Identification of Fodrin as a Major Calmodulin-binding Protein in Postsynaptic Density Preparations

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ABSTRACT A major protein of postsynaptic densities (PSDs), a doublet of 230,000 and 235,000 Mr that becomes enriched in PSDs after treatment of synaptic membranes with 0.5% Triton X-100, has been found to be identical to fodrin (Levine, J., and M. Willard, 1981, J. Cell Biol. 90:631) by the following criteria. The upper bands of the PSD doublet and purified fodrin (alpha-fodrin) were found to be identical since both bands (a) co-migrated on SDS gels, (b) reacted with antifodrin, (c) bound calmodulin, and (d) had identical peptide maps after Staphylococcus aureus protease digestion. The lower bands of the PSD doublet and of purified fodrin (beta-fodrin) were found to be identical since both bands co-migrated on SDS gels and both had identical peptide maps after S. aureus protease digestion. The binding of calmodulin to alpha-fodrin was confirmed by cross-linking azido-125I-calmodulin to fodrin before running the protein on SDS gels. No binding of calmodulin to beta-fodrin was observed with either the gel overlay or azido-calmodulin techniques. A second calmodulin binding protein in the PSD has been found to be the proteolytic product of alpha-fodrin. This band (140,000 Mr), which can be created by treating fodrin with chymotrypsin, both binds calmodulin and reacts with antifodrin.

In our initial paper (13) on the isolation and characterization of postsynaptic densities (PSD) from canine cerebral cortex, we observed a doublet band on denaturing gels of the proteins of the PSD. We pointed out then (13) that this doublet (which we then labeled as Mr 185,000 but have since corrected to Mr 230,000) seems to be an intrinsic protein of the PSD, since it is concentrated in the PSD fraction over that of a synaptic membrane fraction from which the PSD was derived (11, 13). Further evidence for its being intrinsic to the PSD is that the doublet resists solubilization by the ionic detergents, deoxycholate (0.5%) and sarkosyl (1.0%) (31), and that it is also found in the PSD fraction isolated from cerebellum (9). Further work (11) on calmodulin-binding proteins in the PSD showed, by a gel-overlay technique, that this protein binds calmodulin, though at that time we could not tell whether one or both of the bands contain calmodulin. Recently, Davies and Klee (14) purified by calmodulin affinity chromatography a protein from whole brain, which they named CBP-I, and which was found to also bind actin, by sedimentation analysis (14). A somewhat similar result was reported in abstract form by Beach et al. (7), in that a protein doublet, of Mr 245,000, isolated from whole brain, was found to bind actin, and to be a component of the synaptic junction complex. Willard and co-workers (25, 35) had previously shown that among the axonally transported proteins was a doublet of Mr 240,000. They have now purified this protein from whole brain (23) and found that it binds actin by sedimentation analysis. Because they localized it to the cytoplasmic surface of many cell types (23), using immunohistochemistry, they have called it fodrin, from the Greek word for lining, "fodros." Because there is evidence, presented in the Discussion, that the two proteins of this doublet are subunits of fodrin, we have denoted, for convenience, the fodrin doublet as alpha- and beta-fodrin. We present evidence here that a doublet protein of Mr 230,000 and 235,000, which is concentrated in a PSD fraction, is identical to fodrin. It is instructive that both of the proteins known to bind to fodrin, actin (8, 21) and calmodulin (19), have been found to be in the isolated PSD fraction.

MATERIALS AND METHODS

General Methods: Synaptic membranes and postsynaptic densities were isolated by the method of Cohen et al. (13). SDS PAGE was done as described previously (13). Peptide mapping was done by the method of Cleveland.
et al. (12) and the resulting peptide maps were stained with silver stain as described by Morrissey et al. (27).

Purification of Brain Fodrin and Preparation of Antifodrin Antibody: Fodrin was purified from canine cerebral cortex by the method of Levine and Willard (23). Fodrin was then run on SDS gel electrophoresis, and shown to consist of only the two high molecular weight bands. The fodrin doublet was then cut out and homogenized for injection into rabbits for the production of antibodies. Fodrin was mixed with an equal volume of Freund's complete adjuvant and was injected subcutaneously into the back and legs above the knee. After 10 d, fodrin was again injected as described above, except that it was mixed with Freund's incomplete adjuvant. This was repeated again 10 d later. The rabbit was then bled 2 wk later to obtain the antisera.

