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BAND 4.1 ENHANCES SPECTRIN BINDING TO PHOSPHATIDYLSERINE VESICLES

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Erythroid band 4.1 enhances the binding of erythroid spectrin to phosphatidylserine vesicles under conditions of ionic strength and at protein concentrations similar to those in the red cell. The extent of enhancement depends on the concentration of band 4.1; at 2 μM 4.1, spectrin binding increases approximately 10-fold (to 600 μmoles/mole lipid). The K_d is 0.5 μM, as measured by SDS-PAGE of protein-bound vesicles recovered by ficoll gradient centrifugation. The 4.1-enhanced binding of spectrin was also measured by a gel filtration assay. The electrostatic nature of the enhancement of spectrin binding is indicated by its dependence on the phosphatidylserine content of the vesicles.

The elastic properties of the red cell skeleton may be modulated by its interaction with the underlying lipid bilayer (1), a major component of which is phosphatidylserine (2). In support of this suggestion, the major skeletal proteins, band 4.1 (3-5) and spectrin (6-8, R. I. MacDonald, submitted), can bind specifically to vesicles composed of acidic phospholipids, as well as to each other (9-11) and to other red cell surface-associated proteins (12). The K_d for phosphatidylserine binding of spectrin is 0.1 - 1 μM (7-8, R. I. MacDonald, submitted), whereas the K_d for phosphatidylserine binding of band 4.1 is somewhat lower (3,4, this work). Since 4.1 and spectrin associate with each other with a similar affinity (11), it seemed likely that band 4.1 would enhance the binding of spectrin to

Abbreviations: ATP, adenosine 5'-triphosphate; CF, carboxyfluorescein; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_d, dissociation constant; PC, egg yolk phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; PS, bovine brain phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MLV, multilamellar lipid vesicles; LUVETs, large, unilamellar vesicles produced by extrusion technique; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNED, 25 mM Tris, pH 7.6, + 0.1 M NaCl + 0.1 mM EDTA + 0.2 mM DTT; Tris, tris(hydroxymethyl)aminomethane.

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PS vesicles. We report here that 4.1 indeed substantially increases the amount of spectrin binding to PS vesicles.

MATERIALS AND METHODS

Preparation of red cell skeletons. Human red cell ghosts were prepared by a standard procedure (13) within 3 weeks of withdrawal. Chemicals were from Sigma unless otherwise noted. Protease activity was reduced by removing white cells with a leukocyte filter (Pall Biomedical Products Corp.) and by the inclusion of pepstatin (2 μg/ml) and PMSF (34 μg/ml) in the lysis buffer. Skeletons were liberated from ghosts by solubilizing the membrane with an equal volume of 4% (v/v) Triton X-100 in 25 mM HEPES, pH 7.0, + 1 mM EGTA + 0.5 mM DTT + 0.174 mM PMSF (14). To pellet the skeletons stripped of ankyrin and band 3, the detergent-containing mixture was layered over chilled 30% sucrose in 1.5 M NaCl + 25 mM HEPES, pH 7.0, + 0.5 mM ATP + 0.5 mM DTT + 0.5 mM EGTA (15) and centrifuged for 1 h at 112,000 x g in a Beckman SW28 rotor.

Purification of spectrin and band 4.1. Pelleted skeletons were dissociated in 1 M Tris, pH 7.2, (16) and centrifuged for 1 h at 130,000 x g in a Beckman SW50.1 rotor. Solubilized spectrin and band 4.1 were separated on a Sepharose CL-6B column (60 x 2.5 cm) equilibrated with 1 M Tris, pH 7.2, + 0.2 mM EGTA at 4°C. Spectrin was concentrated by precipitation at 50% saturation with $(NH_4)_2SO_4$. The redissolved spectrin was dialyzed against TNED and eluted from a Sepharose CL-4B column (80 x 2.6 cm) with TNED at 4°C to obtain spectrin heterodimer which was concentrated by addition of $(NH_4)_2SO_4$ to 50% saturation and dialysis of the resuspended pellet against TNED. The spectrin concentration was estimated by its absorbance at 280 nm which is $10.7^{1\%}_{1cm}$(17). Band 4.1 was further purified by DEAE-Sephacel chromatography on a 4.5 x 1.4 cm column and concentrated by dialysis against aquacide III (Calbiochem). 4.1 was quantified by the Bradford method (18) or by its absorbance at 280 ($8.0^{1\%}_{1cm}$; 19). Spectrin dimer and band 4.1 were stored at 4°C in TNED and 4.1 was used within a week after purification.

