J. Biochem. 122, 344-351 (1997)

Interaction of Chicken Gizzard Smooth Muscle Calponin with Brain Microtubules

Toshihiro Fujii,*[,1] Tsuneyoshi Hiromori,* Masateru Hamamoto,* and Tatsuo Suzuki†

*Department of Imagination Science (Kansei Engineering), Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386; and †Department of Neuroplasticity, Research Center on Aging and Adaptation, Shinshu University School of Medicine, Matsumoto, Nagano 390

Received for publication, March 4, 1997

Calponin, a major actin-, tropomyosin-, and calmodulin-binding protein in smooth muscle, interacted with tubulin, a main constituent of microtubules, in a concentration-dependent fashion *in vitro*. The apparent K_d value of calponin to tubulin was calculated to be 5.2 μM with 2 mol of calponin maximally bound per 1 mol of tubulin. At low ionic strength, tubulin bound to calponin immobilized on Sepharose 4B, and the bound protein was released at about 270 mM NaCl. Chemical cross-linking experiments showed that a 1:1 molar covalent complex of calponin and tubulin was produced. The amount of calponin bound to microtubules decreased with increasing ionic strength or Ca^{2+} concentration. The addition of calmodulin or S100 to the mixture of calponin and microtubule proteins caused the removal of calponin from microtubules in the presence of Ca^{2+}, but not in the presence of EGTA. Calponin-related proteins including tropomyosin, SM22, and caldesmon had little effect on the calponin binding to microtubules, whereas MAP2 inhibited the binding. Interestingly, there was little, if any, effect of mycalolide B-treated actin on the binding of calponin to microtubules. Furthermore, only about 20% of calponin-F-actin interaction was inhibited in the presence of an excess amount of tubulin (4 mol per mol of calponin), indicating that tubulin binds to calponin at a different site from that of actin. Compared with MAP2, calponin had little effect on microtubule polymerization.

Key words: calcium-binding protein, calponin, calponin-binding protein, interaction, microtubule.

Calponin is an actin-, tropomyosin-, and calmodulin-binding protein found in vertebrate smooth muscle (*1-3*). The binding of calponin to F-actin produces an inhibitory effect on the actin-activated Mg^{2+}-ATPase activity of skeletal muscle and smooth muscle myosin without affecting phosphorylation of the myosin light chain (*4-9*). Both the binding and the ATPase inhibition are calcium-insensitive, but they can be relieved by calmodulin in a calcium-dependent manner (*4, 7-9*). Furthermore, they are modulated by the phosphorylation and dephosphorylation of calponin catalyzed by protein kinase C, kinase II, smooth muscle phosphatase I, type 2A protein phosphatase, or type 2B protein phosphatase (*5, 10-15*). *In vitro* cell motility assays have shown that calponin-like caldesmon is capable of inhibiting the relative movement of actin and myosin (*16-18*).

Calponin is present at approximately the same concentration as tropomyosin (80 μM) in chicken gizzard smooth muscle (*1*). Compared with calmodulin (24-30 μM) and other typical calmodulin-binding proteins such as caldesmon (10 μM) and myosin-light chain kinase (about 5 μM) in smooth muscle (*1, 19, 20*), the level of calponin is relatively high, indicating that this protein may have properties other than binding to actin, tropomyosin, and calmodulin and may participate in functions other than contraction. Szymanski and Tao have reported that calponin can interact with smooth muscle unphosphorylated myosin filaments at low ionic strengths (*21*). Other calcium-binding proteins including S100 (*22*) and caltropin (*23*) have been reported to interact with calponin in a calcium-dependent manner and the interaction causes the recovery of calponin-dependent actomyosin ATPase inhibition. Recently, we have reported that calponin also binds to phospholipids including phosphatidylserine, phosphatidylinositol, and phosphatidylinositol-4,5-bisphosphate, but not to phosphatidylcholine, with apparent K_d values of 0.6-0.8 μM (*24*). These results demonstrate that calponin is a multifunctional protein in smooth muscle.

Cytoplasmic microtubules are fibrous intracellular organelles found in almost all eukaryotic cells and play a role in the maintenance of cell shape, cell division, transport, secretion, and receptor activity (*25, 26*). There is every reason to believe that most functions depend on the architecture of microtubules caused by polymerzation-depolymerization controlled by microtubule-associated proteins (MAPs), temperature, divalent cations, and ionic strength. Several proteins including actin, myosin, and neurofilaments have been reported to interact with microtubules or tubulin (*27-30*). A number of investigations

[1] To whom correspondence should be addressed.

Abbreviations: DTT, dithiothreitol; EDC, 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide; MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

with microtubules have been done using mammalian brains. However, little is known about microtubules and MAPs in smooth muscle.

In this study, we have shown for the first time that calponin interacts with microtubules through its binding to tubulin *in vitro*. Some characteristics of the interaction are described and a regulatory mechanism is proposed. We also examined the effect of calponin on microtubule polymerization.

MATERIALS AND METHODS

Preparation of Proteins—Calponin, tropomyosin, and SM22 were purified from chicken gizzard according to Fujii (*31*), Fujii *et al.* (*32*), and Lees-Miller *et al.* (*33*), respectively. The following proteins were isolated by means of the cited methods: rabbit skeletal muscle actin (*34*), porcine brain calmodulin (*35*), and porcine brain S100 (*35*). Microtubule proteins were prepared from rat brains by two cycles of temperature-dependent assembly and disassembly as described previously (*36*). Tubulin (PC-tubulin) was separated from depolymerized microtubule proteins by phosphocellulose column chromatography (*36*). Microtubule proteins and tubulin were dissolved in 100 mM MES-NaOH (pH 6.6), 0.5 mM $Mg(CH_3COO)_2$, and 1 mM EGTA (buffer A) containing 25% glycerol and stored at −80°C. MAP2 was purified from heat-stable MAPs by Ultrogel A6 (IBF Biotechnics) column chromatography (*37*).

