Ultrastructural Studies of the Interaction of Spectrin With Phosphatidylserine Liposomes

By Amos M. Cohen, Shih-Chun Liu, Laura H. Derick, and Jiri Palek

Spectrin was shown previously to interact with phosphatidylserine and phosphatidylethanolamine, which are preferentially localized in the inner half of the membrane lipid bilayer, but this interaction is not well characterized. In the present study we used electron microscopy of rotary-shadowed platinum replicas of spectrin dimer–phosphatidylserine complexes to study the interaction of spectrin with phosphatidylserine vesicles. At a spectrin concentration of 0.6 mg/mL, 60% of spectrin dimers were associated with phosphatidylserine vesicles and at a spectrin concentration of 1.2 mg/mL, some vesicles were crosslinked by spectrin dimers. The length of the protruding segment of spectrin dimer from the liposome edge ranged from 400 to 960 Å and the contact region to phosphatidylserine extended 272 ± 144 Å from either end of the molecule. Therefore, these data are consistent with multiple binding sites to phosphatidylserine throughout the spectrin dimer molecule. Spectrin tetramers, when bound to phosphatidylserine liposomes, extended 1804 ± 79 Å from the liposome edge and crosslinked liposomes, suggesting that some of the binding sites to phosphatidylserine vesicles is in the proximity of the tail end of spectrin. The association between spectrin dimers to phosphatidylserine was demonstrated by nondenaturing gel electrophoresis. The complexes were separated into multiple bands with molecular weight of 1.4 × 10^6, 1.8 × 10^6, and 2.3 × 10^6. These bands did not represent self-associated spectrin oligomers, since postincubation treatment with Triton-X-100 dissociated them into spectrin dimers. Furthermore, these spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the 14C-phosphatidylserine distribution. These data provide ultrastructural and biochemical evidence that spectrin binds to phosphatidylserine at multiple sites including the tail end region.

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tetramer to 750 exposed phospholipids with an affinity of about 3 x 10^7 mol/L.1,24

In this communication, we provide direct ultrastructural and biochemical evidence that spectrin binds to PS liposomes at multiple sites including the tail end region.

MATERIALS AND METHODS

Materials. 14C-phosphatidylserine, 1,2-dioleoyl, 60 mCi/mmol, was purchased from Amer sham. Protocol, toluidine, and Liquefluor were obtained from New England Nuclear. Bovine brain phosphatidylserine (PS) was purchased from Calbiochem, Avanti, and Pharmacia-PL Biochemicals and were 99% pure by TLC. All other chemicals were obtained from Sigma unless otherwise specified.

Spectrin extraction. Freshly drawn blood was filtered through cellulose and washed to remove white cells. Ghosts were prepared from washed erythrocytes by hypotonic lysis by the method of Dodge et al.24 Ghosts in 3 mmol/L Na3PO4 (pH 8.0) were treated with 0.5 mmol/L disopropyl fluorophosphate (DFP) at 0 °C for ten minutes to inactivate endogenous proteases. For low ionic strength extraction of spectrin, DFP-treated ghosts were washed with 0.1 mmol/L Na3PO4, pH 8.0 and immediately centrifuged to reduce the ionic strength of the medium and obtain a smaller pellet. Ghosts were then incubated at 37 °C for 30 minutes to obtain SpD-enriched extract (or at 0 °C for 16 hours for the study of SpT), with an equal volume of low ionic strength buffer containing 0.1 mmol/L Na3PO4, 0.1 mmol/L EDTA, 0.1 mmol/L dithiothreitol (DTT), and 0.1 mmol/L NaN3, pH 8.0. After incubation, the supernatant containing the crude spectrin extract and ghosts were separated by centrifugation at 250,000 x g, 4 °C, for 35 minutes. Crude spectrin extract was layered onto linear sucrose gradients (5% to 20% w/v) and centrifugation at 200,000 x g for 16 hours at 4 °C to obtain SpD or SpT fractions free of band 4.1. Protein concentration in collected fractions was determined by absorbance at 280 nm. To remove sucrose, the major peak fraction was dialyzed against 0.15 mol/L NaCl, 5 mmol/L TES, 1 mmol/L EDTA, and 0.15 mmol/L NaN3, pH 7.1 for 18 hours. Spectrin purity was tested by 5.6% acrylamide SDS gel electrophoresis. Spectrin dimers and SpT purity were tested by 2.5% acrylamide, 0.3% agarose (without SDS) non-denaturing gel electrophoresis.

