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The binding of nonmuscle caldesmon from brain to microtubules

Regulations by Ca^{2+}-calmodulin and cdc2 kinase

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Nonmuscle caldesmon from bovine brain bound to microtubules with a stoichiometry of five tubulin dimers to one molecule of caldesmon with values of K_a 4.5×10^5 M^{-1}. The binding of caldesmon to microtubules was inhibited in the presence of Ca^{2+} and calmodulin. The phosphorylation of caldesmon by cdc2 kinase also eliminated the microtubule-binding activity. These results suggest that caldesmon may play a physiological role in the functions of microtubules.

Caldesmon; Actin; Microtubule; Calmodulin; cdc2 kinase

1. INTRODUCTION

Caldesmon is an actin-binding protein that regulates the actin–myosin interaction by a Ca^{2+}-calmodulin system in smooth muscle cells ([1] for review) and nonmuscle cells [2]. It has been detected in a variety of vertebrate cells, having an apparent molecular weight on SDS-PAGE of about 80 kDa in nonmuscle cells and about 150 kDa in smooth muscle cells [1]. The actin-binding activity of caldesmon is affected not only by the Ca^{2+}-calmodulin system but also by the phosphorylation with cdc2 kinase [3,4]. Caldesmon also binds directly to tropomyosin [5–7] and to myosin [8,9]. However, there are no reports about the interaction of caldesmon with microtubules.

In this study, we demonstrate a novel property of nonmuscle caldesmon: it can act as a microtubule associated protein that is regulated both by the Ca^{2+}-calmodulin system and by phosphorylation with cdc2 kinase.

2. MATERIALS AND METHODS

2.1 *Proteins*

Nonmuscle caldesmon, 80 kDa on SDS-PAGE and 60 kDa from deduced amino acid sequence of cDNA [1], was purified from bovine brain by the methods of Yamashiro-Matsumura et al. [10] and used as nonmuscle caldesmon. Tubulin was purified from porcine brain by the method of Shelanski et al. [11] and used as microtubule after polymerization. Human cdc2 kinase that was partially purified from HeLa cells [4] and monoclonal antibody that was raised against smooth muscle caldesmon as designated SM12 [10] were generously provided by Dr. Fumio Matsumura (Rutgers University, NJ, USA). Calmodulin from bovine brain was purchased from Sigma (St. Louis, MO, USA).

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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2.2. *Assay for binding to microtubules*

Taxol-stabilized microtubules (10 μM) were mixed with various concentrations of nonmuscle caldesmon (0–8 μM) in 100 mM MES (pH 6.8), 1 mM GTP, 1 mM DTT, 1 mM $MgCl_2$, and 15 mM KCl and then incubated for 30 min at room temperature. The mixtures were centrifuged in an airfuge (Beckman, Palo Alto, CA, USA) at 140,000 × *g* for 20 min. The amounts of the proteins in supernatants and pellets were analyzed by SDS-PAGE and quantitated by densitometry (Joyce Loebl, Malden, MA, USA).

2.3. *Phosphorylation of caldesmon by cdc2 kinase*

Nonmuscle caldesmon (2.5 μM) was incubated with cdc2 kinase at 30°C for 40 min in 10 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT, 10 μg/ml chymostatin, 10 μg/ml leupeptin and 10 μg/ml pepstatin in the presence or absence of 1 mM ATP containing 200 mCi/mmol [γ-^{32}P]ATP (Amersham, Buckingham, UK). Reactions were terminated by boiling the mixtures for 10 min, and the mixtures were then centrifuged at 14,000 × *g* for 10 min. Supernatants were used as phosphorylated caldesmon and control caldesmon, respectively.

2.4. *Other procedures*

Protein concentrations were determined by the methods of Lowry et al. [12], with bovine serum albumin as a standard. SDS-PAGE was performed as described previously [13]. Western blots were performed according to the methods of Towbin et al. [14].

3. RESULTS

3.1. *Binding of nonmuscle caldesmon to microtubules*

We subjected a crude fraction of microtubules to

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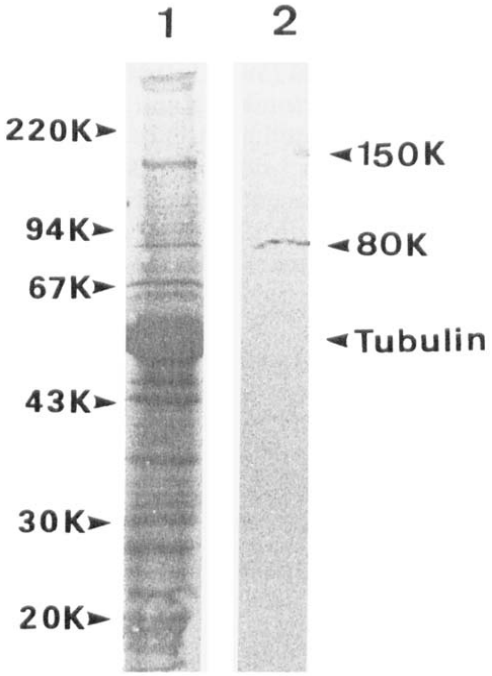

