Phosphatidylserine Binding Sites in Erythroid Spectrin: Location and Implications for Membrane Stability†

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ABSTRACT: The erythrocyte membrane is a composite structure consisting of a lipid bilayer tethered to the spectrin-based membrane skeleton. Two complexes of spectrin with other proteins are known to participate in the attachment. Spectrin has also been shown to interact with phosphatidylserine (PS), a component of the lipid bilayer, which is confined to its inner leaflet. That there may be multiple sites of interaction with PS in the spectrin sequence has been inferred, but they have not hitherto been identified. Here we have explored the interaction of PS-containing liposomes with native α- and β-spectrin chains and with recombinant spectrin fragments encompassing the entire sequences of both chains. We show that both α-spectrin and β-spectrin bind PS and that sites of high affinity are located within 8 of the 38 triple-helical structural repeats which make up the bulk of both chains; these are α8, α9−10, β2, β3, β4, β12, β13, and β14, and PS affinity was also found in the nonhomologous N-terminal domain of the β-chain. No other fragments of either chain showed appreciable binding. Binding of spectrin and its constituent chains to mixed liposomes of PS and phosphatidylcholine (PC) depended on the proportion of PS. Binding of spectrin dimers to PS liposomes was inhibited by single repeats containing PS binding sites. It is noteworthy that the PS binding sites in β-spectrin are grouped in close proximity to the sites of attachment both of ankyrin and of 4.1R, the proteins engaged in attachment of spectrin to the membrane. We conjecture that direct interaction of spectrin with PS in the membrane may modulate its interactions with the proteins and that (considering also the known affinity of 4.1R for PS) the formation of PS-rich lipid domains, which have been observed in the red cell membrane, may be a result.

The membrane of the red blood cell derives its high tolerance of shearing stresses and its capacity to deform elastically during the passage of the cell through the microvasculature from the spectrin-based membrane skeletal network, the membrane skeleton, which covers the cytoplasmic bilayer surface. Spectrin exists as elongated tetramers, their ends constrained by the separation of the network junctions to about one-third of the contour length of 200 nm. The junctions to which the ends of the tetramers are attached consist of F-actin, protein 4.1R, and other proteins (*1*). The primary sites of attachment of the network to the bilayer are, on one hand, through the protein, ankyrin (*2*, *3*), which forms a bridge between each spectrin tetramer and the transmembrane protein, band 3 (*4*, *5*), and on the other, a connection between the 4.1R and the transmembrane protein, glycophorin C (*6*).

The lipid composition of the red cell membrane bilayer differs between its two leaflets, phosphatidylserine (PS),[1] which is anionic at physiological pH, and phosphatidylethanolamine (PE), being confined to the cytoplasmic side by the action of lipid translocating enzymes (*7*−*12*). Spectrin−lipid interactions have been reported in a number of studies (*13*−*16*), and there is reason to suppose that binding of spectrin to PS exerts an influence on the stability of the membrane (*17*).

It is difficult to envisage how relatively sparse protein filaments, coupled to the bilayer through transmembrane proteins some 70 nm apart, can impose grossly transformed mechanical properties on a membrane bilayer, which by itself possesses essentially no elasticity and is so unstable that it breaks up spontaneously into small vesicles when spectrin is dissociated. To gain further insight into the origins of the remarkable properties of the membrane, we have examined the interactions between the bilayer lipid and spectrin, and we consider the possible manner in which they might influence its mechanical character.

EXPERIMENTAL PROCEDURES

Materials

Blood was taken, with informed consent, from healthy human volunteers. Restriction enzymes were from New

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CD, circular dichroism; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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England BioLabs (Beverly, MA). PS and PC from brain were from Sigma (St. Louis, MO) and from Avanti (Alabaster, AL). Synthetic PS and PC were also obtained from Avanti. Reduced form glutathione, thrombin, and IPTG were purchased from Sigma. The pGEX-4T-2 vector and glutathione−Sepharose 4B were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ), SDS−PAGE and electrophoresis reagents were from Bio-Rad (Herculus, CA), and GelCode staining reagent was from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals were of reagent grade and obtained from standard sources.

