

# Identification of a new 84/82 kDa calmodulin-binding protein, which also interacts with actin filaments, tubulin and spectrin, as synapsin I

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A new 84/82 kDa calmodulin-binding protein, which also interacts with actin filaments, tubulin and spectrin, was purified from the bovine synaptosomal membrane. The binding of calmodulin to this protein was  $\text{Ca}^{2+}$ -dependent, and was inhibited by trifluoperazine, the association constant being calculated to be  $2.2 \times 10^6 \text{ M}^{-1}$ . Maximally, 1 mol of calmodulin bound to 1 mol of the purified protein. This protein was phosphorylated by both kinase II ( $\text{Ca}^{2+}$ - and calmodulin-dependent kinase) and cyclic AMP-dependent kinase. In addition, antibody against this protein was demonstrated to have an immunological crossreactivity with synapsin I in the synaptosomal membrane.

Calmodulin-binding protein; Synapsin I; Actin filament; Tubulin; Spectrin;  $\text{Ca}^{2+}$  dependence; Calmodulin dependence; Kinase II

## 1. INTRODUCTION

Calmodulin is one of the ubiquitous intracellular  $\text{Ca}^{2+}$ -mediators in many biological processes. It is now generally accepted that calmodulin activates several intracellular enzymes in a  $\text{Ca}^{2+}$ -dependent manner (reviews [1,2]). In contrast, we purified or identified ten species of calmodulin-binding proteins which were devoid of enzyme activities. These proteins were shown to interact with cytoskeletal elements (actin filaments, tubulin, spectrin and calspectin) and to regulate their functions ([4],

review [3]). Since the synapse is a well-developed and highly specialized cytoarchitecture composed of cytoskeletal elements, it is a promising material to survey for cytoskeletal elements and targets of calmodulin which are involved in the regulation of cytoskeletal function. Actually, we have purified calspectin [5,6] and cytosynalin [7] from the brain synaptosomal membrane. In this paper, we demonstrate the purification of a new calmodulin-binding protein which also interacts with actin filaments, tubulin and spectrin. We then discuss the identification of this protein with synapsin I or erythrocyte protein 4.1.

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## 2. MATERIALS AND METHODS

### 2.1. Materials

Calmodulin was coupled to cyanogen-activated Sepharose 4B by the method of Klee and Krinks [8]. Microtubules were purified from bovine brain [9]. Spectrin [10] and protein 4.1 [11] were purified from human erythrocytes. Kinase II purified from

rat brain was a generous gift from Drs H. Fujisawa and T. Yamauchi [12]. The catalytic subunit of cyclic AMP-dependent kinase was purchased from Sigma. The Triton X-100 treated synaptosomal membrane was prepared from bovine cerebral cortices [13]. F-Actin was prepared from rabbit skeletal muscle as described in [14]. Iodination of a calmodulin-binding protein using  $^{125}\text{I}$  was prepared by the lactoperoxidase method [15]. The production of antibody against the purified calmodulin-binding protein and the preparation of the IgG fraction were carried out according to the method described elsewhere [7].

## 2.2. Analytical methods

The detection of the  $^{125}\text{I}$ -labeled 84/82 kDa protein binding to cytoskeletal elements was carried out by the overlay method [7]. The interaction of the purified protein with F-actin was determined by the cosedimentation method [14]. The assay conditions for phosphorylation of the purified proteins or rat synaptosomal membrane are described in the corresponding legends. SDS-PAGE using 9% acrylamide was carried out in the buffer system of Laemmli [16]. Protein concentrations were determined by the method of Lowry et al. [17].

## 3. RESULTS AND DISCUSSION

The Triton-treated synaptosomal membrane starting with 600 g of bovine cerebral cortices was dispersed with 250 ml of an extraction buffer (0.8 M KCl, 5 mM Mes, pH 7.2, 0.1 mM EGTA, 0.1 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride, 0.1 mM diisopropyl fluorophosphate, 1  $\mu\text{g}/\text{ml}$  of leupeptin and 1  $\mu\text{g}/\text{ml}$  of pepstatin A). After incubation at 37°C for 30 min, the suspension was centrifuged at  $105000 \times g$  for 60 min. The clear supernatant (fig.1A, lane b) was made 1 M KCl and 5 mM  $\text{MgCl}_2$  by the addition of solid KCl and 2 M  $\text{MgCl}_2$ , and applied to a hydroxyapatite column (3  $\times$  3 cm) equilibrated with 1 M KCl, 5 mM Mes, pH 7.2, 5 mM  $\text{MgCl}_2$ , 0.1 mM EGTA and 0.1 mM DTT. The flow-through fractions were collected (fig.1A, lane c), and exhaustively dialyzed against buffer A (5 mM Mes, pH 7.2, 0.1 mM EGTA and 0.1 mM DTT) plus 20 mM KCl. After dialysis, the sample was applied to a phosphocellulose column (1.2  $\times$

