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Interaction of Brain Spectrin (Fodrin) with Phospholipids[†,‡]

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ABSTRACT: Binding of brain spectrin to frozen and thawed liposomes was studied, using a pelleting assay, as a function of lipid composition. Saturable binding was observed for all lipid mixtures that included aminophospholipids, as well as for the total lipid of synaptic plasma membranes. Binding was strong and saturable, with dissociation constants in the nanomolar range. There were two pH optima at ca. 6.0 and 7.5 and a sharp ionic strength optimum, corresponding to physiological solvent conditions. No competition could be detected with extraneous globular proteins, serum albumin, and hemoglobin. The results imply a strong, direct interaction between brain spectrin and the neuronal plasma membrane in the cell.

Brain spectrin (Goodman et al., 1981; Bennett et al.,1982), also known as fodrin (Levine & Willard, 1981), bears a high sequence homology to erythroid spectrin and has the same type of structure with two kinds of polypeptide chains (α and β), each made up of a series of degenerate repeating units with high α helicity (Speicher & Marchesi, 1984; Wassenius et al., 1989). The β chains of both proteins are multiply phosphorylated at their C-terminal ends (Goodman et al., 1984). Fodrin differs from erythroid spectrin in its greater rigidity, as revealed in the electron microscope, and larger stability of the tetramer, generated by head-to-head association of two dimers, but it possesses binding sites for membrane and cytoskeletal proteins, found in brain and analogous to those with which spectrin interacts in the red cell. These include F-actin and proteins closely related to red cell protein 4.1 and ankyrin (Glenney at al., 1983). A further difference between spectrin and fodrin is that the latter binds relatively strongly and the former weakly to calmodulin. This implies that fodrin participates in calcium-regulated processes (Glenney et al., 1983; Harris et al., 1985). Brain spectrin also recognizes a series of other structural proteins of neural tissue, including those of microtubules, especially the important microtubule-associated protein, MAP-τ, neurofilament-associated protein, and glial fibrillary acidic protein (Carlier et al., 1984; Frappier et al., 1987; Ishikawa et al., 1983; Riederer & Goodman, 1990). There is evidence to suggest that brain spectrin may also associate with the plasma membrane by direct interaction with an integral 180 kDa protein, known as neural cell adhesion molecule (Pollerberg et al., 1987). There is also a link through ankyrin to the sodium−potassium ATPase (Nelson & Veshnock, 1987).

We are concerned with the mechanisms by which proteins of the spectrin family, and in particular neuronal spectrin or fodrin, interact with and regulate the properties of membranes. The interaction of erythrocyte spectrin with phospholipids of the membrane bilayer has been studied both on natural (erythrocyte) membranes and on artificial lipid mixtures in the form of phospholipid liposomes (Haest et al., 1978; Sikorski & Kuczek, 1985; Sikorski et al., 1987a; Bitbol et al., 1989) and monolayers (Mombers et al., 1980; Maksymiw et al., 1987) by a variety of techniques. These include monolayer film penetration measurements (Mombers et al., 1980), covalent labeling by hydrophobic reagents (Sikorski & Kuczek, 1985), and pelleting assays (Białkowska et al., 1994; Bitbol et al., 1989). Interaction of spectrin with the erythrocyte membrane affected the fluidity of the membrane as judged by spin label signals (Sikorski & Jezierski, 1986). Erythroid spectrin binds amphipatic compounds, such as brominated fatty acids (Isenberg et al., 1981; Kahana et al.,1992), fatty acids, and anionic, cationic, and zwitterionic detergents (Sikorski et al., 1987b).

We have previously shown that erythroid spectrin binds directly and rather strongly to liposomes containing the aminophospholipids phosphatidylethanolamine (PE)[1] and phosphatidylserine (PS). We have attempted here to determine whether this important property is equally a feature of the brain protein and to characterize the number, specificity, and affinity of the binding sites.