Methods

Preparation of the Spectrin Dimer and Monomers. Spectrin from erythrocytes was prepared as described by Tyler et al. (*18*). Spectrin monomers were isolated essentially as described by Speicher (*19*) with the following modifications: instead of HPLC, a Sephadex 200 (2.5 × 125 cm) gel filtration column was used to purify spectrin monomers. The monomers were concentrated by precipitation with 50% ammonium sulfate and dialyzed against PS binding buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM NaN_3).

Preparation of Recombinant Spectrin Polypeptides and Liposomes. The four α-spectrin recombinant fragments were subcloned into the pGEX-4T-2 vector using *Sma*I and *Not*I cloning sites upstream and downstream, respectively. The four β-spectrin recombinant fragments and all of the single spectrin repeats were subcloned into the pGEX-4T-2 vector, using *Eco*RI and *Sal*I cloning sites upstream and downstream, respectively. Full-length α-spectrin cDNA (kindly provided by Dr. L. Kotula, New York State Institute for Basic Research in Developmental Disabilities) was used as the template for PCR amplification of recombinant α-spectrin polypeptides, and full-length β-spectrin cDNA was used as the template for PCR amplification of β-spectrin polypeptides. The fidelity of all the constructs was confirmed by DNA sequencing. The expression of recombinant proteins was induced by 0.1 mM IPTG at 16 °C for 3−4 h. The GST fusion polypeptides were purified using a glutathione−Sepharose 4B affinity column. For CD measurement, GST was cleaved with thrombin. Protein concentrations were determined spectrophotometrically, using extinction coefficients calculated from the tryptophan and tyrosine contents, taking the molar extinction coefficients of these amino acids at 280 nm as 5500 and 1340, respectively (*20*). Proteins were dialyzed against PS binding buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3) for PS binding assay and against PBS (10 mM phosphate, pH 7.4, and 150 mM NaCl) for CD measurements. All of the proteins were clarified by ultracentrifugation at 230000*g* for 30 min at 4 °C before use. Liposomes were prepared by the sonication method (*21*).

CD Spectroscopy. The far-UV CD spectra were recorded in a Jasco 700 spectropolarimeter, equipped with a thermostated cell housing, in cells of 1 mm path length. The CD data are displayed in terms of mean *r* molar residue ellipticities.

Measurement of Protein−Liposome Interaction by Sedimentation Assay. For sedimentation assays, protein at a concentration of 1 μM was incubated with liposomes at a miminal concentration of 625 μM in a total volume of 100 μL at room temperature for 30 min. The liposomes with bound protein were then collected by centrifugation at 230000*g* for 30 min at 4 °C, with unbound protein remaining in the supernatant. The liposome pellet was washed three times and resuspended in 100 μL of binding buffer. Equal volumes of supernatant and pellet were analyzed by SDS−PAGE. Gels were stained with GelCode staining reagent and evaluated by densitometry.

Table 1: α- and β-Spectrin Polypetides Used in the Present Study

fragment	codons	amino acids
αN−5	1−1752	1−584
α6−11	1722−3540	574−1180
α12−16	3541−5142	1181−1714
α17−C	5128−7290	1710−2430
α6	1732−2046	578−682
α7	2047−2364	683−788
α8	2365−2682	789−894
α9−10	2683−3231	895−1077
α11	3232−3534	1078−1178
βN−4	1−2226	1−742
β5−9	2227−3819	743−1273
β10−14	3820−5388	1274−1796
β15−C	5389−6211	1797−2137
βN	1−903	1−301
β1+	877−1266	293−422
β2	1267−1581	422−527
β3	1582−1908	528−636
β4	1909−2226	637−742
β10	3820−4134	1274−1378
β11	4135−4431	1379−1477
β12	4432−4749	1478−1583
β13	4750−5067	1854−1689
β14	5068−5388	1690−1796

Measurement of Protein−Liposome Interaction by Electrophoretic Migration Shift Assay. The proteins were incubated with liposomes as described above. Samples of 40 μL were mixed with 10 μL of a solution containing bromophenol blue in 50% glycerol, and 15 μL aliquots of these mixtures were applied to a native acrylamide gel, which was run for 5 h in the cold, as described (*22*). The gel was stained with GelCode blue.

