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AFM force measurements on microtubule-associated proteins: the projection domain exerts a long-range repulsive force

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Abstract Microtubule-associated proteins (MAPs) are thought to control spacing between microtubules. We propose that the projection domain is largely unstructured and exerts a long-range repulsive force that is predominantly entropic in origin, providing a physical mechanism for maintaining spacing. To test this hypothesis, we developed an experimental system where MAPs are electrostatically end-attached to a flat surface, such that the projection domains extend away from the surface. Atomic force microscopy force measurements on this system show that projection domains exert a long-range (>100 nm) repulsive force. This force depends on the ionic strength of the solution in a way that is consistent with a polyelectrolyte polymer brush. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Unstructured; Entropic exclusion; Microtubule-associated protein 2; Tau

1. Introduction

Microtubule-associated proteins (MAPs) are a class of proteins that bind to the surface of microtubules in a nucleotide-independent manner. These proteins are known to stabilize microtubules against depolymerization [1]. There is also evidence to suggest that MAPs play a role in maintaining spacing between adjacent microtubules [2,3]. However, the mechanism by which this spacing is created and maintained is not well understood.

There are two major classes of MAPs, type I and type II [4]. Here we are primarily concerned with type II MAPs which include MAP2, tau and MAP4. These MAPs are composed of two distinct domains: a C-terminal microtubule-binding domain and an N-terminal projection domain. The microtubule-binding domain is approximately 400 amino acids in length and is conserved among the type II MAPs. This domain is highly positively charged and electrostatically binds to the negatively charged surface of microtubules. The binding of MAPs is regulated by phosphorylation where phosphorylation causes MAPs to dissociate from the microtubule [5]. The projection domain is not as well conserved as the microtubule-binding domain, and varies in length, from $\sim$300 amino acids in tau to $\sim$1400 amino acids in the high molecular weight isoforms of MAP2. This domain is highly negatively charged; for instance, in MAP2b there are 287 acidic (D and E) and 138 basic (K and R) amino acids [6]. Phosphorylation of the projection domain further increases the amount of negative charge on this part of the molecule [7].

MAPs bound to microtubules produce a structure that resembles a bottle brush where the projection domains extend away from the surface of the microtubule [2,8]. When microtubules with MAPs are sedimented, they form a gel where microtubules are widely spaced [9]. For example, the center to center distance between microtubules with MAP2 bound is $\sim$89 nm, while in the absence of MAPs, the distance is 44 nm [2]. The distance between microtubules is related to the size of the projection domain [10], but the mechanism by which the projection domain maintains this spacing is not known. One hypothesis is that the projection domain acts as a cross-bridge, essentially forming a strut between adjacent microtubules [3,11,12]. Alternatively, Mandelkow and colleagues describe MAPs as acting as 'soft' spacers, based on small angle X-ray scattering of MAP-microtubule gels [13]. A biophysical mechanism for such spacing activity has been proposed [14]. Here the projection domain is highly unstructured, in rapid Brownian motion, and produces a repulsive force that is primarily entropic in origin. This behavior is similar to that of unstructured, non-biological polymers that are widely used to stabilize colloidal suspensions [15]. Thus MAPs bound to the surface of a microtubule can be viewed as a polymer brush, and can maintain the distance between microtubules by entropic repulsion. This is similar to a proposed mechanism by which neurofilament side-arms maintain spacing between neurofilaments [16].

Consistent with this model, there are several lines of evidence that suggest that the MAP projection domain is unstructured. In ^{1}H [17] and ^{13}C [18] nuclear magnetic resonance studies of MAPs, the spectra have narrow resonance peaks that are typical of highly mobile and flexible molecules. Extensive biophysical characterization of MAP2, including circular dichroism, fluorescence spectroscopy and sedimentation equilibrium, has shown the protein to be mostly disordered [19]. In addition, recent analyses of sequences of intrinsically unstructured polypeptides have revealed several features that are characteristic of this class of polypeptides. In particular, unstructured polypeptides tend to be low complexity (i.e. contain a small number of different amino acids) with a high fraction of polar amino acids [20–22]. The projection domain

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Abbreviations: AFM, atomic force microscopy; APTES, 3-aminopropyl-triethoxy-silane; BSA, bovine serum albumin; IgG, immunoglobulin G; MAP, microtubule-associated protein; PIPES, 1,4-piperazine-diethane-sulfonic acid

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of MAPs has a very high content of polar amino acids and the sequence is low complexity [23].

