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Calmodulin-binding proteins that interact with actin filaments in a Ca^{2+}-dependent flip-flop manner: Survey in brain and secretory tissues

(calspectin/spectrin-related protein/microtubule-associated protein)

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ABSTRACT **Regulatory actions of calmodulin on the contractile apparatus and cytoskeleton of smooth muscle and nonmuscle tissue are mediated by a number of specific calmodulin-binding proteins that bind to F-actin in a flip-flop manner—i.e., they bind to calmodulin or F-actin depending on the presence or absence, respectively, of Ca^{2+}. A survey for such proteins in brain, adrenal gland, and pituitary gland identified six polypeptides on polyacrylamide gels—M_r 340,000 (band 1), M_r 240,000/235,000 doublet (band 2), M_r 150,000 (band 3), M_r 129,000 (band 4), M_r 105,000 (band 5), and M_r 94,000 (band 6)—as flip-flop-regulated calmodulin- and F-actin-binding polypeptides. In addition to these polypeptides, a M_r 58,000 non-flip-flop calmodulin-binding actin-binding polypeptide (band 7) was found in all tissues examined. Band 2 was identified as calspectin (spectrin-related protein; fodrin). The flip-flop regulation of calspectin required the presence of a heat-labile nondialyzable factor contained in a supernatant fraction of brain homogenates. Band 1 was distinct from microtubule-associated proteins (MAPs) 1 and 2. However, when band 1 polypeptide was kept on ice 3 days, it converted to a lower molecular weight doublet that migrated with MAP2 on $NaDodSO_4$ gel electrophoresis. Bands 1 and 2 were found in all tissues examined.**

Recent results from our laboratory have led to a view that regulatory actions of Ca^{2+} and calmodulin on the contractile apparatus and cytoskeleton of smooth muscle and nonmuscle tissue are mediated by a number of specific calmodulin-binding proteins that also are able to bind to F-actin filaments or tubulin (1). Caldesmon (2, 3) and M_r 135,000 protein (myosin light chain kinase) (4) from smooth muscle are calmodulin-binding and actin-binding proteins; tau factor (5) from brain microtubules is a calmodulin-binding and tubulin-binding protein. The Ca^{2+}-dependent binding of calmodulin to these proteins inhibits the interaction between these proteins and F-actin (or tubulin). Therefore, the binding of these proteins to calmodulin or cytoskeletal proteins (target proteins) can alternate, depending on the concentration of Ca^{2+} (flip-flop binding; depicted schematically in Fig. 1). The binding of these proteins to the target cytoskeletal proteins alters the function of the latter proteins, and this flip-flop mechanism appears to be a general principle through which the Ca^{2+}-dependent regulatory effect of calmodulin is transmitted to the target cytoskeletal proteins. Examples for this mechanism include the caldesmon-linked flip-flop mechanism in smooth muscle, which regulates F-actin–filamin interaction (gelation) (6) and F-actin–myosin interaction (superprecipitation) (7), and the tau factor-linked flip-flop mechanism in brain microtubules, which regulates tubulin–tau factor interaction (polymerization) (8).

The purpose of the present study is to survey calmodulin-binding proteins that interact with F-actin in a flip-flop manner in brain and secretory tissues. We found six species of such proteins in these tissues. One of these proteins was identified as calspectin. Calspectin (fodrin) is a spectrin-related protein found in brain and other nonerythroid cells (for reviews, see refs. 1 and 9–11). Although this protein binds to both calmodulin and F-actin, it had been thought to be a nonregulated binding protein (12). In the present study, however, flip-flop binding of calspectin to calmodulin or F-actin was observed when a factor contained in a supernatant fraction of brain homogenates was included in the reaction system.