RESULTS

Design and Characterization of Recombinant Spectrin Fragments and Single Spectrin Repeats. To map the PS binding sites in spectrin, we began by constructing four α-spectrin fragments (αN−5, α6−11, α12−16, and α17−C, where N and C denote the chain termini and the numbers refer to the homologous triple-helical repeats) and four β-spectrin fragments (βN−4, β5−9, β10−14, and β15−C), which span the entire sequences of the α- and β-chains. After the fragments that could bind PS were identified, the search for the binding sites was narrowed by isolating the single repeats contained within those fragments. The boundaries of all repeats were defined by the SMART database (*23*) (http://smart.embl-heidelberg.de/) (see Table 1) with two exceptions: β-spectrin repeat 1 (here designated β1+), which starts 8 residues before the predicted repetitive segment (*24*), and α-spectrin fragment 9−10. Here, since segment 10 is not a complete repeat, repeat 9 and segment 10 were expressed as a single piece. The precise locations of the repeat boundaries have been a matter of some debate, but that the results delivered by interrogating the SMART

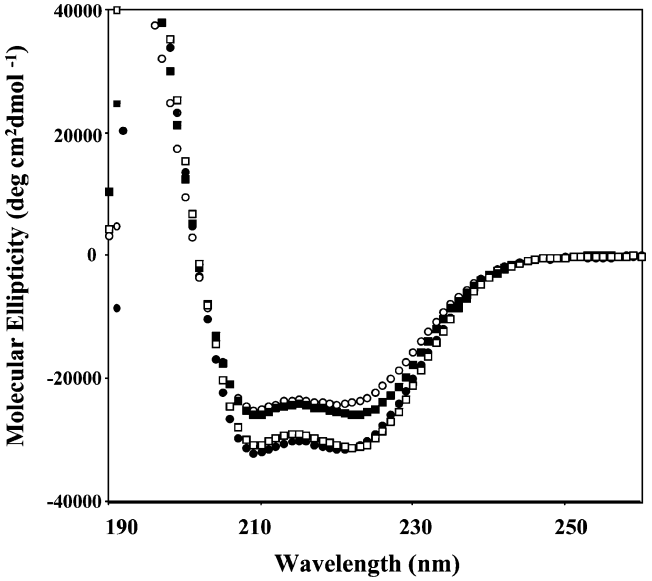

FIGURE 1: Representative circular dichroism spectra of recombinant spectrin polypeptides. The spectra were recorded at 20 °C. The solvent was PBS. Key: closed circles, αN−5; open circles, α6−11; closed squares, *β*5−9; open squares, *β*10−14.

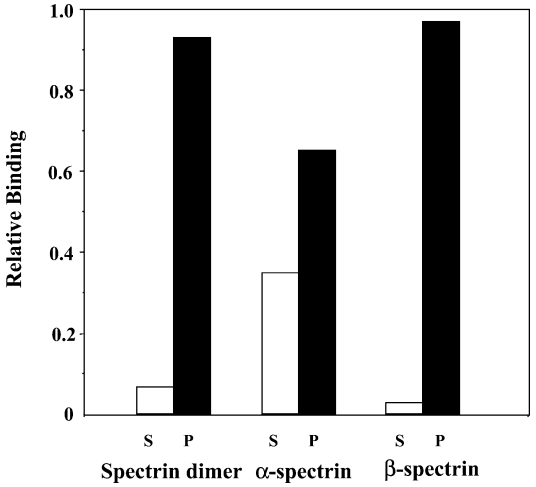

FIGURE 2: Binding of PS to the spectrin dimer and spectrin subunits. Following incubation of spectrin or its subunits with PS liposomes, the bound and unbound proteins were separated by ultracentrifugation (see text). Proteins in the supernatant (S) and pellet (P) were resolved by 10% SDS−PAGE and evaluated by densitometry. The bulk of the protein was found in each case in the lipid pellet.

