-to-bead ratios. Each data point was the average from 10 fields. In the presence of ATP, the number of moving (*a*), diffusing (*b*), and stationary (*c*) beads increased linearly as the molar ratio of cytoplasmic dynein to bead increased. The presence of AMP-PNP inhibited bead diffusion and movement but enhanced bead binding to the microtubules.

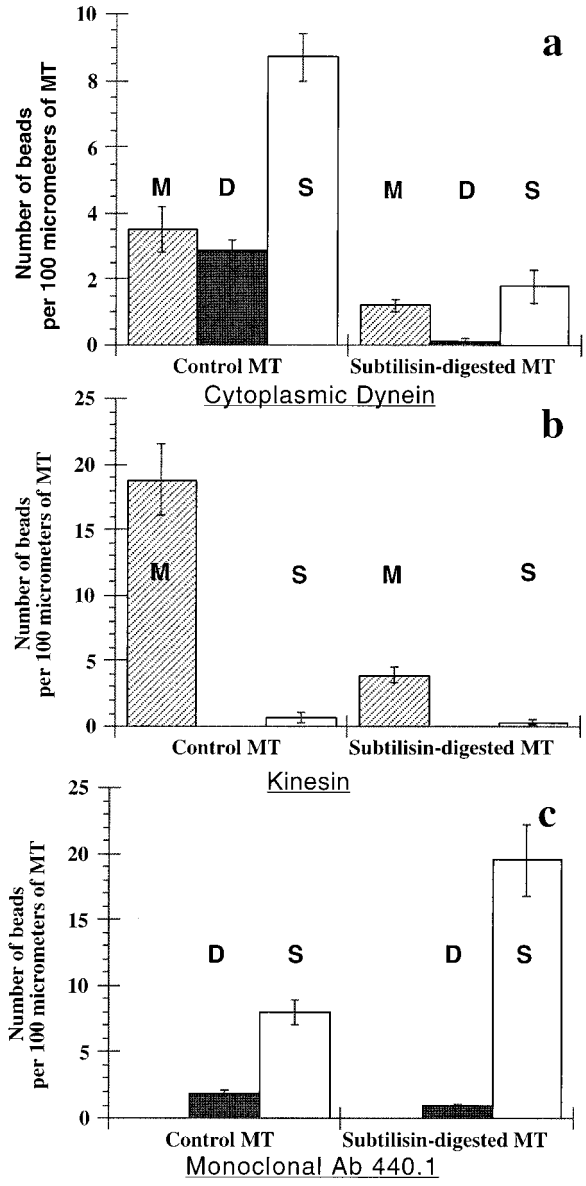

FIGURE 3 Decreased amount of binding of motor- and antibody-coated beads on subtilisin-digested microtubules. The numbers of moving (M), diffusing (D), and stationary (S) beads on control or subtilisin-digested microtubules are given for cytoplasmic dynein-, kinesin-, and antibody 440.1-coated beads in *a, b,* and *c,* respectively. The data were collected from six randomly chosen fields under each condition. Each field was observed for 1 min. The total microtubule length in each field was measured. The number of beads was calculated using 100 μm of microtubules as the unit length. The error bar shows the standard error of the mean of the data from six fields. The molar ratios of proteins to beads was ~60 for cytoplasmic dynein, ~100 for kinesin, and ~390 for antibody 440.1. The ATP concentration was 1 mM.

previous observations that the fraction of diffusing beads on microtubules was very low with kinesin-coated beads. Thus we find that removal of the anionic C-terminal of tubulin dramatically decreases cytoplasmic dynein diffusion and decreases active movement of both kinesin and cytoplasmic dynein.

Effect of salt on motility

To test the effect of changing charge-charge interactions on motility, we increased the salt concentration. As the concentration of potassium chloride in the buffer was increased,

there was a decrease in the total number of bound and moving kinesin-coated beads (Fig. 4b). In the presence of 150 mM KCl, the number of bound and moving cytoplasmic dynein-coated beads also decreased to one-third of control levels. Unlike in the case with subtilisin cleavage, however, the fraction of beads that diffused remained constant with increasing ionic strength. In the presence of 150 mM KCl, we found that 20% of the dynein-coated beads diffused, even though the total number of beads binding to the microtubules had been decreased to 30% of the control level.

