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Review

Interaction of membrane skeletal proteins with membrane lipid domain★✪

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The object of this paper is to review briefly the studies on the interaction of red blood cell membrane skeletal proteins and their non-erythroid analogues with lipids in model systems as well as in natural membranes. An important question to be addressed is the physiological significance and possible regulatory molecular mechanisms in which these interactions are engaged.

Membrane lipid bilayer–membrane skeleton interactions are thought to be responsible for the membrane integrity and its mechanical properties (e.g. very high linear elasticity while maintaining negligible extensibility) and several models were proposed to explain these membrane properties [1–3]. One of the most studied biological membranes is that of the red blood cell. During its 120 day life time in the circulation this 8 μm diameter cell has to pass repeatedly through 2 μm capillaries and hence withstand and respond to very

★75th Anniversary of Membrane Lipid Bilayer Concept.

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Abbreviations: CTAB, cetyltrimethylammonium bromide; FAT-liposomes, frozen and thawed liposomes; IP_3, inositol 1,4,5-triphosphate; LDAO, dimethyldodecylamine oxide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH, pleckstrin homology; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PL, phospholipid; PS, phosphatidylserine.

strong mechanical stresses. This makes the red cell an appealing model to study. Another reason is the simplicity of the cell and its membrane, i.e., mature mammalian red blood cells are devoid of a nucleus and all organelles. Membranes prepared from these cells by hypotonic lysis comprise the plasmalemma associated with which are some 20 polypeptide chains as visualised by simple Coomassie blue stained SDS/polyacrylamide gel electrophoregrams. This attractively simple system has provided several generations of students with a grounding in practical membrane and cell biology.

The remarkable mechanical properties of the red cell membrane stem from the presence on the cytoplasmic surface of a dense, well organised protein network called the membrane skeleton. Its major component is spectrin, a high molecular mass flexible rod-like protein formed by head-to-head association of two heterodimers composed of α (280 kDa) [4] and β (247 kDa) [5] subunits. Spectrin heterodimers are formed by antiparallel double helical association of α and β subunits, each of which form predominantly a segmental triple helical molecule. The detailed structure of the triple-helical segment, which is 106 amino acids long, was first solved by X-ray crystallography and also by NMR studies on an expressed fragment of insect spectrin [6]. Five to six spectrin tetramers interact with a short (37 nm) actin protofilament, formed of 13–14 G-actin molecules [7] to form a structure known as the junctional complex [7, 8]. Several other proteins: protein 4.1, adducin, dematin, p55, tropomyosin and tropomodulin are also involved in forming the junction. Both protein 4.1 and adducin are known to promote high affinity binding of spectrin to protofilaments (F-actin in the test tube) which otherwise would bind very weakly. Thus the formation of spectrin tetramers (i.e., the dimer–dimer interaction) and the junctional complex are responsible for the planar integrity of the membrane skeleton, and these cytoskeletons can be isolated from either intact cells or from membranes by extraction with a non-ionic detergent solution [9].

The membrane skeleton of the red blood cell (and quite probably membrane skeletons in other cells) is attached to the intrinsic membrane domain (lipid bilayer embedded with integral membrane proteins) through two pathways of interaction with transmembrane proteins, i.e., spectrin–ankyrin–protein AE1 (band 3) [10–13] and protein 4.1–glycophorins C and D [14] and the ternary interaction protein 4.1–p55–glycophorin C [15]. The structure of the membrane skeleton, the mutual interactions of its components and its interactions with membrane proteins in non-erythroid cells are known to a much lesser extent, largely because of their much higher stuctural complexity. However, since many animal cell membranes contain spectrin and spectrin-binding protein analogues (of erythrocyte membrane proteins), together with novel spectrin-binding proteins, the existence of a similar protein network, tightly associated with membrane proteins, is anticipated [16].

The above mentioned high affinity protein–protein interactions responsible for membrane skeleton attachment to the membrane have been the subject of many excellent reviews (e.g. [17]). There are, however, many indications coming from various studies on cells, isolated membranes, and model systems that direct protein–lipid interactions contribute to the attachment of the membrane skeleton to the membrane hydrophobic domain. Progress in this field has been rather slow, and the significance of these interactions remains unclear even though the binding of erythrocyte spectrin to phospholipid in model system has been known for more than 25 years.