Monitoring the quality of spectrin and band 4.1. 1) Spectrin and band 4.1 isolated from the stripped skeletons contained only the α and β subunits and a single band, respectively, on SDS-PAGE. The integrity of proteins was regularly verified by SDS-PAGE. 2) Spectrin and band 4.1 formed a gel when incubated with muscle actin (20). 3) The spectrin dimer-tetramer equilibrium K_d was 1.2 μM at 30°C, which compares well with the 2.5 μM obtained by electrophoretic separation of dimer and tetramer (21). 4) Others (3-5) have reported PS binding of band 4.1 isolated by 1 M KCl extraction of spectrin-depleted ghosts, rather than the method of Ohanian and Gratzer (16). We therefore tested 4.1 obtained by the latter method for binding to PS vesicles and found maximal binding of 4.1 to the outer monolayer of PS MLV to be 3.6 mmole/mole PS, 40% of the 9 mmole/mole PS found by Cohen et al. (3) and about 3 times higher than the 1.25 mmole/mole PS reported by Rybicki et al. (4). The dissociation constant for band 4.1 and PS vesicles was found to be 0.3 μM, corresponding to that obtained previously (4). The binding of band 4.1 to LUVETs of PS, phosphatidic acid, phosphatidylinositol, PC-phosphatidylethanolamine (1:1) or PC was in the ratio 1:2.2:1.1:0.2:0.08, i.e. parallel with the lipid charge.

Preparation of lipid vesicles. MLVs were prepared by shaking dried lipid with the appropriate buffer under N_2 and freezing and thawing nine times. The fraction of PS in the outer monolayer was measured with TNBS (Fluka) [22] and found to be 11% of the total. LUVETs were prepared as described (23). Phospholipid was measured according to (24).

Ficoll-SDS PAGE assay of protein binding to lipid vesicles. Proteins were incubated with MLVs in 100 μl of 152 mM NaCl + 5 mM Tris, pH 7.2, + 1 mM NaN_3 at room temperature for 1 hr. To isolate the vesicles from the protein, the incubated sample was mixed with 100 μl of 24% ficoll-400 in buffer and overlaid with 500 μl of 8% ficoll-400 in

buffer and 20 μl of buffer alone. The gradient was centrifuged at 28,000 x g in a Sorvall HB-4 rotor for 40 min at 4°C, after which 50 μl of the top layer was removed. 25 μl of this layer was analyzed by SDS-PAGE (25) on 8% gels and the amounts of Coomassie blue or silver-stained (26) protein quantified in an LKB Gelscan densitometer with known amounts of similarly stained spectrin and 4.1 as internal standards. Phosphate in the remaining 25 μl of the top layer from the gradient was measured (24) to determine lipid content.

Gel filtration assay of CF-spectrin binding to LUVETs. To corroborate binding data obtained with the ficoll:SDS-PAGE assay and to expedite data gathering, we measured spectrin binding to PS by an alternative assay, in which bound and free spectrin were separated by gel filtration on Sepharose CL-2B (R.I. MacDonald, submitted). Spectrin at μM concentrations and labeled with carboxyfluorescein (final dye:protein ratio close to 1:1) by reaction with 5-(and) 6-carboxyfluorescein, succinimidyl ester (Molecular Probes), was incubated with 250 μM PS LUVETs with or without 1 μM 4.1 in 0.2 ml TNED for 30 min at room temperature. Vesicle bound CF-spectrin was separated from free CF-spectrin on a Sepharose CL-2B column (1 x 11 cm) by elution at room temperature with TNED at 1 ml/min. Spectrin in the eluant was detected by its carboxyfluorescein fluorescence. The bound CF-spectrin was determined from the area under the free and vesicle-bound peaks.