Protein concentrations were determined according to Bradford (*38*) using bovine serum albumin as the standard or by spectrophotometric measurements using A_{290} of 6.3 for actin, A_{278} of 1.8 for tropomyosin, A_{276} of 3.0 for caldesmon, A_{275} of 1.8 for calmodulin, and A_{280} of 3.44 for S100.

Sedimentation Assay for Calponin-Microtubules and Calponin-F-Actin Interactions—The standard reaction mixture contained, in 60 mM MES-NaOH (pH 6.8), 0.2 mM $Mg(CH_3COO)_2$, 0.1 mM DTT, 0.4 mM EGTA, 25 mM NaCl, and 10% glycerol (buffer B) in a final volume of 100 μl. The assembly was initiated by adding 20 μM taxol and 1 mM GTP. The samples were incubated for 30 min at 37°C. F-Actin-binding assay was performed in buffer B and the solution was incubated for 20 min at 25°C. The samples were centrifuged at 100,000 × *g* for 30 min at 25°C for microtubule-binding and 4°C for F-actin-binding assays. Both supernatants and pellets were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the amounts of calponin, tubulin, and actin were determined by densitometry. SDS-PAGE was carried out in 12.5% polyacrylamide gel in a discontinuous Tris-glycine buffer system according to Laemmli (*39*).

Affinity Chromatography—Calponin was coupled to CNBr-activated Sepharose 4B following the procedures outlined by Pharmacia. About 1 mg of calponin was linked to 0.3 ml of Sepharose. Rat brain microtubule proteins were loaded on a calponin-Sepharose column (0.7 × 6 cm) equilibrated with 25 mM MES-NaOH (pH 6.8), 0.2 mM DTT, 0.5 mM EGTA, 1 μg/ml pepstatin, and 40 mM NaCl (buffer C) at a flow rate of 3-5 ml/h. The column was washed with 2 column volumes of buffer C, and the bound protein was eluted with a salt gradient, 40-500 mM NaCl in buffer C.

Chemical Cross-Linking—The standard chemical cross-linking assay was performed in 40 mM MES-NaOH (pH 6.8), 0.15 mM $Mg(CH_3COO)_2$, 0.1 mM DTT, 0.3 mM EGTA, 20 mM NaCl, and 8% glycerol in a final volume of 30 μl. The reaction was started by adding 3 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) which was freshly prepared. After incubation for 30 min at 30°C, the reaction was stopped by the addition of one-fifth volume of a 5 × electrophoresis sample buffer (155 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 0.75% β-mercaptoethanol, and 0.0025% pyronin Y), then heated to 95°C for 2 min. The samples were subjected to electrophoresis.

Microtubule Assembly—Microtubule polymerization experiments were carried out in the reaction mixture containing 70 mM MES-NaOH (pH 6.8), 0.5 mM $Mg(CH_3COO)_2$, 0.4 mM EGTA, 10% glycerol, and 1 mM GTP. The polymerization was monitored in terms of the change in absorbance at 350 nm in a Hitachi U-2001 spectrophotometer fitted with a temperature-controlled compartment.

RESULTS

Interaction of Calponin with Microtubule Proteins—We examined the interaction between calponin and microtubule protein *in vitro*. The binding of calponin to microtubule proteins was investigated by measuring the ability of the protein to cosediment with microtubules during high-speed centrifugation; calponin itself was not sedimented by this centrifugation. Taxol is an effective drug for microtubule assembly, because it can promote tubulin polymerization and decrease the critical concentration for microtubule assembly (*41, 42*). When rat brain microtubule proteins were incubated with 20 μM taxol and 1 mM GTP, most of the protein was recovered in the pellet (Fig. 1A). Calponin was also sedimented with taxol-stabilized microtubules in a concentration-dependent manner. Tubulin, a main constituent of microtubule proteins, was used in place of microtubule proteins to examine whether calponin bound to either tubulin or MAPs. Calponin also bound to taxol-polymerized microtubules in the absence of MAPs (data not shown). A Scatchard plot showed that the apparent K_d value of calponin for tubulin was 5.2 μM, when the tubulin content in the microtubule preparation was estimated to be 75%. The binding was saturated at a molar ratio of 2 calponin molecules to 1 molecule of tubulin dimer (Fig. 1B).

Evidence for the binding of calponin to microtubule proteins was also obtained using affinity chromatography (Fig. 2). When microtubule proteins were applied to a calponin-Sepharose column, most of the protein was retained by the column and the bound protein was eluted at 250-300 mM NaCl under the conditions used. Electrophoretic analysis showed that a large portion of tubulin was recovered in the bound fraction, while high-molecular-weight MAPs were eluted in both the unbound and bound fractions. All procedures were carried out at 4°C. These results indicate that calponin can bind to unpolymerized microtubule proteins, as well as the polymerized form.

The binding of calponin to microtubule proteins was also examined using the zero-length cross-linker EDC. As shown in Fig. 3, when calponin and PC-tubulin were mixed with EDC, a cross-linking product with an apparent