ERYTHROCYTE SPECTRIN–LIPID INTERACTIONS

The earliest published studies [18, 19] on the interaction of spectrin with phospholipids pre-

dicted the discovery of ankyrin, the protein that links the cytoskeleton to the transmembrane protein AE1 (band 3) in erythrocytes. In these early studies, high ionic strength was found to impede the binding of spectrin (in fact a spectrin–actin complex was used in these experiments) to the phospholipid monolayer, the implication being that hydrophobic domains exist on spectrin. Further studies showed that purified spectrin had the ability to bind hydrophobic and amphipathic ligands such as: brominated stearic acid [20, 21], fatty acids, and anionic (SDS), cationic (CTAB), and zwitterionic (LDAO) detergents [22], all of which supported the view that spectrin contains a number of hydrophobic sites. This has been confirmed by analyses of binding isotherms using brominated fatty acids and phospholipid vesicles [21, 23]. Also, isolated erythrocyte spectrin was found to bind strongly to hydrophobic agaroses, again reflecting the presence of hydrophobic regions in the molecule. In particular, a 65 kDa proteolytic fragment (the βII domain) generated after limited trypsin digestion of spectrin was inferred to be rich in such regions [24].

Numerous studies on the interaction of erythrocyte spectrin with membrane bilayer phospholipids from natural (erythrocyte) membranes, liposomes, or monolayer lipid films have been carried out by a variety of techniques (for a review of the older literature see [25]). The observation that spectrin can be labelled in the membrane by the hydrophobic arylisothiocyanates suggests that it may partially penetrate the lipid bilayer, i.e., small regions of this molecule may interact directly with the hydrophobic region of the lipid bilayer [26, 27]. The interaction of spectrin with the erythrocyte membrane can also affect the membrane fluidity, hence removal of spectrin induced an increase in the spin-label order parameter, while re-association of such a membrane with purified spectrin resulted in a decrease of this parameter [28]. Similar results were obtained when the fluorescent membrane probe, diphenylhexatriene, was used to assess membrane fluidity [29].

Mombers and colleagues [30] observed a decrease in the enthalpy change associated with the phase transition of anionic phospholipids such as PS, PG or cardiolipin in the presence of spectrin. Also, monolayer penetration studies [31] indicated a specificity of spectrin for anionic phospholipids such as phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylglycerol (PG). This interaction was pH-dependent, being optimal at pH 5.5. Further specificity studies on the interaction of anionic phospholipid monolayers with sepa-

Table 1. Interactions of erythrocyte spectrin with phospholipids in model systems.

Examples of equilibrium dissociation constants obtained by different laboratories.

Lipid composition of vesicles	K_D [nM]		Conditions	Ref.
PC	111 ± 17,	99 ± 13	pH 6.0, pH 7.6	[38]
		100	pH 7.6	[23]
		170 ± 11	pH 7.6	[39]
PE/PC	89 ± 14,	577 ± 132	pH 6.0, pH 7.6	[38]
PS/PC (3:2)	206 ± 39,	146 ± 15	pH 6.0, pH 7.6	[38]
PS/PC (3:2)	140 ± 120		10 mM phosphate + 100 mM NaCl,	[39]
	1220 ± 480		10 mM phosphate	[39]
DMPS[a]/DMPC[a] (1:1)	100			[23]
PS/PC	2700			[42]
PS/PE	700			[42]

[a]DM, dimyristoyl

rated α or β spectrin subunits indicated preferential binding by the β subunit [32]. A similar conclusion was reached when phosphatidylethanolamine (PE)/phosphatidylcholine (PC) monolayers were used [33].

It has been suggested by several groups that spectrin binds preferentially to membrane monolayers or bilayers that contain PS. Indeed, some experimental data seem to confirm this hypothesis [31, 34–36]. On the other hand, our data [26, 37, 38] and those of others [23, 39] showed that the affinity of purified spectrin for PS-containing vesicles was not significantly different from that for PC vesicles. There is a limited number of studies that provide kinetic data characterising the affinity of interactions of spectrin with phospholipids. However, none of these data showed an increased affinity of spectrin towards membranes containing PS. Some examples of the available data on spectrin binding to phospholipids are presented in Table 1 [23, 38–42]. It should be noted that even though the data are obtained by a variety of methods, at varying PS/PC ratios, and under different conditions, the discrepancies between them are rather small.