Immunological Detection of Proteins on Nitrocellulose: Proteins subjected to PAGE in SDS were electrophoretically transferred to nitrocellulose sheets by the method of Towbin et al. (34). The nitrocellulose blots were washed in a solution containing 2.5% BSA, 10 mM Tris, pH 7.5, 0.9% NaCl for 2 h at 23°C. Rabbit antiserum was diluted in the Tris-saline solution and was incubated with the blot for 2 h at 23°C followed by 16 h at 0-4°C. The blots were washed two times for 10 min each in the Tris-saline solution, Tris-saline-0.1% Triton X-100, followed by Tris-saline. Antibody binding was detected by incubation for 2 h at 23°C with 2 μg/ml 125I protein A (2.5 x 10⁶ cpm/μg). The nitrocellulose transfers were washed as described above, dried on a gel drying plate under vacuum at 23°C, and subjected to autoradiography at -80°C with an intensifying screen for 1-2 d. Following autoradiography, transfers were stained with amido black to locate the protein bands.

Detection of Calmodulin-binding Proteins: Calmodulin was iodinated by the lactoperoxidase method of Richman and Klee (28) as slightly modified by Carlin et al. (10, 11). Iodinated calmodulin was bound on gels to proteins renatured after SDS gel electrophoresis by the method of Carlin et al. (10, 11). Azido-iodinated calmodulin was prepared and used as described by Andreasen et al. (1).

RESULTS

The data indicating the probable identity of the PSD doublet as fodrin are shown in Figs. 1-3. In Fig. 1, lanes 1 and 2 demonstrate the similarity of migration on SDS gel electrophoresis of both bands of fodrin purified from whole brain with the doublet bands of an isolated PSD fraction. In data not shown, fodrin obtained from M. Willard and CBP-I obtained from C. Klee also migrated with the PSD doublet. Antifodrin serum, obtained by us after injecting both bands of purified whole brain fodrin, binds only to the upper band of purified brain fodrin (Fig. 1, lane 3) and of the PSD doublet (Fig. 1, lane 4). We also found that the antisera obtained from M.
Willard (cf. reference 23) and C. Klee (cf. reference 14) bound to the same upper band, even though in both cases, antibody was prepared to both bands. We have injected each band separately into rabbits and have found again that we could only obtain antisera to the upper band and not to the lower band of fodrin. Nevertheless the data indicate the immunological similarity between the upper band of the PSD doublet and the upper band of whole brain fodrin. As can be seen in Fig. 1, lanes 3 and 4, another band on the gels, $M_r$ 140,000, also bound the antisera. As is shown in Fig. 3 this band is probably a proteolytic breakdown product of the upper band of fodrin. No indication of binding to fodrin or to the PSD doublet was observed when preimmune sera was used (Fig. 1, lanes 5 and 6).

We examined the binding of radioiodinated calmodulin to fodrin and to the PSD doublet by our gel-overlay technique (10, 11). Fig. 1, lane 7 indicates the binding of calmodulin to the upper, and not to the lower, band of fodrin, while Fig. 1, lane 8 shows the same result in the case of the PSD fraction, while also showing binding to the 140,000 $M_r$ band. In addition, calmodulin binds to other PSD proteins as was shown before (10, 11). We had previously observed that calmodulin binds to the PSD doublet (10, 11), but we were unable to ascertain to what band; now we show that it binds to the upper band. Data not shown indicate that the upper band of fodrin obtained from M. Willard, as well as a $M_r$ 140,000 band, also bound calmodulin. Recently, Glenney et al. (15) have also shown that only the upper band of fodrin binds calmodulin. The absence of binding to the lower band of fodrin is not due to the lack of renaturation of this protein in the gel-overlay technique. When fodrin was incubated with azido-iodinated calmodulin prepared by the method of Andreasen et al. (1) followed by cross-linking of the calmodulin to fodrin by uv light, again only the upper band of fodrin bound calmodulin (Fig. 1, lane 11). As a control the binding of calmodulin and of azido-calmodulin was performed in the presence of 1 mM EGTA, and no binding was observed (Fig. 1, lanes 9, 10, and 12). Thus, by the criterion of calmodulin binding, the upper band of the PSD doublet seems to be identical to the upper band of purified brain fodrin, and in addition the $M_r$ 140,000 band seems to be related to fodrin.

Another method of examining identities of protein is through the use of limited proteolysis. Fig. 2 gives the results when S. aureus protease digests of the separated upper and lower bands of both purified fodrin and of the PSD doublet are run on one-dimensional SDS gel electrophoresis. In both cases, a marked similarity was observed when a digest of the upper band of fodrin was compared to a digest of the upper band of the PSD.

