THE CALMODULIN-BINDING PROTEIN IN MICROTUBULES IS TAU FACTOR

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1. Introduction

The discovery of Ca^{2+}-activatable cyclic nucleotide phosphodiesterase [1] and subsequent demonstration of a protein factor which confers Ca^{2+}-sensitivity upon this enzyme [2,3] coincided with the discovery of a protein activator of brain phosphodiesterase [4]. Since then, this protein factor, nowadays called calmodulin, has been shown to cause Ca^{2+}-dependent activation of a variety of enzymes [5]. Moreover, calmodulin has been shown to interact with a number of proteins apparently devoid of enzyme activities by forming complexes with them [6–10]. Although possibilities are raised that these calmodulin-binding proteins may perform yet unknown functions in the cell, the biological activities of these proteins have not been clarified, in spite of various attempts.

We have purified a 150 000-M_r calmodulin-binding protein (caldesmon) from chicken gizzard muscle [11,12]. Caldesmon associates with F-actin when not interacting with calmodulin. Therefore, in the presence of the 3 protein species, calmodulin, caldesmon and F-actin, the concentration of Ca^{2+} acts as a flip–flop switch toward the formation of the caldesmon · calmodulin complex at the increased level ($>10^{-6}$ M) and toward the formation of the caldesmon · F-actin complex at the decreased level. This is the first demonstration of the Ca^{2+}-dependent regulation of calmodulin on the cytoskeleton and contractile system of eucaryotic cells other than striated muscles. This communication deals with the interaction of calmodulin with another cellular fibrous structure, microtubules. Calmodulin was shown to associate in a Ca^{2+}-dependent fashion with one species of microtubule-associated proteins called 'tau' [13].

Abbreviations: MES, 2-(*N*-morpholino)ethane sulfonic acid; EGTA, ethyleneglycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPs, microtubule-associated proteins; HM_r-MAPs, higher molecular mass microtubule-associated proteins; M_r, relative molecular mass; PC-tubulin, phosphocellulose-purified tubulin; SDS, sodium dodecyl sulfate

2. Method

2.1. *Preparation of proteins from the microtubules*

The following procedures were carried out at 4°C unless otherwise specified. Microtubule protein was obtained from bovine brain by the temperature-dependent polymerization–depolymerization cycles in [14]. The second cycled-pellet of microtubules were frozen and stored at −70°C until use. The purified microtubule protein (3XMT) was prepared by an additional depolymerization–polymerization cycle of the frozen sample without the addition of glycerol. Crude MAPs fraction was prepared by a modification of the method in [15] as follows: 3XMT pellets were resuspended in polymerization medium (100 mM MES buffer (pH 6.8), 1 mM EGTA and 1 mM $MgCl_2$) containing 1 M NaCl and 2 mM dithiothreitol. The suspension was kept in ice for 30 min and then immersed in a boiling water bath for 5 min. It was then quickly chilled down and centrifuged at 200 000 × *g* for 20 min. The supernatant thus obtained was concentrated to ~8 mg protein/ml through an Amicon PM-10 membrane filter and then the solution medium changed to 20 mM MES (pH 6.8), 80 mM KCl, 1 mM 2-mercaptoethanol and 1 mM $MgCl_2$ (medium A) plus 0.1 mM GTP and 1 mM EGTA through a column of Sephadex G-25. The final solution (crude MAPs) was stored at −70°C. The crude MAPs contained HM_r-MAPs and tau factor as the major constituents (fig.1). These two protein species (HM_r-MAPs and tau) were separated from each other by column chromatog-

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raphy as follows: The crude MAPs (~8 mg protein) were applied to a 1.2 × 44.5 cm column of Sephadex G-200 equilibrated with medium A. The column was eluted with medium A and two proteins peaks were obtained. Peak 1 eluted near the void volume contained HM_r-MAPs and peak 2 eluted thereafter consisted mainly of tau proteins. HM_r-MAPs were further purified from peak 1 by an additional gel filtration using a column of Sepharose 4B. Tau proteins were purified by a rechromatography of the peak 2 using the same column of Sephadex G-200. Tubulin was purified from the 3XMT by a phosphocellulose column chromatography as in [16]. PC-tubulin thus obtained was further purified by gel filtration as follows: 1 ml PC-tubulin (5–10 mg protein/ml) was applied to a 1.2 × 50 cm column of Sephadex G-200 equilibrated with medium A plus 0.1 mM GTP and 1 mM EGTA. The column was eluted with the same medium and 2 peaks were yielded. The second peak which appeared at M_r ~ 120 000 was collected, concentrated and used as the tubulin dimer.

