Mapping the Microtubule Binding Regions of Calponin†

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ABSTRACT: The smooth muscle basic calponin interacts with F-actin and inhibits the actomyosin ATPase in a calmodulin or phosphorylation modulated manner. It also binds in vitro to microtubules and its acidic isoform, present in nonmuscle cells, and co-localizes with microfilaments and microtubules in cultured neurons. To assess the physiological significance and the molecular basis of the calponin−microtubule interaction, we have first studied the solution binding of recombinant acidic calponin to microtubules using quantitative cosedimentation analyses. We have also characterized, for the first time, the ability of both calponin isoforms to induce the inhibition of the microtubule-stimulated ATPase activity of the cytoskeletal, kinesin-related nonclaret disjunctional motor protein (ncd) and the abolition of this effect by calcium calmodulin. This property makes calponin a potent inhibitor of all filament-activated motor ATPases and, therefore, a potential regulatory factor of many motor-based biological events. By combining the enzymatic measurements of the ncd-microtubules system with various in vitro binding assays employing proteolytic, recombinant and synthetic fragments of basic calponin, we further unambiguously identified the interaction of microtubules at two distinct calponin sites. One is inhibitory and resides in the segment 145−182, which also binds F-actin and calmodulin. The other one is noninhibitory, specific for microtubules, and is located on the COOH-terminal repeat-containing region 183−292. Finally, quantitative fluorescence studies of the binding of basic calponin to the skeletal pyrenyl F-actin in the presence of microtubules did not reveal a noticeable competition between the two sets of filaments for calponin. This result implies that calponin undergoes a concomitant binding to both F-actin and microtubules by interaction at the former site with actin and at the second site with microtubules. Thus, in the living cells, calponin could potentially behave as a cross-linking protein between the two major cytoskeletal filaments.

Calponins are F-actin- and calcium calmodulin-binding proteins thought to be involved in a variety of biological processes including the regulation of smooth muscle contraction by inhibiting the actomyosin ATPase activity (*1*, *2*), the organization of the actin cytoskeleton in smooth muscle and nonmuscle cells by stabilizing the actin networks (*3*−*5*), and cell signaling at the surface membrane of vascular smooth muscle (*6*, *7*). There are three well-known genetic calponin variants in mammalian tissues. The basic and neutral calponin isoforms, designated h1 and h2, respectively, are specifically expressed in smooth muscle cells (*8*, *9*), whereas the third isoform, termed acidic calponin h3, is the only calponin present in both smooth muscle and nonmuscle cells (*10*). All isoforms of calponin are single polypeptide chain proteins which exhibit an NH_2-terminal conserved region, encompassing residues 32−127 (numbering of the chicken gizzard calponin h1 sequence (*11*)) and called the calponin homology domain. When present in multiple copies, this module has been implicated in actin binding of several cytoskeletal proteins. But in the case of calponin, it does not express any actin-binding ability (*12*, *13*). Previous structural studies from this and other laboratories using proteolytic, synthetic, and recombinant fragments of basic calponin have led to the identification of the central region spanning residues 145−182 as the primary actin-binding and inhibitory segment of calponin as well as the regulatory domain harboring the subsites involved in the recognition of calmodulin and other calcium binding proteins, the interaction of which dissociates calponin from F-actin (*12*−*16*). In each calponin, this functionally crucial stretch is followed by three 30-residue tandem repeats extending between amino acids 172 and 273 with the NH_2-terminal part of the first repeat (residues 172−187) forming a secondary, noninhibitory actin-binding site (*13*, *17*) and includes serine/threonine residues whose enzymatic phosphorylation induces the dissociation of the calponin−F-actin complex (*18*, *19*). The three calponin forms contain a COOH-terminal tail of variable length and primary structure whose function would be to exert an isoform specific influence on calponin binding to actin (*20*).

More recently, the basic calponin was shown to also interact in vitro with microtubules in a calcium calmodulin-modulated manner (*21*, *22*). The binding of microtubules to calponin involved the acidic COOH-terminal regions of the α-and *β*-tubulin subunits as well as other nonidentified tubulin segments (*22*). The physiological relevance of this association between the basic calponin and microtubules

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could not conclusively be appreciated, as it was not known whether acidic calponin, the most represented isoform in nonmuscle cells and in particular in brain (*23*), also has the microtubule-binding competence. However, a subcellular colocalization of this isoform and α-tubulin has been observed in cultured neuronal cells (*24*) and in rat hippocampus (*25*), suggesting a possible in vivo interaction of calponin with microtubules. A dual interaction of calponin with the two major F-actin- and microtubule-based cytoskeletal elements would be of particular interest, as it could confer to calponin the property to communicate between the microfilament and microtubule networks and, consequently, to contribute to the coordination and regulation of their functioning in nonmuscle cells.

To gain more insight into the microtubule−calponin interactions, we have identified, in the present investigation, the direct solution binding of microtubules to recombinant acidic calponin, using cosedimentation analyses. We have also characterized, for the first time, the property of both the basic and the acidic calponins to promote the inhibition of the microtubule-stimulated ATPase activity of the cytoskeletal, kinesin-like motor protein, nonclaret disjunctional (ncd),[1] in a calcium calmodulin-regulated manner. This feature makes calponin a general inhibitory factor of the filament-activated motor ATPases. Furthermore, enzymatic measurements combined with in vitro binding assays, employing proteolytic, synthetic, and recombinant calponin peptides, revealed that the binding of microtubules takes place at two distinct calponin sites. One is inhibitory and resides in the critical actin-and calmodulin-binding domain of residues 145−182, and the other is noninhibitory and lies in the COOH-terminal segment of residues 183−292. Finally, quantitative spectrofluorometric analyses of the binding of basic calponin to pyrenyl F-actin in the presence of microtubules did not indicate any competition between the two classes of filaments which would be able to interact concomitantly at different sites on calponin; this finding suggests that calponin may also potentially provide a structural link between the actin and microtubule cytoskeletons.

EXPERIMENTAL PROCEDURES

Materials. Taxol, GTP, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS), and rat brain protein kinase C were purchased from Sigma. *N*-(1-Pyrenyl)-iodoacetamide was obtained from Interchim. Chymotrypsin was supplied by Worthington.