The reason for the discrepancies between the qualitative effects and quantitative data for spectrin binding to PS-containing membranes remains unknown, although, as we suggest below, one of the possible explanations could be the different lipid-binding sites in the spectrin molecule. Another possibility is that the different experimental set-ups and techniques measure different types of lipid binding.

The addition of liposome suspensions to spectrin solutions causes quenching of its intrinsic tryptophan fluorescence [29, 37]. Similarly, changes of the degree of polarisation of emission of isoindole, a fluorescent probe, attached to the protein [40], as well as changes in quenching of the protein intrinsic and isoindole fluorescence by acrylamide were observed [29]. These interactions were only moderately affected by ionic strength and pH or by the presence of high concentrations of urea. The largest reduction of the intrinsic fluorescence of spectrin was induced by PE and PE/PL combinations [29, 37]. Certain trypsin or pronase proteolytic sites on spectrin became inaccessible in the presence of phospholipid liposomes [41]. Aqueous suspensions of phospholipids, particularly those containing PE, display a stabilising effect on spectrin structure and thus reduce the fluorescence intensity changes induced by ionic strength and temperature [37].

We have found that, for the interaction of erythrocyte spectrin with PE-containing lipid aggregates, the bilayer structure may be important. Thus, lyso-PE and H_{II}-phase-forming dioleyl-PE suspensions in water showed little or no capacity to quench the intrinsic fluorescence of spectrin in solution [43].

INTERACTIONS OF NON-ERYTHROID SPECTRINS WITH MEMBRANE LIPIDS

There are indications that lipids interact with non-erythroid spectrins, e.g. in the membranes of lymphoid and myeloid cells. Here, fatty acids or phorbol myristate acetate induced a reversible alteration in the spectrin distribution between the so called cytoplasmic aggregate and the plasma membrane [44, 45]. While the mechanism how these changes occur remains unknown, they are almost certainly related to the physical properties of the membrane lipid bilayer, i.e., fluidity, domain formation, etc.

Mammalian brain spectrin comprises a mixture of several spectrin isoforms, the most prominent of which is neuronal αIIΣ1/βIIΣ1 [46–48] spectrin. These bear a high sequence homology to erythroid spectrins and have the α and β subunits of 283 and 275 kDa, respectively [49–52]. Each monomer contains a series of 106 amino acid repeats of high α-helicity [6, 53]. Almost all the proteins that bind spectrin in the erythrocyte membrane have non-erythroid counterparts. Non-erythroid

spectrin has been found to interact with many other elements of cell membranes e.g., neural cell adhesion molecules [54, 55], cytoskeleton-forming proteins such as MAP-τ [56], and neurofilament-associated protein or glial acidic protein [57, 58]. As well as being a component of the membrane skeleton non-erythroid spectrin is thought to be involved in the regulation of exocytosis [59] and in particular in the regulation of neurotransmitter release, in which its interaction with small synaptic vesicles *via* synapsin was shown to play a crucial role [60–63]. Numerous interactions of spectrin with other proteins have been recently inferred using the yeast two hybrid system (interaction trap) [64], as reviewed in [65].

In our laboratory, we addressed the question of whether non-erythroid (brain) spectrin also binds to membrane phospholipids. We used a total brain spectrin preparation, the great majority of which was the neuronal, non-erythroid type isoforms (αIIΣ1/βIIΣ1). In our initial experiments we used a pelleting assay in which soluble purified brain spectrin was bound to dextran-filled frozen and thawed liposomes (FAT-liposomes). Saturable binding was observed for liposomes prepared from the following lipid mixtures: PC, PE/PC 2:3, PS/PC 2:3, PE/PC 2:3, as well as for liposomes prepared from the total lipid extract from synaptic plasma membranes [66, 67]. Binding was strong and saturable with K_D's in the nanomolar range (i.e., from 16 nM at pH 7.5 for liposomes prepared from the total lipid mixture to about 500 nM for PC liposomes at pH 6.0) [67].