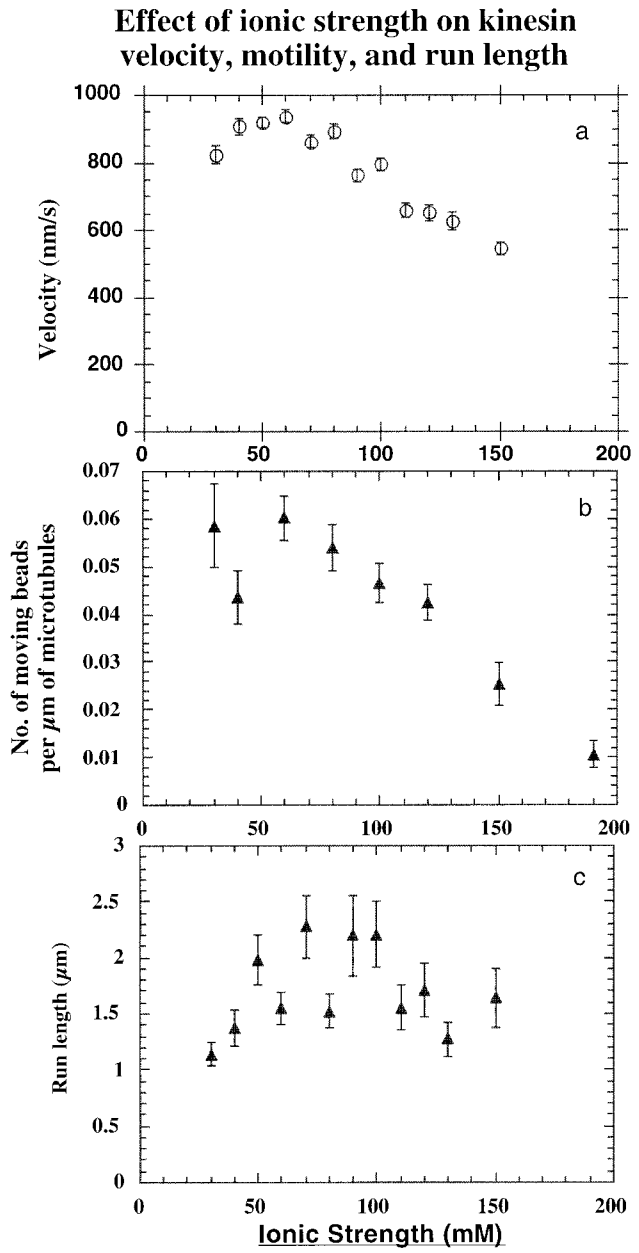

FIGURE 4 Effect of ionic strength on the number of moving kinesin beads (*a*), the average run length (*b*), and average velocity (*c*). Beads were coated with a low concentration of kinesin (~4 kinesin/bead), and the level of motility was assayed on normal microtubules. The average run length was calculated from the tracks of all beads with a clear history that moved more than 0.2 μm along a microtubule. Velocity was calculated by measuring the distance traveled by beads in a continuous run and dividing by the time that the bead was moving.

Effect of subtilisin cleavage on antibody diffusion

From earlier studies (Wang and Sheetz, 1999) we determined that a monoclonal antibody against cytoplasmic dynein heavy chain, 440.1, would cause bead binding to and diffusion on microtubules in the absence of cytoplasmic dynein. Other monoclonal antibodies that we tested did not cause binding. Because the diffusion of the dynein beads was dramatically inhibited by the carboxy-terminal cleavage, a similar effect may occur with the antibody-coated particles. The number of diffusing 440.1-coated beads decreased from 1.8 ± 0.7 on control microtubules to 0.9 ± 0.6 on subtilisin-digested ones (Fig. 3 *c*). A *t*-test showed that the two numbers were different at a level of significance, $p = 0.01$. However, the number of stationary 440.1-coated beads increased from 8 ± 0.9 on control to 20 ± 2.8 per 100 μm of MT length on subtilisin-digested microtubules, indicating that cleavage increased the affinity of the antibody beads for microtubules. Thus there is not a nonspecific decrease in microtubule affinity of proteins that catalyze bead diffusion after the truncation of the C-terminal domain.