MATERIALS AND METHODS

Materials. Bovine tissues were obtained from a local slaughterhouse and transported to the laboratory on ice. The sources of commercial materials used in this work were as follows: DEAE-cellulose (DE-52) was from Whatman, Sepharose 4B was from Pharmacia Japan (Tokyo), and phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate were from Sigma. Calmodulin and calcineurin were prepared from bovine brain as described in refs. 13 and 14, respectively. Calmodulin-Sepharose 4B was prepared by the method of Klee and Krinks (15). Calspectin was prepared from bovine brain by a modification (16) of the method of Kakiuchi *et al.* (17). G-actin was prepared from chicken gizzard smooth muscle as described in ref. 18 and then converted to F-actin as described in ref. 6. Microtubule protein was prepared from bovine brain by temperature-dependent polymerization–depolymerization cycles (19). The third-cycle pellet of microtubules was used.

Preparation of Calmodulin-Binding Protein Fraction. The following procedures were carried out at 4°C. Fresh bovine brain, adrenal medulla, adrenal cortex, posterior pituitary, or anterior pituitary (8 g each) were homogenized in a Polytron PT 10 homogenizer (Kinematica, Luzern, Switzerland) by three 10-sec operations at an output setting of 7 with 5 vol of buffer A (0.3 M KCl/2 mM ATP/0.5 mM $MgCl_2$/0.5 mM dithiothreitol/50 mM imidazole·HCl, pH 6.9/0.25 mM phenylmethylsulfonyl fluoride/0.1 mM diisopropyl fluorophosphate). The homogenate of each tissue was centrifuged for 30 min at 105,000 × *g*. The supernatant was adjusted to 60% saturation of ammonium sulfate, and the resulting precipitate was collected by centrifugation and dissolved in a minimum volume (about 5 ml) of buffer B (20 mM Tris·HCl, pH 7.5/0.1 mM dithiothreitol/0.1 mM EGTA) plus 0.6 M KCl. The solution was clarified by centrifugation for 30 min at 105,000 × *g*, and the resulting clear su-

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Abbreviation: MAP, microtubule-associated protein.

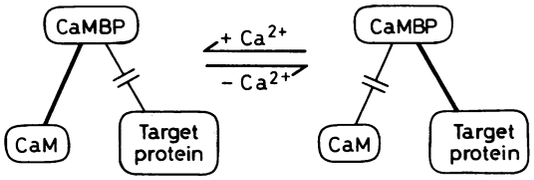

FIG. 1. Schematic representation of a calmodulin-binding protein-linked flip-flop mechanism that transmits the regulatory action of calmodulin to its target cytoskeletal protein. CaM, calmodulin; CaMBP, calmodulin-binding protein.

pernatant was applied to a column (35 × 3 cm) of Sepharose 4B that had been equilibrated with buffer B plus 0.6 M KCl. Material was eluted with buffer B plus 0.6 M KCl and a fraction corresponding to M_r 100,000–1,000,000 was collected. This fraction was dialyzed overnight against buffer B plus 0.1 M KCl. The solution was made 0.2 mM in Ca^{2+} by adding $CaCl_2$ and then clarified by centrifugation for 30 min at 200,000 × *g*. The clear supernatant was subjected to affinity column chromatography on calmodulin-Sepharose. The column (6 × 2 cm) was washed first with buffer C (20 mM Tris·HCl, pH 7.5/0.1 mM dithiothreitol/0.2 mM $CaCl_2$) plus 0.1 M KCl, and then with buffer C plus 0.5 M KCl. The calmodulin-binding protein fraction was eluted with 20 mM Tris·HCl, pH 7.5/0.1 mM dithiothreitol/0.1 M KCl/1 mM EGTA. The fraction thus eluted was diluted with 4 vol of buffer B to decrease the salt concentration. The diluted solution was applied to a column (2 × 1.2 cm) of DEAE-cellulose that had been equilibrated with buffer B plus 20 mM KCl. The column was washed with the same medium and the calmodulin-binding protein fraction was eluted with buffer B plus 0.5 M KCl. The fraction was dialyzed overnight against buffer B plus 0.1 M KCl and then clarified by centrifugation for 60 min at 105,000 × *g*. The clear supernatant thus obtained (calmodulin-binding protein fraction) was stored in ice and used within a day (or two days at most).