EXPERIMENTAL PROCEDURES

Materials. Sephacryl S-500-HR, acrylamide, MES, TRIS, PE, PS, EGTA were from Sigma Chemical Co. (St. Louis, MO). DTT, Coomassie brilliant blue R-250, *N,N'*-methylenebis(acrylamide), and EDTA were from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). SDS was from BDH Laboratory Supplies (Poole, England). Tween 20 was from Schuchardt (Munchen, Germany). HPTLC plates, silica gel 60 no. 5641, dimethyl sulfoxide, and Folin−Ciocalteu's phenol reagent were from Merck (Darmstadt, Germany). Phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL).

Bovine Brain Spectrin Isolation. Bovine brains, obtained from the local slaughter house within 30 min of death, were

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[1] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FAT-liposomes, frozen and thawed liposomes; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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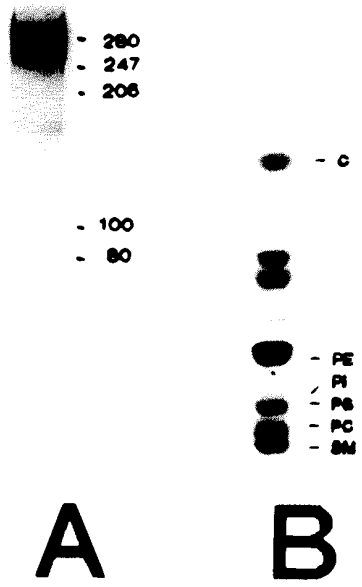

FIGURE 1: SDS (5%)−polyacrylamide gel electrophoresis of isolated brain spectrin stained with 0.01% Coomassie blue in 5% acetic acid and 10% ethanol and destained with 5% acetic acid and 10% ethanol in water (A) and total phospholipids extracted from bovine brain synaptic plasma membranes (B) with a mixture of chloroform:methanol (1:1) separated by thin-layer chromatography on HPTLC plates (Merck no. 5641) in two successive steps; the first in the solvent system: methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25/25/28/10/7) and the second in hexane/diethyl ether/acetic acid (75/23/2) (Yao & Rastetter, 1985), developed in iodine vapor. Other details are as in the Experimental Procedures.

processed according to Bennett et al. (1986) and stored for up to 3 months at −70 °C. Spectrin was purified according to the same authors, except that the last DEAE-cellulose column chromatography step was omitted (Sikorski et al., 1991). Isolated protein was dialyzed overnight against 5 mM sodium phosphate buffer containing 50 mM NaCl, 0.1 mM DTT, and 0.1 mM EDTA (pH 7.4). The resulting preparation contained only the two spectrin subunits of apparent molecular weight 280 and 267 kDa, with traces of a proteolytic 160 kDa fragment of the α-spectrin subunit, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) (Figure 1A). No phospholipid could be detected in the protein preparation by phospholipid phosphorus analysis (limit of detection 0.1% by weight).

Preparation of FAT-Liposomes. Frozen and thawed (FAT) liposomes were prepared according to Hope et al. (1986), using the "assay buffer" (Mombers, 1982) consisting of 5 mM MES, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM DTT, and 1 mM NaN_3, pH 5.0−7.0, containing 20% Dextran T 40. At pH 7.5 and 8.0, 5 mM TRIS/HCl was substituted for MES. The liposome suspension was diluted with assay buffer without Dextran and centrifuged at 30 000*g* to remove small vesicles and external Dextran.

Binding Assay. Purified bovine brain spectrin was dialyzed against the assay buffer. The incubation mixture (110 μl) contained liposomes (usually 200−300 mg of phospholipid) and purified spectrin. After 30 min incubation at room temperature (20 °C), the sample (90 μl) was layered on a 150 μl cushion of 0.5% Dextran in the assay buffer and centrifuged at 17 000*g* for 6 min. To test the effect of ionic strength on binding, the sodium chloride concentration of the assay buffer was varied. Protein in the pellets was determined by the method of Dulley and Grieve (1975), using bovine serum albumin as a standard. For each experimental point, a control with no liposomes was performed. The amount of spectrin in the control pellet, which did not exceed 25−30% of that in the liposome pellet, was subtracted. The control binding assays for bovine serum albumin, hemoglobin, and ovalbumin were carried out in exactly the same way. The competition assays using haemoglobin or bovine serum albumin were carried out similarly, except that pellets were analyzed by SDS (5%)−polyacrylamide gel electrophoresis as above. Quantitation of bound protein in the pellets was performed by elution of Coomassie blue stain from the excised zones with dimethyl sulfoxide and measuring the absorbance at 595 nm.