To test the hypothesis that the projection domain of MAPs is unstructured and forms a polymer brush on the surface of microtubules, we have developed a system to study forces produced by the projection domains of MAPs. This system exploits the positive charge on the microtubule-binding domains to electrostatically end-attach MAPs to a negatively charged substrate (mica). With the microtubule-binding domains attached to the mica, the projection domains extend away from the mica surface and, at sufficient density, form a polymer brush. Forces exerted by the projection domains can then be studied by direct atomic force microscopy (AFM) measurements (e.g. [16,24,25]). Our measurements demonstrate the presence of a long-range repulsive force that is consistent with a polyelectrolyte brush.

2. Materials and methods

2.1. Protein adsorption to mica

A whole MAP fraction from adult bovine brain (Cytoskeleton Inc., Denver, CO, USA), composed of 70% MAP2a/b and 30% other MAPs, including tau, was used. This preparation was restored from a lyophilized powder with water to a 1 mg/ml stock solution in 10 mM 1,4-piperazine-diethane-sulfonic acid (PIPES), 0.3 mM EGTA and 0.3% sucrose. 10 μl of the stock solution was added to 40 μl of 1 mM PIPES, pH 7.2 (Sigma Aldrich, St. Louis, MO, USA). The 50 μl aliquot was incubated on a freshly cleaved mica disk (Asheville-Schoonmaker, Newport News, VA, USA) for 30 min at room temperature. The mica was then thoroughly washed with 1 mM PIPES, pH 7.2. All solutions were prepared in deionized (>18 MΩ) and UV-treated water (Milli-Q UV, Millipore Corp., Bedford, MA, USA). Care was taken to keep the sample submerged in buffer during sample preparation and throughout the experiment.

Control proteins included bovine serum albumin (BSA; Sigma Aldrich), type II histone (Sigma Aldrich), skeletal muscle myosin (Sigma Aldrich) and an immunoglobulin G (IgG; Sigma Aldrich). A 1 mg/ml solution of each protein, in 1 mM PIPES, pH 7.2, was prepared. A 10 μl aliquot of the protein solution was added to 40 μl of 1 mM PIPES, pH 7.2. The 50 μl aliquot was incubated on freshly cleaved mica for 30 min, rinsed thoroughly and used in AFM force measurements.

3-Aminopropyl-triethoxy-silane (APTES)-modified mica surfaces were prepared essentially as previously described [26]. Briefly, 100 μl of neat APTES (Sigma Aldrich) was diluted into 10 ml of 1 mM acetic acid. The mica stubs were immersed in the APTES solution for 10 min, then thoroughly rinsed with distilled water and blown dry with compressed gas (Vari-Air, Peca Products, Janesville, WI, USA). These substrates were used in place of regular mica where indicated.

2.2. AFM

A Nanoscope III or IIIa controller equipped with a Multimode AFM, a J type scanner (*xy* scan range ~150 μm; *z* range ~6 μm) and conventional fluid cell (Digital Instruments, Santa Barbara, CA, USA) were used. Unsharpened 'D' type silicon nitride cantilevers (Microlevers; nominal end-radius ~50 nm; Thermomicroscopes, Sunnyvale, CA, USA) with a nominal force constant of 0.03 N/m were used. Cantilevers were cleaned for 20 min by exposure to high-intensity UV light (UVO-Cleaner, Jelight Co. Inc., Laguna Hills, CA, USA). Force curves were typically collected over 1–2 μm with 2048 data points per curve, at a rate of 0.1 Hz. Relative triggers of 0.3–3 nN were used to prevent the tip from gouging the surface. A closed fluid cell was used to allow for fluid exchange during an experiment. When exchanging solutions of different ionic strength, the fluid cell was rinsed with several ml (>20 times the fluid cell volume) of the new solution prior to a measurement.

2.3. Quantitation of MAPs bound to mica

To estimate the density of MAPs bound to the mica surface, known quantities of MAPs were adsorbed to mica and rinsed as described above. MAPs bound to mica in each sample were then solubilized in 33 μl Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 25% glycerol, 0.01% bromophenol blue, 20% β-mercaptoethanol). A 25 μl aliquot of solubilized MAPs from each sample was run on a 7.5% acrylamide gel, together with MAPs of known amounts. The gel was silver-stained (Bio-Rad) and densitometry analysis was performed using Fujifilm LAS-1000 and Fuji Image Gauge V3.3 imaging software (Fujifilm Medical Systems USA, Inc., Edison, NJ, USA).