Other Methods. Electrophoresis of proteins was carried out on polyacrylamide gels in the presence of 0.1% $NaDodSO_4$ by the procedure of Laemmli (20). Proteins were then localized on the gels by Coomassie brilliant blue staining. Protein concentrations were determined by the method of Lowry *et al.* (21).

RESULTS AND DISCUSSION

Calmodulin-binding protein fractions were prepared from bovine tissue by a combination of ammonium sulfate precipitation, gel filtration, calmodulin-affinity chromatography, and DEAE-cellulose column chromatography. Fig. 2 reveals electrophoretic resolution of polypeptides contained in the calmodulin-binding protein fractions from brain and adrenal medulla on $NaDodSO_4$/polyacrylamide gels (10% acrylamide). Some of these polypeptides (designated bands 1–7) were able to bind F-actin. Fig. 3 *A* and *B* shows the binding of brain and adrenal medulla proteins, respectively, to F-actin in the presence or absence of Ca^{2+} and calmodulin. Polypeptides coprecipitated with F-actin are shown (lanes a, c, e, and g). Six polypeptides (bands 1–6) were found in which binding to F-actin was eliminated in the presence of both Ca^{2+} and calmodulin (see lane c)—i.e., they alternately bound to calmodulin or F-actin in the presence or absence, respectively, of Ca^{2+} (flip-flop binding). These polypeptides are M_r 340,000 (band 1), M_r 240,000/235,000 doublet (band 2), M_r 150,000 (band 3), M_r 129,000 (band 4), M_r 105,000 (band 5), and M_r 94,000 (band 6). Bands 2, 4, and 5 predominated in brain; bands 1, 3, and 6 predominated in adrenal medulla (Fig. 2). Band 7 (M_r, 58,000), which was found not only in brain and adrenal medulla but also in adrenal cortex and posterior and anterior lobes of pituitary gland (not shown),

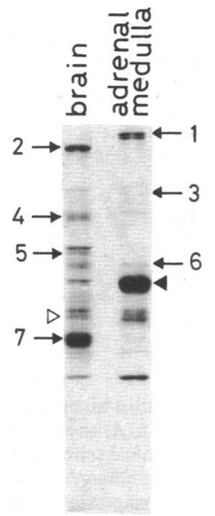

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of calmodulin-binding protein fractions from brain and adrenal medulla. The concentration of acrylamide was 10%. Bands 1–6 are flip-flop-regulated calmodulin- or F-actin-binding proteins (see also Fig. 3). ▷, Calcineurin; ◀, M_r 80,000 calmodulin-binding protein.

was a calmodulin- and actin-binding protein, but its binding to F-actin was not regulated by Ca^{2+} and calmodulin (non-flip-flop binding). The concentrations of band 7, as judged from protein staining intensities, were highest in brain and posterior pituitary followed by anterior pituitary. Band 7 was distinct from calcineurin because it migrated slightly faster on $NaDodSO_4$ gels (Fig. 2). The calcineurin band was identified by comparison with a purified calcineurin sample. Adrenal medulla contained a M_r 80,000 calmodulin-binding protein (Fig. 2), which was unable to bind to F-actin (Fig. 3*B*). Because this protein was by far the most abundant among calmodulin-binding proteins in adrenal medulla, we purified it from this tissue.

We collected the calmodulin-binding protein fraction of M_r 100,000–1,000,000 from a Sepharose 4B column. But band 6 (M_r, 94,000) and band 7 (M_r, 58,000) and the M_r 80,000 band were also found in this fraction. The possibility may be considered that these polypeptides existed as polymers, or alternatively, formed complexes with other proteins. Indeed, native forms of band 6 and the M_r 80,000 band, which were purified recently from bovine adrenal medulla, appeared to be a dimer (M_r, 200,000) and a polymer (M_r, 600,000) composed of 7–8 subunit polypeptides, respectively (unpublished data).