Preparation of 14C-phosphatidylserine liposomes. Purified bovine brain phosphatidylserine (PS) was used for electron microscopic studies or was mixed (9:1) with 14C-phosphatidylserine 1,2 dioleoyl for electroforetic studies and sonicated (Heat System Ultrasconics, Plainview, NY, model 185 F, 200 watt) for 30 minutes at 0 °C under N2, to prevent oxidation. Under these conditions, small unilamellar vesicles are formed.27 Metal debris and undispersed lipid were removed by centrifugation at 200,000 x g for 30 minutes at 4 °C. PS concentration was determined by phosphate analysis by the method of Ames.28 The liposomes were kept under N2 atmosphere at 0 °C, and used within four hours after preparation to prevent oxidation and aggregation.

Ultrastructural analysis of spectrin-PS complexes by low angle rotary shadowing. Spectrin dimers or spectrin tetramers (0.6 to 1.2 mg/mL) were incubated with PS vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C (a temperature at which spectrin dimer- tetramer transformation is kinetically "frozen") for 120 minutes. After incubation, aliquots were added directly to glycerol at 0 °C to yield a glycerol concentration of 60%. Samples of spectrin or spectrin-PS complexes were sprayed onto freshly cleaved mica, dried under vacuum, and shadowed on a rotating stage with platinum-carbon from an electron bombardment gun (Balzers BAF 400D) at a shadow angle of 6° as described.28 Replicas were coated with a support film of carbon, floated onto distilled water, mounted on 400-mesh copper grids, and examined with a JEOL 100S electron microscope at 60 kV. Samples of pure PS vesicles in 60% glycerol were sprayed onto freshly cleaved mica which was previously prepared by immersion into boiling 0.1% Alicaline blue 8GFX for two minutes, rinsed with distilled water, and air dried, to promote lipid vesicle adhesion to the mica.29 The sprayed PS vesicles were dried and shadowed in the same way as pure spectrin or spectrin-PS complexes.29 Pure PS vesicle replicas were floated off the mica onto 5% sodium hypochlorite, rinsed with distilled water, cleaned with chromic acid followed by distilled water rinses, and mounted on 400-mesh copper grids and studied in the same way as pure spectrin or spectrin-PS complexes. Assessment of spectrin binding sites to PS vesicles was made by examination of SpD-PS and SpT-PS complexes at x 125,000 by 0.1 mm graduated X7 measuring magnifier (Bausch and Lomb). The distance between SpD or SpT end and PS vesicle edge was measured and subtracted from SpT native length (1000 PA and 2000 PA, respectively), which yielded spectrin binding region length and an unbound segment of spectrin.