Proteins and Peptides. F-Actin from rabbit skeletal muscle was prepared by the procedure described in ref *12*. Basic calponin was isolated from fresch turkey gizzards as reported earlier (*12*). Recombinant rat acidic calponin was a generous gift from Mario Gimona (Salzburg, Austria) and was expressed as described (*20*). Bovine brain calmodulin was purified as described (*26*). Standard pig brain tubulin was prepared by cycles of polymerization−depolymerization, followed by phosphocellulose chromatography (*27*). Ultrapure tubulin (free of traces of MAPS), used for quantitative binding experiments, was obtained according to ref *28*. The tubulin was equilibrated by overnight dialysis in the cold against either of the three buffers employed in the present work, and the three final tubulin solutions were subjected to polymerization initiated at 37 °C by 1 mM GTP and 5 mM $MgCl_2$ in the presence of 20 μM taxol. The stock microtubule preparations were stored frozen in aliquots of 10 mg/mL at − 80 °C. The construct nonclaret disjunction motor domain of residues 335−700 (ncd 335) was prepared as described in ref *29*. Basic calponin was labeled at Cys 273 with 1,5-IAEDANS as reported (*30*), and the fluorescent protein was purified by gel filtration over a PD10 column in 2 mM $MgCl_2$, 30 mM KCl, 25 mM Hepes (pH 7.0). The labeling ratio, estimated spectrophotometrically at 340 nm, using a molar extinction coefficent of 6.2 mM^{-1} cm^{-1}, was 0.70 mol of dye/mol of calponin. F-Actin was conjugated to pyrene idoacetamide as described (*31*), and the protein labeled at Cys 374 was isolated by ultracentrifugation at 150 000*g* for 30 min. The labeling stoichiometry, measured spectrophotometrically at 339 nm, using a molar extinction coefficient of 26 mM^{-1} cm^{-1}, was 0.60 mol of dye/mol of actin. All labeled protein samples were used within 3 days after centrifugation before each experiment at 12 000*g* for 15 min. Phosphorylated basic calponin was obtained following the procedure described in ref *18*, using protein kinase C in 20 mM imidazole−HCl, 100 mM KCl, 0.5 mM EGTA, 2 mM $MgCl_2$, 2 mM dithiotreitol, 1 mM ATP (pH 7.2), in the presence of 50 μg/mL phosphatidylserine and 1.5 mM $CaCl_2$. The proteolytic NH_2-terminal 22 kDa and COOH-terminal 13 kDa fragments of basic calponin (residues 7−182 and 183−292, respectively) were produced by digestion of the protein with chymotrypsin at a protease to substrate weight ratio of 1:1000 followed by affinity chromatography of the digest over immobilized calmodulin under the conditions reported earlier (*12*, *14*). The short NH_2-terminal 13 kDa fragment (residues 7−144) was prepared similarly using a chymotryptic digest of calponin at a protease to substrate weight ratio of 1:100. The polypeptide corresponding to residues Ala 145−Tyr 182 of basic calponin was synthesized on a 9050 Milligen PepSynthesizer (Millipore) and purified by reverse-phase HPLC. Its identity and purity were confirmed by amino acid analysis, sequence determination, and electrospray ionization mass spectrometry (*14*). The recombinant fragment spanning the COOH-terminal segment of basic calponin between Arg 164 and Gln 292 was a generous gift from Mario Gimona and was obtained as described (*13*). Protein concentrations were determined spectrophotometrically at 280 nm with an extinction coefficient A1% of 11.7 cm^{-1} for actin, 7.6 cm^{-1} for basic calponin, 2.0 cm^{-1} for calmodulin (*12*, *14*), and 8.9 cm^{-1} for acidic calponin, as deduced from its amino acid composition (*10*). The concentration of tubulin was estimated by the Bradford assay (*32*, *33*) with bovine serum albumin as the standard. This assay is accurate and reproducible for the measurement of tubulin concentrations as assessed by control determinations we performed using automated amino acid analyses of 24 h 6N HCl hydrolyzates of tubulin. The Bradford procedure was also used for the determination of the concentration of modified calponin and of the fragments or synthetic peptides of calponin with the native protein as the standard.

Chymotryptic Digestions. Restricted proteolysis of basic calponin (1 mg/mL) in the absence or presence of micro-

[1] Abbreviations: CaP, calponin; Tub, tubulin; CaM, calmodulin; Ncd, nonclaret disjunction; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine.

tubules (molar ratio = 1:1) was performed at 25 °C in 10 mM imidazole−HCl, 30 mM NaCl, 2 mM $MgCl_2$, 4 mM NaN_3, 20 μM taxol (pH 7.0), using a protease to substrate weight ratio of 1:1000. At the desired time intervals (0−30 min), protein aliquots were mixed with an equal volume of boiling Laemli sample buffer and subjected to gel electrophoresis.

Cosedimentation Assays. Mixtures of microtubules corresponding to 4 μM polymerized tubulin and acidic calponin or phosphorylated basic calponin or the fragments of basic calponin (4 μM) were incubated in 10 mM imidazole−HCl, 30 mM NaCl, 2 mM Mg Cl_2, 20 μM taxol (pH 7.0) for 30 min at 25 °C. When present, calcium calmodulin was added at a 6-fold molar excess over calponin or the calponin fragments. After centrifugation of the protein mixtures in a Beckman Airfuge at 150 000*g*, for 30 min at 25 °C, the pellets were homogenized in the same buffer and submitted to gel electrophoresis.

Electrophoresis. SDS−polyacrylamide gel electrophoresis was carried out in 5−18% or 7.5−20% gradient acrylamide gels (*34*). The running buffer was 50 mM Tris, 100 mM boric acid (pH 8.0). The gels were stained with Coomassie blue R-250 and destained with 7% acetic acid.

ATPase Measurements. The microtubule-stimulated ATPase activity of ncd 335 (1 μM) was assayed at 25 °C in 25 mM Pipes, 40 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 20 μM taxol (pH 6.9), using a microtubule concentration corresponding to 10 μM polymerized tubulin. The reaction was initiated by adding 2 mM ATP and terminated after 15 min by 15% trichloroacetic acid. Pi was determined colorimetrically as described previously (*12*, *14*).