3. Results and discussion

To test the hypothesis that the projection domain of MAPs behaves as an unstructured polymer, we developed a system for studying forces exerted by the projection domain (Fig. 1). Our approach was to end-attach MAPs to mica via the microtubule-binding domain. This domain is highly positively charged and should electrostatically bind to the negatively charged surface of mica, leaving the projection domain free to extend away from the surface. At a sufficient density of proteins, a polymer brush should form. The properties of this brush could then be examined by direct AFM force measurements.

Force measurements on the MAP system showed a long-range repulsive force (Fig. 2). This long-range repulsive force could be detected 100–150 nm away from the surface. Within a given preparation, forces measured at different points on the surface did not vary significantly. When small compressive forces were used (<0.3 nN), the retracting curve retraced

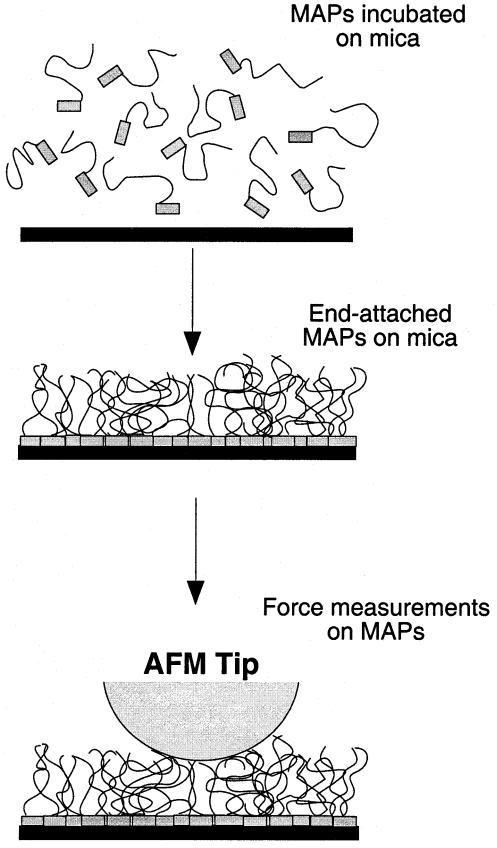

Fig. 1. Schematic of the experimental system of MAPs end-attached to mica. Positively charged microtubule-binding domains (rectangles) electrostatically interact with negatively charged mica (thick horizontal line). The MAPs self assemble into a polymer brush on the mica surface, with the projection domains extending away from the surface. The properties of the projection domain are investigated by direct AFM force measurements.

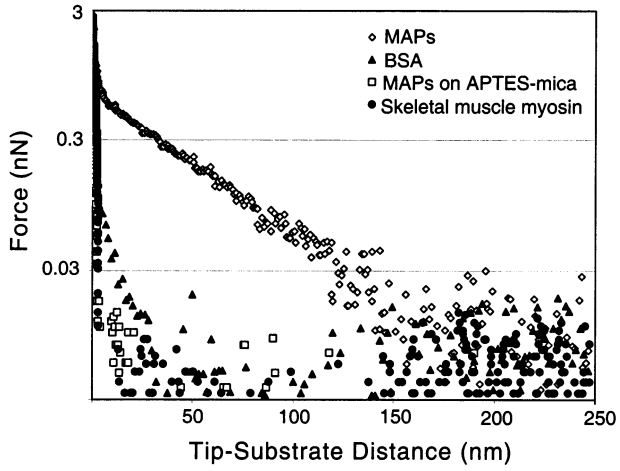

Fig. 2. Force measurement on the MAP polymer brush. A long-range, roughly exponential, repulsive force can be detected up to 150 nm away from the surface in the presence of MAPs. In each of the controls, BSA, type II histone, IgG and skeletal muscle myosin on mica, and MAPs bound to APTES mica, the long-range repulsive force is absent. For clarity, data for type II histone and IgG are not shown. In both those cases the measured forces were no greater than those for BSA.

the advancing curve exactly in $>90\%$ of the curves. In the remaining $<10\%$ a negative deflection away from the surface, typical of a trapped polymer, was observed. When high compressive forces were used, the approach curve overlapped the low force measurements but complex retracting curves were commonly observed. These retracting curves were highly irregular and suggested multiple interactions between molecules on the surface and the tip. Approach curves were reproducible for hundreds of measurements, suggesting that protein was not accumulating on the tip with time. These measurements are consistent with an entropic force originating from a polymer brush [16,24,25].