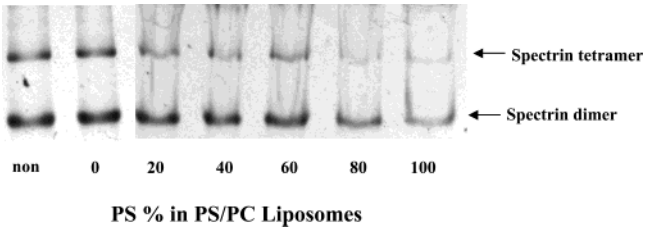

FIGURE 3: Binding of spectrin to PS/PC liposomes. Spectrin was incubated with liposomes containing varying ratios of PS and PC. The binding of spectrin to liposomes was judged by retardation of its electrophoretic migration in polyacrylamide gels containing no denaturant. PC liposomes had no effect on spectrin migration, while the amount of spectrin enetering the gel fell with increasing proportion of PS in the mixed PS/PC liposomes.

database are satisfactory for our purposes is shown by the formation of the stable, highly α-helical fold by the expressed products (see below).

Because some of the polypeptides were insoluble when expressed at 37 °C, and expression at lower temperature greatly improved the yield of soluble product, all of the polypeptides were expressed at 16 °C. Figure 1 shows the CD spectra of some representative recombinant polypeptides, demonstrating typical highly α-helical profiles: molar residue ellipticities at the three extrema at 222, 208, and 192 nm corresponded to α-helix contents between 65% and 85% (*25*). Moreover, thermal melting profiles all showed a plateau region at low temperature, followed by a sigmoidal cooperative transition, terminating in a second plateau. This established that the proteins were properly folded. At the temperature of the lipid binding assays all of the fragments were below, or almost entirely below, the start of their unfolding transitions.

Binding of the Spectrin Dimer and α- and β-Spectrin Chains to PS Liposomes. As a first step toward locating the PS binding sites, the binding of PS liposomes to the spectrin dimer and to the separated α- and *β*-chains was measured by the ultracentrifuge sedimentation assay. As shown in Figure 2, all three showed abundant binding by this criterion.

Binding of Spectrin to PS/PC Liposomes. To examine the specificity of the interaction with PS, binding assays of spectrin to PS/PC liposomes were performed, using an electrophoretic migration shift assay (*14*, *26*). In this procedure, binding of lipid to protein is detected by retardation of protein migration in the electrophoretic gel. Figure 3 shows that while pure PC liposomes did not alter spectrin migration in native gels, increasing the content of PS in the mixed liposomes caused a progressive diminution in the proportion of spectrin entering the gel, indicating that the binding of spectrin to PS/PC liposomes is a function of the concentration of PS.

Mapping the PS Binding Sites in the α-Spectrin Chain. To define the PS binding sites in α-spectrin, four recombinant α-spectrin polypeptides (αN−5, α6−11, α12−16, and α17−C) were first constructed and examined for binding in the sedimentation assay. Figure 4A shows that in the conditions of these experiments more than 95% of the fragment comprising repeats 6 through 11 cosedimented with PS liposomes. By contrast, spectrin fragments αN−5 and α12−16 remained in the supernatant. Some 30% of the α17−C polypeptide appeared in the pellet, indicative of a weak interaction with PS. To locate binding sites in the α6−11 segment more precisely, the single constituent repeats of this sequence were prepared. Figure 4B shows that while more than 95% of α8 and α9−10 cosedimented with PS liposomes, repeats 6, 7, and 11 remained in the supernatant. Thus, we have localized a distinct PS binding site to repeats 8−10 in the α-spectrin chain.

Mapping the PS Binding Sites in β-Spectrin Chain. The same approaches as above were followed in identifying the PS binding sites in the spectrin *β*-chain. As shown in Figure 5A, two *β*-spectrin fragments, *β*N−4 and *β*10−14, cosedimented with PS liposomes, while the other two fragments, *β*5−9 and *β*15−C, remained in the supernatant. Of the constituent structural elements of *β*N−4, the N-terminus and *β*2, *β*3, and *β*4 all cosedimented with PS liposomes, while *β*1+ remained in the supernatant. Three subfragments