Electrophoresis of spectrin-PS complexes. Quantitation of protein-lipid binding by direct methods requires the separation of the unbound protein from the protein-lipid complexes. A previous method that employed sedimentation of dextran loaded vesicles to quantitate spectrin-lipid binding was found in our hands to show low recovery of the SpD-PS complexes, due to leakage of the PS vesicles (unpublished data). To evaluate SpD-PS binding we subjected SpD-PS mixture to non-denaturing gel electrophoresis to separate free SpD and SpD bound to PS. Purified SpD (0.1 mg/mL) was incubated with 1.0 mg/mL 14C-PS liposomes at isotonic conditions 5 mmol/L TES, pH 7.1, 0.15 mol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L DTT, and 0.15 mmol/L NaN3, pH 7.1 for 120 minutes. To separate SpD-PS species by non-denaturing gel electrophoresis, samples that contained 10 ug SpD were mixed with one-ninth volume of concentrated buffer to give a final solution of 40 mmol/L Tris-HCl, pH 7.4, 20 mmol/L Na acetate, 2 mmol/L DTT, 200 mmol/L sucrose, and 2 mmol/L EDTA, and electrophoresed in a 0.3% agarose to 2.5% acrylamide gels (without SDS) at 20 volts for 22 hours as previously described.30 Triton-X-100, a nonionic detergent, was used to dissociate spectrin species from PS vesicles for evaluation of SpD self-association into SpT and SpO. Control Sp-D-PS mixture, SpD or SpO were further incubated with 0.1% Triton-X-100 for ten minutes at 0 °C and electrophoresed under the same conditions. For tracing spectrin, gels were stained with Coomassie blue, frozen, and sliced into 1 mm sections. Each slice was then extracted by pyridine (25% v/v) and the absorbance at 605 nm was read for each slice. For counting 14C-PS duplicate gels were frozen immediately and sliced into 1 mm sections. Each slice was then soaked in 5 mL scintillation fluid (toluene, Protocol, Liquefluor) and counted for 14C (Beckman, gamma 4000, Irvine, Calif) as described.32

RESULTS

Ultrastructure of spectrin dimer-phosphatidylserine complexes. As shown on Fig 1A and 1B, the length (1000 PA for dimer) and thickness (50 to 80 PA) of the SpD molecules are consistent with the published values.4 The diameter of the PS liposomes (less than 500 PA) as measured by low angle rotary shadowing was consistent with measurements of the liposomes by negative staining (data not shown) and with published values for the sonicated small unilamellar vesicles.27 Spectrin dimer-PS complexes are shown in Fig 1A and 1B. In order to determine whether the SpD-PS complexes are genuine or artifact, we examined by low angle

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Table 1. Number of Spectrin Dimers Bound per Phosphatidylserine Vesicles

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<th>Number of Spectrin Dimer Molecules Bound per a PS Vesicle</th>
<th>Percentage of Total Complexes</th>
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Spectrin dimers (0.6 mg/mL) were incubated with phosphatidylserine vesicles in isotonic conditions (pH 7.1) at 0 °C for 120 minutes. Electron micrographs of low angle shadowed platinum-carbon replicas of spectrin-PS complexes were studied for the number of spectrin dimer molecules bound per vesicle.

rotary shadowing the ultrastructure of actin, ankyrin, albumin, and protein 4.1, which were incubated with PS vesicles. The ultrastructure results showed that actin, ankyrin, and albumin did not associate with PS vesicles (data not shown), therefore suggesting that SpD-PS complexes are genuine. In contrast, protein 4.1 associated with PS vesicles, which is the subject of another communication (Cohen AM, Liu SC, Palek J, manuscript in preparation). At an SpD concentration of 0.6 mg/mL, and a PS concentration of 0.3 mg/mL, 68% (out of 318 SpD molecules counted) were bound to PS vesicles. This SpD concentration of 0.6 mg/mL was the highest at which no crosslinking of PS vesicles by SpD took place. The number of SpD molecules bound per PS vesicle was analyzed in 61 SpD-PS complexes and ranged between 1 and 7 SpD molecules bound per PS liposome. As shown in Table 1, complexes of SpD-PS that contained 3 SpD were most prevalent (26%). However, quantitation of the number of SpD molecules bound per lipid vesicle provided only an approximate estimation because of a relatively limited number of SpD-PS complexes available for quantitation, as well as a possible variation of adhesion SpD-PS complexes to the mica surface and fragmentation of crosslinked complexes during sample spraying. Most of the SpD molecules' contact point to PS consisted of less than half of the SpD molecule length (Fig 2A to 2C). This observation enabled a crude estimation of the location of the spectrin binding sites for PS vesicles which was made by subtraction of the distance of the SpD that extended beyond the liposome edge from the native SpD length. Analysis of 63 SpD molecules bound to PS vesicles revealed the length range of the protruding segment of SpD from the liposome edge to be 400 to 960 Å (Fig 3). The contact region of SpD with PS liposomes was calculated to be 272 ± 144 Å (1 SD) from...
were incubated with phosphatidylserine vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. Samples were then diluted in glycerol and treated as in Fig 1. The electron micrographs (×125,000) of 63 spectrin dimer molecules extending from lipidome edge were measured by a ×7 measuring magnifier.