Several controls were performed to support this interpretation. First, different proteins that are thought to be well folded were adsorbed to mica and the forces measured. BSA and type II histone are known to be folded from X-ray crystallography [27,28]. Type II histone is positively charged and should bind electrostatically to mica, similar to the microtubule-binding domain. We note that a short stretch of type II histone has been proposed to be unstructured [20,21]; however the unstructured portion of the molecule is positively charged and should be immobilized on the mica. Other control molecules included skeletal muscle myosin and IgG. None of the control proteins exhibited the type of long-range repulsion seen for MAPs (Fig. 2). Second, forces from MAPs incubated on APTES-modified mica were measured. APTES modification of mica creates a positively charged surface [26]. This surface should bind the negatively charged projection domain and prevent the formation of a polymer brush. In agreement with that prediction, the APTES-bound MAPs did not produce a long-range force (Fig. 2).

The amount of MAPs bound to the surface was estimated by removing the MAPs from the surface and quantitating them by gel electrophoresis and densitometry. Samples, prepared identically to those used in the force measurements, had a MAP concentration on the mica surface of approximately 100 ng/cm^2. This corresponds to a monolayer of MAPs with an average intermolecular distance of 20 nm. For an ideal polymer of the length of the MAP2 projection domain ($\sim$500 nm) in a theta solvent, the radius of gyration is $\sim$22 nm. This is larger for a polymer in a good solvent, as would be the case for a polyelectrolyte in water. Thus the measured grafting density is well within the brush limit and is consistent with the picture presented in Fig. 1. In addition, the amount of protein bound to the mica saturates, with an increasing amount of protein used in the initial incubation, near 100 ng/cm^2, indicating that the protein does not form multilayers (data not shown).

Given the large net negative charge of the projection domain, it might be expected to behave as a polyelectrolyte. One characteristic property of polyelectrolyte polymer brushes is that the degree to which they are expanded is sensitive to the ionic strength of an aqueous medium [29]. Such sensitivity to ionic strength depends on ionic screening of intramolecular interactions. This prediction was supported by force measurements on the MAP polymer brush in solutions of different ionic strength (NaCl concentration), which show that as ionic strength increases, the measured force decreases (Fig. 3). The ionic strength sensitivity also demonstrates that the forces measured are not simply an electrostatic force between the layer of protein at the surface and the AFM tip. The decay length of the force measured in 100 mM NaCl is $\sim$7 nm. This is a factor of seven larger than would be expected for a purely electrostatic interaction (e.g. [30,31]). It should be noted that changes in intramolecular interactions in a polyelectrolyte could also be achieved by modifying the intrinsic charge, which can be accomplished in a biological context by phosphorylation and dephosphorylation (discussed below).

One concern regarding the ionic strength sensitivity is that the decrease in force could result from loss of MAPs from the surface, due to ionic effects on the interaction of the microtubule-binding domain with the mica. Indeed, high ionic strength solutions are used to disrupt the interaction of the microtubule-binding domain with negatively charged chromatography media (phosphocellulose) during MAP purification [32,33]. To ensure that the change in force was not due to a loss in MAPs from the surface, we tested the reversibility of the ionic strength effect. Following an experiment where ionic strength had been increased from the 1 mM PIPES to 1 mM

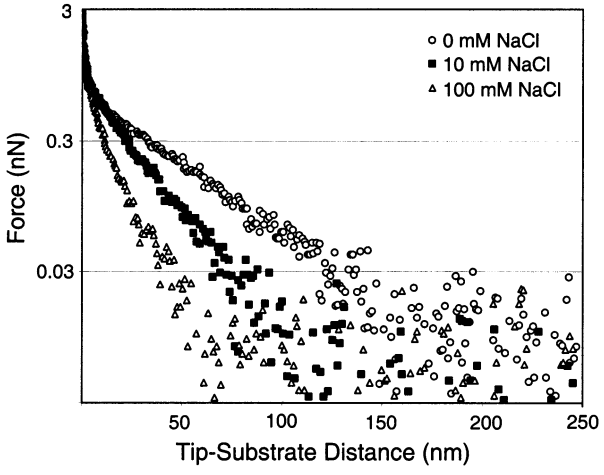

Fig. 3. Force measurement on the MAP polymer brush as a function of ionic strength. The long-range repulsive force decreases with increasing ionic strength. This sensitivity to ionic strength is consistent with a polyelectrolyte polymer brush.