![Limited proteolysis of fodrin and the PSD doublet.](image)

**FIGURE 2** Limited proteolysis of fodrin and the PSD doublet. Limited proteolysis of fodrin and the PSD doublet were done with *S. aureus* protease using the method of Cleveland et al. (12). Fodrin and PSDs were run on 5% SDS gel electrophoresis; the bands of interest were cut out and placed in a second 5-15% SDS polyacrylamide gel. The samples were then overlaid with *S. aureus* protease, the samples were run into the stacking gel, left for 2 h, and the gel was run to completion. Lanes 1, alpha-fodrin; lanes 2, PSD doublet upper band; lanes 3, purified beta-fodrin; lanes 4, PSD doublet lower band; lanes 5, *S. aureus* protease alone. Panel a, no protease; panel b, 100 ng *S. aureus* protease; panel c, 1 µg *S. aureus* protease. The small arrows mark the position of alpha- and beta-fodrin and the large arrow marks the position of the *S. aureus* protease.
doublet (Fig. 2, lanes 1 and 2, respectively), and when a digest of the lower band from fodrin was compared to a digest of the lower band of the PSD doublet (Fig. 2, lanes 3 and 4 respectively). Panel a shows the purified fodrin and PSD doublet subunits before treatment with proteases. Panel b shows the peptide map of the purified subunits after treatment with small amounts of protease. A few of the bands did not reproduce well on panel b, lanes 1 and 2, since at low concentrations the proteases cleave at slightly different rates in different lanes of the gel, but there was a corresponding band in lane 2 for every band in lane 1. Panel c shows the result of treatment of the proteins with large amounts of S. aureus protease, showing identical peptide maps of the upper (lanes 1 and 2) and of the lower (lanes 3 and 4) bands. All these results clearly indicate the virtually identical nature of the PSD doublet and fodrin.

Proteolysis with chymotrypsin indicates that the 140,000 Mr band, previously shown to bind antifodrin (Fig. 1, lanes 3 and 4) and to calmodulin (Fig. 1, lane 8), is a breakdown product of fodrin. When purified fodrin, whose two bands are shown in Fig. 3 (lane 1, panel a), was digested with increasing concentrations of chymotrypsin, only the upper band disappeared with a corresponding increase occurring in the Mr 140,000 band (Fig. 3, lanes 1-4, panel a). When these preparations were incubated with antifodrin on a western blot, the upper band of whole fodrin showed binding that disappeared with increasing concentrations of chymotrypsin coupled with a corresponding increase in the binding of the antibody to the Mr 140,000 band (Fig. 3, lanes 1-4, panel b).

DISCUSSION

By the following criteria we have concluded that the PSD doublet is identical to the purified whole brain fodrin doublet:

(a) both bands of each of the doublets had identical migration on SDS polyacrylamide gels, (b) the upper bands of both react with antibody raised against purified fodrin, (c) both of the upper bands of these two preparations bind calmodulin by the gel-overlay technique, and (d) both proteins showed nearly identical co-migration of peptides on SDS gels after limited S. aureus protease digestion of the separated bands of each. In addition, Beach et al. (7) have shown by sedimentation that a protein in synaptic junctions, which appears to be the same protein as the PSD doublet, binds actin, which has also been shown for purified fodrin by Levine and Willard (22). The calmodulin- and actin-binding doublet (CBP-I) described by Davies and Klee (14) also appears to be identical to fodrin and the PSD doublet since they all have identical migration on SDS polyacrylamide gels, they all react with antibody raised against purified CBP-I, and they all bind calmodulin (data not shown). In addition, CBP-I has been shown to bind to actin (14). Taken together, all the above data strongly indicate that the PSD doublet, fodrin, and CBP-I are one and the same protein.

For convenience, we would like to use the terminology of alpha- and beta-fodrin, since both seem to be subunits of the same protein. The evidence for this is that the two bands are always purified together in approximately equimolar amounts (14, 15, 21, and Fig. 1, lane 1), elution of fodrin from a calmodulin-affinity column gives two bands (14) even though only the upper band binds calmodulin, the two protein filaments appear to wrap around one another (15), and antibody to only the upper band of fodrin precipitates both bands of fodrin (23, unpublished observations). Furthermore, there are indications that beta-fodrin is not a breakdown of alpha-fodrin, since the two bands of purified fodrin always appear in approximately equimolar amounts (14, 15, 21), since anti-human spectrin reacts only with the lower band of the PSD doublet (4) which we have now shown is probably identical to beta fodrin, and since digestion by small amounts of chymotrypsin (Fig. 3), trypsin, papain, or S. aureus protease (unpublished data) does not reveal an increase in the lower band concomitant with a breakdown of the upper band. Alpha-fodrin will be used to designate the slower migrating band of fodrin on SDS gels, the band which binds calmodulin, while the faster-moving band not binding calmodulin will be labeled beta-fodrin.