RESULTS

Enhancement by 4.1 of spectrin binding to PS vesicles. Several reports have indicated the K_d of spectrin binding to PS to be 0.1 μM - 1 μM (7-8, R.I. MacDonald, submitted), which is in the same range of the K_d of 4.1 binding to PS vesicles--i.e., 0.3 μM (3-4, this work). Since 4.1 and spectrin associate (9-11), we tested whether 4.1 could enhance spectrin binding to PS vesicles. Figure 1A shows that at a total concentration of 0.6 μM, spectrin binding to 2.1 mM PS MLVs, as measured by the ficoll gradient method, increases from 2.5 μmoles of spectrin/mole lipid (20 μmoles spectrin/mole of lipid in the outer monolayer) of MLV in the absence of 4.1 (Figure 1A, filled circles) to 30 μmoles of spectrin/mole lipid (270 μmoles of spectrin/mole of lipid in the outer monolayer) of MLV with a K_d of 0.5 μM in the presence of 4.1 at 0.9 μM (Figure 1A, open circles). The K_d obtained in the absence of 4.1 was similar to that obtained in the presence of 4.1 although there is greater uncertainty in the former case because of the much smaller amounts bound. The apparent primary effect of band 4.1 is thus to increase the extent rather than the strength of spectrin binding to PS vesicles.

Figure 1B shows the effect of the concentration of band 4.1 on the binding of spectrin to PS vesicles, measured as for Figure 1A. In confirmation of the data of Figure 1A, binding of spectrin at 0.73 μM to 2.4 mM PS MLVs is strongly dependent on the amount of band 4.1 and increases to 600 μmoles/mole lipid as the band 4.1 concentration is increased to near 2 μM.

Effect of PS content on binding of spectrin to PS vesicles depends on the presence of 4.1. The binding of 4.1 to PS-PC vesicles increases in proportion to their PS content (4;

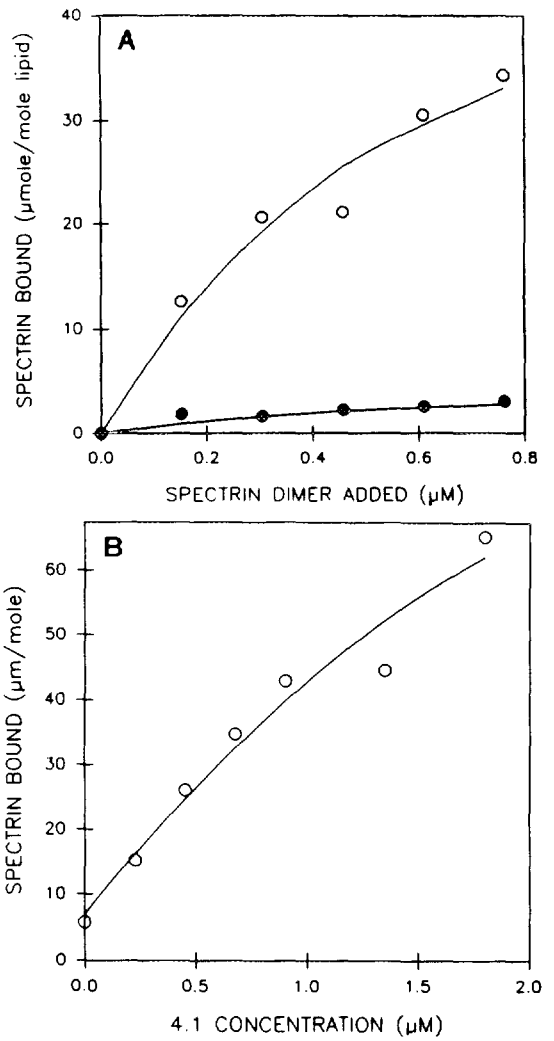

Figure 1. A) Effect of 4.1 on spectrin binding to PS vesicles. Spectrin was incubated at concentrations shown with 2.1 mM PS MLVs in the presence (open circles) or absence (filled circles) of 0.9 μM band 4.1 in 100 μl of 5 mM Tris, pH 7.2, + 0.152 M NaCl + 1 mM NaN_3 for 1 hr at 25°C. Bound spectrin was measured as described under MATERIALS AND METHODS. Each point is the mean of duplicate samples except for the 2nd, 3rd and 5th points in the lower curve, which represent single measurements. The upper curve corresponds to an adsorption isotherm with the parameters, K_d = 0.5 μM and maximum binding = 400 μmoles/mole lipid in the outer monolayer of the MLVs. The lower curve is characterized by the same K_d and a maximum binding of 40 μmoles/mole lipid in the outer monolayer of the MLVs. B) Spectrin binding to PS vesicles as a function of band 4.1 concentration. The spectrin concentration, incubation conditions and procedures for measuring bound spectrin were the same as in A) and the band 4.1 concentration was varied as shown. Each point is the mean of duplicate samples.