Lipid Extraction from Bovine Brain Synaptic Plasma Membranes. Lipids from bovine brain synaptic plasma membranes remaining after the extraction of brain spectrin (see above) were prepared according to Bligh and Dyer (1959) with the small modifications of Kates (1972). Phospholipid:cholesterol ratio in the resulting lipid mixture was 35:10. The major components were cholesterol, PE, PS, PC, and sphingomyelin. The qualitative composition of this mixture was analyzed by thin-layer chromatography (Figure 1B) according to Yao and Rastetter (1985) in solvent systems A [methyl acetate/*n*-propanol/chloroform/methanol/0.25% KCl (25/25/28/10/7] and B [hexane/diethyl ether/acetic acid (75/23/2)]. The composition of the mixture was PC, 23.90%; PE, 21.12%, phosphatidylinositol, 6.95%; PS, 9.72%; sphingomyelin, 7.78%; and cholesterol, 15.24%, respectively. Two unidentified spots (Figure 1B) accounting for 9.75% and 5.56% of total lipid contained 11.47% and 6.55% of total phosphorus. Phospholipid phosphorus and cholesterol were determined by the methods of Rouser et al. (1966) and Courchaine et al. (1959), respectively.

Succinylation of Brain Spectrin. Succinylation of purified brain spectrin was carried out according to Klotz (1967) using 1 mg of succinic anhydride/2.2 mg of protein in 20 mL of distilled water at pH 7.4 for 30 min at room temperature.

All binding measurements were made at least in duplicate with an average variation of ±10%. For determinations repeated more than five times, standard deviations were calculated and included as error bars in the graphs.

RESULTS

To measure binding of brain spectrin to lipid membranes, we used a sedimentation method of the kind first described by Mombers (1982), using liposomes filled with 20% Dextran. With liposomes composed of PE and PC in a 3:2 molar ratio, saturable binding was observed. The amount of spectrin carried down increased linearly with increasing lipid concentration up to 4 mM in the assay (data not shown). At a lipid:spectrin weight ratio above ca. 10:1, more than 95% of spectrin was bound. All subsequent experiments were carried out at a constant concentration of liposome phospholipid of 3.3 mM (2.5 mg/mL).

To ensure that we were observing binding of brain spectrin and not proteolytic fragments and impurities, the protein brought down under standard assay conditions was analyzed by SDS−gel electrophoresis. The results are shown in Figure 2.

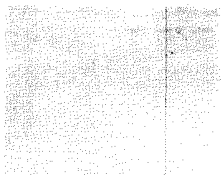

FIGURE 2: Binding of isolated brain spectrin to FAT-liposomes prepared from a PE/PC (60:40) mixture at pH 6.0. Indicated concentrations of protein were incubated with liposomes (0.225 mg) at room temperature. After centrifugation, pellets were subjected to SDS–polyacrylamide gel electrophoresis as described in the Experimental Procedures.

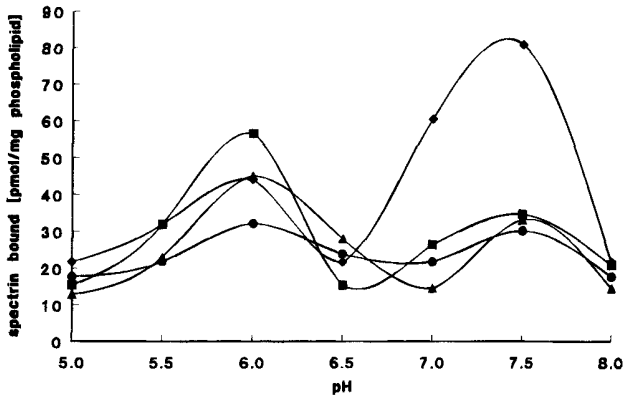

FIGURE 3: Effect pH of the incubation mixture on brain spectrin–FAT-liposome interaction. Liposomes were prepared from (■) PC, (◆) PE/PC (60:40), (▲) PS/PC (60:40), and (●) PE/PS (60:40). Assay buffer: 5 mM MES or TRIS, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM DTT, and 1 mM NaN_3, pH as indicated. The results are for brain spectrin concentration of 110–140 nM. Identical pH optima were obtained at brain spectrin concentrations of 160 and 200 nM. Details are in the Experimental Procedures.