Purified brain spectrin induced an increase in surface pressure in lipid monolayers (at low initial surface pressure) composed of PE/PC, PS/PC (3:2) and PC. The maximal effect ($\Delta\pi$) was observed when monolayers contained PE, in particular when PE was 50–60% of the lipid mixture [68]. This interaction occurred optimally at pH 6.0 (coincidental with the spectrin isoelectric point) and pH 7.5, both in the pelleting assays and in monolayer experiments. There was also an ionic strength optimum, corresponding to 0.15 M NaCl [67, 68]. These conditions (pH 7.5 and 0.15 M NaCl ionic strength) are essentially physiological. Monolayer experiments similarly revealed, as in the case of red blood cell spectrin, that the major lipid-binding site is located in the β-subunit of the brain protein. Small unilammellar phospholipid vesicles caused quenching of the intrinsic tryptophan fluorescence of brain spectrin in solution. These changes, which were generally smaller (20–30% maximally) than those described above for the changes in fluorescence of erythrocyte spectrin, and were larger when vesicles were prepared from lipid mixtures containing PE [68].

One of the known specific lipid-binding sites in non-erythroid spectrins is the PH (pleckstrin homology) domain which is present both in the βIΣ2 and in βIIΣ2 isoforms [69, 70]. The PH domain is approximately 100 amino acids in length and is present in a variety of proteins, e.g., kinases isoforms of phospholipase C, GTPases, GTPase-activating proteins, and nucleotide exchange factors of Ras, Vav, Dbl and Bcr. Many PH domain-containing proteins interact with GTP-binding proteins indicating that this domain may be involved in regulatory/signalling pathways [71]. Wang & Shaw [72] found that bacterially expressed β-spectrin PH domain bound to the following membrane preparations: brain membranes, vesicles made of lipids extracted from brain membranes, and to PIP_2-containing lipid vesicles. Moreover, they found that IP_3 binds specifically to the PH domain and inhibits binding of this polypeptide to brain membranes. When this domain (of βIΣ2 spectrin) was fused with green fluorescent protein and expressed in mammalian COS7 cells, the specific fluorescence was localised at the plasma membrane [73], perhaps indicating a regulatory role for this protein.

Our preliminary monolayer experiments on PI and PIP_2 binding to purified brain spectrin showed that a monolayer formed of PI or PI/PC bound brain spectrin efficiently. This is

similar to the binding of spectrin to PE/PC monolayers; however, monolayers of PIP_2 or PIP_2/PC did not bind purified brain spectrin at all (Diakowski, W., unpublished results), suggesting that spectrin's affinity is for anionic phospholipids. This result is consistent with the low levels of $\beta I\Sigma 2$ spectrin isoform found in brain spectrin preparations [74].

In our re-binding assays with brain spectrin and either synaptic plasma membranes or erythrocyte membranes, we found that membranes treated in such a way as to remove protein receptors (i.e., low ionic strength extraction, NaOH extraction and treatment with proteases), still bind spectrin [75], thus indicating the presence of a protein-independent receptor. The affinities of these stripped membrane preparations for spectrin are similar to those found in other model systems. Moreover, this binding is competitively inhibited by lipid vesicles (Diakowski, W., Szopa, J. & Sikorski, A.F., submitted).

INTERACTION OF PROTEIN 4.1 WITH MEMBRANE LIPID BILAYER

Protein 4.1 is a multifunctional extrinsic, globular membrane protein existing in the erythrocyte membrane as two forms 4.1a and 4.1b. They are products of the same gene (*4.1R*), whereas in non-erythroid cells the family of 4.1 proteins are products of the *4.1B* gene [76]. There are many isoforms of this protein; in developing erythroid cells as many as 7 major and 11 minor splice variants were detected [77]. Mutant forms in mice and humans are usually connected to erythrocyte membrane disorders. Protein 4.1 interacts with both actin [78] and spectrin to promote the formation of a complex between these proteins (K_D of this complex formation in the presence of protein 4.1 decreases by 5–6 orders of magnitude) and is essential for membrane skeleton integrity. The second known function of protein 4.1 is to attach the distal ends of spectrin tetramers and entire junctional complexes *via* a direct interaction with the membrane intrinsic domain. Many studies were devoted to this interaction and it seems proven that protein 4.1 binds glycophorins C and D [14], products of a single gene, as well as, through an alternative binding site to glycophorin C *via* the p55 protein, which is one of the band 4.9 polypeptides [15]. A detailed study [79] revealed the existence of two binding sites on glycophorins C and D, one for 4.1 (lower affinity), the other (high affinity) for p55 *via* PDZ-domain of p55 [80]. Binding sites for glycophorins C and D and p55 are located in the membrane-binding 30 kDa domain of protein 4.1 [79].