Fluorescence Analyses. All fluorescence emission spectra were recorded at 22 °C in solutions containing 2 mM $MgCl_2$, 30 mM KCl, 10 μM taxol, 25 mM Hepes (pH 7.0), using a LS 50 Perkin-Elmer Luminescence spectrometer. The fluorescence value was deduced from the area of the emission spectrum. Fluorescence polarization measurements (P) were performed with basic AEDANS−calponin (1.1 μM) plus increasing concentrations of ultrapure microtubules (0−3.0 μM) using an excitation wavelength of 340 nm and emission wavelength from 450 to 530 nm. The polarization of calponin alone ($P°$) was first determined. The polarization value at an infinite microtubule concentration ($P\infty$) was computed by autoregressive extrapolation (*35*) from which the relative polarization change, corresponding to the fraction of calponin bound to microtubules, was solved as $X = (P - P°)(P\infty - P°)$. The number of binding sites (n) and the affinity constant K_a were determined by two approaches (*36*−*38*), using the two following equations:

$$1/(1 - X) = K_a(C/(XE) - n) \quad (1)$$

$$\log(C/X) = f(\log C) \quad (2)$$

where C and E are total concentrations of microtubules and calponin, respectively. Plots of either $1/(1 - X)$ versus $C/(XE)$ (eq 1) or $\log(C/X)$ versus $\log C$ (eq 2) were drawn. The former plot gives the number of binding sites, which is the value of $C/(XE)$ for $1/(1 - X) = 0$. The slope of the same curve directly gives the value of the affinity constant. In the later plot, the intersection of the two tangents gives the value of $\log(nE)$ from which n is calculated. Fluorescence changes were recorded for the binding of increasing concentrations of basic calponin (0−3.6 μM) to pyrene labeled F-actin (1 μM) in the absence or presence of a concentration of ultrapure microtubules corresponding to 4.7 μM polymerized tubulin, using excitation wavelength at 365 nm and emission wavelength from 385 to 395 nm. The fluorescence of the preformed complex between pyrenyl F-actin (1 μM) and calponin (1 μM) was also measured in the presence of increasing concentrations of microtubules corresponding to 0−5.3 μM polymerized tubulin.

RESULTS

Calponin Inhibits the Microtubule-Activated ATPase of the Ncd Motor. To assess the physiological significance of the reported interaction between microtubules and the smooth muscle basic calponin, we first examined whether the cytoplasmic acidic calponin is also able to recognize in vitro microtubules. Recombinant rat acidic calponin and ultrapure brain microtubules (each protein at 4.0 μM) were mixed at pH 7.0, 22 °C, and their association was followed by high-speed centrifugation. The amount of calponin that sedimented together with the microtubules was estimated by densitometry following SDS−PAGE. As illustrated in Figure 1A,B, 95% ± 5% ($n = 3$) of the starting soluble calponin material (lanes 1) was found in the microtubule-containing pellet (lanes 3). The employed microtubules contained only traces of unpolymerized tubulin (lanes 2). The molar ratio of the calponin and microtubule materials which underwent cosedimentation was nearly 1:1. This finding suggests that the acidic calponin also binds to miocrotubules. In the next step, we tested its influence as well as that of the basic calponin isoform on the microtubule-stimulated ATPase activity of the cytoplasmic, kinesin-related, and minus-end directed motor, ncd. As shown in Figure 2, increasing concentrations of either calponin were added to ATPase assays, including 10 μM microtubules and the expressed monomeric motor domain, ncd 335 (1 μM), which represents residues 335−700 and whose interaction with microtubules or the tubulin heterodimer has been described earlier (*29*). The data presented in Figure 2 demonstrate the property of each calponin tested to decrease, in a concentration-dependent manner, the microtubule-activated ATPase of the motor to the same level corresponding to about 25% of the initial value of this enzymatic activity. In contrast, when the ATPase measurements were performed in the presence of calcium calmodulin, which was reported to dissociate the complex between microtubules and basic calponin (*21*), no change could be noticed in the microtubule-stimulated ncd 335 ATPase, using 3- or 4-fold molar excess of calponin over tubulin. These results strongly suggest that the observed ATPase inhibition was specific and was caused only by the attachment of the calponins to microtubules and not at all by any other secondary effect of these proteins on the motor active site. Also, in control experiments the low Mg-ATPase activity of ncd in the absence of microtubules was not affected by calponin (data not shown). The inhibitory region of the calponin molecule could be envisioned by analyzing, under the same experimental conditions, the microtubule-activated ncd 335 ATPase in the presence of either of the two separated chymotryptic fragments spanning residues 7−182 and 183−292, respectively, of basic calponin and accounting for almost its entire covalent structure (*12*). As shown in Figure 2,

