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INVOLVEMENT OF FODRIN-BINDING PROTEINS IN THE STRUCTURE OF THE NEURONAL POSTSYNAPTIC DENSITY AND REGULATION BY PHOSPHORYLATION

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SUMMARY. Novel polypeptides with M_r values about 140,000 bind fodrin and spectrin and are enriched in the postsynaptic density (PSD) compared to other tissues or subcellular fractions. ^{125}I-fodrin binding to these polypeptides is competed for by unlabeled spectrin. These polypeptides are distinct from ankyrin and its proteolytic fragments and from band 4.1 which also bind fodrin. Phosphorylation of PSDs by the endogenous calmodulin-dependent protein kinase markedly reduces ^{125}I-fodrin binding to the transblotted preparation. Such an event may play a regulatory role in governing protein-protein interactions among elements of the PSD. © 1986 Academic Press, Inc.

The protein-protein interactions involved in the assembly of the neuronal cytoskeleton, in particular the PSD, may be important in the control of intercellular signaling events among neurons and perhaps between neurons and their supporting glia. While the relative proportions of the major proteins present in the PSD structure such as actin, tubulin, fodrin, neurofilaments, and the subunits of the Type II calmodulin-dependent protein kinase have been investigated, (reviewed in (1)), little is known about how these and other proteins present in significant quantities associate with one another to form this complex. Calmodulin, under the appropriate conditions, has been shown to bind to specific proteins after denaturation and separation of PSD components by SDS-PAGE (2). Moreover, by the use of a non-diffusible oxidant, surface iodination detected an apparent "shell" of actin occluding the other, more abundant proteins (3). Such a model is consistent with the large number of 5 - 7 nm filaments observed projecting from PSD's *in vitro* and *in vivo* (4 - 6). However, the relative insolubility of the PSD element

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; AppNHp, 5'-adenylyl-imidodiphosphate; PSD, postsynaptic density; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid.

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has hampered the necessary solubilization and reconstitution experiments. Yet, the seemingly "insoluble" PSD element can be surprisingly plastic under physiologic stress, both growing and dividing or degenerating under pathological conditions, as well as during the synaptic remodeling and consolidation involved in development and regeneration (7,8).

One protein readily detected in PSDs and exhibiting many of the properties expected for a cross-linking element involved in organizing the junctional complex is the calmodulin binding protein, fodrin (9). Fodrin, a spectrin-like M_r 240,000/235,000 alpha/beta heterodimer, by analogy with its erythrocyte counterpart, would be expected to participate in the submembrane cytoskeletal structure of neurons and glia in brain. The cytoskeletal network would be anchored to the lipid bilayer through the interaction of the elongate fodrin molecule with protein elements similar to erythrocyte ankyrin, and band 4.1. These two proteins have been detected in a number of tissues, including brain by both immunologic and biochemical methods, demonstrating similar biochemical and physical properties to their red cell homologs (10-12).

Proteins that bind ^{125}I-fodrin can be detected by overlay of SDS-PAGE separated polypeptides electrophoretically transferred to nitrocellulose (transblots) (13). A group of major fodrin binding polypeptides of M_r 135,000 -147,000 (P-140) is enriched in PSDs and in cytoskeletal elements derived from the P3 (microsomal/plasma membrane) fraction of brain comprised in large proportion of the calmodulin-dependent protein kinase Type II. In this communication, we describe the fodrin-binding properties of P-140 which is concentrated in neuronal cytoskeletal preparations, especially in PSDs. Fodrin binding to this component is regulated by phosphorylation of P-140 by the endogenous calmodulin-dependent protein kinase Type II and may represent a physiologically relevant mechanism to modulate synaptic structure, perhaps by allowing rearrangement of PSD components.

MATERIALS AND METHODS

^{125}I-Bolton Hunter reagent (2200 Ci/mmol) was obtained from ICN. All SDS-PAGE components and the transblot apparatus were from Biorad. Nitro-

cellulose (0.45 μm) sheets were purchased from Millipore. Sephacryl S-500, Fraction V bovine serum albumin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and pepstatin A were acquired from Sigma. Fodrin was purified from rat brain through the Sephacryl S-500 step (13) and iodinated according to Davis and Bennett (14). Spectrin was purified (14) and similarly iodinated, and the purity of the iodinated products was assessed by SDS-PAGE. The iodinated ligands bind appropriately to inside out erythrocyte vesicles. 7% acrylamide - 0.187% bisacrylamide SDS-PAGE was performed with a Laemmli buffer system (15) and transblots were run at 60 V for 2.5 hours in 25 mM Tris-HCl - 0.192 M glycine - 0.01% SDS, pH 8.3 with water cooling. The transblots were blocked and overlaid with 7 nM dimer (100,000 cpm/ml). ^{125}I-fodrin binding was also assessed in the presence of an excess (1 μM) of erythrocyte spectrin. Synaptosomes and PSDs were isolated according to Cohen et al. (16) in the presence of 100 μg/ml PMSF, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A in all homogenization steps.