Protein 4.1 was found to contain a hydrophobic region as its intrinsic fluorescence could be quenched by 2-bromostearate [21] indicating that it could bind phospholipids. The earliest data on the binding of phospholipid by protein 4.1 were provided by Sato & Ohnishi [81]. They found that purified erythrocyte protein 4.1 bound vesicles prepared from phospholipid mixtures, in particular those containing PS. They found that treatment of inside-out-oriented vesicles of the erythrocyte membrane with PS decarboxylase decreased their ability to bind protein 4.1. Protein 4.1 induced permeability changes of large vesicles prepared from lipid mixtures containing PS. The interaction of protein 4.1 with PS-containing membranes was inhibited by Ca^{2+}. Others investigators [82], analysing protein 4.1-deficient elliptocytic erythrocytes, found an increased concentration of PS in the outer leaflet of these membranes and also demonstrated preferential binding of protein 4.1 to vesicles prepared from PS containing lipid mixtures. The equilibrium dissociation constant (K_D), determined for ^{125}I-labelled protein 4.1 on PS vesicles, amounted to 3.3×10^{-7} M [83]. Protein 4.1 penetrated monolayers prepared from a mixture of PS and PC, even at surface pressures over 30 mN/m. Protein 4.1 increased the permeability of negatively charged PS, but not PC liposomes. The interaction of protein 4.1 with large

unilamellar vesicles made of PS increased as pH and ionic strength were lowered, and decreased as Ca^{2+} or Mg^{2+} concentrations and ionic strength were raised [84]. Kinetic data also confirm the preference of this protein for PS-containing lipid mixtures [85].

INTERACTION OF ANKYRIN WITH MEMBRANE LIPID BILAYER

Ankyrins are a very well known group of erythroid and non-erythroid peripheral membrane proteins. As many as 12 different isoforms encoded either by separate genes (3 in humans) or being a result of alternative splicing of primary transcript have been discovered to date (for a review see [65]). Their role in erythroid as well as in numerous non-erythroid animal membranes has been studied in substantial detail [86–88].

In contrast, its interaction with membrane lipids has not been characterised to the level deserving a separate publication. Gratzer's group [21] showed, by intrinsic fluorescence quenching experiments, that ankyrin and its spectrin-binding domain reveal an affinity (in the micromolar concentration range) for hydrophobic compounds. In our experiments, we found that purified bovine erythrocyte ankyrin bound to phospholipid monolayers and bilayers, but with a much lower affinity than spectrins [89] i.e., at micromolar concentrations a linear dependence of bound ankyrin on concentration was observed.

ROLE OF INTERACTIONS OF ANKYRIN AND PHOSPHOLIPIDS WITH SPECTRIN. ANKYRIN:PE MODEL

An attempt to identify the amphipathic compound binding site of spectrin revealed its close proximity to the ankyrin-binding domain [24]. This implies that a functional relationship exists between ankyrin and lipid binding by spectrin. Indeed, when the effect of ankyrin on spectrin binding to phospholipid vesicles was tested, an inhibition of this interaction was observed [38, 90]. The effect was greater for vesicles containing PE (PE/PC 3:2) for which 60% inhibition was found compared to 10–20% inhibition for PS/PC vesicles. Almost identical results were obtained using a monolayer technique. Dixon-type analysis indicated a competitive mechanism of inhibition by ankyrin of PE/PC vesicle or monolayer binding to spectrin. Tetrameric spectrin bound similarly to a PE/PC monolayer but inhibition with ankyrin suggested that only one of the two possible binding sites is engaged in this interaction [33]. Moreover, when interactions of brain spectrin with PE/PC monolayers in the presence of ankyrin were analysed, a similar level of inhibition of these interactions by ankyrin was observed [68]. Also, when isolated erythroid spectrin β-subunit was introduced into the subphase of PE/PC monolayers in the presence of ankyrin, the inhibition was even stronger, i.e. a three-fold lower concentration of ankyrin was needed to induce the same effect. If the α-subunit was used instead of β, its effect on the monolayer surface pressure was small and entirely insensitive to ankyrin treatment [33].