2.2. *Other methods*

Calmodulin was purified from bovine brain as in [17]. Calmodulin–Sepharose 4B conjugate was prepared as in [18]. [^{3}H]Calmodulin was prepared from bovine brain calmodulin as in [19]. The concentrations of calmodulin and protein were determined as detailed in [20]. Electrophoreses of proteins were carried out following [21] in 0.1% SDS–7.5% polyacrylamide gels. Proteins were located by staining with Coomassie brilliant blue.

3. Results

HM_r-MAPs, tau factor and the purified tubulin (PC-tubulin or tubulin dimer) were prepared from microtubules purified by assembly–disassembly cycles as in [14]. The result is summarized in fig.1. As reported in [16,22], HM_r-MAPs were resolved into several related proteins with M_r ~ 300 000 on SDS–polyacrylamide gel electrophoresis. Four closely spaced protein bands of app. M_r 55 000–62 000 have been reported for tau factor [13,23]. Our results are consistent with those observations. Each of the protein species obtained was completely free from the contamination of the other species.

We then examined the binding of these protein species with calmodulin. In fig.2, each of these protein species as well as crude MAPs was mixed with [^{3}H]calmodulin and then applied to a Sephadex G-100 column. When the column was eluted with a medium containing EGTA, only one peak of the unbound [^{3}H]calmodulin appeared in each of the cases. With Ca^{2+} in the medium, however, a shift of the [^{3}H]calmodulin peak toward the higher M_r region occurred only with tau factor (fig.2B) but not with HM_r-MAPs (fig.2C) or PC-tubulin (fig.2D). The result indicates the Ca^{2+}-dependent formation of a protein complex between the tau factor and calmodulin. With the crude MAPs containing both HM_r-MAPs and tau factor, essentially the same result was obtained (fig.2A). The ability of the crude MAPs to interact with [^{3}H]calmodulin was completely abolished after the crude MAPs were treated with trypsin (450 μg crude MAPs were incubated with 50 μg trypsin for 30 min at 30°C, then 150 μg soybean trypsin inhibitor added to the mixture) (not shown). The Ca^{2+}-dependent binding of tau factor with calmodulin was further confirmed by an affinity chromatography using calmodulin–Sepharose. Only tau factor but not HM_r-MAPs or PC-tubulin was retained in the column with a medium containing Ca^{2+}, and subsequently eluted

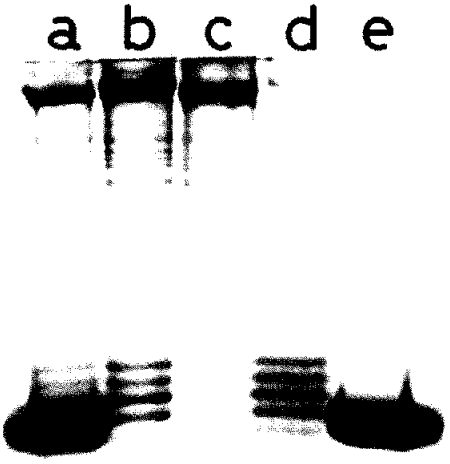

Fig.1. SDS–Polyacrylamide gel electrophoresis of the microtubule proteins: (a) microtubules, purified by 3 assembly–disassembly cycles (3XMT); (b) crude MAPs fraction containing both HM_r-MAPs and tau factor; (c) HM_r-MAPs; (d) tau factor; (e) tubulin dimer.