To obtain better resolution of the high molecular weight region, protein samples were electrophoresed on 5% acrylamide gels. Fig. 4 shows results with calmodulin-binding protein fractions from brain, adrenal medulla, adrenal cortex, and posterior and anterior lobes of pituitary gland. On the gel, band 2 was identified as calspectin (spectrin-related protein, fodrin) as it comigrated with purified calspectin. Although brain contained the highest concentration of calspectin, the same protein was found in all other tissues examined. The presence of calspectin in rat adrenal gland has been reported (22). It should be noted that the results shown in Fig. 4 represent the concentration of calspectin in the cytosol fractions not the total amount in the tissues. Our previous study showed that about 30% of calspectin in brain was distributed in the cytosol fraction; the rest was associated with membranes (17).

It was of interest to check the possible identity of band 1 with microtubule-associated protein (MAP)1 or MAP2 because MAP2 was reported to be a calmodulin-binding (23) and actin-binding (24) protein. We also obtained results compatible with

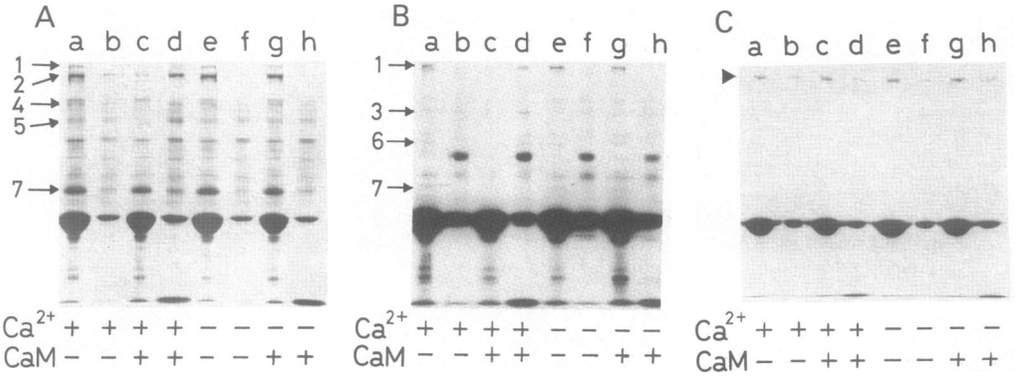

FIG. 3. Binding of calmodulin-binding proteins to F-actin in the presence or absence of Ca^{2+} and calmodulin. The calmodulin-binding protein fraction (0.4 mg/ml) from brain (*A*) or adrenal medulla (*B*) was incubated with gizzard F-actin (1.3 mg/ml) for 30 min at 30°C in 20 mM Tris·HCl, pH 7.5/0.1 M KCl/0.1 mM dithiothreitol/1 mM $MgCl_2$/0.2 mM ATP and either 0.2 mM $CaCl_2$ or 1 mM EGTA, as indicated in the figure, in a final volume of 0.25 ml. Calmodulin (100 μg/ml) was included in the mixture where indicated. At the end of the incubation, the mixtures were centrifuged for 60 min at 105,000 × *g* to precipitate F-actin and proteins bound to F-actin. The temperature was maintained at 25°C during the centrifugation. The protein contents of both the pellets (lanes a, c, e, and g) and supernatants (lanes b, d, f, and h) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. (*C*) Purified calspectin (95 μg/ml) was incubated with F-actin. CaM, calmodulin. ►, Calspectin.