Run length of motor-coated beads

We next characterized the run length of the motor-coated beads that were actively moving on the microtubules. Run length reflects the probability that the bead detaches from the microtubule at every step of the movement. Dramatic decreases in bead run length were observed, even at high motor-to-bead ratios. To know the run length of a bead, the distance between the point of bead binding to and the point of release from the microtubule should be recorded. In the measurements of run length we have used the criterion that movements were over 0.2 μm in length (one bead diameter), but we do find many events that could represent shorter movements. Shorter displacements could represent bead rolling on the microtubule. In the case of cytoplasmic dynein, the short movements are not analyzable because active and diffusive bead movements are not easily distinguishable at such short distances. In long movements, the beads were described as having a "clear history" when both attachment and release points were observed. There were many moving beads without a clear history that moved into or out of the observation field from or to an adjacent field, making it impossible to know the binding or release positions, respectively. The measured distance of movement of beads with an "unclear history" was necessarily shorter than the run length.

For movement on subtilisin-digested microtubules, ~83% of the cytoplasmic dynein-coated beads that were observed to be moving had a clear history, and so had 85% of kinesin-coated beads. The average run length of cytoplasmic dynein-coated beads with a "clear history" was 1.1 μm (n = 44, Fig. 5 *b*). Kinesin-coated beads had a run length of 1.2 μm (n = 46, Fig. 6 *b*). The small fraction of beads with an "unclear history" resulted from bead binding to microtubules at the edge of the field of view.

For movement on control microtubules, only half of the moving beads (49% for cytoplasmic dynein-coated beads and 53% for kinesin-coated beads) showed a clear history. The average run length of beads with a clear history was 2.6 μm for cytoplasmic dynein (n = 31, Fig. 5 *a*) and 3.1 μm for kinesin (n = 30, Fig. 6 *a*). However, those values underestimated the true run length of the moving bead population. Some of the beads moved out of the field after moving more than 10 μm (the average length of a microtubule). Therefore, the low percentage of beads with a "clear history" was inevitable. The average displacement of beads with an "unclear history" on control microtubules was 5.6 μm for cytoplasmic dynein (n = 33) and 9.6 μm for kinesin (n = 20). For reasons discussed above, the actual decrease in run length should be higher than the two- to threefold decrease that was obtained from beads with a "clear history." For both motor proteins, the distribution of run lengths on subtilisin-digested microtubules (Figs. 5 *b* and 6 *b*) was more shifted toward the left than that of the control microtubules (Figs. 5 *a* and 6 *a*). If all movements are averaged, the decrease in run length on the subtilisin microtubules was approximately fourfold for cytoplasmic dynein-coated beads and fivefold for kinesin-coated beads.

The run lengths of both kinesin- and cytoplasmic dynein-coated beads on subtilisin microtubules were similar to the run lengths of single motors under these ionic conditions (Wang and Sheetz, 1995). It was therefore important to determine if the run length of single motors was decreased

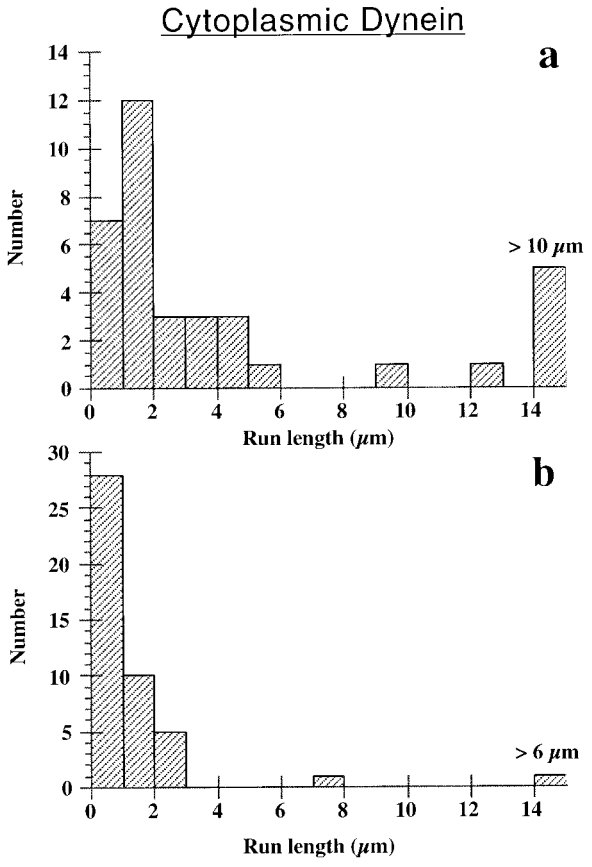

FIGURE 5 Decrease in run length of cytoplasmic dynein-coated beads moving on subtilisin-digested microtubules. The run lengths of cytoplasmic dynein-coated beads moving on control and C-terminal truncated microtubules with a "clear history" are given in *a* and *b*, respectively. Data were collected from experiments with two different motor preparations and pooled together. The cytoplasmic dynein-to-bead ratios were ~60, and the ATP concentration was 1 mM. The mean run length is 2.6 μm for movements on control microtubules (n = 31) and 1.1 μm for subtilisin-digested microtubules (n = 44).