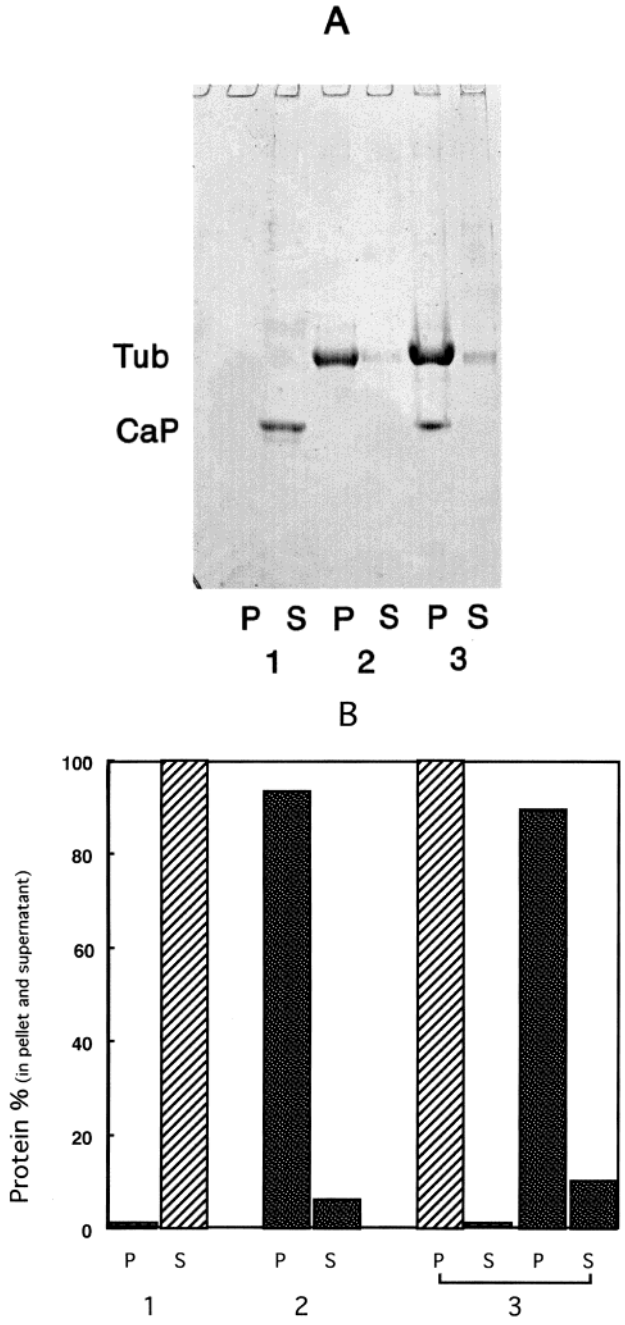

FIGURE 1: Interaction of acidic calponin and microtubules assessed by cosedimentation. (A) Expressed rat acidic calponin (4 μM) and ultrapure microtubules at a final concentration corresponding to 4 μM polymerized tubulin were incubated together for 30 min at 25 °C in 10 mM imidazole−HCl, 30 mM NaCl, 2 mM Mg Cl_2, and 20 μM taxol (pH 7.0) and then ultracentrifuged as indicated under Experimental Procedures. Aliquots of the supernatant (S) and solubilized pellet (P) were subjected to a 5−18% polyacrylamide gradient SDS−PAGE (lanes 3). The same analyses were performed for calponin (lanes 1) or microtubules (lanes 2) solutions centrifuged separately. (B) The amounts of calponin (hatched bars) or tubulin (dark bars) in each lane were quantified by densitometry. Note that for lanes 3, four measurements are shown corresponding to the amounts of calponin or tubulin in the pellet and to the amounts of either protein in the supernatant. Results are typical of those observed in three independent experiments. CaP = calponin. Tub = tubulin.

increasing concentrations of the former NH_2-terminal 22 kDa peptide elicited, like the parent protein, a progressive ATPase inactivation the degree of which was, however, slightly less pronounced than that observed with the intact protein. In

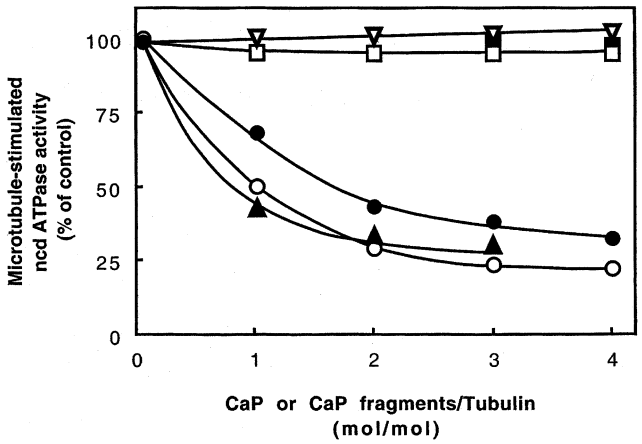

FIGURE 2: Inhibition of the microtubule-activated ncd ATPase by acidic calponin or basic calponin or its NH_2-terminal 22 kDa fragment. The microtubule-stimulated ATPase activity of ncd 335 was measured in the presence of 1−4-fold molar excess of acid calponin (▲) or basic calponin (○) or its NH_2-terminal 22 kDa fragment (residues 7−182) (●) over tubulin. ATPase assays were similarly performed in the presence of the shorter NH_2-terminal 13 kDa fragment (residues 7−144) (□) or the COOH-terminal 13 kDa fragment (residues 183−292) (▽) of basic calponin. ATPases were also estimated in the presence of basic calponin + 6-fold molar excess of calmodulin and 1 mM $CaCl_2$ (■). All ATPase measurements were conducted as specified under Experimental Procedures. 100% ATPase = 2.8 s^{-1}.

contrast, the COOH-terminal 13 kDa fragment did not induce any change in the motor ATPase. Remarkably, the shorter NH_2-terminal fragment encompassing amino acids 7−144 also did not affect the activation of the ncd 335 ATPase by microtubules. Together, the findings point to the stretch 145−182, as the calponin region required for the inhibition of the microtubule-activated ncd 335 ATPase and its direct binding to microtubules will be demonstrated below. Earlier, this 38-residue segment was shown to contain both the inhibitory actin binding and regulatory calmodulin recognition sites (*14*). In addition, it harbors Ser 175, the phosphorylation of which as well as that of the adjacent Thr 184 dissociates the calponin−actin complex and is thought to modulate in vivo the calponin−F-actin interactions (*18*, *19*). While the dissociation of the calponin−microtubule complex by calmodulin has been documented (*21*), the influence of the calponin phosphorylation on its binding to microtubules is unknown. To highlight this important point, we studied the interaction of microtubules with basic calponin phosphorylated with protein kinase C, using cosedimentation assays and gel electrophoresis. Most of the phosphorylated protein failed to sediment with microtubules (about 80% as estimated by densitometry) and remained in the supernatant after centrifugation (Figure 3, lane c and lane d), in contrast to the unphosphorylated protein which was essentially associated with microtubules in the pellet (Figure 3, lane a and lane b). Thus, the phosphorylation process decreases the affinity of calponin for both F-actin (*1*) and microtubules.