the observations reported in refs. 23 and 24 (not shown). As shown in Fig. 4 (lane h), brain microtubules purified by cycles of polymerization and depolymerization revealed a polypeptide band of M_r 400,000 (MAP1) and a M_r 300,000 doublet (MAP2). Resolution of MAP2 into a closely spaced doublet on 4–5% acrylamide gels was consistent with published reports (25, 26). Freshly prepared band 1 polypeptide was distinct from MAP1 or MAP2 (Fig. 4, lane i); however, upon aging, band 1 yielded doublet bands that migrated with MAP2 on the $NaDodSO_4$ gel. One such example is shown in lane k, where a sample of adrenal medulla band 1 kept on ice for 3 days was electrophoresed. The coincidence of the aged band 1 and MAP2 was also shown by coelectrophoresis of both samples (not shown). Moreover, we found that the binding of MAP2 to calmodulin and F-actin occurred in a flip-flop fashion (unpublished data). These facts, taken together with the extremely rapid conversion rate of band 1 to a lower molecular weight doublet even at 0°C, suggest that the MAP2 reported in the literature as a M_r 300,000 polypeptide doublet may have been derived from the band 1 protein. Band 1 was found in all tissues examined. The order of its relative concentrations in the calmodulin-binding protein fractions of these tissues is adrenal medulla > anterior pituitary > adrenal cortex > posterior pituitary > brain.

Calspectin deserves further comments. Calspectin is a spectrin-related protein able to bind to calmodulin and F-actin (for reviews, see refs. 1 and 9–11). In our previous studies using purified brain calspectin, the binding of calspectin to F-actin was not influenced by the presence of both Ca^{2+} and calmodulin—i.e., calspectin was bound to F-actin irrespective of the binding of this protein to calmodulin (non-flip-flop binding) (12). This result was reproduced in the present study as shown in Fig. 3C. However, in Fig. 3 *A* and *B*, in which the calmodulin-binding protein fractions from brain and adrenal medulla were used instead of the purified calspectin, the binding of calspectin to F-actin was inhibited by the presence of both Ca^{2+} and calmodulin (flip-flop binding). The results suggest that an additional factor(s), which confers flip-flop regulation on the

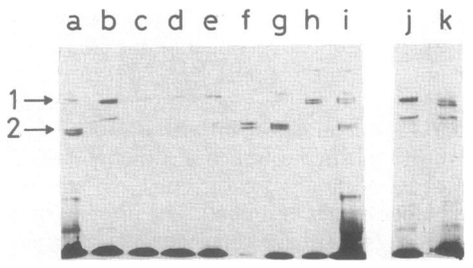

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of calmodulin-binding proteins. Acrylamide concentration was 5%. Lanes a–e: calmodulin-binding protein fractions from brain (a), adrenal medulla (b), adrenal cortex (c), posterior pituitary (d), and anterior pituitary (e). Lanes f–i: purified brain calspectin (f), coelectrophoresis of material in lanes a and f (g); purified brain microtubules (h); coelectrophoresis of material in lanes a and h (i). Lanes j and k: fresh calmodulin-binding protein fraction from adrenal medulla (j); and material as in lane j but after storage for 3 days on ice (k).