MacDonald, unpublished). To ascertain whether 4.1 would affect the dependence of spectrin binding on the PS content of the vesicles, we measured spectrin binding to vesicles containing different amounts of PS in the presence and absence of 4.1. Spectrin binding was

measured by a gel filtration assay by which we have obtained results similar to those obtained by the ficoll gradient assay (R.I. MacDonald, submitted). Table I gives the μmoles CF-spectrin bound/mole phospholipid in the outer monolayer in the presence and absence of band 4.1 at 25°C. Next to this value and in parentheses is the % represented by this value of that for 100% PS vesicles, i.e. [spectrin bound to PC-PS vesicles] x 100 divided by [spectrin bound to PS vesicles]. The amount of spectrin binding is closely related to the PS content of the vesicles and an increase from 50% to 100% PS leads to somewhat more than a doubling of the amount of bound spectrin. The amounts of spectrin bound to 100% PS LUVETs in the absence of 4.1 were about the same, whether measured by gel filtration or by ficoll gradient centrifugation. The order of magnitude difference between data in Table I and in Figures 1A and 1B is due to the latter representing spectrin bound to MLV PS, only 11% of which is accessible for binding. The smaller 4.1-mediated increase in spectrin binding shown in Table I compared with Figures 1A and 1B, however, is real and may be due to some enhancement of the 4.1 effect by ficoll used in the latter case.

DISCUSSION

We report that band 4.1 substantially enhances the binding of spectrin to PS vesicles. Although others have demonstrated that band 4.1 (3-5) and spectrin (6-8, R. I. MacDonald, submitted) individually bind to vesicles composed of acidic phospholipid, it had not been shown previously that band 4.1 can enhance the binding of spectrin to phosphatidylserine

Table I

Enhancement by band 4.1 of CF-spectrin binding to vesicles of various PS:PC ratios*

	%PS	without band 4.1	with band 4.1
Exp. 1	100	21+ (100%)	75 (100%)
	69	15 (71%)	43 (57%)
	44	6 (29%)	21 (28%)
Exp. 2	100	40 (100%)	100 (100%)
	65	25 (63%)	50 (50%)
	39	9 (23%)	--

*0.55 (Exp. 1) or 0.44 (Exp. 2) μM CF-spectrin was incubated with about 1 mM phospholipid with or without 1 μM band 4.1 in 0.2 ml of TNED buffer for 30 min at room temperature. The bound spectrin was measured as described under MATERIALS AND METHODS.

+μmole CF-spectrin bound/mole phospholipid in outermost monolayer. Values in parentheses indicate % of spectrin bound relative to that bound to 100% PS vesicles.

vesicles and to an extent of potential physiological importance (Figures 1A and 1B). This result shows that the interaction of spectrin with band 4.1 need not preclude its interaction with phosphatidylserine. This observation is also promising of the reconstitution of a skeleton on a lipid bilayer, which should further understanding of the role(s) of skeletal components in membrane mechanics (27-28).

In contrast to the well-characterized protein-protein interactions responsible for the integrity of the skeleton and its attachment to the red cell membrane, the properties and roles of the weaker protein-lipid interactions are more obscure, precisely because they are weaker and, therefore, not amenable to study in the presence of the higher affinity protein-protein interactions. The significance of attaining a degree of binding of the major skeletal component, spectrin, to defined, model membranes which is comparable to that in the cell, nevertheless, depends on whether the in vitro conditions approximate in vivo conditions. The concentration of spectrin at the inner surface of the red cell is calculated to be 0.92 x 10^{-15} g/μm^2, or 230 mg/ml (29), which considerably exceeds the 0.5 μM K_d of spectrin-PS binding at room temperature (Figure 1A) and even the 3 μM K_d determined for the spectrin-PS interaction at 37°C (R.I. MacDonald, submitted). In the presence of 4.1, spectrin lying on the surface of the vesicle could be as concentrated as 0.41 x 10^{-15} g/μm^2 (Figure 1A), given that 11% of the PS in MLV is in the outer monolayer and that one PS molecule occupies 60 $Å^2$. Moreover, this density was attained at a bulk spectrin concentration of about 0.3 μM, an order of magnitude lower than the cellular concentration.