We have presented evidence that fodrin is an intrinsic protein of the PSD by its enrichment in PSDs after treatment of synaptic membranes with 0.5% Triton X-100 (11, 13) and its resistance to solubilization by ionic detergents (31). It may be a protein that helps to both bind the PSD to the membrane and to bind the large quantities of actin found in the PSD (8, 21). A definitive localization of this protein to the PSD will require immuno-electron microscopy. However, fodrin is found in other parts of the nervous system other than the PSD (23). It represents 0.3–0.4% of total brain protein (14) and is found in all major axoplasmic transport groups (23, 25, 35). The function of fodrin in the PSD is unknown, but we do know that both of the proteins that bind to fodrin, actin and calmodulin, are present in quantity in the PSD. Calmodulin has been identified in the PSD both biochemically (19) and by immunohistochemistry (24, 36), while actin has also been shown by various criteria to be in the PSD (8, 21, 26). We have previously found (9) that cerebellar PSDs, which differ in structure from cerebral cortex PSDs, contain the high molecular weight doublet in similar amounts. The cerebellar PSDs contain actin in similar amounts but only contain about one-half the calmodulin found in cerebral cortex PSDs (9). Thus it could be that...
fodrin is the major calmodulin- and actin-binding protein in cerebellar PSDs. Cerebral cortex PSDs contain another calmodulin-binding protein, the major 51,000 M_r protein. This protein, which is present in much reduced amounts in cerebral PSDs, could well bind the increased amounts of calmodulin found in cerebral cortex PSDs. How all these proteins interact in the functioning of the PSD remains to be determined.

Not only do fodrin, CBP-I, and the PSD doublet appear to be identical but some protein doublets described by other laboratories appear to be the same as, or similar to, fodrin. Fodrin has been previously shown to be present in a wide variety of cells (23). With this information, the following can be summarized about fodrin. First, fodrin can be traced at least as far back as dogfish in evolution and is present in the cell surface-associated cytoskeleton of nonmammalian erythrocytes (2-5). The beta-fodrin reacts weakly with antispectrin (4), which is morphologically similar to it (15), and thus may be the spectrinlike protein recently identified in nonerythroid cultured cells (18). Recently, Glenney et al. (15) found a marked relatedness among spectrin, fodrin, and an intestinal brush border protein, TW 240/260. The relationship of fodrin and spectrin both in localization to the cell periphery and possibly in origin suggests that fodrin may be playing a role similar to spectrin in nonerythroid cells. Second, a protein that binds actin and is composed of a doublet with molecular weights corresponding to fodrin has been found to stimulate an actomyosin ATPase (30). The ability of this protein, which may be fodrin, to stimulate actomyosin contraction suggests that fodrin may be important in intracellular movement of molecules. In this connection, fodrin has been identified as a major component in axoplasmic transport (23, 25). Third, fodrin has been shown to be associated with the cap of lymphocytes during capping (22), indicating that fodrin may anchor cell surface molecules via its attachment to cytoskeletal actin. In addition, two laboratories (15-17, 20) have found, using a iodinated calmodulin overlay technique similar to ours, that calmodulin binds to a protein of M_r 250,000 in intestinal brush borders, but not to microvilli. However, this protein, labeled TW 260/240 (15, 16), which is morphologically and immunologically similar to fodrin (15, 16), has a different subunit structure. Fourth, a protein doublet that binds nonfilamentous actin in the periacrosomal region of echnoderm sperm was found to be similar to spectrin (33). If this protein is indeed fodrin, then fodrin would have the ability to bind nonfilamentous actin as well as polymerized actin (14, 23).

A major question to be resolved is the function of fodrin and the roles of actin and calmodulin in this function. Calmodulin does not seem to be involved in the attachment of actin to fodrin. Davies and Klee (14) found that their CBP-I protein cosedimented with F-actin independent of the presence of calcium and calmodulin. After capping in lymphocytes, calmodulin has been localized in the caps (29) in what appears to be specific for fodrin (6). Although many observations have been made concerning fodrin, it is clear that the role of fodrin in cell function, as well as the effects of actin- and calmodulin-binding, remain to be elucidated.

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