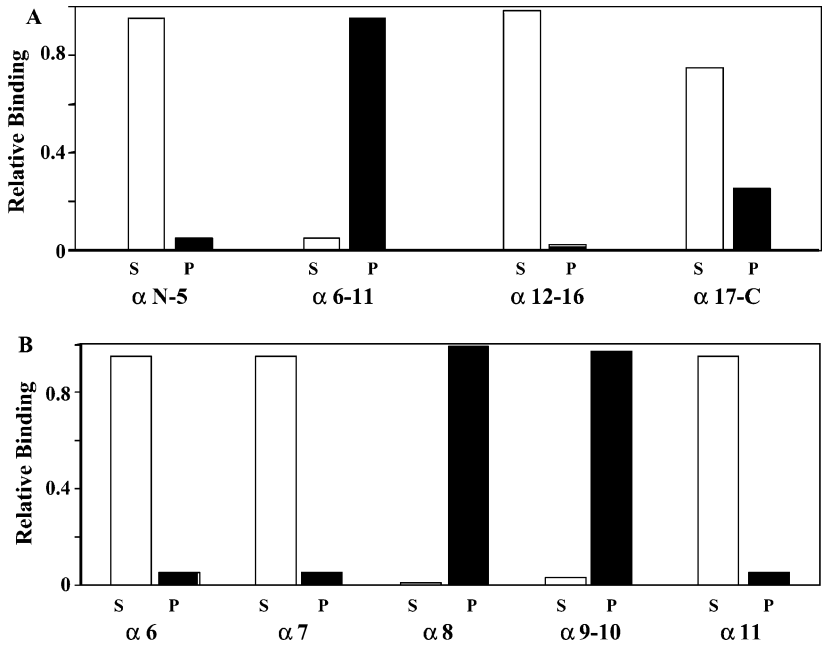

FIGURE 4: PS binding sites in the α-spectrin chain. (A) Four α-spectrin fragments were incubated with PS liposomes, and the bound and free protein fractions were separated by ultracentrifugation. Protein in the supernatant (S) and pellet (P) was analyzed by electrophoresis in 10% SDS gels and densitometry. (B) Single α-spectrin repeats making up segment α6−11 were incubated with PS liposomes, and binding was evaluated in the same way.

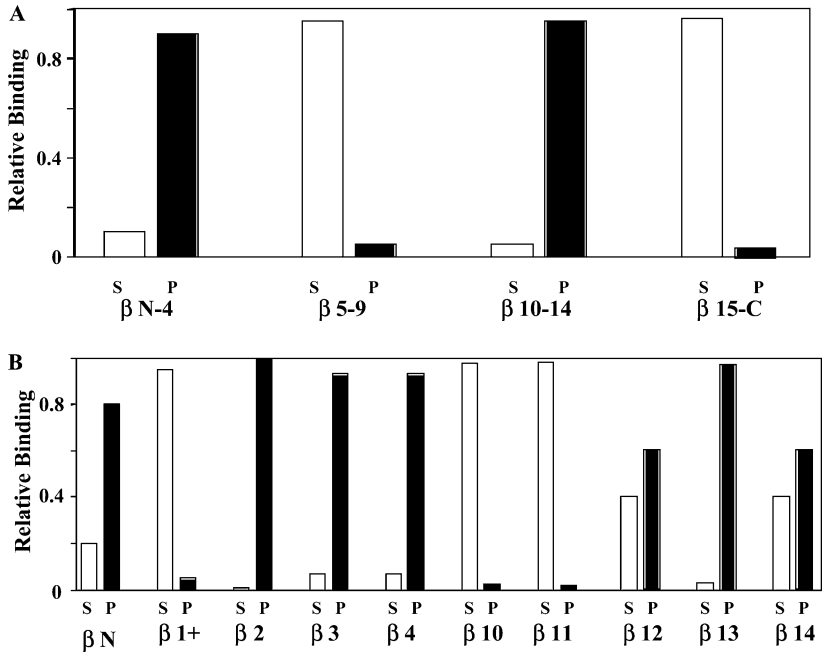

FIGURE 5: PS binding sites in the spectrin β-chain. (A) Four β-spectrin fragments were incubated with PS liposomes. Bound and unbound fractions were separated by ultracentrifugation. Protein in the supernatant (S) and pellet (P) was estimated by SDS−PAGE and densitometry. (B) Single β-spectrin repeats, making up segments βN−4 and β10−14, were analyzed for PS binding activity as above.

of β10−14 cosedimented with PS liposomes; these were β12, β13, and β14 (of which β13 showed the strongest binding), while β10 and β11 remained in the supernatant (Figure 5B). Thus there are two regions in the β-spectrin chain that contain grouped PS binding sites, one at the N-terminal end, and thus close to the 4.1R and actin binding site, the other in the segment of repeats 12−14, which is close to the ankyrin binding site.