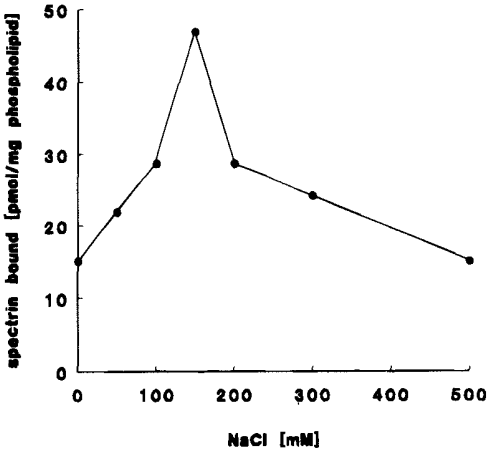

FIGURE 4: Effect of NaCl concentration on brain spectrin binding to FAT-liposomes prepared from PE/PC (60:40) at pH 7.5. Brain spectrin (40 nM) was incubated with filled with 20% Dextran T 40 in the "test buffer" liposomes. At each point, the "assay buffer" contained the indicated concentrations of NaCl. Identical optima were obtained for 20, 60, and 80 nM spectrin in the sample. Other details are in the Experimental Procedures.

Figure 3 shows the effect of pH of the incubation medium on binding of spectrin to liposomes of varying composition, viz., PC alone, PE/PC (60:40), PS/PC (60:40), and PE/PS (60:40). For liposomes prepared from all the lipid mixtures tested, two pH optima at ca. 6 and 7.5 can be observed. These optima were independent of protein concentration (results not shown). It should be noted that the binding is greater at pH 6, except to liposomes containing PE, which show equal (PE/PS) or more (PE/PC) binding at pH 7.5.

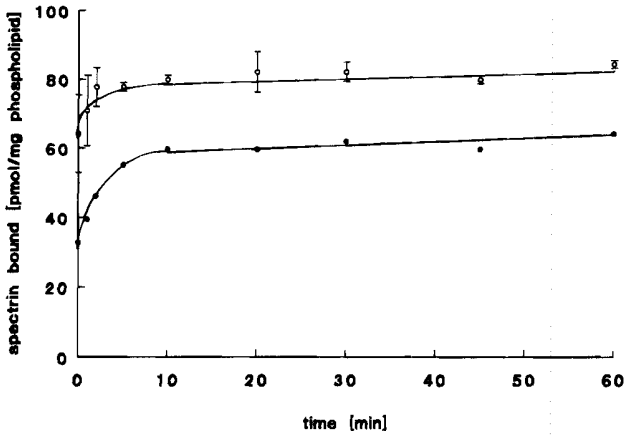

FIGURE 5: Time course of the interaction of isolated brain spectrin with FAT-liposomes prepared from PE/PC (60:40). Brain spectrin was incubated for the indicated time periods with liposomes at pH 6.0 (72 nM spectrin) (●) and at pH 7.5 (60 nM spectrin) (○). The liposomes and unbound spectrin were then separated by centrifugation on a cushion of 0.5% Dextran in the "assay buffer" at 17000*g* for 6 min. Other details are as in the Experimental Procedures.

Table 1: Equilibrium Dissociation Constants (K_D) and Maximal Binding Capacities (B_{max}) of the Interaction of Purified Brain Spectrin with FAT-Liposomes Prepared from Different Lipids[a]

liposome composition	pH	$K_D \pm$ SE (nM)	$B_{max} \pm$ SE (pmol/mg of lipid)
PC	6.0	531 ± 122	204 ± 35.2
	7.5	118 ± 12.4	84 ± 4.5
PE/PC	6.0	21 ± 4	41 ± 2.8
	7.5	63 ± 9.3	110 ± 8.5
PS/PC	6.0	77 ± 14.8	55 ± 4.3
	7.5	245 ± 34	74 ± 6.3
PE/PS	6.0	94 ± 11	60 ± 3.9
	7.5	83 ± 12.9	57 ± 4.5
total lipids of synaptic membrane[b]	6.0	50 ± 8	246 ± 20.5
	7.5	16 ± 2.9	184 ± 11.4