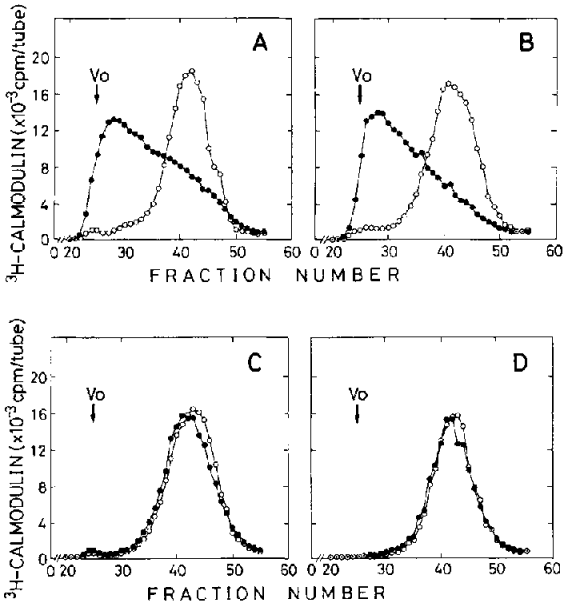

Fig.2. Test for the binding of calmodulin with microtubule proteins using Sephadex G-100. Microtubule proteins to be examined (450 μg) were incubated with 10 μg [^{3}H]calmodulin (spec. act. 500 mCi/mmol) for 30 min at 30°C in a medium consisting of 20 mM MES (pH 6.8), 80 mM KCl, 1 mM $MgCl_2$, 0.1 mM GTP, 1 mM 2-mercaptoethanol in the presence of either 1 mM $CaCl_2$ plus 1 mM EGTA (free $Ca^{2+} > 10^{-5}$ M) (–•–), or 1 mM EGTA (–○–). At the end of the incubation, the mixture was chilled down in ice and applied to a column (0.9 × 35 cm) of Sephadex G-100 equilibrated with the corresponding medium used for the incubation. The column was eluted with the same medium and fractions (0.4 ml each) thus eluted were determined for their radioactivity. (A) Crude MAPs fraction; (B) tau factor; (C) HM_r-MAPs; (D) PC-tubulin.

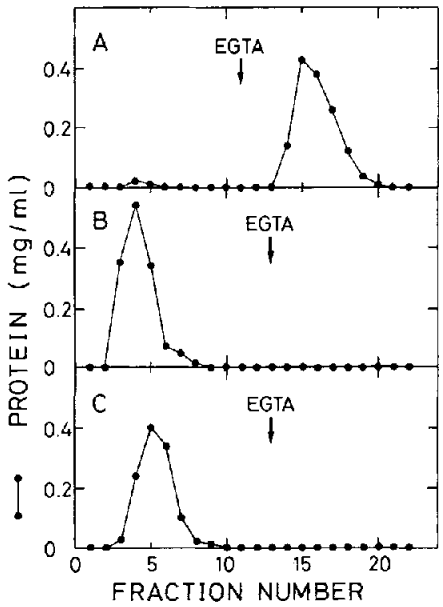

Fig.3. Test for the binding of calmodulin with microtubule proteins using a calmodulin–Sepharose column: 360 μg each of tau factor (A); HM_r-MAPs (B) and tubulin dimer (C), dissolved in a medium consisting of 20 mM MES (pH 6.8), 80 mM KCl, 1 mM $MgCl_2$, 0.1 mM GTA, 1 mM mercaptoethanol and 1 mM $CaCl_2$ plus 1 mM EGTA (free $Ca^{2+} > 10^{-5}$ M), was applied to a column (0.5 × 2 cm) of calmodulin–Sepharose 4B equilibrated with the above medium. The column was eluted with the same medium and at an arrow indicated in the figure, 2 mM EGTA in place of 1 mM $CaCl_2$ plus 1 mM EGTA was added. Fractions of 0.2 ml each were collected and their protein concentrations were determined as in [22].

from the column with EGTA (fig.3). The 4 protein bands (M_r 55 000–62 000) corresponding to tau factor were retained by calmodulin–Sepharose and eluted with EGTA (not shown).

4. Discussion

In [23] anti-calmodulin immunofluorescence was concentrated in the half-spindle of the mitotic apparatus during metaphase–anaphase and a role suggested for calmodulin in the control of microtubule assembly–disassembly. Therefore, it is of particular importance to investigate the interaction of calmodulin with the microtubule proteins.

Accumulating evidence now suggests that the microtubules in vivo are constituted of not only tubulin but also several non-tubulin accessory proteins:

(i) These accessory proteins are present in roughly constant stoichiometry with tubulin during assembly–disassembly cycles of the microtubules in vitro [24,25].

(ii) Immunofluorescent localizations of these proteins coincided with cytoplasmic microtubules [26–28].

For these non-tubulin accessory proteins, two species have been characterized; HM_r-MAPs of ~300 000 M_r [24] and a family of 4 closely related lower M_r (55 000–62 000) proteins named tau (τ) factor [25].

Here, three protein species from the microtubules, i.e., HM_r-MAPs, tau and tubulin, were isolated and separated from each other by column chromatographies and each was examined for its ability to bind calmodulin in the presence of Ca^{2+}. Special precau-

tions were taken against cross contaminations between each protein species. Ca^{2+}-dependent binding between protein and [^{3}H]calmodulin was studied by two different means, i.e., a gel filtration column chromatography using Sephadex G-100 and an affinity column chromatography using calmodulin–Sepharose. The results clearly establish that only tau factor but not purified tubulin or HM_r-MAPs is capable of binding to calmodulin in a Ca^{2+}-dependent manner. This finding is inconsistent with reports [29,30] in which the purified tubulin dimer was shown to be the calmodulin-binding protein. The reason for this discrepancy is still unclear.

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