Binding of Spectrin Fragments to PS/PC Liposomes. To determine how the binding of spectrin fragments to liposomes varies with PS content, we used the electrophoretic mobility shift assay to examine the retardation by PS/PC liposomes of two α-chain fragments and one β-chain fragment and one β-chain repeat. As Figure 6A shows, the migration of fragment αN−5, which did not bind PS in the sedimentation assay, was unaffected by PS/PC and indeed even by pure PS liposomes, showing that there is no significant interaction with either. By contrast (Figure 6B), the migration of α6−11, which bound PS in the sedimentation assay, was increasingly retarded with increasing proportion of PS in the liposomes. Similarly, the liposomes had no effect on β5−9 migration but greatly retarded that of β13, depending again

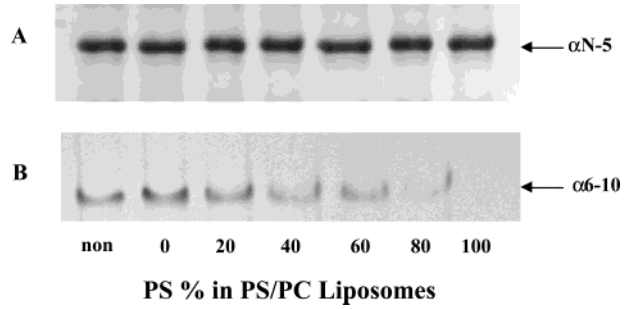

FIGURE 6: Binding of spectrin fragments to PS/PC liposomes. The polypeptides were incubated with liposomes containing varying ratios of PS:PC. Binding was detected by the migration shift assay (see text). (A) αN−5; (B) α6−11.

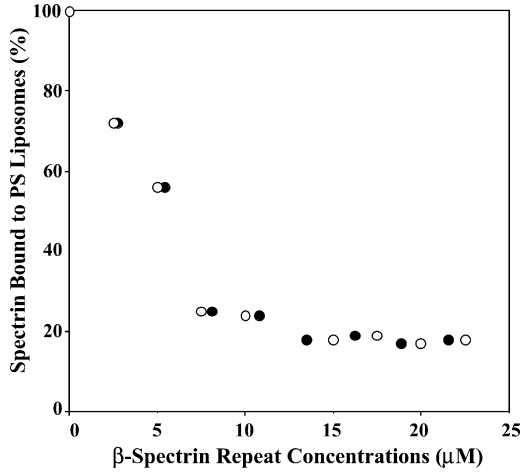

FIGURE 7: Inhibition of binding of spectrin dimers to PS liposomes by single spectrin repeats. PS liposomes were preincubated with increasing concentrations of β2 (solid circles) or β13 (open circles) at room temperature for 30 min. Spectrin dimer (0.77 M) was added, and its binding to the liposomes was measured by the ultracentrifugation assay. Note inhibition of binding of the dimer by both peptides.

on PS concentration (data not shown). This again is consistent with the results of the sedimentation assays.

Inhibition of Binding of the Spectrin Dimer to PS Liposomes by Single Spectrin Repeats. To confirm the specificity of interaction at the PS binding sites defined above, we carried out competition experiments between spectrin dimers and single PS binding repeats. Figure 7 shows the results of such an experiment, in which spectrin was added to PS liposomes preincubated with varying concentrations of β2 or β13. Inhibition was observed as predicted, but an unexpected, and so far unexplained, feature of the results was that the degree of inhibition terminated in a plateau at about 80%, even at a molar ratio of fragment:spectrin of 30:1.