[a] Data presented here were derived from results of the experiments shown in Figures 6A–E with nonlinear regression with the use of the EZ-Fit computer program (F. W. Perrella, E. I. DuPont de Nemours & Co., Glenolden Laboratory, Glenolden, PA; obtained from NETSERV EMBL, Heidelberg, Germany). SE = standard error. [b] Estimated as phospholipid phosphorus.

Figure 4 shows the dependence of binding of brain spectrin to liposomes composed of PE/PC (60:40) on NaCl concentration. All subsequent studies were carried out at optimal NaCl concentration (0.15 M).

Figure 5 shows the time course of the interaction of brain spectrin with liposomes prepared from a PE/PC (60:40) mixture at pH 6.0 and 7.5. It can be seen that the reaction reaches equilibrium in less than 10 min, so that a 30 min incubation should ensure that the system has reached equilibrium.

Figure 6A–D shows the binding isotherms at pH 6.0 and 7.5 to liposomes of the following phospholipid compositions: PC, PE/PC (60:40), PS/PC (60:40), and PE/PS (60:40). Saturable binding of spectrin was observed in all cases. The binding isotherms can be satisfactorily fitted with a single binding constant, i.e., one class of independent binding sites. The equilibrium dissociation constants and maximal binding capacities resulting from these analyses are given in Table 1.