RESULTS

^{125}I-fodrin binding to M_r 135,000 - 147,000 polypeptides (P-140).

^{125}I-fodrin and ^{125}I-spectrin bind to a spectrum of proteins from erythrocyte inside-out vesicles, brain microsomal cytoskeletal preparations, and PSDs separated by SDS-PAGE and transblotted onto nitrocellulose (Fig. 1). Heating the labeled ligand diminishes fodrin binding to PSDs, but spectrin binding to PSDs is not reproducibly sensitive to heat. Human erythrocyte inside-out

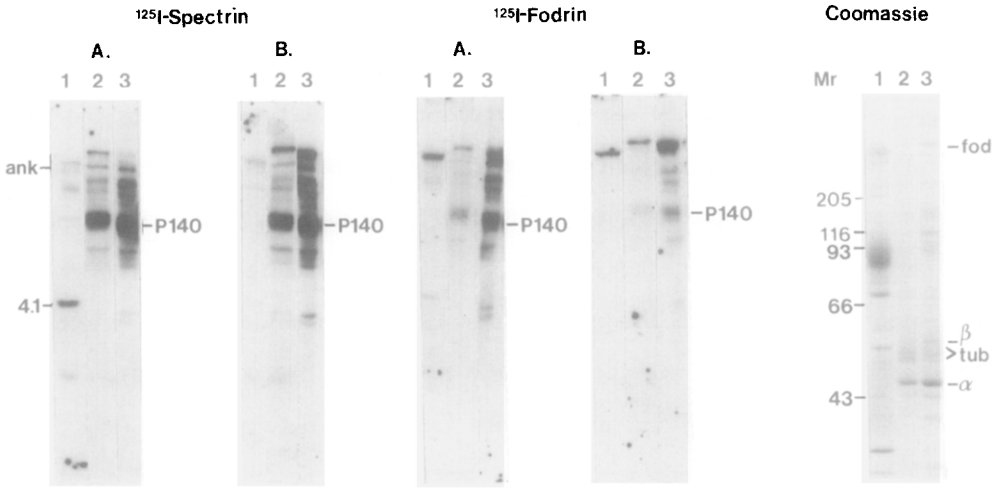

Fig. 1. ^{125}I-Fodrin and ^{125}I-Spectrin Bind to Similar Proteins. Fifteen μg protein from spectrin-depleted human erythrocyte vesicles (lane 1), rat brain microsomal cytoskeletal preparation (lane 2), and rat brain PSDs (lane 3), were subjected to SDS-PAGE, transblotted, and overlaid with 7 nM (expressed as dimer) of either ^{125}I-spectrin or ^{125}I-fodrin as described in (13). Group A ligand was untreated while Group B ligand was heated at 60°C for 10 minutes before overlay. Virtually all of the protein was transferred to the nitrocellulose, including high molecular weight proteins up through M_r 270,000. M_r values correspond to the Coomassie blue-stained gel fod = fodrin, ank = ankyrin, 4.1 = band 4.1, tub = tubulin, α and β = subunits of calmodulin dependent kinase type II.

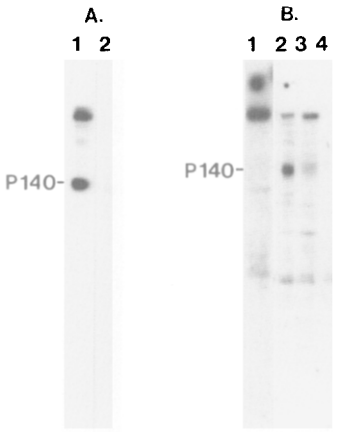

Fig. 2. Specificity and Distribution of P-140 ^{125}I-Fodrin Binding Proteins. A. Displacement of ^{125}I-fodrin binding by unlabeled spectrin. Fifteen μg of a PSD preparation was separated, transblotted, and overlaid with either 7 nM ^{125}I-fodrin (lane 1); or 7 nM ^{125}I-fodrin + 1 μM (dimer) unlabeled erythrocyte spectrin (lane 2). B. Distribution of P-140 ^{125}I-fodrin binding protein. Fifteen μg of protein from brain cytosol (lane 1), synaptosomes (lane 2), 250,000 x G synaptosomal supernatant after solubilization with 1% Triton X-100 (lane 3), and PSD glycoproteins (lane 4).

vesicles demonstrate ^{125}I-fodrin binding to ankyrin and band 4.1. PSDs contain several fodrin binding proteins including M_r 215,000 and M_r 80,000 polypeptides which have the same electrophoretic mobility as ankyrin and band 4.1. A number of smaller M_r fodrin binding proteins are also visible which may represent proteolytic fragments of ankyrin or band 4.1, and they tend to increase with storage of the PSD preparation. In addition, a significant proportion of the fodrin binding of the PSD is associated with a closely spaced group of proteins migrating between M_r 135,000-147,000 (P-140).