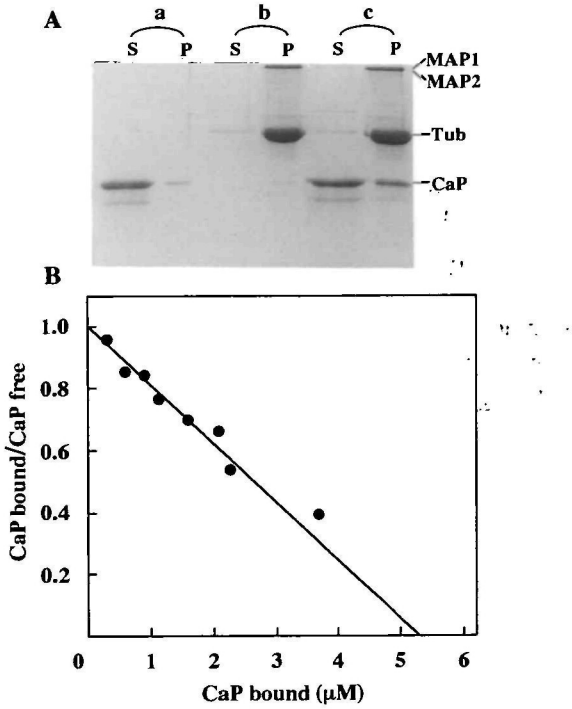

Fig. 1. **Sedimentation analysis of the interaction of calponin with microtubules and the Scatchard analysis.** (A) The binding of calponin to microtubules was examined by the high-speed centrifugation method, described in "MATERIALS AND METHODS," in which the protein compositions of supernatants (S) and pellets (P) were analyzed by 12.5% SDS-PAGE. The concentrations of calponin and microtubule proteins were 5.5 μM and 0.38 mg/ml, respectively. Pair a, calponin; pair b, microtubules; pair c, calponin + microtubules. (B) Microtubules were incubated with various concentrations of calponin. The amounts of calponin were determined by densitometry. The concentration of microtubule proteins was 0.38 mg/ml, in which the tubulin content was estimated to be 2.6 μM. CaP, calponin; Tub, tubulin.

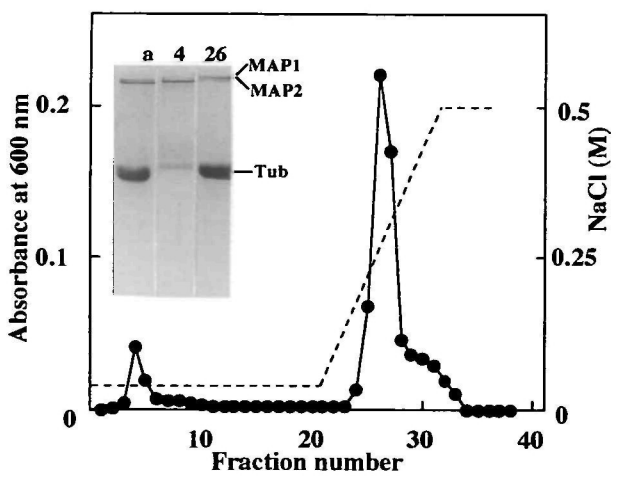

Fig. 2. **Calponin-Sepharose 4B chromatography.** Rat brain microtubule proteins (2 mg) were applied to a calponin Sepharose 4B column. Elution was performed with a NaCl gradient (40-500 mM). Fractions (0.7 ml) were collected and analyzed for protein. The inset shows SDS polyacrylamide gel profiles of the applied sample (a), and fractions 4 and 26.

molecular mass of 86 kDa was generated in a concentration-dependent manner. The 86 kDa band was immunostained with anti-calponin and anti-tubulin antibodies (data not shown). We interpret these results as indicating that the 86 kDa band represents the 1:1 molar covalent complex of calponin and the tubulin subunit. Similar results were observed when microtubule proteins were used instead of PC-tubulin, indicating that calponin directly interacts with tubulin subunit.

To determine the concentration dependence of salt and divalent cations on calponin-microtubule interaction using co-sedimentation and chemical cross-linking experiments, various concentrations of NaCl, $MgCl_2$, and $CaCl_2$ were added to the standard reaction mixtures. At physiological ionic conditions (50-100 mM NaCl), considerable amounts of calponin cosedimented with microtubules and the formation of an 86 kDa band was observed. The calponin binding to microtubules was maximum at around 0.5 mM Mg^{2+}, whereas the binding was slightly inhibited by adding Ca^{2+}.

Effects of Calmodulin on the Binding of Calponin to Microtubules—Because calmodulin can interact with calponin in a Ca^{2+}-dependent manner (*7-9, 23*), the effect of calmodulin on the binding of calponin to microtubules in the presence of Ca^{2+} or EGTA was examined. As can be seen in Fig. 4, calmodulin inhibited the calponin binding to micro-

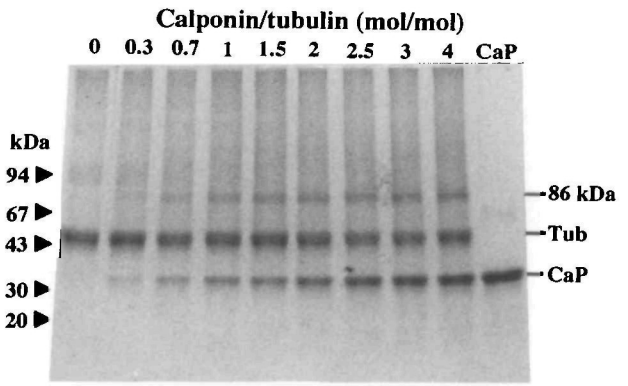

Fig. 3. **Cross-linking of calponin and tubulin.** The cross-linking reaction was performed with 3 mM EDC for 30 min at 30°C as described in "MATERIALS AND METHODS," in the standard assay containing 1.9 μM PC-tubulin and various concentrations of calponin as indicated. The cross-linked materials were separated by 5-15% SDS-PAGE.

TABLE I. **Effects of NaCl, $MgCl_2$, and $CaCl_2$ on the binding of calponin to microtubules.** The bindings were assayed as indicated in the legends to Fig. 1 (sedimentation) and Fig. 3 (EDC), in various concentrations of NaCl, $MgCl_2$, and $CaCl_2$ as shown. The concentrations of calponin, microtubule proteins, and PC-tubulin were 5.5 μM, 0.38 mg/ml, and 1.9 μM, respectively.

	Conc. (mM)	Sedimentation (%)	EDC (%)
Control		100	100
NaCl	50	47	60
	100	39	36
	200	33	21
$MgCl_2$	0.5	140	120
	2	116	108
$CaCl_2$	0.5	94	97
	2	81	70

tubules in the presence of Ca^{2+}, though it had little effect on the binding in the absence of Ca^{2+}. Half-maximal inhibition occurred at a molar ratio of calmodulin to calponin of 1.7, showing that calmodulin is a potent inhibitor of calponin-microtubule interaction, as well as calponin-F-actin and calponin-phospholipid interactions (*7-9, 24*).