Microtubules Bind at Two Distinct Sites on Calponin. To characterize the calponin segments which directly recognize the microtubules, we first determined the changes in the chymotryptic susceptibility of basic calponin when it was complexed to microtubules. As depicted in Figure 4A, calponin used alone was specifically split by chymotrypsin (protease to substrate weight ratio = 1:1000) at the peptide bond Tyr 182−Gly 183, giving rise to its two constituting

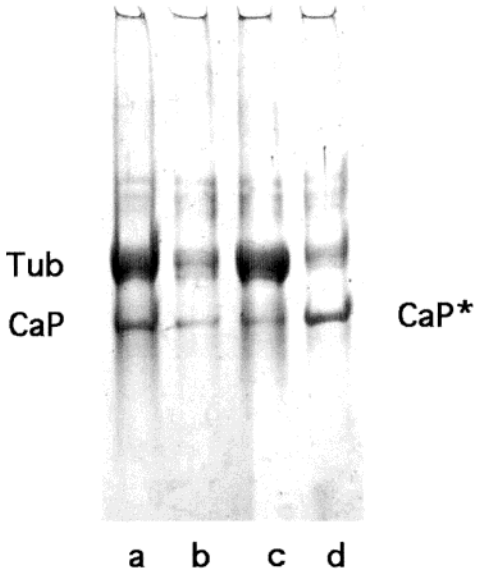

FIGURE 3: Dissociation of basic calponin from microtubules by phosphorylation. Phosphorylated basic calponin (4 μM) and microtubules at a concentration corresponding to 4 μM polymerized tubulin were incubated and then centrifuged as in Figure 1. The pellet (lane c) and supernatant (lane d) were analyzed over a 5−18% gradient acrylamide gel. The pellet (lane a) and supernatant (lane b) corresponding to a mixture of native calponin+microtubules were used as control. CaP* = phosphorylated calponin. CaP = native calponin. Tub = tubulin.

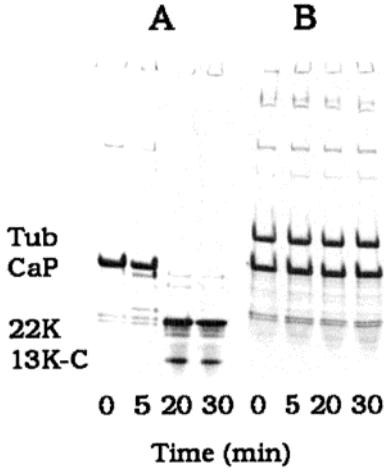

FIGURE 4: Influence of microtubules on the chymotryptic susceptibility of basic calponin. The protein was treated with chymotrysin in the absence (A) or presence (B) of microtubules using the experimental conditions depicted under Experimental Procedures. At the indicated time intervals, aliquots of the digest were submitted to 5−18% polyacrylamide gel electrophoresis. 22 K = NH_2-terminal fragment 7−182; 13 K-C = COOH-terminal fragment 183−292. Tub = tubulin. CaP = calponin.

moieties, the NH_2-terminal 22 kDa fragment (residues 7−182) and the COOH-terminal 13 kDa peptide (residues 183−292), which were earlier identified (*12*). However, the addition of an equimolar concentration of microtubules prior to digestion afforded a complete protection against the scission of this peptide bond, and only the intact calponin accumulated in the digest even when the proteolytic reaction was prolonged up to 30 min (Figure 4B). It should be noticed that, under the experimental conditions employed, the microtubule proteins were refractory to the protease and did not seem to compete with calponin for chymotrypsin. Consequently, the suppression of the calponin cleavage was likely resulting from a microtubule-induced change in the conformation and/or accessibility of the region around residues 182−183 of calponin. When the chymotryptic digest of the native calponin was subjected to ultracentrifugation in the presence of added microtubules followed by gel electrophoresis, the two main calponin fragments were found in the pellet (Figure 5A). Their binding to microtubules was not due to an intermolecular association by non covalent forces between the two peptides since, as illustrated in Figure 5B, lanes b and lanes c, the microtubules also cosedimented with either peptide used separately. We stress that no fragment could be sedimented when it was centrifuged without microtubules (data not shown). Remarkably, the breakdown of the 22 kDa NH_2-terminal fragment of residues 7−182 into the shorter 13 kDa fragment of residues 7−144, by a further limited chymotryptic digestion, completely abolished its pelleting with microtubules (Figure 5B, lane d). On the other hand, the cosedimentation studies, presented in Figure 6, show that only the former NH_2-terminal fragment could be dissociated from microtubules by calcium calmodulin (lanes 1) but not at all the COOH-terminal 13 kDa peptide of residues 183−292 (lanes 2) nor the longer COOH-terminal recombinant fragment R 164 representing residues 164−292 (lanes 3 and lanes 4). These observations are essentially in agreement with the fact that the calmodulin-dependent regulatory site of calponin is confined to the stretch 153−163 (*14*), present only in the 22 kDa fragment. The overall binding data match very well those, described above, using the ncd 335 ATPase measurements, and from the combination of both sets of results, we conclude that microtubules bind at two different calponin sites. One is inhibitory residing in the critical central segment of residues 145−182, and the other is noninhibitory lying in the COOH-terminal repeat-containing region 183−292. To ensure that the former site was not acting indirectly by influencing the actual binding of microtubules to the adjacent calponin homology domain, we subjected a mixture of microtubules and the synthetic peptide consisting of residues 145−182 to sedimentation and gel electrophoresis. The results, shown in Figure 7, illustrate the extensive association of the soluble peptide (lanes 2) with the microtubule pellet (lanes 3) and provide an unambiguous evidence of its direct involvement in calponin binding to microtubules.

Calponin May Interact Concomitantly with Microtubules and F-Actin. To compare the strength of the calponin−microtubule interaction with that known for the calponin−F-actin complex, we determined the steady-state fluorescence polarization of AEDANS-labeled basic calponin (1.1 μM) upon titration with increasing concentrations of isolated ultrapure microtubules devoid of MAPS corresponding to 0−3.0 μM polymerized tubulin. Use was made of the latter microtubule preparation to avoid competition between calponin and MAPS which both interact at the COOH-terminus of tubulin (*21*). As depicted in Figure 8A, a concentration-dependent increase of the polarization value of the labeled calponin was observed that approached saturation at high concentrations of added microtubules. Employing two different methods for the quantitative analysis of the data (Figure 8B), we obtained an apparent dissociation constant value of 0.3 μM ± 0.05. A K_d value of 5.2 μM was previously obtained under similar ionic conditions but using