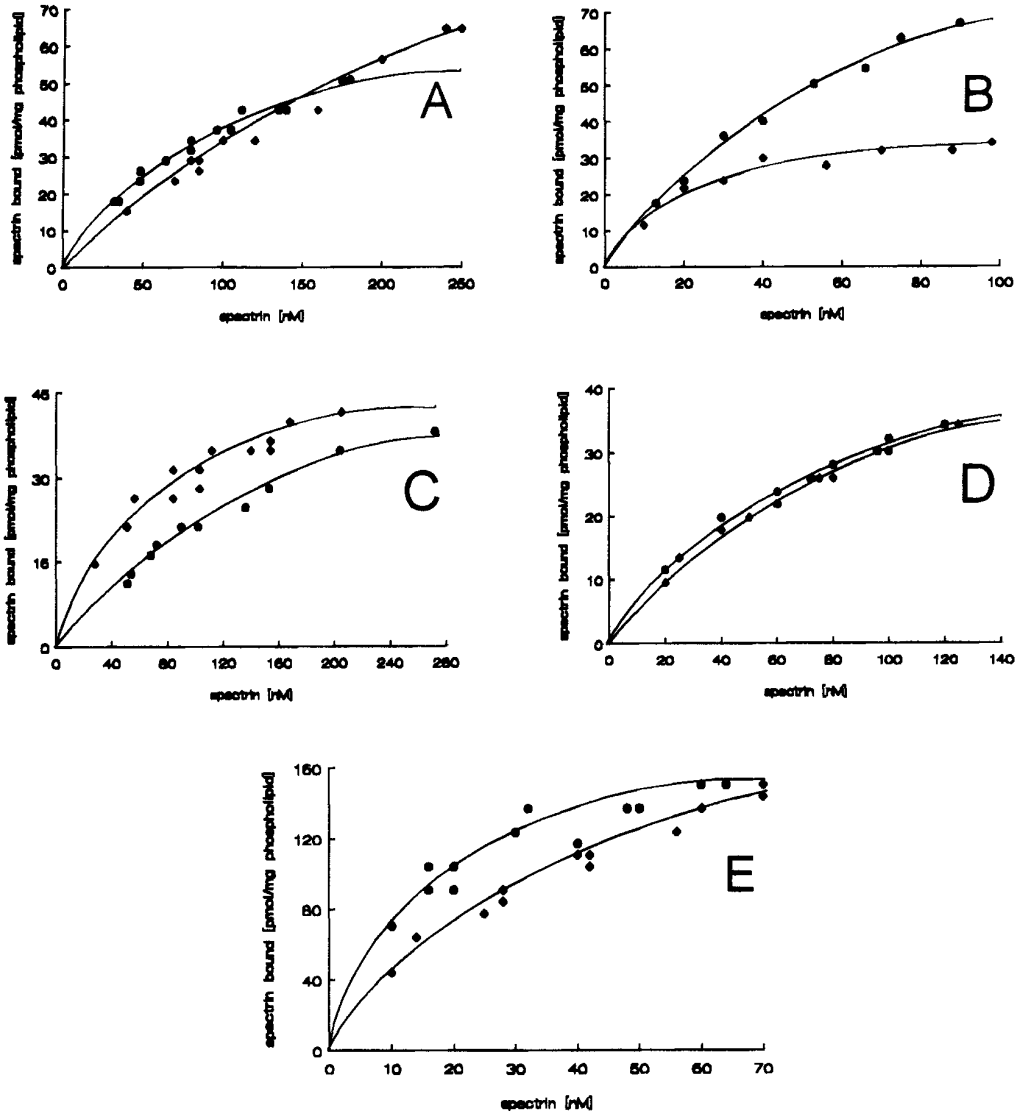

FIGURE 6: Binding of increasing concentrations of purified brain spectrin by FAT-liposomes prepared from: (A) PC, (B) PE/PC (60:40), (C) PS/PC (60:40), (D) PE/PS (60:40), and (E) total phospholipids extracted from bovine brain synaptic plasma membrane. Lipid isolation and characterization were as in the Experimental Procedures and Figure 1B. Lipid concentration (2.05 mM) was estimated as phospholipid phosphorus. Binding data obtained at pH 7.5 (●) and at pH 6.0 (◆). Other details are in the Experimental Procedures.

We sought to determine whether the lipid binding was exclusively a property of the native conformation of brain spectrin and whether other, unrelated proteins also show binding to the phospholipid vesicles. The first question presents the problem that heat-denatured spectrin largely sediments in the conditions of the pelleting assay, whereas the undenatured fraction that remains after heat treatment (67 °C, 15 min) binds with unchanged affinity (data not shown). We therefore succinylated the protein to bring about unfolding without reducing the solubility. This did not abolish the binding capacity of brain spectrin toward phospholipids (Figure 7C), giving a K_D of 135 ± 46 nM (compared to 63 nM for native spectrin on the same liposomes at the same pH) and a maximal binding capacity of 184 ± 44 pmol of protein/mg of lipid, instead of 110 pmol of protein/mg of lipid (compare Table 1, PE/PC liposomes, pH 7.5). Thus lipid-binding sites evidently persist in the modified, unfolded protein. These may correspond to clusters of hydrophobic and/or charged amino acid residues, the affinity of which is largely independent of tertiary structure. However, it should be noted that succinylation induced some changes in binding characteristics. Moreover we have observed that as measured by quenching of intrinsic protein fluorescence and in monolayer penetration, fodrin preparations, aggregated by storage, showed an evidence of reduced phospholipid binding (Diakowski and Sikorski, unpublished data).

To examine whether typical globular proteins bind to our liposomes, we performed binding assays with freshly isolated bovine hemoglobin. Essentially no binding was observed in the molar concentration range of the spectrin-binding experiments (0−300 nM). Some binding was observed at much higher protein concentrations (>2 mM), but there was no indication of saturation up to 10 mM hemoglobin (not shown). The results of binding experiments with bovine serum albumin and ovalbumin are shown in Figure 7A. As can be seen in the concentration range studied (0−1400 nM), no saturation in binding occurred. Figure 7B shows the results of the experiments in which either hemoglobin or bovine serum albumin was added as a possible competitor