Effects of Calponin-Related Proteins and MAP2 on the Binding of Calponin to Microtubules—We examined the effects of S100, tropomyosin, caldesmon, SM22, and MAP2 on calponin-microtubule interaction by sedimentation analysis (Fig. 5). These proteins were not sedimented by themselves in this centrifugation. S100 is an acidic Ca^{2+}-binding protein which belongs to a family of EF-hand-type Ca^{2+}-binding proteins and is distributed in a variety of tissues (*43*). Previously, we found that S100 as well as calmodulin can modulate both calponin-F-actin and calponin-phospholipid interactions (*22, 24*). Like calmodulin, S100 inhibited calponin binding to microtubules in the presence of Ca^{2+}. Addition of tropomyosin, which weakly interacts with calponin, did not affect the binding. Among calmodulin- and actin-binding proteins in smooth muscle, caldesmon is believed to play a significant role in thin and thick filament interaction (*44, 45*) and is also known to be a microtubule-binding protein (*46, 47*). Compared with calponin, only a small amount of caldesmon was found in the microtubule pellets under our experimental conditions (data not shown). The presence of caldesmon had little

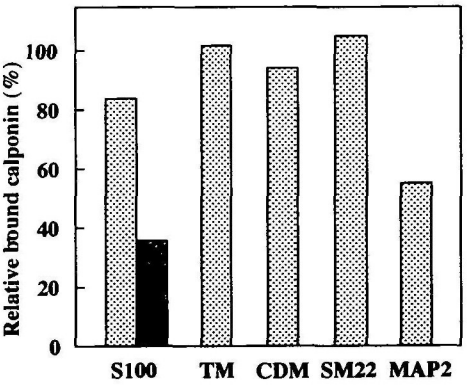

Fig. 5. **Effects of calponin-related proteins and MAP2 on the binding of calponin to microtubules.** The assay conditions were the same as those in Fig. 5 in the presence of 0.2 mM EGTA (▨) or 0.5 mM $CaCl_2$ (■). The concentrations of calponin, microtubule proteins, S100, tropomyosin, caldesmon, SM22, and MAP2 were 5.5 μM, 0.38 mg/ml, 16.5 μM, 16.5 μM, 16.5 μM, 16.5 μM, and 5.5 μM, respectively. TM, tropomyosin; CDM, caldesmon.

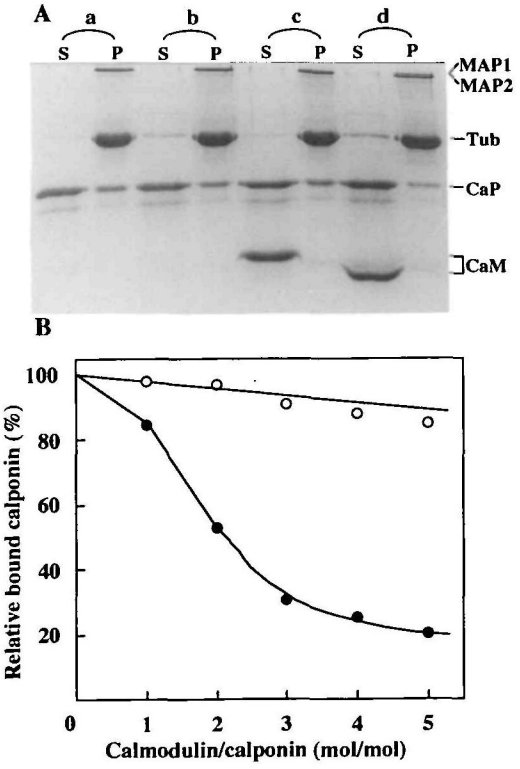

Fig. 4. **Sedimentation analysis of the interaction between calponin and microtubules in the presence or absence of calcium and calmodulin.** (A) The concentrations of calponin, microtubule proteins, and calmodulin were 5.5 μM, 0.38 mg/ml, and 16.5 μM, respectively. CaM, calmodulin. Pair a, 0.2 mM EGTA-calmodulin; pair b, 0.5 mM $CaCl_2$-calmodulin; pair c, 0.2 mM EGTA + calmodulin; pair d, 0.5 mM $CaCl_2$ + calmodulin. (B) The binding of calponin to microtubules was examined in the presence of 0.5 mM $CaCl_2$ (●) or 0.2 mM EGTA (○), and various concentrations of calmodulin as indicated. The amounts of calponin bound to microtubules were determined from densitometric scans of SDS-gels.

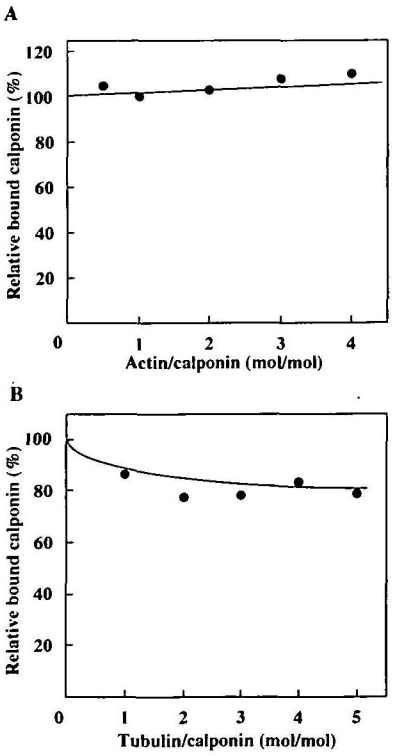

Fig. 6. **Effects of G-actin and PC-tubulin on the binding of calponin to microtubules and F-actin.** (A) Actin (90 μM) was preincubated with 100 μM mycalolide B (Wako Chemicals) in the sedimentation assay solution for 20 min at 25°C. The sample was centrifuged at 100,000 × *g* for 30 min at 25°C, and the obtained supernatant was used as non-polymerized actin. (B) PC-tubulin (0-9 μM) was incubated with 1.8 μM calponin and 5.6 μM F-actin for 20 min at 25°C. The assay procedure was described in "MATERIALS AND METHODS." The amount of calponin bound to microtubules or F-actin was measured by densitometry of Coomassie Blue-stained gels.