Phosphorylation of red blood cell spectrin either *in vitro* with cAMP-dependent protein kinase or metabolically in intact cells, resulted in a reduced ability to affect the surface pressure of the PE/PC monolayer by dimers or tetramers of this protein. This effect in the presence of ankyrin was almost completely eliminated. When isolated ankyrin was phosphorylated, its ability to inhibit the spectrin–PE/PC monolayer interaction was completely eliminated [33].

Taking into account the physiological significance of this observation, Sikorski and colleagues have proposed [38, 91] an ankyrin:PE model. It is well known that, at least in erythrocyte membranes, there are situations in which ankyrin is either deficient or its affinity for spectrin is reduced. The spectrin

tetramers bind ankyrin at the highest affinity site, but when there is not enough functional ankyrin to accommodate all of them, the tetramers will bind to PE-rich domains. Known examples of such physiological situations are: ankyrin is among the last components synthesised and assembled into the membrane skeleton (for review a see [92]). Erythrocytes of mutant mice, whose erythroblasts fail to synthesise ankyrin, still accumulate half of the normal amount of spectrin [93] and moreover, their fetal red blood cells are morphologically similar to fetal erythrocytes of normal mice [94]. Another case is erythrocytes of ankyrin-deficient mice which contain normal membrane skeletons but lack AE1 (band 3) tetramers [95]. Reduced affinity of ankyrin for spectrin occurs when ankyrin is phosphorylated [96]. In all these situations PE-rich domains of the inner leaflet of the membrane would serve as "anchors" substituting for ankyrin and ensuring the preservation of the mechanical properties of spectrin tetramers in the skeletal lattice. This would also explain why mice with a disrupted *AE1* gene, expressing no AE1, are characterised by severe spherocytosis despite the presence of a normal erythrocyte membrane skeletons [97], in contrast to the above mentioned cases of ankyrin deficiency; possibly in this case ankyrin inhibits the binding of spectrin to membrane lipids.

The nature of lipid-binding by spectrin seems to be controlled by phosphorylation, since phosphorylation reduces the binding to lipid monolayers, and furthermore, this reduced binding became insensitive to ankyrin inhibition [33]. It should be noted, however, that not all lipid-binding sites in the spectrin molecule are sensitive to inhibition by ankyrin; as mentioned above, the PS-binding sites are less sensitive, while PC binding is insensitive to this inhibition [33]. Very specific PIP_2 and IP_3 binding by PH domains of non-erythroid spectrins is probably also insensitive. The same is for the "general" phospholipid-binding site (also aminophospholipid-specific), which is sensitive to proper folding as described for the rod domain repeat unit of dystrophin [98].

CONCLUDING REMARKS

As outlined above, some of the facts concerning direct interactions of membrane skeletal proteins with membrane lipids have been known for many years. There is, however, increasing amount of data indicating the importance of these interactions as possible mechanisms of regulatory events within the cell. One example of such events is the dependence of a certain (possibly major) type of lipid-binding sites on ankyrin, another is the binding of PIP_2 and IP_3 by certain spectrin isoforms. Further studies on the details of this binding, as well as the identification of the lipid-binding domain(s) on the proteins should be undertaken. It has also been known for a long time that other membrane skeletal elements, such as protein 4.1, are capable of phospholipid binding. Since this protein is also "polyfunctional" and a target for regulatory modifications (e.g., phosphorylation and Ca^{2+}-calmodulin binding [99]) it would be interesting to determine whether lipid-binding could substitute for one or more of those interactions, as is the case with ankyrin-binding by spectrins. The knowledge of the regulatory mechanisms, such as the dependence of some of the interactions with other proteins, as well as the dependence of lipid binding on phosphorylation, Ca^{2+}-calmodulin etc., may answer many important questions concerning membrane stability and dynamics. It would also be of interest to see whether newly described isoforms of β-spectrin [100–102] have similar lipid-binding properties.

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