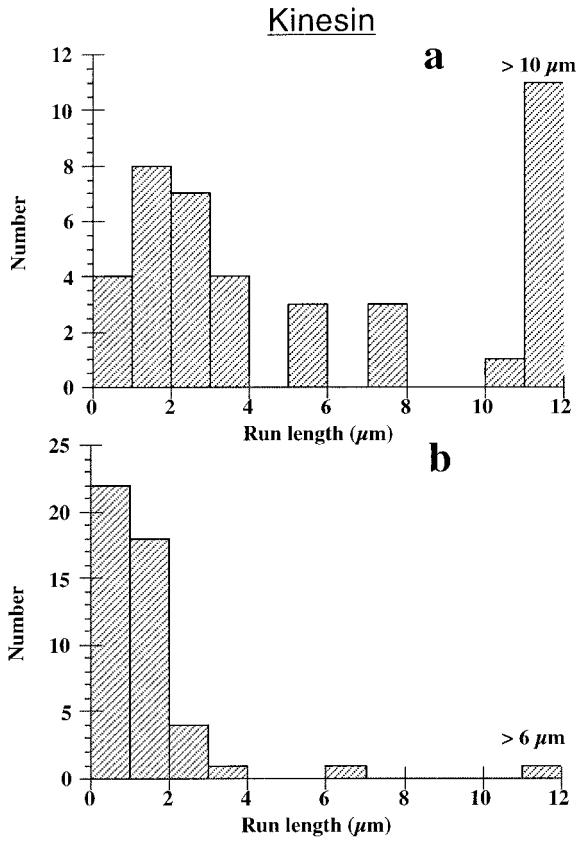

FIGURE 6 Decrease in run length of kinesin-coated beads moving on subtilisin-digested microtubules. The run lengths of kinesin-coated beads moving on control and C-terminal truncated microtubules with a "clear history" are given in *a* and *b*, respectively. The mean run length is 3.1 μm for movements on control microtubules (n = 30) and 1.2 for subtilisin-digested microtubules (n = 46). The kinesin-to-bead ratio was ~100, and the ATP concentration was 1 mM.

on subtilisin microtubules. The average run length of kinesin beads with a low ratio of kinesin to beads (less than four motors per bead) was decreased to 0.6 μm as compared to 1.6 μm for control microtubules. Thus the processivity of individual motors is decreased on subtilisin-digested microtubules compared with controls.

Effect of salt on run length

The decreased run length could result from a decreased charge-charge interaction between the subtilisin-digested microtubules and the motors. We therefore tested the effect of salt on the average run length of kinesin beads at low kinesin concentration and found no change with increasing ionic strength (Fig. 4 *c*).

Velocity of motor-coated beads

We also measured the effect that the C-terminal domain of tubulin has on the velocity of bead movement. Bead velocity was measured in two ways. One was to measure the distance moved and divide it by the elapsed time, as mentioned in the last section. To control for possible differences in the stationary periods, we also measured instantaneous velocities as a function of the period of measurement as described in Materials and Methods. We calculated the average instantaneous velocity for periods of 0.3–2 s (the time between the bead positions used to compute the velocity histogram) for both motors. The histogram of velocities showed a sharp peak for a period of 0.3 s but not for longer periods for both motors. This suggests that most frequently the velocity was only sustained for 300 ms. We did not find major differences in the velocity distributions on control versus subtilisin-digested microtubules. The weighted average velocity from the instantaneous histogram plots was the same as that determined from the beginning and ending points of a movement trace. Therefore, only the peak in the histogram was used to calculate the velocity of movement.