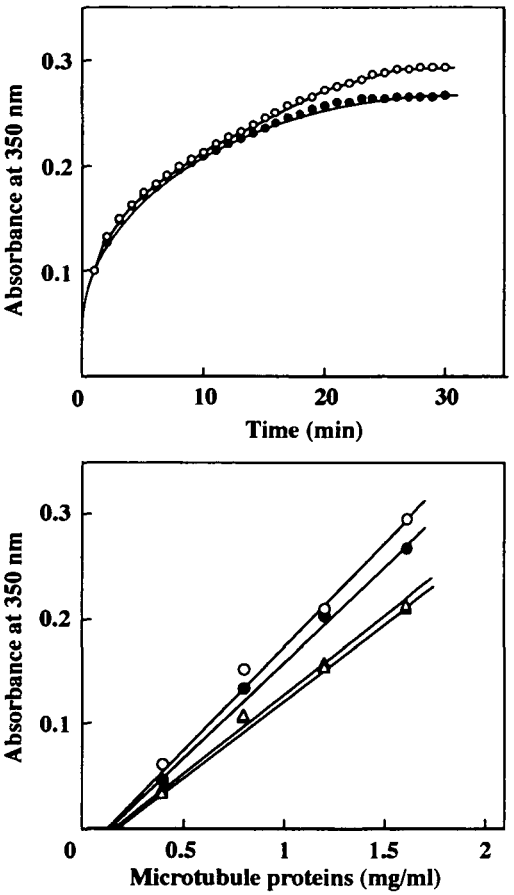

Fig. 7. **Effect of calponin on microtubule polymerization.** (A) Microtubule protein (1.6 mg/ml) in MES buffer containing 1 mM GTP, was incubated at 37°C either in the absence (○) or presence of 5.8 μM calponin (●). The assembly was monitored in terms of the change in absorbance at 350 nm. (B) Polymerization in the absence (○, △) or presence of (●, ▲) was expressed as absorbance at 350 nm for the initial 10 min (△, ▲) and 30 min (○, ●).

effect on calponin binding to microtubules. SM22, a 22-kDa basic protein whose amino acid sequence has a high degree of homology to that of calponin (41%), is expressed exclusively in smooth muscle (*33, 48*). SM22 did not interact with microtubules with significant affinity (data not shown) and had little effect on the interaction of calponin with microtubules. MAP2 is one of the best characterized MAPs (*25, 26*) and was contained in the microtubule preparation used. As shown in Fig. 1, the binding of calponin to microtubules did not remove the MAPs from the microtubules. On the other hand, the amount of calponin bound to microtubules decreased upon the addition of exogenous MAP2.

Effects of Tubulin and Actin on the Binding of Calponin to F-Actin and to Microtubules—The calponin-microtubule and calponin-F-actin interactions were compared by measuring the amount of calponin displacement from calponin-microtubules or calponin-F-actin complexes as a function of G-actin or PC-tubulin concentration (Fig. 6). When G-actin was incubated with a mixture of calponin and microtubule proteins, most of the G-actin was converted to F-actin and was recovered in the pellets as well as the microtubules under the present experimental conditions. Recently, mh B isolated from marine sponge has been reported to be a selective and powerful depolymerizing agent of F-actin which binds to G-actin in a 1:1 molar ratio (*49*). Addition of actin prepared by mycalolide B treatment, which did not sediment by itself during this centrifugation, had little effect on the binding of calponin to microtubules when the molar ratio of actin to calponin was 4 (Fig. 6A). Actin treated with mycalolide B retained the ability to produce a cross-linked complex with calponin (data not shown), like untreated actin (*50*). We next examined the effect of PC-tubulin on calponin-F-actin interaction (Fig. 6B). When calponin (1.8 μM) was mixed with 5.5 μM F-actin, most of the calponin bound to actin filaments. In the presence of PC-tubulin (0-9 μM), the bound calponin was slightly decreased to about 80%.

Microtubule Polymerization in the Presence of Calponin—We examined the effect of calponin on microtubule polymerization (Fig. 7). Microtubule polymerization was monitored by measuring the turbidity change. Addition of calponin did not affect the initial rate of microtubule polymerization, but this protein slightly inhibited the final level of polymerization (Fig. 7A). When the concentration of microtubule proteins was varied in the presence and absence of calponin to estimate the concentration required for microtubule polymerization, the critical concentration (0.13-0.2 mg/ml) in the absence of calponin was not affected by its presence (Fig. 7B).

DISCUSSION

The present results provide convincing evidence for the interaction between calponin and microtubules. The calponin-F-actin interaction is believed to be physiologically important in the regulation of smooth muscle contraction. The apparent K_d value of calponin for actin has been estimated to be 0.66-6 μM by sedimentation analysis using native and [^{14}C]iodoacetamide-labeled calponin, and the binding is saturated at about 1 mol calponin per 3 mol of actin monomer (*31, 51*). The estimated K_d value for the binding of calponin to microtubules is 5.2 μM, and the binding is saturated at about 2 mol calponin per 1 mol of tubulin dimer (Fig. 1). Like the calponin-actin and calponin-phospholipid interactions (*7-9, 24*), calponin-microtubule interaction was modulated by calcium-binding proteins, including calmodulin and S100, in a calcium-dependent manner (Figs. 4 and 5). The amounts of calmodulin required for 50% inhibition of calponin binding to F-actin and for 50% restoration of calponin-induced inhibition of actomyosin-ATPase activity are 2.1-5.2 times (*7-9, 22*) and 1.2-5 times (*4, 7*) the molar level of calponin, respectively. Half-maximal inhibition of calponin-microtubule interaction by calmodulin occurred at a molar ratio of calmodulin to calponin of 1.7, indicating that calmodulin at a relatively low level is also capable of modulating the calponin-microtubule interaction.