either end of the SpD molecule. These results appear to be considerably more variable than the site of spectrin-ankyrin interaction on β spectrin, which resides approximately 200 Å from spectrin head end.32,33 Thus, these data are consistent with the presence of multiple binding sites to PS throughout the SpD molecule length.

Crosslinking of phosphatidylserine vesicles by spectrin dimers. At spectrin concentration of 0.6 mg/mL, SpD decorated PS vesicles (Fig 2A to 2C) and at a concentration of 1.2 mg/mL, SpD produced considerable crosslinking of PS vesicles (Fig 2D, 2E). This suggests that both spectrin head and tail end regions bind to PS liposomes. Some SpD-PS complexes crosslinked by SpD had a distance of up to 720 Å between bound vesicles (range 256 to 720 Å) (Fig 2D, 2E). Furthermore, SpD crosslinked PS vesicles into small aggregates (Fig 1B and 2F), indicating that SpD molecule has several binding sites to PS.

Interaction of spectrin tetramers with phosphatidylserine. Electron micrographs of SpT-enriched preparations (with less than 10% SpD and SpO) and PS liposomes are shown in Fig 4A and 4B. The structure of SpT (2000 Å length, 50 to 80 Å thickness) was consistent with previously published values.3 Spectrin tetramers were bound to PS vesicles mainly at one of their ends, constituting the SpD tail end (Fig 4C, Fig 5A to 5C). The average distance for 24 SpT molecules from liposome edge was 1804 ± 79 Å (range 1760 to 1920 Å), which suggested a binding site for PS located in a region within 196 ± 79 Å from the spectrin tail end. Spectrin tetramers crosslinked PS vesicles with a distance of up to 1400 Å between two bound vesicles (range 760 to 1400 Å), which confirmed the involvement of several binding sites of SpT to PS (Fig 5D, 5E). In addition, incubation of SpT with PS vesicles leads to an extensive crosslinking of PS vesicles (Fig 5F). Both SpT and SpD, at the same mass concentration (1 mg/mL), crosslinked PS vesicles by a similar amount.