Data obtained by a gel filtration assay in Table I affirm the ability of 4.1 to enhance the binding of spectrin to PS vesicles as first measured by the ficoll gradient assay (Figures 1A and 1B). The increase in spectrin bound to PS in the presence of band 4.1 apparently occurs without a correspondingly large increase in binding affinity (Figure 1A). An increase in amount but not affinity of binding is unexpected in a situation involving single, non-interacting sites. Since the present case may involve more complicated interactions, its treatment by adsorption isotherm formalism may be an approximation. In particular, it is unlikely that the binding site area or the dissociation constant are completely independent of the extent of coverage of the PS surface by spectrin. An alternative possibility is that spectrin occupies a smaller area on the vesicle surface when interacting with band 4.1 than when interacting with the PS surface in the absence of band 4.1--perhaps because band 4.1 may affect spectrin flexibility or cause spectrin to attach perpendicular, rather than parallel, to the lipid surface at high spectrin surface density. Further experiments are necessary to determine whether 4.1 enhances spectrin binding to PS vesicles by simultaneously interacting with spectrin and with the lipid vesicle surface--the simplest explanation of the effect reported here.

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REFERENCES

1. Markin, V. S. and Kozlov, M. M. (1988) J. Theoret. Biol. 133, 147-167.
2. Ferrell, J. E. and Huestis, W. H. (1984) J. Cell Biol. 98, 1992-1998.
3. Cohen, A. M., Liu, S. C., Lawler, J., Derick, L. and Palek, J. (1988) Biochemistry 27, 614-619.
4. Rybicki, A. C., Heath, R., Lubin, B. and Schwartz, R. S. (1988) J. Clin. Invest. 81, 255-260.
5. Sato, S. B. and Ohnishi, S. (1983) Eur. J. Biochem. 138, 19-25.
6. Maksymiw, R., Sui, S., Gaub, H. and Sackman, E. (1987) Biochemistry 26, 2983-2990.
7. Mombers, C. A. M. (1982) "Investigation on Spectrin-Lipid Interaction in Model Systems," p. 47, Ph.D. Dissertation at the University of Utrecht.
8. Bonet, D. and Begard, E. (1984) Biochem. Biophys. Res. Commun. (1984) 120, 344-350.
9. Cohen, C. and Foley, S. F. (1984) Biochemistry 23, 6091-6098.
10. Coleman, T. R., Harris, A. S., Mische, S. M., Mooseker, M. S. and Morrow, J. S. (1987) J. Cell Biol. 104, 519-526.
11. Podgorski, A. and Elbaum, D. (1985) Biochemistry 24, 7871-7876.
12. Bennett, V. (1990) Physiological Reviews 70, 1029-1065.
13. Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119-130.
14. Sheetz, M. P. (1979) Biochim. Biophys. Acta 557, 122-134.
15. Shen, B. W., Josephs, R. and Steck, T. (1984) J. Cell Biol. 99, 810-821.
16. Ohanian, V. and Gratzer, W. (1984) Eur. J. Biochem. 144, 375-379.
17. Kam, Z., Josephs, R., Eisenberg, H. and Gratzer, W. B. (1977) Biochemistry 16, 5568-5572.
18. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
19. Tyler, J. M., Reinhardt, B. N. and Branton, D. (1980) J. Biol. Chem. 255, 7034-7039.
20. Spudich, J. A. and Watt, S. J. (1977) J. Biol. Chem. 246, 4866-4871.
21. Morrow, J. S., Speicher, D. W., Knowles, W. J., Hsu, C. J. and Marchesi, V. T. (1980) Proc. Natl. Acad. Sci. USA 77, 6592-6596.
22. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E. and Carlson, F. D. (1977) Biochemistry 16, 2806-2810.
23. MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K. and Hu, L.-r. (1991) Biochim. Biophys. Acta 1061, 297-303.
24. Bartlett, G. R. (1959) J. Biol. Chem. 234, 446-468.
25. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685.
26. Merril, C. R., Goldman, D. and Van Keuren, M. I. (1983) Methods in Enzymology 96, 230-239.
27. Steck, T. L. (1989) in Cell Shape: Determinants, Regulation, and Regulatory Role (Stein, W. D. and Bronner, F., eds.) Ch. 5, Academic Press, N. Y.
28. Elgsaeter, A. and Mikkelsen, A. (1991) Biochim. Biophys. Acta 1071, 273-290.
29. Lux, S. (1979) Semin. Hematol. 16, 21-51.