DISCUSSION

Erythrocyte spectrin has previously been found to interact with PS (*13*−*16*). Cohen et al. (*14*) provided ultrastructural and biochemical evidence that the protein contains multiple binding sites for the lipid. There were even suggestions that binding sites for phospholipids, or at least for anionic amphipaths, might be distributed along the entire length of the chains (*27*). In the present study, we have identified the locations of the specific binding sites in α- and β-spectrin for PS.

We have not here considered the wider question of lipid specificity of the binding sites. Many anionic lipophiles have been reported at various times to bind to spectrin, as have nonmembrane anionic lipids, and PE has also been stated to bind (*16*). The affinity of spectrin for PC in a supported planar bilayer was reported by McKiernan et al. (*28*) to be comparable to that for PS, whereas Maksymiw et al. (*13*) could detect no interaction with PC. Similarly, an affinity of spectrin for PC liposomes has been reported by one laboratory (*29*), while others observed no such effect (*15*, *16*). We, at all events, could detect no binding of spectrin or its fragments to unilamellar PC liposomes under conditions in which there is abundant interaction with PS.

A striking feature of the distribution of the PS binding sites on the spectrin chains, shown schematically in Figure 8, is that they coincide with, or are at least very close to, the sites of attachment of the proteins (ankyrin and 4.1R) that form linkages to the lipid bilayer. This leads us to conjecture that a direct interaction between spectrin and the bilayer may be coupled to that with proteins. It should be remarked that Bialkowska et al. (*30*) have found that an interaction between spectrin and lipid monolayers at low surface pressures is inhibited by the attachment of ankyrin to the spectrin. Conversely, protein 4.1R has been seen to increase the affinity of spectrin for PS (*31*).

The stoichiometry of binding of spectrin to PS-containing liposomes is unusual, for, although the interaction is by no means weak (association constants of ca. 3×10^6 to 3×10^5 M^{-1}, depending on the temperature), saturation occurs at a molar ratio of PS:spectrin of about 30000−1000 (depending again on temperature) (*15*). This implies that spectrin binding sites on the lipid surface are made up of sizable patches of PS. Whether such patches preexist in the lipid mixture of the red cell membrane inner leaflet or are generated by the attachment of the spectrin is unclear, but phase separation has been observed in the native cell membrane (*32*, *33*), and even artificial lipid mixtures of the same composition are reportedly in a state of incipient phase separation (*34*). It is at all events possible that spectrin regulates the state of the bilayer. Whether spectrin−lipid

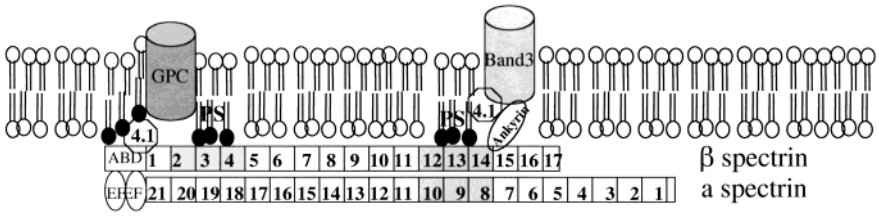

FIGURE 8: Schematic representation of the disposition of PS-binding sites in spectrin. The boxes represent the structural repeats making up most of the two spectrin chains. The repeats containing PS binding sites are shown shaded. The positions of the ankyrin and 4.1R attachment sites are indicated. ABD = actin binding domain; EF = EF hand.

interactions contribute significantly to the preservation of phospholipid asymmetry in the cell [predominantly controlled by lipid translocating enzymes (see ref *8* for review)] has been a matter of debate (*35*, *36*); there is, however, evidence that spectrin does interact with one or both of the inner-leaflet lipids (PS and PE) in situ and that this interaction plays an important part in stabilizing the membrane (*17*).

We have no definitive explanation for the incomplete competition between spectrin and single-repeat binding fragments (Figure 7), since presumably only one type of binding site (PS or clusters thereof) exists in the liposomes. Conceivably the resistance of a fraction of the bound spectrin to competition by the smaller fragments is determined by the size and flexibility of the long chains of the intact dimer, which may require binding sites comprising many more molecules of PS than those for the small fragments.

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