Separation of spectrin dimer-phosphatidylserine complexes by nondenaturing gel electrophoresis. In order to quantitate SpD binding to PS vesicles, we employed nondenaturing gel electrophoresis. We have previously used this method to separate the various size spectrin oligomers from membrane extracts.31 Originally we employed large multilamellar PS vesicles, but these vesicles barely entered the gels and decreased the mobility of unbound SpD, thus preventing valid quantitation of binding. Small unilamellar PS vesicles moved freely through the gel and concentrated behind the dye front region and therefore were selected to study SpD-PS association. Complexes of SpD with 14C-PS liposomes were separated by nondenaturing gel electrophoresis into multiple high molecular weight bands (Fig 6A). A short postincubation treatment with Triton-X-100 dissociated these bands predominantly into SpD, which exhibited a slightly faster mobility than the control SpD. This change in mobility was not a consequence of degradation of the spectrin, as confirmed by SDS gel electrophoresis of the SpD-PS complexes, dissociated by Triton-X-100, which showed that spectrin mobility was unchanged. Furthermore, spectrin oligomers were not dissociated by 1% Triton-X-100, as documented by nondenaturing electrophoresis (data not shown) which is consistent with a previous report.32 Spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the 14C-PS distribution (Fig 6B). Thus, these 1.4 × 106, 1.8 × 106, 2.3 × 106,
and higher molecular weight bands that formed following SpD incubation with PS represent SpD-PS complexes as indicated by their complete disappearance upon treatment with Triton-X-100 and by the comigration of spectrin (Coomassie blue) and 14C-PS in the gels. The spectrin to PS molar ratio varied throughout the high molecular bands region (0.8 × 10⁻¹ to 6.5 × 10⁻²) with a trend of higher molar ratios to coincide with the spectrin bands. This variability of the SpD to PS molar ratio probably reflected PS vesicles' mass heterogeneity and prevented quantitation of SpD to PS binding or estimation of the number of SpD molecules bound per PS vesicle. This pattern of high molecular band formation of SpD-PS complexes by nondenaturing gel electrophoresis was remarkably reproducible when three different sources of purified PS were tested, thus making the possibility of SpD interaction with a contaminant highly unlikely. We have also examined the SpD association with sphingomyelin, PC, and PE. Sphingomyelin and PC, the major constituents of the outer lipid bilayer, did not associate to a significant degree with SpD. In contrast, SpD formed similar high molecular weight complexes with the inner lipid bilayer constituents PE and a mixture of PE/PS (3:2 molar ratio). Both of these data are in agreement with previously published data.¹⁴,³⁹

**DISCUSSION**

Previous reports of the interaction of spectrin with the membrane lipids are mainly confined to indirect evidence. These data were obtained through studies on the effect of spectrin on lipid vesicle phase transition, lipid monolayer surface area, lipid bilayer rearrangement, and identification of hydrophobic domains of this protein. In this report, we provide direct ultrastructural evidence that spectrin interacts with PS vesicles. The ultrastructural results of spectrin-PS interaction suggest that the binding is relatively specific for spectrin, since under the same experimental conditions, actin, ankyrin, and albumin did not associate with PS vesicles. In contrast, protein 4.1 was found to associate with PS vesicles, which is the subject of another communication (Cohen AM, Liu SC, Palek J, manuscript in preparation). We find various regions of the spectrin molecule involved in SpD association with PS vesicles, as documented by the considerable variability of the contact regions (272 ± 144Å² from either side of the spectrin molecule). In addition, at higher SpD concentration, SpD can crosslink PS vesicles, suggesting that both ends of the spectrin molecule
have binding sites for PS. Spectrin tetramers are shown to bind and crosslink PS vesicles. Thus, the result is consistent with the hypothesis that some of the PS binding sites are located at the distal end region of the spectrin molecule. The exact number of PS binding sites and their location on the spectrin molecule are presently not determined.

In addition to the ultrastructural evidence, we documented the interaction by employing nondenaturing gel electrophoresis. Spectrin dimers incubated with 14C-PS vesicles were separated by nondenaturing gel electrophoresis into multiple high molecular bands. These spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the 14C-PS distribution, suggesting that they are complexes of SpD-PS. These bands did not represent oligomeric forms of spectrin, as indicated by their complete solubilization by Triton-X-100.

Spectrin is anchored to the membrane both at its head and tail ends by high affinity interactions with ankyrin and protein 4.1, respectively. Based on the direct demonstration of PS-spectrin interaction reported here, as well as previously published, largely indirect data, we speculate that additional low affinity interactions of spectrin and possibly other skeletal proteins with lipids of the inner lipid bilayer might exist, thereby further stabilizing the lipid bilayer. Furthermore, the possible biological significance of such low affinity interactions between spectrin and PS is enhanced by the estimated high local concentration of spectrin (230 mg/mL) close to the inner leaflet of the membrane lipid bilayer. The role of the spectrin-lipid interactions in the maintenance of lipid bilayer stability in certain hemolytic disorders, such as hereditary spherocytosis, which is characterized by membrane instability leading to a loss of membrane lipid, remains to be elucidated.

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