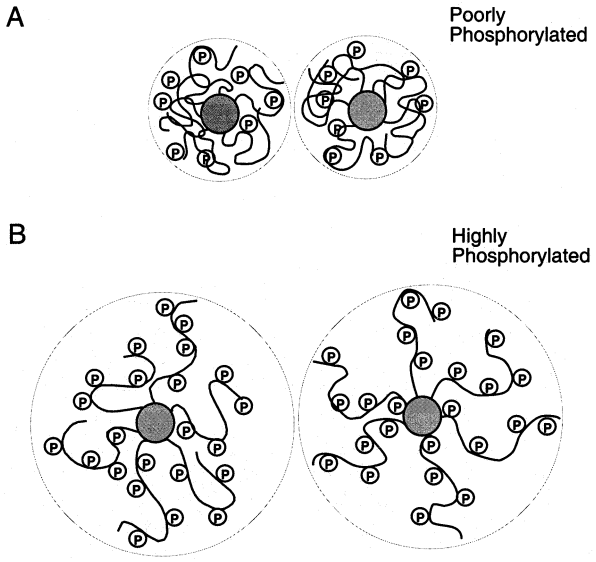

Fig. 4. Proposed regulation of MAP polymer brush by phosphorylation and dephosphorylation. A: Dephosphorylation reduces the intramolecular repulsion, causing the projection domains to become more compact and allows the microtubules to move closer. B: Phosphorylation of the projection domains causes the projection domains to expand due to an increase in intramolecular repulsion. This in turn causes the distance between adjacent microtubules to increase.

PIPES with 100 mM NaCl, the ionic strength was returned to 1 mM PIPES. This fully restored the original force, indicating that there was no detectable loss of MAPs from the surface (data not shown).

The focus of the work presented here is to provide evidence that the projection domains of MAPs behave as unstructured polymers, and can give rise to a long-range repulsive force that is predominantly entropic in origin. By predominantly entropic we mean that the protein is mostly unfolded, and that the force arises from restrictions to the space available for the protein to explore. This is not to exclude intramolecular interactions, such as electrostatic interactions, that are particularly critical in polyelectrolytes. Furthermore, it is not to say that some parts of the projection domain might not have some structure, but overall, the projection domain is sufficiently unstructured to give rise to the measured forces. The data presented clearly support this hypothesis, and provide a biophysical mechanism for MAPs maintaining spacing between microtubules. In this role, the polymer brush model for MAPs on microtubules advanced here suggests that MAPs should be highly compressible (compared to a folded protein). This is in agreement with the finding by Mandelkow and colleagues, who showed that MAPs behave as compressible spacers between microtubules [13]. However, the results presented here do not strictly exclude MAPs cross-linking microtubules. The free end of the projection domain could in principle bind to an adjacent microtubule, leaving the remainder of the projection domain unstructured and free to move. The results also do not exclude the binding of other molecules to the MAP projection domain, as has been proposed (e.g. [34]).

The polymer brush model for MAPs suggests a general mechanism for regulation of projection domain function and a specific mechanism for the effect of phosphorylation on microtubule packing (Fig. 4). Small angle X-ray scattering has been used to show that phosphorylation of MAPs increases the distance between microtubules in MAP-microtubule gels [13]. Increasing the charge of a polyelectrolyte in a good solvent produces an increase in the radius of gyration through increased intramolecular repulsion [29]. Therefore, in the model presented here, phosphorylation of MAPs would increase the intramolecular repulsion and cause the MAP polymer brush to expand.

Beyond providing a mechanism for maintaining spacing between microtubules, the question of what the biological significance of the long-range force reported here might be arises. To begin with, the MAPs may contribute to cellular mechanics in a way identical to that proposed for neurofilaments [16]. The unstructured projection domain could act as an entropic spring between microtubules, or between microtubules and other cytoskeletal components (e.g. neurofilaments). This spring would resist mechanical compression and could serve to maintain cell shape. The unstructured projection domain would also occupy a very large volume, compared to folded protein of the same length, and tend to exclude other macromolecules in a size-dependent manner (the larger the molecule the more it would be excluded). This in turn could lead to a significant increase in the effective concentration of excluded molecules and thereby influence their intracellular biochemical activity. It should be noted that the presence of the polymer brush does not exclude the binding of other proteins to the surface of microtubules. For example, MAPs have been proposed to regulate motor protein interactions with microtubules [35]. Interestingly, Mandelkow and colleagues [36] have shown that tau affects the attachment and detachment of motor proteins but not the transport speed. Finally, unstructured polymers grafted to the surface of colloidal-sized particles are commonly used to prevent aggregation [15]. Microtubules themselves are highly soluble, and hence it would not appear that MAPs are involved in maintaining their solubility. However, it is interesting to note that some MAPs, in particular tau, are known to aggregate under pathological conditions [37,38]. Such aggregation might be expected if the polymer brush behavior of MAPs described here failed.

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