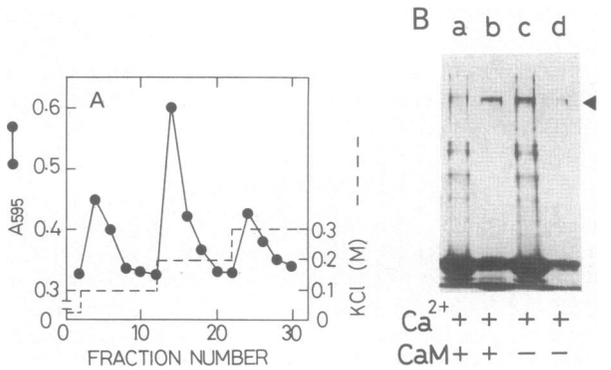

FIG. 5. Demonstration in the calmodulin-binding protein fraction of a factor that confers flip-flop regulation on purified calspectin. Calmodulin-binding protein fraction (10 mg of protein) from brain was diluted with 5 vol of buffer B and applied to a column (2 × 1.2 cm) of DEAE-cellulose that had been equilibrated with buffer B plus 20 mM KCl. Material was eluted with buffer B and increasing concentrations of KCl as indicated in the figure, and 2-ml fractions were collected (*A*). ●, Protein concentration determined by the method of Bradford (27). Samples (80 μl each) from tubes 4, 14, and 24 were incubated with purified calspectin (100 μg/ml) and F-actin (1.3 mg/ml). After the incubation, the mixtures were centrifuged and both pellets and supernatants were analyzed for their protein content by $NaDodSO_4$/polyacrylamide gel electrophoresis (7.5% acrylamide). These procedures are described in the legend to Fig. 3. The sample from tube 14 (200 mM KCl fraction) but not from other tubes inhibited the binding of calspectin to F-actin in the presence of both Ca^{2+} and calmodulin. (*B*) Results from tube 14. The effect was observed at concentrations of the column fraction of 0.2–0.3 mg of protein per ml. Lanes: a and c, pellets; b and d, supernatants. ◄, Calspectin.

calspectin-actin-calmodulin interaction, was present in the calmodulin-binding protein fractions from these tissues. This possibility was substantiated by the experiment shown in Fig. 5, in which the brain calmodulin-binding protein fraction was further fractionated on a DEAE-cellulose column by increasing concentrations of KCl, and the active factor conferring flip-flop regulation on purified calspectin was monitored in eluted fractions. The presence of such a factor in a fraction eluted with 200 mM KCl was demonstrated by five repeated runs of column chromatography of each of the separate batches of the brain calmodulin-binding protein fraction. A typical result is depicted in Fig. 5. The factor, which will be tentatively referred to as DE-200 factor, was nondialyzable and heat labile: under conditions used for Fig. 5*B*, heated DE-200 factor (3 min in boiling water) was incapable of conferring flip-flop regulation on purified calspectin, the result being consistent in two independent experiments. DE-200 factor was devoid of calspectin kinase activity by the assay as described in ref. 16. The nature of DE-200 factor and the mechanism by which the factor confers flip-flop regulation on calspectin are yet to be elucidated.

In addition to calspectin (1, 9–11), several calmodulin-binding proteins have been purified from brain recently. These include calcineurin, a protein composed of M_r 61,000 and M_r 15,000 subunit polypeptides (14, 28), a heat-stable M_r 70,000 protein (29), and a M_r 50,000 protein (30). Coated vesicle proteins from brain (31) and adrenal medulla (32) have been shown to be calmodulin-binding proteins. A number of other calmodulin-binding proteins have been detected but not characterized in brain (33–35) and adrenal medulla (32). However, there are no reports of calmodulin-binding proteins that interact with F-actin in a flip-flop fashion. The present study identified bands 1–6 (M_rs 340,000, 240,000/235,000 doublet, 150,000, 129,000, 105,000, and 94,000, respectively) as such proteins. Among these, band 2 was identified as calspectin. Band 6 protein has recently been purified from bovine adrenal medulla, and the flip-flop binding of band 6 to calmodulin or F-actin was confirmed by using this purified protein (unpublished data). In addition, band 7 (M_r, 58,000) was a calmodulin- and F-actin-binding protein, but its binding to F-actin was not regulated by calmodulin and Ca^{2+} (non-flip-flop binding). Bands 1–7 were generally found in brain, adrenal medulla, adrenal cortex, and anterior and posterior lobes of pituitary gland. As suggested by Itano *et al.* (36), calmodulin-binding proteins of basic nature may be of questionable physiological significance. However, the bands 1–7 reported here are acidic (or neutral) proteins as judged from their elution profiles on DEAE-cellulose chromatography. We propose that bands 1–7, together with caldesmon (2, 3), M_r 135,000 protein (4), and tau factor (5), be collectively termed "cytocalbin" from cytoskeleton-related calmodulin-binding protein.

There is accumulating evidence that the release of transmitter substances at neural synapses and the secretion of hormones from secretory cells are triggered by an increase in the Ca^{2+} concentration in the cells (37). It is generally accepted that Ca^{2+}-dependent effects of calmodulin on the cytoskeletal systems of these cells is intimately involved in the release mechanism. Studies on the flip-flop regulation of calmodulin- and F-actin-binding proteins (cytocalbins) reported here provide an important clue for the elucidation of this mechanism.

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