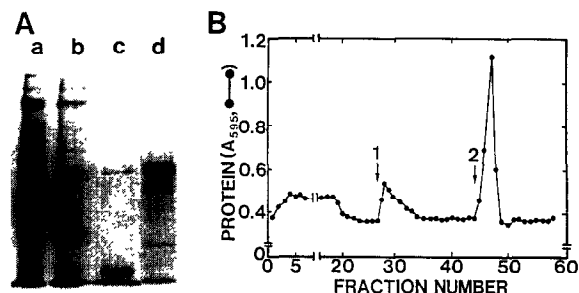


Fig.1. (A) SDS-PAGE of the purification procedures. Lanes: a, Triton-treated synaptosomal membrane; b, high salt extract; c, flow-through fraction of the hydroxyapatite column chromatography; d, after the phosphocellulose column chromatography. (B) Purification of the 84/82 kDa protein by the calmodulin-affinity chromatography. (1) Washing with buffer A plus 0.3 mM  $\text{CaCl}_2$  and 500 mM KCl; (2) elution with buffer A plus 1 mM EGTA and 500 mM KCl. (Inset) SDS-PAGE of the purified protein (fraction no.47).

2 cm) equilibrated with buffer A plus 20 mM KCl. Then, the column was developed with a linear gradient of 20–600 mM KCl in buffer A. The fractions corresponding to 400 mM KCl were collected (fig.1A, lane d), and dialyzed against buffer A plus 100 mM KCl. The dialyzed sample was adjusted to 0.2 mM  $\text{CaCl}_2$  by the addition of 100 mM  $\text{CaCl}_2$  and applied to a calmodulin-coupled Sepharose 4B column (fig.1B). Finally, the protein retained on the column was eluted with buffer A plus 1 mM EGTA and 500 mM KCl. The purified protein was composed of the doublet with molecular masses of 84 and 82 kDa determined by SDS-PAGE (inset of fig.1B). The molar ratio of 84 and 82 kDa polypeptides in seven different preparations was 0.45–0.55:1. Ultimately, 1–2 mg of this protein was obtained from 600 g of bovine cerebral cortices.

We confirmed the interaction of the 84/82 kDa protein with calmodulin using the zero-length crosslinker (1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide, EDC) [18]. The analysis of the crosslinked products on SDS gels revealed that the complex of  $^{125}\text{I}$ -labeled calmodulin with the 84/82 kDa protein is formed only in the presence of  $\text{Ca}^{2+}$ , but not in the absence of  $\text{Ca}^{2+}$  or in the presence of both  $\text{Ca}^{2+}$  and trifluoperazine (not shown). Based on Scatchard plot analysis (fig.2), the association constant of calmodulin to synapsin

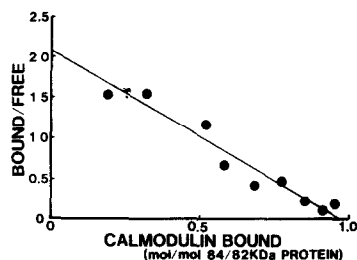


Fig.2. Scatchard plot analysis for the calmodulin binding to the 84/82 kDa protein. Assay conditions were as follows: 20 mM imidazole·HCl, pH 7.2, 100 mM KCl, 0.1 mM DTT, 2 mM  $\text{MgCl}_2$ , 0.2 mM  $\text{CaCl}_2$ , 75  $\mu\text{g/ml}$  of the 84/82 kDa protein and various concentrations of  $^3\text{H}$ -labeled calmodulin (7–115  $\mu\text{g/ml}$ ). The binding assays were carried out according to our method [19].