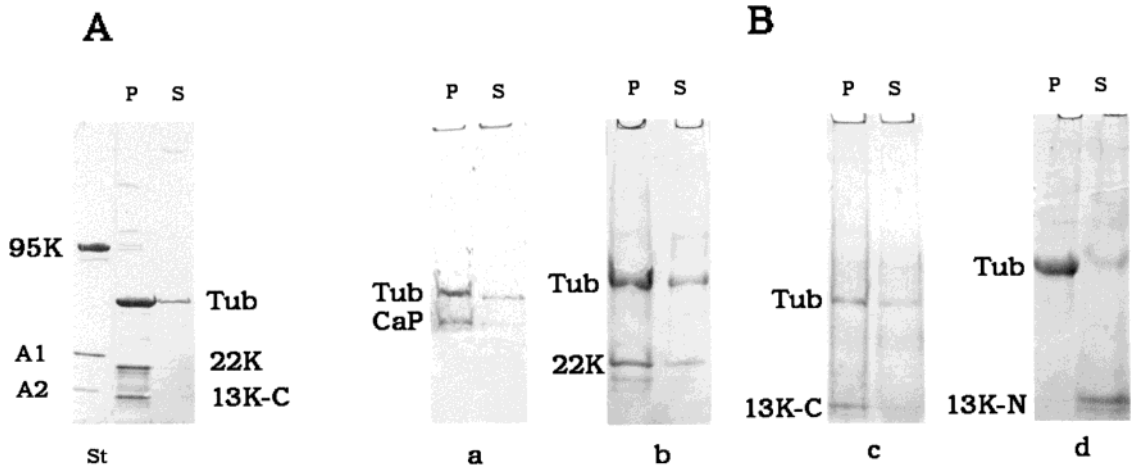

FIGURE 5: Cosedimentation analyses of the interaction of microtubules with the chymotryptic fragments of basic calponin. (A) After a 30 min limited digestion of calponin with chymotrysin, the reaction medium was brought to 2 mM phenylmethansulfonylfluoride and supplemented with polymerized tubulin added at equimolar amounts relative to calponin. The protein mixture was then incubated and centrifuged as in Figure 1. The pellet (P) and supernatant (S) were subjected to 5−18% polyacrylamide gel electrophoresis. St = protein markers, including the 95 kDa heavy chain and the A1 + A2 alkali light chains of rabbit skeletal myosin subfragment-1. (B) The electrophoretic patterns are presented for the pellets (P) and supernatants (S) obtained when microtubules at a concentration corresponding to 4 μM polymerized tubulin were incubated and centrifuged in the presence of an equimolar concentration of the isolated NH_2-terminal 22 kDa fragment 7−182 (lanes b), COOH-terminal 13 kDa fragment 183−292 (lanes c), or short NH_2-terminal 13 kDa fragment 7−144 (lanes d). The three fragments were produced and purified as reported under Experimental Procedures. The patterns corresponding to cosedimented microtubules + intact calponin (lanes a) are also shown.

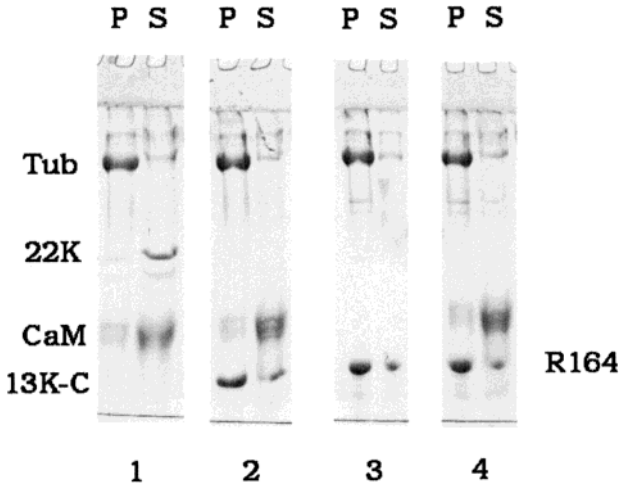

FIGURE 6: Modulation of the binding of the NH_2-terminal fragment 7−182 to microtubules by calmodulin. The electrophoretic profiles on a 5−18% polyacrylamide gel were established for the pellets (P) and supernatants (S) after sedimentation of microtubules at a concentration corresponding to 4 μM polymerized tubulin plus an equimolar concentration of the NH_2-terminal 22 kDa fragment 7−182 (lanes 1) or the COOH-terminal 13 kDa fragment 183−292 (lanes 2) in the presence of 1 mM $CaCl_2$ and a 6-fold molar excess of calmodulin over each fragment. Cosedimentation analyses are also shown for the mixture of microtubules + the recombinant fragment R-164 (residues 164−292) in the absence (lanes 3) or presence (lanes 4) of calmodulin. The electrophoresis was run for a shorter time than usually in order to achieve a better separation between calmodulin and the 13 K-C or R-164 fragments. CaM = calmodulin.

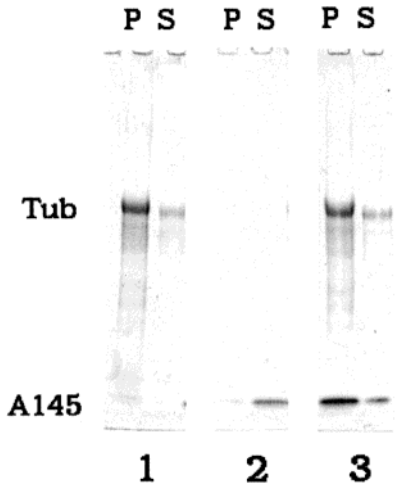

FIGURE 7: Interaction of microtubules with the segment of residues 145−182 of basic calponin. The synthetic peptide spanning amino acids 145−182 (4 μM) in the 10 mM imidazole−HCl (pH 7.0) buffer was centrifuged in the absence (lanes 2) or in the presence of microtubules at a concentration corresponding to 4 μM polymerized tubulin (lanes 3). The pellet (P) and supernatant (S) obtained after each sedimentation were analyzed by electrophoresis over a 7.5−20% gradient acrylamide gel. Lanes 1: the pellet and supernatant fractions of microtubules centrifuged alone. A 145 = the synthetic peptide of residues A145−Y182.

cosedimentation of calponin and partially polymerized microtubules (*21*). Our fluorometric approach and the use of ultrapure and fully polymerized microtubules likely yield a more accurate K_d value. This value compares well with that known for the calponin−skeletal F-actin complex (K_d = in the range 0.1−1 μM), suggesting that calponin binds to microtubules with virtually the same affinity as to F-actin. This observation encouraged us to assess whether the two kinds of filaments could compete for binding to calponin. For this purpose, we employed a quantitative fluorometric procedure exploiting the previously reported specific property of calponin binding to pyrenyl F-actin to promote a 60% quenching of the fluorescence intensity of the chromophore attached to Cys 374 of the actin subunit (*39*, *40*). Thus, any microtubule-induced displacement of calponin from F-actin can precisely be followed owing to the decrease of the calponin quenching effect on the pyrenyl group fluorescence. In Figure 9, we present the titration curve for the binding of a fixed concentration of pyrenyl F-actin (1 μM) to increasing concentrations of basic calponin (0−4 μM). A concentration-