On the basis of the isoelectric point of calponin, three types of calponin isoforms, acidic (pI=5-6), neutral (pI=7-8), and basic (pI=8-10), have been identified (*52-56*). Furthermore, basic calponin consisted of α- and β-isoforms produced by alternative mRNA splicing. Because basic calponin is predominantly expressed in smooth muscle, the species of calponin that interacts with microtubules should be the basic one. Two-dimensional electrophoresis indicat-

ed that both α- and β-isoforms of basic calponin were found in the precipitate with microtubules (data not shown).

In the mammalian brain, microtubules are composed of tubulin and several other proteins designated MAPs, including MAP1, MAP2, and tau (*25, 26*). The microtubule-binding fragments of MAP2 and tau are basic with an isoelectric point of 10-11, and the projection region is acidic with an isoelectric point of 4-5 (*57-59*). Moreover, some cations including poly(L-lysine), poly(L-arginine), and histone are capable of inducing tubulin polymerization (*60, 61*). The MAP2 binding region in the tubulin molecule is located within the highly acidic 4 kDa fragment of the C-terminal moiety (*62, 63*). Like MAP2, tau, and caldesmon, calponin is heat-stable and its biological activities to bind to actin, calmodulin, myosin, phospholipids, and microtubules are retained after incubation at 95-100°C for 4-5 min, though calponin and these proteins have few common or homologous amino acid sequences. The isoelectric point of chicken gizzard calponin is around 10 (*52*). Calponin-microtubule interaction is sensitive to ionic conditions in the reaction mixture (Table I). Taking these results into account, we propose that electrostatic interactions play an important role in the interaction between calponin and microtubules. We think that the binding of calponin to microtubules is specific. This is supported by the following observations. Both calmodulin and S100 can block calponin-microtubule interaction only in the presence of Ca^{2+}. SM22, a basic protein (pI, around 9.3) which has a remarkable degree of homology in amino acid sequence with the N-terminal region of calponin (*33, 48, 52*), did not bind to microtubules and, further, had little effect on calponin-microtubule interaction (Fig. 5). Interestingly, addition of actin to the calponin-microtubule complex did not cause the displacement of calponin. These results suggest that the tubulin-binding site on the calponin molecule is different from its actin-binding site, and the calponin-binding site on the tubulin molecule may overlap at the C-terminal domain with the site at which MAP2 interacts with microtubules. The detailed structures of the domains where calponin and tubulin bind to each other are under investigation.

In smooth muscle, caldesmon, an actin-, tropomyosin-, and calmodulin-binding protein, has been reported to modulate actin-myosin filament interaction (*44, 45*). Recently, Ishikawa *et al.* (*46, 47*) have shown that high- and low-molecular-weight caldesmons from chicken gizzard and bovine brain, respectively, can interact with microtubules with an apparent K_d value of 0.9-2.2 μM and this binding is saturated at about 1 mol caldesmon per 5 mol of tubulin dimer. These results suggest that both calponin and caldesmon may be microtubule-associated proteins, as well as actin-, tropomyosin-, and calmodulin-binding proteins, in smooth muscle.

Representative cytoskeletal components are microfilaments, microtubules, and intermediate filaments. In the brain, the interactions of microtubules with microfilaments and of microtubules and neurofilaments have been reported, and these interactions are regulated by calcium/calcium-binding protein and phosphorylation-dephosphorylation (*28, 29, 64-66*). This is the first report describing the interaction of calponin with microtubules through calponin binding to tubulin dimer *in vitro*. The physiological significance of this is still unknown. However, these data demonstrate that calponin may be a candidate for a mediator and regulator of the interaction between microfilaments and microtubules in smooth muscle.

We wish to thank Dr. J. Johnson (National Cancer Institute, NIH) for the gift of taxol. We also thank Dr. T. Arai (Science University of Tokyo) for providing anti-tubulin monoclonal antibody, and H. Tada for a critical reading of the manuscript.