No change in velocity was found in cytoplasmic dynein-coated beads moving on subtilisin-digested and control microtubules (Fig. 7). The mean velocity was 422 nm/s for control microtubules (n = 46, Fig. 7 *a*) and 379 nm/s for subtilisin microtubules (n = 49, Fig. 7 *b*). There was a small but significant decrease in velocity for kinesin-coated beads when they moved on subtilisin-digested microtubules. The mean velocity decreased from 836 nm/s for control microtubules (n = 50, Fig. 8 *a*) to 647 nm/s for subtilisin-digested microtubules (n = 50, Fig. 8 *b*). Thus, although there are significant decreases in the amount of motility, the velocity of movement is altered only for kinesin and then by a small amount (23% decrease).

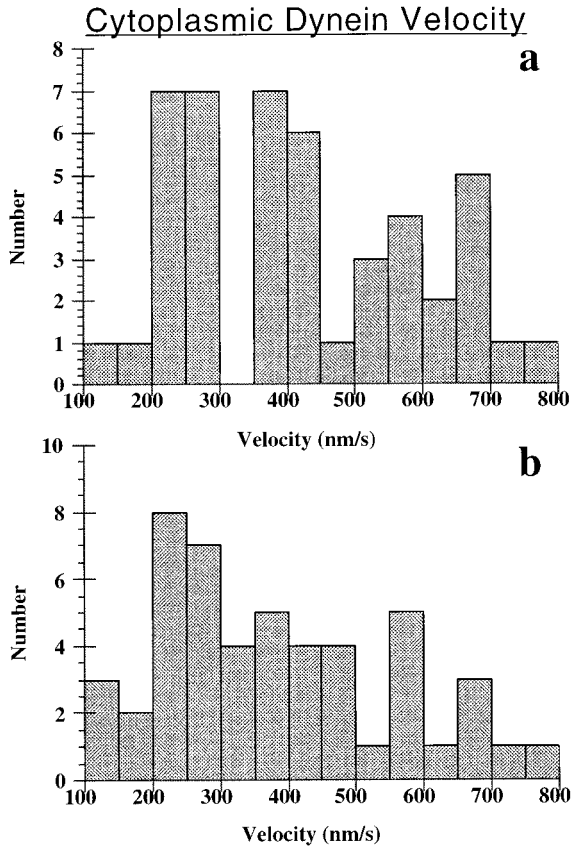

FIGURE 7 The velocity of cytoplasmic dynein-coated beads did not change when moving on subtilisin-digested microtubules. The velocities of cytoplasmic dynein-coated beads moving on control and C-terminal truncated microtubules are given in *a* and *b*, respectively. Data were collected under the same condition as in Fig. 3. The mean velocity was 422 nm/s for control microtubules (n = 46) and 379 nm/s for subtilisin-digested microtubules (n = 49).

DISCUSSION

Removal of the C-terminal charged domain from tubulin causes dramatic changes in the motility of both kinesin and cytoplasmic dynein. The changes in motility are related to changes in affinity of motors for the microtubules, but it appears that specific steps in the motility cycle are more affected than others. Characterization of the motility highlights the steps in the cycle in which motor interactions with the C-terminal tail might have an important influence on motor function. The loss of the C-terminus particularly affected processive movement, which points to a critical role for the C-terminus in maintaining contact.

Changes in size and charge of the cleaved tubulin were evident from SDS-PAGE and isoelectric focusing gel analyses. Because the tubulin was digested after it was assembled into microtubules, there was minimal loss of the polymer; although the digestion pattern by subtilisin was the same for tubulin assembled in polymer and free tubulin dimer (Serrano et al., 1984b; Sackett et al., 1985). The subtilisin-digested microtubules were shorter; but there were no apparent morphological or contrast changes in the digested microtubules as viewed by DIC microscopy. When the spacing of tubulin subunits in polymers assembled from