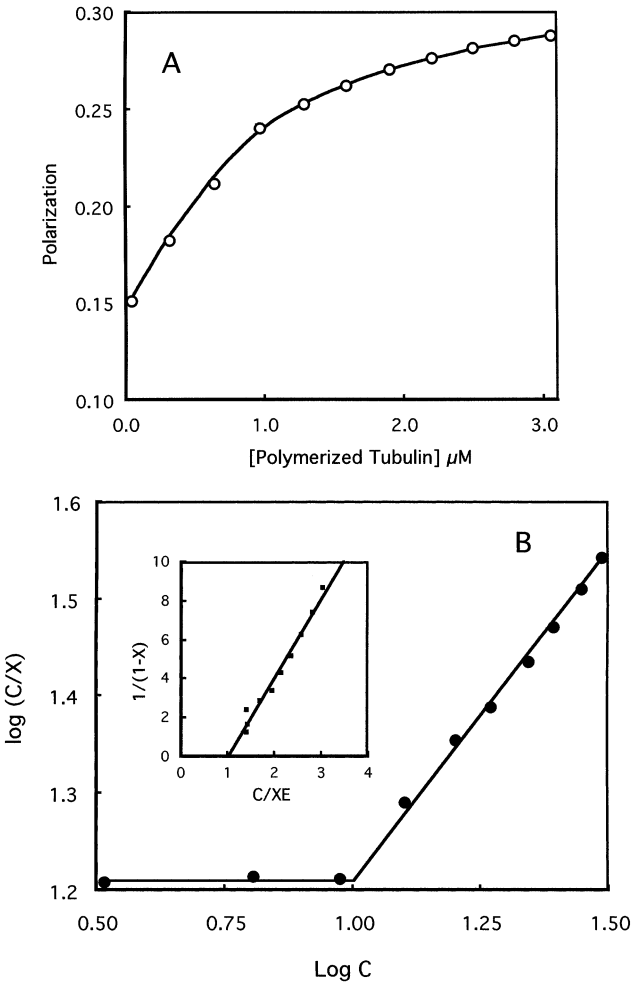

FIGURE 8: Binding of microtubules to basic calponin monitored by fluorescence polarization. (A) Titration of AEDANS−calponin (1.1 μM) with increasing concentrations of ultrapure microtubules corresponding to 0−3 μM polymerized tubulin in 2 mM $MgCl_2$, 30 mM KCl, 10 μM taxol, 25 mM Hepes (pH 7.0). Polarization was plotted versus microtubule concentrations. The data are the average from at least three separate analyses. The fluorometric experiments were performed as detailed under Experimental Procedures. (B) The quantitative analysis of the data was achieved by plotting log(*C*/*X*) vs log *C* or 1/(1 − *X*) vs *C*/(*XE*) (insert), where *C* corresponds to the microtubule concentration expressed in 10^{-7}M polymerized tubulin and *E* to the labeled calponin concentration fixed at 1.1 μM. *X* binding ratio was determined from polarization measurements.

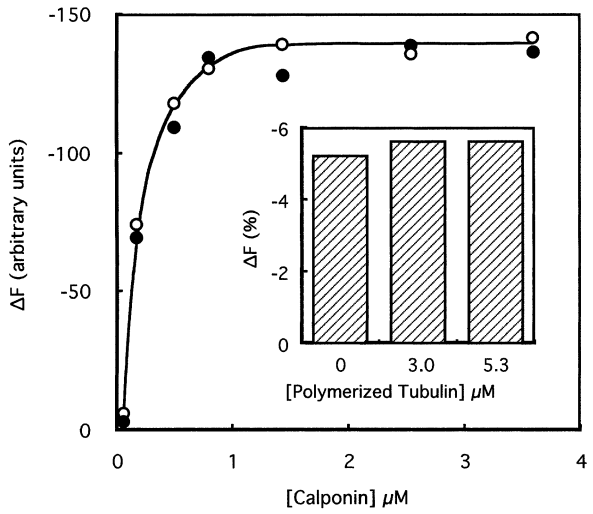

FIGURE 9: Dual interaction of basic calponin with F-actin and microtubules. Increasing concentrations of basic calponin (0−3.6 μM) were added to pyrenyl F-actin (1 μM) in the absence (●) or presence (○) of a microtubule concentration corresponding to 4.7 μM polymerized tubulin. The fluorescence changes were measured in 2 mM $MgCl_2$, 30 mM KCl, 10 μM taxol, 25 mM Hepes (pH 7.0) at 22 °C, using wavelengths of 365 and 385−395 nm for excitation and emission, respectively. Inset: fluorescence measurements for the complex of pyrenyl F-actin (1 μM) and calponin (1 μM) in the presence of increasing concentrations of microtubules corresponding to 0−5.3 μM polymerized tubulin. The data are expressed as percent of the initial fluorescence of pyrenyl actin alone. Experimental details are given under Experimental Procedures.

dependent and saturable decrease of the label's fluorescence intensity was produced and the curve plateaued at nearly 1:1 molar ratio of pyrenyl actin and calponin. However, an identical fluorescence titration curve was obtained when calponin was added to pyrenyl F-actin mixed with microtubules at a concentration corresponding to 4.7 μM polymerized tubulin. Also, the fluorescence intensity value of the preformed calponin−pyrenyl F-actin complex (protein molar ratio = 1:1) did not significantly change upon the addition of increasing concentrations of microtubules corresponding to 0−5.3 μM polymerized tubulin (Figure 9, inset). These data suggest that microtubules may bind to calponin without abolishing the concomitant interaction of the protein with F-actin. Earlier, it was also reported that the addition of an excess of G-actin to the calponin−microtubules complex or an excess of tubulin to the calponin−F-actin complex did not cause the displacement of calponin from its filamentous partner (*21*). However, because the behavior of the soluble actin and tubulin could differ from that of the corresponding polymerized forms, these observations remain questionable. Our present experiments, using only the filamentous cytoskeletal proteins, provide a more convincing evidence of the lack of competition between F-actin and microtubules for binding to calponin. Thus, in nonmuscle cells, calponin could not only interact with either F-actin or microtubules, but also it may potentially bind to both filaments acting as a cross-linker protein. Of course, further studies are needed to directly demonstrate the latter novel calponin property.