REFERENCES

1. Takahashi, K., Hiwada, K., and Kokubu, T. (1986) Isolation and characterization of a 34000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle. *Biochem. Biophys. Res. Commun.* **141**, 20-26
2. Takahashi, K., Hiwada, K., and Kokubu, T. (1987) Occurrence of anti-gizzard P34K antibody cross-reactive components in bovine smooth muscles and non-smooth muscle tissues. *Life Sci.* **41**, 291-296
3. Takahashi, K., Abe, M., Hiwada, K., and Kokubu, T. (1988) A novel troponin T-like protein (calponin) in vascular smooth muscle: Interaction with tropomyosin paracrystals. *J. Hypertension* **6**, S40-S43
4. Abe, M., Takahashi, K., and Hiwada, K. (1990) Effect of calponin on actin-activated myosin ATPase activity. *J. Biochem.* **108**, 835-838
5. Winder, S.J. and Walsh, M.P. (1990) Smooth muscle calponin. *J. Biol. Chem.* **265**, 10148-10155
6. Horiuchi, K.Y. and Chacko, S. (1991) The mechanism for the inhibition of actin-activated ATPase of smooth muscle heavy meromyosin by calponin. *Biochem. Biophys. Res. Commun.* **176**, 1487-1493
7. Makuch, R., Birukov, K., Shirinsky, V., and Dabrowska, R. (1991) Functional interrelationship between calponin and caldesmon. *Biochem. J.* **280**, 33-38
8. Winder, S.J., Walsh, M.P., Vasulka, C., and Johnson, J.D. (1993) Calponin-calmodulin interaction: Properties and effects on smooth and skeletal muscle actin binding and actomyosin ATPases. *Biochemistry* **32**, 13327-13333
9. Kolakowski, J., Makuch, R., Stepkowski, D., and Dabrowska, R. (1995) Interaction of calponin with actin and its functional implications. *Biochem. J.* **306**, 199-204
10. Naka, M., Kureishi, Y., Muroga, Y., Takahashi, K., Ito, M., and Tanaka, T. (1990) Modulation of smooth muscle calponin by protein kinase C and calmodulin. *Biochem. Biophys. Res. Commun.* **171**, 933-937
11. Nakamura, F., Mino, T., Yamamoto, J., Naka, M., and Tanaka, T. (1993) Identification of the regulatory site in smooth muscle calponin that is phosphorylated by protein kinase C. *J. Biol. Chem.* **268**, 6194-6201
12. Winder, S.J., Allen, B.G., Fraser, E.D., Kang, H.-M., Kargacin, G.J., and Walsh, M.P. (1993) Calponin phosphorylation *in vitro* and in intact muscle. *Biochem. J.* **296**, 827-836
13. Ichikawa, K., Ito, M., Okubo, S., Konishi, T., Nakano, T., Mino, T., Nakamura, F., Naka, M., and Tanaka, T. (1993) Calponin phosphatase from smooth muscle: A possible role of type 1 protein phosphatase in smooth muscle relaxation. *Biochem. Biophys. Res. Commun.* **193**, 827-833
14. Nagumo, H., Sakurada, K., Seto, M., and Sasaki, Y. (1994) Phosphorylation of calponin by PKC is blocked by F-actin *in vitro*. *Biochem. Biophys. Res. Commun.* **203**, 1502-1507
15. Fraser, E.D. and Walsh, M.P. (1995) Dephosphorylation of calponin by type 2B protein phosphatase. *Biochemistry* **34**, 9151-9158
16. Shirinsky, V.P., Biryukov, K.G., Hettasch, J.M., and Sellers, J.R. (1992) Inhibition of the relative movement of actin and myosin by caldesmon and calponin. *J. Biol. Chem.* **267**, 15886-15892
17. Haeberle, J.R. (1994) Calponin decreases the rate of cross-bridge cycling and increases maximum force production by smooth muscle myosin in an *in vitro* motility assay. *J. Biol. Chem.* **269**, 12424-12431