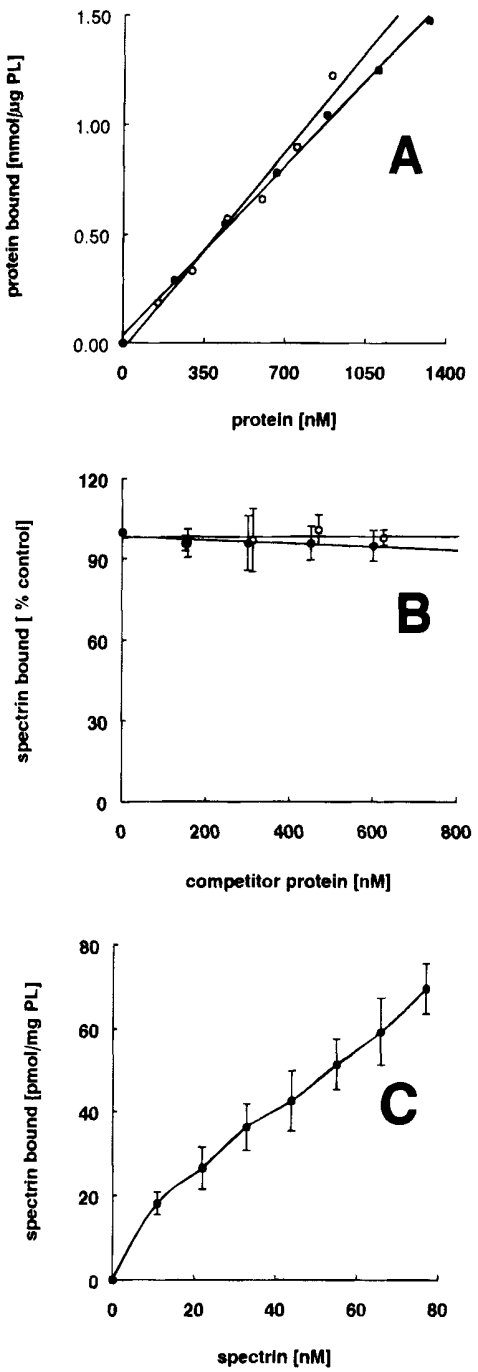

FIGURE 7: Control experiments. (A) Binding of bovine serum albumin (○) and ovalbumin (●) by FAT-liposomes prepared from PE/PC (60:40). Binding assays were carried out at pH 6.0. Other details are as in the Experimental Procedures. (B) Effect of hemoglobin or bovine serum albumin on binding of brain spectrin to liposomes prepared from PE/PC (60:40). Binding assays were performed at pH 6.0 in the presence of the indicated concentrations of bovine serum albumin (●) and hemoglobin (○). Error bar = ±SD. (C) Binding of succinylated brain spectrin to FAT-liposomes prepared from the PE/PC (60:40) mixture. Binding assay carried out at pH 7.5. Error bar = ±SD. Other details are as in the Experimental Procedures.

of brain spectrin binding to liposomes prepared from PE/PC (60:40) mixture. The quantitative analyses of the liposome pellets for the presence of brain spectrin and the extraneous protein (bovine serum albumin or hemoglobin) by means of SDS−gel electrophoresis and quantitation of the dye bound revealed that at 60 nM spectrin (well above the saturation level, see Figure 6B and Table 1, PE/PC, pH 6.0) no inhibition occurred even at a 10-fold excess of the competitor protein.

Figure 6E shows the results of binding assays carried out at a series of brain spectrin concentrations to liposomes prepared from the total lipid extracted with a mixture of chloroform and methanol from the synaptic plasma membrane. The binding isotherms are similar to those for liposomes prepared from purified phospholipids and their mixtures. The parameters (K_D and B_{max}) obtained from these data are also included in Table 1.

In conclusion, we may state that liposomes prepared from all the mixtures used, including total lipid from synaptic plasma membrane, bind brain spectrin in a saturable manner with K_D values in the nanomolar range and B_{max} values corresponding to 5300−31 000 lipid molecules/spectrin tetramer.

DISCUSSION

Our method of determining the binding of brain spectrin to liposomes has the advantage of simplicity and in particular not requiring chemical modification of the protein by a radioactive-labeling agent.