Fig. 1. A crude fraction of microtubules from bovine brain contains 150 kDa and 80 kDa peptides that cross-react with an antibody raised against smooth muscle caldesmon. Bovine brain was homogenized with 0.8 volumes of 100 mM MES (pH 6.8), 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP, and the homogenate was centrifuged at 12,000 × *g* for 10 min. The supernatant was incubated at 37°C for 30 min to polymerize the tubulin and then centrifuged at 100,000 × *g* for 1 h. The pellet was used as the crude microtubule fraction. The crude microtubule fraction (30 μg) was analyzed by SDS-PAGE and blotted. (Lane 1) Amido black staining; (lane 2) corresponding immunoblot stained with a caldesmon-specific antibody.

analysis by Western blotting using an antibody raised against smooth muscle caldesmon. As shown in Fig. 1, 150 kDa and 80 kDa polypeptides cross-reacted with the antibody. The molecular weights of these peptides were identical to those of brain caldesmons [15], indicating that brain caldesmons are co-purified with microtubules.

To confirm whether brain caldesmons belong to the family of proteins that are associated with microtubules, we examined the binding of caldesmon to microtubules in vitro. As shown in Fig. 2, the amount of nonmuscle caldesmon that bound to microtubules gradually increased as the concentration of caldesmon was increased. The binding constant was $4.5 \times 10^5\ M^{-1}$. The binding was saturated at a stoichiometry of nonmuscle caldesmon to tubulin dimers of 0.18:1, if we assume the molecular weight of the tubulin dimers and nonmuscle caldesmon are 100 kDa [16] and 60 kDa [1], respectively.

3.2. *Regulation by Ca^{2+}-calmodulin of the microtubule-binding activity of nonmuscle caldesmon*

When we mixed microtubules with nonmuscle caldesmon and calmodulin in the presence of EGTA, 56%

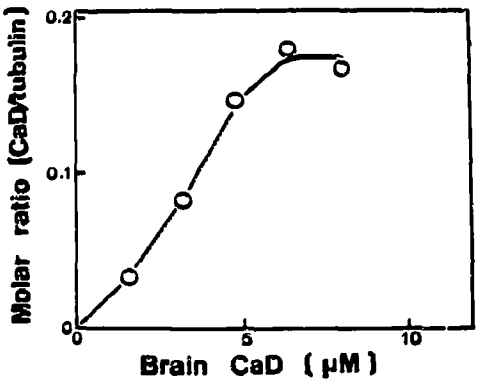

Fig. 2. Nonmuscle caldesmon (CaD) from bovine brain binds to microtubules. Microtubules were mixed CaD, and then the mixtures were centrifuged at 140,000 × *g* for 20 min. Supernatants and pellets were subjected to SDS-PAGE and the amounts of CaD and tubulin were determined by densitometry. Ordinate: molar ratio of the amount of nonmuscle caldesmon that co-sedimented with microtubules. Abscissa: the concentration of nonmuscle caldesmon that was mixed with microtubules.

of the caldesmon bound to the microtubules (Fig. 3, lane 2), a level similar to that observed in the absence of calmodulin. By contrast, only 3% of the caldesmon bound to microtubules in the presence of Ca^{2+} (Fig. 3, lane 4). These results suggest that the binding activity of caldesmon to microtubules is inhibited by the Ca^{2+}-calmodulin system.