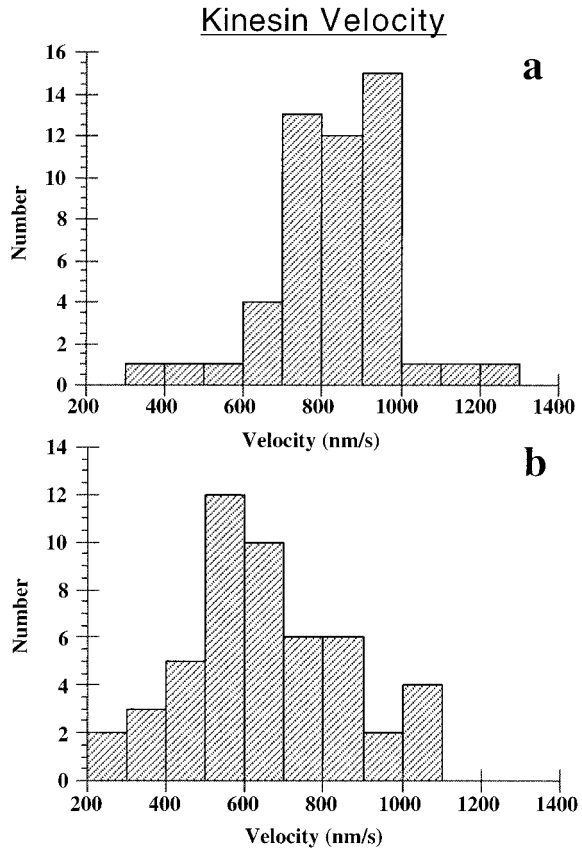

FIGURE 8 The velocity of kinesin-coated beads decreased when they moved on subtilisin-digested microtubules. The velocities of kinesin-coated beads moving on control and C-terminal truncated microtubules are given in *a* and *b*, respectively. Data were collected under the same conditions as in Fig. 4. The mean velocity was 836 nm/s for control microtubules ($n = 50$) and 647 nm/s for subtilisin-digested microtubules ($n = 50$).

subtilisin-digested tubulin was analyzed, it was the same as that of regular microtubules (Serrano et al., 1984b). Furthermore, motion analysis revealed no significant alteration in the pattern of on-axis or off-axis movement of beads on the digested microtubules. Thus we find no evidence that a gross structural change in the microtubule caused the changes in motility.

In interpreting the three- to fourfold decrease in the number of moving beads coated with cytoplasmic dynein or kinesin, it is extremely unlikely that the remaining 25–33% of the movements were on undigested microtubules or undigested tubulin subunits. All microtubules supported motility, which shows that a particularly motile subpopulation did not exist. On SDS-PAGE, no band was visible in the intact tubulin position after 1 h of digestion (less than 5%). With even 5% of normal tubulin, the motor would have to reach on average over 24 nm between intact tubulin subunits, which is beyond the reach of kinesin in particular. Therefore, subtilisin-digested tubulin can support motor movement. Because we count any bead movement over 0.5 μm as only one movement, the decrease in the number of movements represents a decrease in the on-rate of motor-microtubule interaction. Increasing the ionic strength also decreases the on-rate of the motor-microtubule interaction. Cleavage of the C-terminal domain of tubulin decreases the on-rate of bead binding to the microtubule in a manner similar to that of salt, which is consistent with a decrease in the charge-charge interactions between the motors and the microtubule inhibiting binding.

Previous studies have indicated that the C-terminal domain of tubulin is not the binding site for cytoplasmic dynein or kinesin but is involved in their movement. It has previously been reported that kinesin binds to subtilisin-digested microtubules (Hagiwara et al., 1994; Marya et al., 1994). Analysis of the activation of kinesin ATPase by subtilisin microtubules indicated that the affinity of the motor for subtilisin microtubules is fourfold lower, which agrees well with the three- to fourfold decrease in bead binding to subtilisin microtubules (Tucker and Goldstein, 1997). Similarly, the C-terminal region of tubulin was implicated in the activation of cytoplasmic dynein's ATPase activity, and a five- to sixfold decrease in the microtubule-activated ATPase activity of cytoplasmic dynein was found with subtilisin-digested microtubules (Paschal et al., 1989). The binding of stationary cytoplasmic dynein-coated beads to subtilisin-digested microtubules decreased as much as motility did. But diffusion of cytoplasmic dynein-coated beads was almost completely inhibited by the cleavage of the C-terminal domain. Therefore, the C-terminal domain may mediate a weak interaction between cytoplasmic dynein and microtubules. This weak binding site is distinct from the strong binding site that exists between cytoplasmic dynein and microtubules during movement or stationary binding. Because of the low number of events, the data for stationary kinesin-coated beads were less convincing. No diffusing beads were observed in the kinesin motility assay. A diffusive binding state was found with truncations of the kinesin-like protein, ncd, but not with kinesin alone (Chandra et al., 1993). The inhibition of the cytoplasmic dynein diffusional state by C-terminal cleavage is clearly distinct from the effect of added salt and implies that the weak interaction supporting diffusion is not particularly salt dependent.