DISCUSSION

Our data show that microtubules bind in vitro to either of the two major calponin isoforms, the basic and the acidic calponins. An important consequence of this interaction is the specific inhibition of the microtubule-stimulated ATPase activity of a kinesin-related motor. This property is likely resulting from the observed ability of calponin to bind at the COOH-terminal regions of the tubulin subunits that also interact with kinesin (*21*). In nonmuscle cells, the microtubule−calponin association combined with the F-actin−calponin interaction would make the calponin molecule a general inhibitory protein of all filament-activated motor ATPases. Most interestingly, this potential cytoskeletal function may help to understand the reported capacity of calponin to affect the cell cycle at mitosis by blocking the G1/S-phase transition (*41*) and by depressing cell proliferation rates acting as an antitumor agent within a variety of cells such as vascular smooth muscles, fibroblasts, or osteosarcoma tissues (*42*, *43*). So far, the calponin action was thought to be related to the known influence of the

protein on the organization of the actin cytoskeleton and the expression of the actomyosin ATPase activity (*42*). However, because the microtubule motors play an essential role in cell division, the mechanism of the calponin effect could additionally and/or alternatively involve the regulation of the interaction of these motors with microtubules during critical steps of mitosis.

The binding of microtubules takes place at two different calponin segments. One comprises residues 145−182, and the other corresponds to the stretch spanning amino acids 183−292. It is unlikely that the COOH-terminal tail of varying length and amino acid sequence, located between residues 274 and 292 in basic calponin, could directly participate in microtubule recognition. Thus, microtubules should bind at the conserved repeat-containing region of residues 183−273. In this regard, it is noteworthy that microtubule binding to proteins of the MAP2/tau family and MAP4 is also mediated by up to four repeats of a conserved 31 amino acid sequence (*44*−*46*). However, there is no sequence homology between the latter repeats and those of calponin. The occurrence of these two microtubule binding sites on calponin is in agreement with the reported conclusion that the tubulin subunit exhibits one set of binding sites for calponin on the acidic COOH-terminal region and a second set of binding sites on another unknown portion of the tubulin monomer (*21*). The first calponin segment 145−182 certainly binds at the former tubulin sites, which also serve for the binding of kinesin and kinesin-related motors, and promotes the inhibition of the microtubule-activated ncd ATPase as observed with the 22 kDa fragment 7−182. The second calponin segment 183−273 must interact with the latter tubulin sites without change in the ncd ATPase activation by microtubules as observed with the 13 kDa fragment 184−292. Full binding of calponin to microtubules most likely involves the attachement of the protein at both tubulin sites. However, a significant interaction can still take place upon binding of calponin at a single tubulin site as we observed using its two separated NH_2- or COOH-terminal chymotryptic fragments and as indicated by the earlier finding that the proteolytic excision of the acidic COOH-terminal ends of tubulin did weaken but did not at all abolish the complexation of calponin to microtubules (*22*). The inhibitory calponin stretch 145−182 is a multifunctional region which binds F-actin and calmodulin (*14*, *16*), the myosin rod (*47*), and, as illustrated in this study, the microtubule filament. It lies immediately adjacent to the COOH-terminus of the calponin homology domain which did not bind microtubules. Its NH_2-terminal 19-residue portion 145−163 was earlier shown to represent the principal actin binding site in calponin that confers to the protein its ability to inhibit the actomyosin ATPase activity in a calcium calmodulin- and phosphorylation-modulated manner (*16*). In a recent fitting of the calponin homology domain in calponin to the three-dimensional helical reconstruction of the F-actin−calponin complex, this particular segment appeared to be well oriented toward the region of Glu 334 in the actin subdomain 1 (*48*). We propose that the same segment is operating in the calponin−microtubule complex being stereospecifically directed toward the carboxylic residues in the COOH-terminal portion of the tubulin subunit. Such an interaction accounts for the reported carbodiimide-catalyzed cross-linking of calponin to synthetic peptides representing the latter tubulin region as well as to intact microtubules (*21*, *22*). Earlier, the critical calponin segment was also shown to directly cross-link to Glu 334 of actin (*14*). Thus, the calponin peptide 145−163 would behave as a general filament-interacting motif. In this regard, it is noteworthy that the intermediate filament, desmin, was previously reported to interact with calponin in a calmodulin regulated manner and by binding only to the NH_2-terminal 22 kDa fragment (*49*). We surmise that this interaction also involves the calponin module 145−163. The interaction of this region with filamentous proteins of different structure would be made possible by its ability to adopt various conformations, as suggested by its flexibility and the lack of secondary structure predicted from its primary sequence (*11*).

In contrast to the inhibitory site 145−163, the second noninhibitory microtubule binding site on the segment 183−273 seems to be quite different from the secondary non-inhibitory actin binding site involving residues 172−187 in the first repeat motif of calponin. This proposal is supported by two major observations. First, the chymotryptic cleavage at the peptide bond 182−183 did not suppress microtubule binding to the resulting COOH-terminal 13 kDa fragment 183−292, but it did abolish the interaction of actin with the latter fragment (*14*). Second, and most importantly, the lack of competition found between F-actin and microtubules for binding to calponin requires that at least one specific microtubule recognition site resides in calponin. Since the association of microtubules to a single calponin site is possible as discussed above, they would interact mainly at this specific site located on the second and/or third repeat motif of calponin in the presence of F-actin. As a result, no competition between the two filaments is apparent, and calponin becomes able to cross-link the microtubules to F-actin. In support of this proposal is the previous finding that calponin induces in vitro the cross-linking and bundling of microtubules (*22*). This feature could readily be explained by the ability of the protein to interact with each microtubule filament at either of the two microtubule binding sites we have identified in the present study.

In conclusion, calponin in the living cells may reversibly inhibit any filament-activated motor ATPase, thereby regulating many motor-based biological processes. The protein may also function to link the microtubules to the microfilaments the two major components of the cell cytoskeleton.

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