The affinity of this interaction is rather high (K_D values in the nanomolar range), and analysis of the binding isotherms, whether by curve fitting or Scatchard (1949) analysis (not shown), is well fitted in terms of a single class of independent binding sites. The above data are similar to those obtained for red blood cell spectrin by the same method (Białkowska et al., 1994). The results shown above were obtained by titration of a fixed concentration of liposome with increasing brain spectrin concentrations. In experiments in which the brain spectrin concentration was kept constant (not shown) while lipid concentration varied, much higher values of K_D were obtained. The same problem arises in regard to the number of phospholipid molecules bound, which appears to be rather high (27−150 molecules/nm of spectrin tetramer contour length), but this does not reflect the true capacity of each binding site: because the liposomes are quite large and not unilamellar, the number of available binding sites for spectrin must be limited by steric factors. Thus, when the spectrin is in excess, the stoichiometry will be determined by the number of spectrin molecules that can find access to sites on the surface of each liposome, whereas when the liposomes are in excess, each tetramer will bind the maximum number of liposomes that can fit along the rod. Steric factors may then affect the apparent binding constants correspondingly. The same is true when the data for binding of red blood spectrin to phospholipid vesicles obtained at constant concentration of liposomes were compared to those obtained at constant spectrin concentrations (Białkowska et al., 1994; Michalak et al., 1993). It should also be noted that the affinity of spectrin toward different amphipatic compounds may vary, being lower than for phospholipids, e.g., brominated fatty acids (Kahana et al., 1992), or detergents, particularly SDS (Sikorski et al., 1987b). Moreover, red blood cell spectrin did not bind lysophosphatidylethanolamine but did bind PE with high affinity (Michalak et al., 1994).

Optimal conditions for the interaction are in the range of physiological conditions (0.15 M NaCl, pH 7.5). The existence of a double pH optimum could be explained by

the properties of brain spectrin; the lower pH optimum is not far from the isoelectric point of the protein. For some lipid mixtures, binding is higher at pH 6.0 than at pH 7.5, but the differences are not large (see Figure 3). Increased binding of erythrocyte spectrin to phospholipid mono- and bilayers at pH 5.5 was observed previously (Mombers et al., 1980). The existence of the optimum at ca. pH 7.5 seems to be a feature of brain spectrin (in contrast to red blood cell spectrin).

The control experiments indicated the lack of binding of hemoglobin at molar concentrations comparable to those of brain spectrin and also no inhibition of brain spectrin binding to liposomes even by a 10-fold molar excess of either hemoglobin or bovine serum albumin in the assay. The binding of bovine serum albumin and ovalbumin, although of high capacity, did not show saturation in the range of 0−1400 nM.

We consider finally the possible physiological role of this interaction. In the first place it appears that proteins of the spectrin family exert a dominant effect on the mechanical properties of the membranes to which they are bound. To bring about such a gross transformation in the mechanical properties of the lipid bilayer, the protein (spectrin) must interact extensively with the lipids, giving the membrane the character of a laminar composite (Evans & Hochmuth, 1978; Stokke et al., 1986a,b).

It should be mentioned that the affinities of interaction of brain spectrin for phospholipid vesicles are about 1 order of magnitude lower than that of the ankyrin−fodrin complex (Davis & Bennett, 1984) or ankyrin-independent fodrin membrane interactions (K_D 3−50 nM; Steiner & Bennett, 1988). It is possible that interactions of fodrin with phospholipids are of importance in certain, particularly, early stages of membrane biogenesis, before stable linkages between peripheral and integral membrane proteins have been established. Asymmetrical distribution of aminophospholipids, i.e., enrichment of the cytoplasmic leaflet of the synaptosomal membrane bilayer (Fontaine et al., 1980), may also favor such interactions. The ability of non-erythroid spectrin to interact directly with lipids of the membrane seems to be particularly important since there are indications from work on lymphocytes and stable lymphoid cell lines that the interaction of spectrin with the membrane of the intact cell depends on the fluidity of lipids in the plasma membrane (Pauly et al., 1986; Langner et al., 1992). Agents modifying physical properties of membrane lipids, such as fatty acids or phorbol ester, caused a reversible alteration in spectrin distribution (plasma membrane → cytoplasmic aggregate) (Pauly et al., 1986; Langner et al., 1992) in lymphoid and myeloid cells.

Future studies should be aimed at clarifying the effect of this interaction on the properties of the membrane lipid bilayer. A further priority will be to establish whether this interaction is a property of the whole molecule, i.e., whether there are many lipid-binding domains distributed evenly along the α and β subunits of this protein or only a few hydrophobic domains with high binding capacity.

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