^{125}I-fodrin binding is displacable by unlabeled spectrin (Fig. 2A). Fodrin binding is thus considered to be specific by the criteria of displacement by unlabeled ligand and the observation that the binding does not parallel the Coomassie blue staining profile, e.g., the major staining species, the alpha subunit of the calmadulin-dependent protein kinase, does not bind fodrin. The presence of albumin, Triton X-100, and a high ionic strength in the overlay incubation should also reduce nonspecific adsorption of ligand.

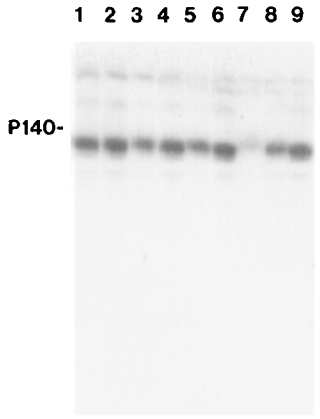

Fig. 3. Effect of Phosphorylation of PSD Proteins by the Endogenous Calmodulin-Dependent Protein Kinase. Fifty µg of PSD protein were incubated for 10 min at 30°C in a reaction mixture containing 50 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM DTT, and the following additions in a total of 150 µl: none (lane 1), 1 mM EGTA (lane 2), 1 mM EGTA + 1 mM ATP (lane 3), 100 µM $CaCl_2$ (lane 4), 100 µM $CaCl_2$ + 1 mM ATP (lane 5), 100 µM $CaCl_2$ + 10 µg/ml calmodulin (lane 6), 100 µM $CaCl_2$ + 10 µg/ml calmodulin + 1 mM ATP (lane 7), 100 µM $CaCl_2$ + 10 µg/ml calmodulin + 1 mM App(NH)p (lane 8), none (lane 9) (kept at 0°C throughout the incubation). The reaction was stopped by the addition of 50 µl of SDS sample buffer to each tube. 50 µl aliquots were electrophoresed, transblotted, and overlaid with 7 nM ^{125}I-fodrin.

^{125}I-Fodrin binding shows P-140 to be present in synaptosomes and to be concentrated in PSD preparations (Fig. 2B); binding is not detectable in liver cytoskeleton, brain cytosol, or primary neuronal or glial cultures (data not shown). PSD glycoproteins of M_r 230,000, 180,000, 130,000, and 110,000 purified by chromatography on Concanavalin-A Sepharose (17) failed to bind fodrin in the overlay assay. Thus, the fodrin binding entity is distinct from the glycoproteins previously described in synaptosomes and PSDs (18). Furthermore, P-140 was not released from PSDs by extraction with 0.05% Tween 20 + 0.8 M KI (19), and did not comigrate with the chymotryptic fragments of ankyrin (M_r 72,000) or fodrin (M_r 140,000).

Modulation of Fodrin Binding in PSDs by Phosphorylation. Since a major protein component of the PSD is a Type II calmodulin-dependent protein kinase (20-22) which is highly active on endogenous PSD substrates, the possibility that phosphorylation of some PSD element might perturb its interaction with other components such as fodrin was tested. Figure 3 shows the effect of incubating PSDs under conditions that activate phosphorylation of PSD

proteins by the calmodulin-dependent kinase prior to separation by SDS-PAGE. Treatment in the presence of Ca^{+2}/calmodulin and ATP, but not AppNHp, reduced binding of ^{125}I-fodrin in the transblot overlay assay. ^{32}P is incorporated into protein in the M_r 140,000 region when [γ-^{32}P]ATP is present under the same conditions (data not shown).

DISCUSSION

The majority of ^{125}I-fodrin binding to PSDs is accounted for by a group of proteins clustered around M_r 140,000, as determined by overlay of the separated, transblotted polypeptides. Direct binding of fodrin to native or low ionic strength-treated PSDs was not demonstrable, possibly because of the presence of endogenous fodrin and because steric and denaturation factors may be important *in situ*. The specificity of the fodrin/spectrin interaction with P-140 was inferred from displacement of radioligand by excess unlabeled ligand, the selectivity of the tissue distribution, and the general lack of correspondance with the protein distribution by Coomassie blue staining.

Binding of P-140 to fodrin and its enrichment in PSDs suggests that these polypeptides may serve a substantial role in consolidation of the synapse in addition to that possibly performed by ankyrin and band 4.1, if they are localized in the PSD. Regulation of fodrin-binding activity by phosphorylation may be an expression of synaptic plasticity at the level of the PSD.

Covalent modification of synaptic proteins by the endogenous Type II calmodulin-dependent protein kinase might be expected to alter several elements of the synapse such as neurotransmitter receptors, ion channels, and cytoskeletal components. The regulation of cytoskeletal interactions by protein kinase-mediated phosphorylation of cross-linking and bundling components has been described for the tubulin-MAP-2 (23) and the spectrin-ankyrin systems (24). Previously, autophosphorylation of the calmodulin-dependent protein kinase had been shown to decrease the apparent affinity of the enzyme for calmodulin (25). In the present case, a decrease in fodrin

binding induced by P-140 phosphorylation could serve to relax the PSD structure, perhaps increasing the turnover rate of some of the comonents *in vivo* thereby contributing to synaptic plasticity.

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