18. Ye, L.-H., Hayakawa, K., Lin, Y., Okagaki, T., Fujita, K., and Kohama, K. (1994) The regulatory role of myosin light chain kinase as an actin-binding protein. *J. Biochem.* **116**, 1377-1382
19. Grand, R.J.A., Perry, S.V., and Weeks, R.A. (1979) Troponin C-like proteins (calmodulins) from mammalian smooth muscle and other tissues. *Biochem. J.* **177**, 521-529
20. Sobue, K., Kanda, K., Tanaka, T., and Ueki, N. (1988) Caldesmon: A common actin-linked regulatory protein in the smooth muscle and nonmuscle contractile system. *J. Cell. Biochem.* **37**, 317-325
21. Szymanski, P.T. and Tao, T. (1993) Interaction between calponin and smooth muscle myosin. *FEBS Lett.* **331**, 256-259
22. Fujii, T., Oomatsuzawa, A., Kuzumaki, N., and Kondo, Y. (1994) Calcium-dependent regulation of smooth muscle calponin by S100. *J. Biochem.* **116**, 121-127
23. Wills, F.L., McCubbin, W.D., and Kay, C.M. (1994) Smooth muscle calponin-caltropin interaction: Effect on biological activity and stability of calponin. *Biochemistry* **33**, 5562-5569
24. Fujii, T., Yamana, K., Ogoma, Y., and Kondo, Y. (1995) Interaction of calponin with phospholipids. *J. Biochem.* **117**, 999-1003
25. Olmsted, J.B. (1986) Microtubule-associated proteins. *Annu. Rev. Cell Biol.* **2**, 421-457
26. Wiche, G., Oberkanins, C., and Himmler, A. (1991) Molecular structure and function of microtubule-associated proteins. *Int. Rev. Cytol.* **124**, 217-273
27. Fujii, T. and Tanaka, R. (1979) Interaction of rat brain microtubule proteins and 6S tubulin with rabbit skeletal muscle actomyosin. *Life Sci.* **24**, 1683-1690
28. Aamodt, E.J. and Williams, R.C., Jr. (1984) Microtubule-associated proteins connect microtubules and neurofilaments in vitro. *Biochemistry* **23**, 6023-6031
29. Yamauchi, P.S. and Purich, D.L. (1993) Microtubule-associated protein interactions with actin filaments: Evidence for differential behavior of neuronal MAP-2 and tau in the presence of phosphatidyl-inositol. *Biochem. Biophys. Res. Commun.* **190**, 710-715
30. Best, A., Ahmed, S., Kozma, R., and Lim, L. (1996) The ras-related GTPase rac1 binds tubulin. *J. Biol. Chem.* **271**, 3756-3762
31. Fujii, T. (1991) Purification and characterization of an actin-, calmodulin- and tropomyosin-binding protein from chicken gizzard smooth muscle. *Chem. Pharm. Bull.* **39**, 2622-2626
32. Fujii, T., Ozawa, J., Ogoma, Y., and Kondo, Y. (1988) Interaction between chicken gizzard caldesmon and tropomyosin. *J. Biochem.* **104**, 734-737
33. Lees-Miller, J.P., Heeley, D.H., Smillie, L.B., and Kay, C.M. (1987) Isolation and characterization of an abundant and novel 22-kDa protein (SM22) from chicken gizzard smooth muscle. *J. Biol. Chem.* **262**, 2988-2993
34. MacLean-Fletcher, S. and Pollard, T.D. (1980) Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochem. Biophys. Res. Commun.* **96**, 18-27
35. Isobe, T., Nakajima, T., and Okuyama, T. (1977) Reinvestigation of extremely acidic proteins in bovine brain. *Biochim. Biophys. Acta* **494**, 222-232
36. Ihara, Y., Fujii, T., Arai, T., Tanaka, R., and Kaziro, Y. (1979) The presence of an adenosine-5′-triphosphatase dependent on 6S tubulin and calcium ions in rat brain microtubules. *J. Biochem.* **86**, 587-590
37. Nakamura, A., Arai, T., Kondo, Y., and Fujii, T. (1989) Inhibitory effects of poly(L-aspartic acid) on the assembly of brain microtubules and the interaction of microtubule-associated protein 2 with F-actin *in vitro*. *J. Biochem.* **106**, 93-97
38. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
39. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
40. Fujii, T., Imai, M., Rosenfeld, G.C., and Bryan, J. (1987) Domain mapping of chicken gizzard caldesmon. *J. Biol. Chem.* **262**, 2757-2763
41. Schiff, P.B., Fant, J., and Horwitz, S.B. (1979) Promotion of microtubule assembly *in vitro* by taxol. *Nature* **277**, 665-667
42. Kumar, N. (1981) Taxol-induced polymerization of purified tubulin. *J. Biol. Chem.* **256**, 10435-10441
43. Donato, R. (1986) S-100 proteins. *Cell Calcium* **7**, 123-145
44. Sobue, K. and Sellers, J.R. (1991) Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems. *J. Biol. Chem.* **266**, 12115-12118
45. Marston, S.B. and Redwood, C.S. (1991) The molecular anatomy of caldesmon. *Biochem. J.* **279**, 1-16
46. Ishikawa, R., Kagami, O., Hayashi, C., and Kohama, K. (1992) The binding of nonmuscle caldesmon from brain to microtubules: Regulations by Ca^{2+}-calmodulin and cdc2 kinase. *FEBS Lett.* **299**, 54-56
47. Ishikawa, R., Kagami, O., Hayashi, C., and Kohama, K. (1992) Characterization of smooth muscle caldesmon as a microtubule-associated protein. *Cell Motil. Cytoskeleton* **23**, 244-251
48. Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morrisey, E.E., Ip, H.S., and Parmacek, M.S. (1995) Structure and expression of a smooth muscle cell-specific gene, SM22α. *J. Biol. Chem.* **270**, 13460-13469
49. Saito, S., Watabe, S., Ozaki, H., Fusetani, N., and Karaki, H. (1994) Mycalolide B, a novel actin depolymerizing agent. *J. Biol. Chem.* **269**, 29710-29714
50. Mezgueldi, M., Fattoum, A., Derancourt, J., and Kassab, R. (1992) Mapping of the functional domains in the amino-terminal region of calponin. *J. Biol. Chem.* **267**, 15943-15951
51. Lu, F.W.M., Freedman, M.V., and Chalovich, J.M. (1995) Characterization of calponin binding to actin. *Biochemistry* **34**, 11864-11871
52. Takahashi, K. and Nadal-Ginard, B. (1991) Molecular cloning and sequence analysis of smooth muscle calponin. *J. Biol. Chem.* **266**, 13284-13288
53. Strasser, P., Gimona, M., Moessler, H., Herzog, M., and Small, J.V. (1993) Mammalian calponin: Identification and expression of genetic variants. *FEBS Lett.* **330**, 13-18
54. Applegate, D., Feng, W., Green, R.S., and Taubman, M.B. (1994) Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J. Biol. Chem.* **269**, 10683-10690
55. Maguchi, M., Nishida, W., Kohara, K., Kuwano, A., Kondo, I., and Hiwada, K. (1995) Molecular cloning and gene mapping of human basic and acidic calponins. *Biochem. Biophys. Res. Commun.* **217**, 238-244
56. Masuda, H., Tanaka, K., Takagi, M., Ohgami, K., Sakamaki, T., Shibata, N., and Takahashi, K. (1996) Molecular cloning and characterization of human non-smooth muscle calponin. *J. Biochem.* **120**, 415-424
57. Flynn, G., Joly, J.C., and Purich, D.L. (1987) The 28,000 M_r microtubule-binding domain of microtubule-associated protein-2 also contains a neurofilament-binding site. *Biochem. Biophys. Res. Commun.* **148**, 1453-1459
58. Lewis, S.A., Wang, D., and Cowan, N.J. (1988) Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. *Science* **242**, 936-939
59. Aizawa, H., Emori, Y., Murofushi, H., Kawasaki, H., Sakai, H., and Suzuki, K. (1990) Molecular cloning of a ubiquitously distributed microtubule-associated protein with M_r 190,000. *J. Biol. Chem.* **265**, 13849-13855
60. Lee, J.C., Tweedy, N., and Timasheff, S.N. (1978) In vitro reconstitution of calf brain microtubules: Effects of macromolecules. *Biochemistry* **17**, 2783-2790
61. Lopez, R.A., Arce, C.A., and Barra, H.S. (1990) Effect of polyanions and polycations on detyrosination of tubulin and microtubules at steady state. *Biochim. Biophys. Acta* **1039**, 209-217
62. Serrano, L., Avila, J., and Maccioni, R.B. (1984) Controlled proteolysis of tubulin by subtilisin: Localization of the site for MAP2 interaction. *Biochemistry* **23**, 4675-4681
63. Vera, J.C., Rivas, C.I., and Maccioni, R.B. (1988) Antibodies to synthetic peptides from the tubulin regulatory domain interact with tubulin and microtubules. *Proc. Natl. Acad. Sci. USA* **85**,

6763-6767
64. Kotani, S., Nishida, E., Kumagai, H., and Sakai, H. (1985) Calmodulin inhibits interaction of actin with MAP2 and tau, two major microtubule-associated proteins. *J. Biol. Chem.* **260**, 10779-10783
65. Yamauchi, T. and Fujisawa, H. (1988) Regulation of the interaction of actin filaments with microtubule-associated protein 2 by calmodulin-dependent protein kinase II. *Biochim. Biophys. Acta* **968**, 77-85
66. Miyasaka, H., Okabe, S., Ishiguro, K., Uchida, T., and Hirokawa, N. (1993) Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by τ protein kinase II. *J. Biol. Chem.* **268**, 22695-22702