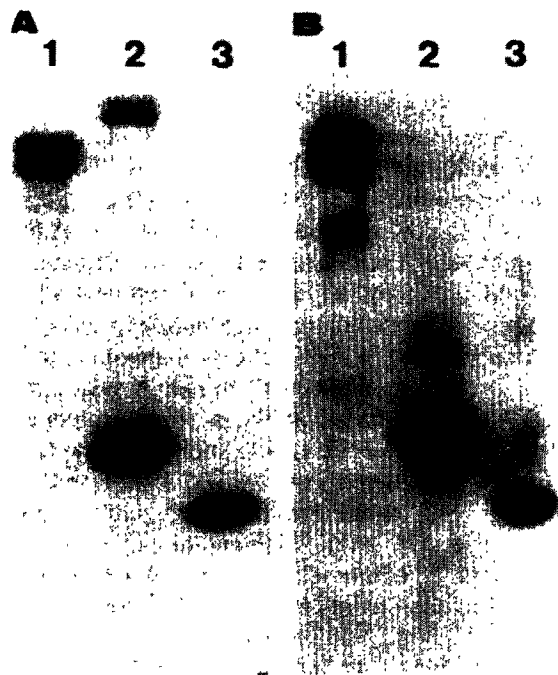


Fig.3. Binding of the  $^{125}\text{I}$ -labeled 84/82 kDa protein to cytoskeletal elements. The proteins separated by SDS gel were electrophoretically transferred to nitrocellulose membrane. The  $^{125}\text{I}$ -labeled 84/82 kDa protein binding was determined by the overlay method. The  $^{125}\text{I}$ -labeled 84/82 kDa protein bound to the transblotted cytoskeletal proteins was detected by autoradiography. (A) Coomassie brilliant blue-stained SDS gels; (B) autoradiograms of the  $^{125}\text{I}$ -labeled 84/82 kDa protein binding. Lanes: 1, spectrin; 2, microtubules; 3, actin.

I was calculated to be  $2.2 \times 10^6 \text{ M}^{-1}$  by the alkali PAGE binding assay using  $^3\text{H}$ -labeled calmodulin as a ligand [19]. Maximally, 1 mol of calmodulin bound to 1 mol of synapsin I (not shown). The 84/82 kDa protein binding to cytoskeletal elements was also investigated. The  $^{125}\text{I}$ -labeled 84/82 kDa protein was bound to transblotted spectrin, tubulin and actin, respectively (fig.3). However, an excess amount of calmodulin had no effect on these bindings regardless of the  $\text{Ca}^{2+}$  concentrations (not shown). In our preliminary experiment, the 84/82 kDa protein possessed the binding ability with brain caldesmon determined by the overlay method. Furthermore, we investigated the 84/82 kDa protein-actin filament interaction by the cosedimentation method. The 84/82 kDa protein bound to F-actin in a dose-dependent manner, and 1 mol of the 84/82 kDa protein maximally bound to 7 mol of actin monomer (fig.4).  $\text{Ca}^{2+}$  and/or calmodulin had no effect on the bindings. By low-shear viscometry and electron microscopy, the 84/82 kDa protein did not show the crosslinking activity of actin filaments.

Considering the similarity with regard to the localization in the synaptosomal membrane, molecular masses of the doublet and binding abilities to spectrin and tubulin, we made an attempt to identify the purified doublet as synapsin

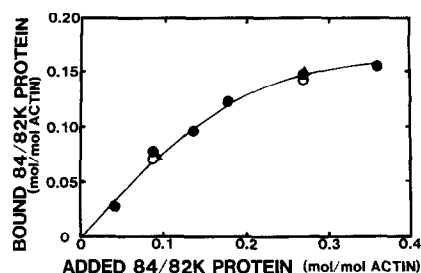
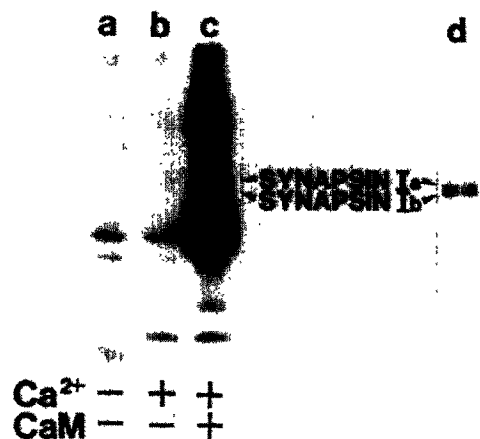


Fig.4. Binding of the 84/82 kDa protein to F-actin. Assay conditions were as follows: 0.11 mg/ml of F-actin, 20 mM imidazole·HCl, pH 7.2, 100 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.1 mM DTT and indicated amounts of the 84/82 kDa protein in the presence of 1 mM EGTA (●), 0.2 mM  $\text{CaCl}_2$  (○), or 0.2 mM  $\text{CaCl}_2$ /40  $\mu\text{g}$  per ml of calmodulin (▲). After incubation at  $30^\circ\text{C}$  for 30 min, the reaction mixtures were centrifuged at  $150000 \times g$  for 30 min. The precipitated fractions were subjected to SDS-PAGE. The amount of the 84/82 kDa protein bound to F-actin was quantified by densitometric scanning of SDS gel.