A further difference between the effects of salt and C-terminal cleavage is with regard to run lengths. Run length reflects the dissociation probability of motors from microtubules, and the values obtained from bead and single molecule measurements agree remarkably well, in that a run length of $\sim$1.5 μm is observed for kinesin in all cases (Block et al., 1990; Vale et al., 1996). We found that the run lengths of both cytoplasmic dynein- and kinesin-coated beads decreased on subtilisin-digested microtubules. This indicated that the cleavage of the C-terminal domain of tubulin enhanced the dissociation of motor proteins from microtubules. A similar decrease in the run length of single

motor molecules was observed with alterations in the neck domain of kinesin (Romberg et al., 1998). It is possible that there is weak interaction between the C-terminal domain and motor proteins, particularly the neck region of kinesin. When a cationic portion of the neck is duplicated, the run length is doubled, further indicating that a local interaction involving anionic charges on the microtubule and cationic charges on the motor may be involved (Romberg et al., 1998). If those charge interactions involve groups that are spaced closer than the Debye length, then increased salt concentration may only have a secondary effect. A reasonable model is that the motor neck and the C-terminal region of tubulin interact closely and serve to hold the motor near the filament as it steps from one tubulin to the next.

The four- to five-fold change in run length is in sharp contrast to the small changes in velocity of kinesin and cytoplasmic dynein movement on subtilisin-digested microtubules. Marya et al. also found that subtilisin-digested microtubules were moved by kinesin at a rate comparable to that of control microtubules (1994). This suggests that the frequency of steps is not strongly related to the probability that the motor will fall off when taking a step. The small decrease in velocity on subtilisin microtubules can be explained by a weakening of the motor-microtubule interaction during the step such that the orientation of the motor to the microtubule is more random during movement. A similar weak interaction has been observed for myosin and actin (Van Dijk et al., 1998; Roopnarine and Thomas, 1996). The weak interaction could orient the head, making it easier to find the next strong binding site for the motor. When the weak interaction is inhibited, it would take the motor longer to find the next site, thus slowing down the movement. The weak interaction is not essential for motor movement, even though it greatly enhances the distance of movement.

The effect of subtilisin digestion on motor movement could be caused by the change in microtubule morphology. However, there was no discernible change in the polymer morphology, and the spacing between subunits in subtilisin-digested microtubules was the same as for normal microtubules (Serrano et al., 1984b). The movement of motors should be sensitive to tubulin arrangement in and between protofilaments, but no differences have been found for digested and control microtubules. The cleaved C-terminal small fragment came off of microtubules, as shown by gel filtration and cross-linking analysis (White et al., 1987; Serrano et al., 1984b). We think that the absence of the C-terminal domain results directly in the change in motor movement.

We feel that these findings are consistent with a hand-over-hand model for motor movement that was proposed from the early biophysical data on kinesin (Gelles et al., 1988; Howard et al., 1989; Block et al., 1990) and confirmed by subsequent analyses (Berliner et al., 1995; Hackney, 1995; Hancock and Howard, 1998; Tripet et al., 1997). The number of such hand-over-hand cycles that occur before a motor falls off the filament is going to be critically dependent upon the weakest step in the motility cycle. Interestingly, the run length was found to decrease by only 30% when the velocity of kinesin movement was decreased by nearly 100-fold at lower ATP concentrations (Lopez and Sheetz, unpublished results). Thus processivity is a function of the number of steps and not the time needed for each step. Although processivity is insensitive to salt, it is dramatically affected by the removal of the C-terminus of tubulin. One explanation for this behavior is that in the motility cycle the highest probability for motor release occurs during a period when the strongest interactions between the motor and filament depend upon a weak interaction between the C-terminal tail of tubulin and the neck domain of kinesin. That interaction would not have to be sensitive to salt, e.g., if the C-terminus were closely opposed to the motor. In terms of the motility cycle, such a complex could stabilize the transition as the next head binds after the release of the last head. Although it may not be related, the dramatic decrease in the probability of cytoplasmic dynein diffusion along the microtubule with the C-terminal cleavage could reflect the change in a similar weakly bound state. Because both kinesin and cytoplasmic dynein behave similarly, we suggest that a similar weakly bound state is involved in the motilities of both motors on microtubules.

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