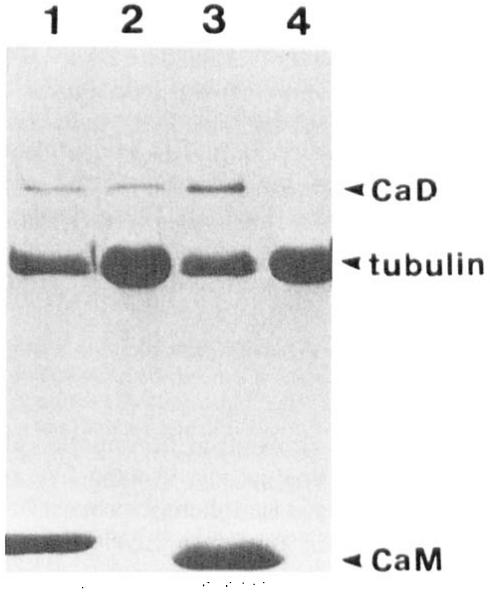

Fig. 3. The binding of nonmuscle caldesmon (CaD) to microtubules is inhibited in the presence of Ca^{2+} and calmodulin (CaM). Taxol-stabilized microtubules (10 μM) were incubated with 1.6 μM CaD and 10 μM CaM in the presence of EGTA or Ca^{2+}, and then mixtures were centrifuged at 140,000 × *g* for 20 min. The supernatant and pellets were separated and analyzed by SDS-PAGE. (Lanes 1 and 2) Supernatant and pellet, respectively, obtained with 1 mM EGTA; (lanes 3 and 4) supernatant and pellet, respectively, obtained with 0.1 mM Ca^{2+}.

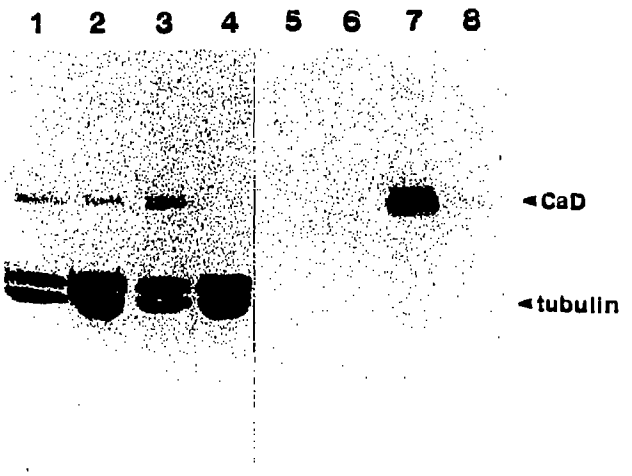

Fig. 4. Phosphorylation of nonmuscle caldesmon (CaD) by cdc2 kinase inhibits the microtubule-binding activity of caldesmon. CaD was phosphorylated by human cdc2 kinase with [γ-^{32}P]ATP. As a control, CaD was incubated with cdc2 kinase in the absence of ATP. The control and phosphorylated caldesmons (1.6 μM) were mixed with 10 μM microtubules. Mixtures were centrifuged at 140,000 × g for 20 min and subjected to SDS-PAGE. (Lanes 1 and 2) Staining with Coomassie blue of supernatant and pellet, respectively, obtained with the phosphorylated caldesmon; (lanes 3 and 4) staining with Coomassie blue of supernatant and precipitate, respectively, obtained with the control caldesmon; (lanes 5–8) autoradiography corresponding to lanes 1–4.

3.3. *Effects of phosphorylation of nonmuscle caldesmon on the binding of caldesmon to microtubules*

We examined the effects of phosphorylation by cdc2 kinase on the binding of nonmuscle caldesmon to microtubules. Nonmuscle caldesmon was phosphorylated by cdc2 kinase, and then the phosphorylated caldesmon was mixed with microtubules and pelleted with the microtubules by centrifugation. As shown in Fig. 4, 50% of the control caldesmon bound to microtubules (lane 2) while only 4% of the phosphorylated caldesmon bound to microtubules (lane 4). These results suggest that phosphorylation of caldesmon by cdc2 kinase causes nonmuscle caldesmon to dissociate from microtubules.

4. DISCUSSION

The present study shows that nonmuscle caldesmon has microtubule-binding activity in vitro. We are confident that this activity is an inherent property of native caldesmon because it is subject to physiological ways of control such as Ca^{2+}-calmodulin system ([17] for review) and phosphorylation by cdc2 kinase ([18] for review).

There are some indications of interactions between actin filaments and microtubules in vivo. For example, the disruption of actin networks by cytochalasin D inhibits the motility of centrosomes in human leukocytes [19]. MAP2 is suggested to be a cross-linker between microtubules and actin filaments because MAP2 can bind both to microtubules and to actin filaments in vitro [20,21]. Thus, it is provable that nonmuscle caldesmon may also work as a microtubule-associated protein to cross-link between microtubules and actin filaments.

However, the immunofluorescence studies with nonmuscle cells show that caldesmon is detectable in association with actin filaments but not with microtubules [1]. At this point, we do not know the reason of the disagreement of the studies with the present results in vitro. Further characterization of the microtubule-binding activity of caldesmon both in situ and in vitro remains to be demonstrated.

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