I by biochemical and immunological methods. Greengard and his associates [20,21] first reported that synapsin I is one of the major substrates for kinase II and cyclic AMP-dependent kinase in the synaptosomal membrane. In agreement with their

Fig.5. Identification of the 84/82 kDa protein as synapsin I. The rat synaptosomal membrane prepared according to the method of Schulman and Greengard [21] was endogenously phosphorylated. Buffer conditions were the same as described in table 1. Protein concentration used were as follows: the rat synaptosomal membrane (0.5 mg/ml) and calmodulin (40  $\mu$ g/ml). After incubation at 30°C for 1 min, the synaptosomal membrane proteins were separated by SDS gel and the phosphoproteins were detected by autoradiography. The immunoprecipitation with anti-84/82 kDa protein antibody was carried out [25] using the phosphorylated synaptosomal membrane proteins under the same assay conditions in the presence of  $\text{Ca}^{2+}$  and calmodulin (corresponding to lane c). The immunoprecipitated phosphoproteins were detected by autoradiography (lane d).

observation, synapsin I (Ia and Ib) in the synaptosomal membrane was phosphorylated by the endogenous kinase in a  $\text{Ca}^{2+}$ - and calmodulin-dependent manner (fig.5, lanes a-c). Phosphorylated synapsin I (Ia and Ib) in the synaptosomal

Table 1

Phosphorylation of the 84/82 kDa protein and protein 4.1 by kinase II and catalytic subunit of cyclic AMP-dependent kinase

Kinase	Substrate	Assay condition		$^{32}\text{P}$ incorporation (mol/mol)
		$\text{Ca}^{2+}$	Calmodulin	
Kinase II	84/82 kDa protein	-	-	$0.03 \pm 0.004$
		+	-	$0.04 \pm 0.003$
		+	+	$3.29 \pm 0.13$
	protein 4.1	-	-	0.00
		+	-	$0.01 \pm 0.002$
		+	+	$0.02 \pm 0.06$
Cyclic AMP-dependent kinase	84/82 kDa protein	-	-	$1.28 \pm 0.06$
	protein 4.1	-	-	$1.18 \pm 0.04$

The incubation medium was as follows: 5 mM Mes, pH 7.2, 10 mM  $\text{MgCl}_2$ , 1 mM DTT, 60 mM KCl, 0.1 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (1 Ci/mmol) and 0.2 mM  $\text{CaCl}_2$  or 1 mM EGTA. Protein concentrations used were: 84/82 kDa protein (90  $\mu$ g/ml), protein 4.1 (90  $\mu$ g/ml), kinase II (2  $\mu$ g/ml), calmodulin (1  $\mu$ g/ml) and catalytic subunit of cyclic AMP-dependent kinase (5  $\mu$ g/ml). After incubation for 30 min at 37°C, the reaction was terminated by addition of SDS stop solution [26]. The proteins in the mixture were separated by SDS-PAGE and stained with Coomassie brilliant blue. The gel area corresponding to the 84/82 kDa protein or protein 4.1 was cut out, and counted for radioactive phosphate on a liquid scintillation spectrometer

membrane (lane c) was specifically immunoprecipitated with anti-84/82 kDa protein antibody (fig.5, lane d). The purified protein reported here was also demonstrated to be a good substrate for both kinases. Maximally, 3.3 and 1.3 mol of phosphate were incorporated into 1 mol of this protein by kinase II and cyclic AMP-dependent kinase, respectively (table 1). Based on these results, we have clearly demonstrated that a new calmodulin-binding protein purified from the synaptosomal membrane which interacts with a broad range of cytoskeletal elements is synapsin I itself. Baines and Bennett [22] have recently reported that synapsin I is a protein immunologically related to erythrocyte protein 4.1. However, protein 4.1 could be phosphorylated by cyclic AMP-dependent kinase, but not kinase II (table 1) and anti-synapsin I antibody showed no crossreactivity with protein 4.1 and vice versa [4]. Although protein 4.1 enhanced the spectrin (or caldesmon)-actin filament interaction, synapsin I did not show this activity [4]. These results suggest the idea that synapsin I might be functionally and immunologically different from protein 4.1.

Baines and Bennett [22,23] have recently demonstrated that synapsin I is a spectrin-binding and microtubule-bundling protein, and Huttner et al. [24] have reported that phosphorylation of synapsin I by kinase II appeared to induce the dissociation of synapsin I from synaptic vesicles. In this paper, we have demonstrated that synapsin I also interacts with calmodulin and actin filaments. These observations lead to the consideration that synapsin I may play some role in the neurotransmitter release mediated by the synaptic vesicle-cytoskeleton interaction.

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