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Ankyrin shares a binding site with phospholipid vesicles on erythrocyte spectrin*

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The spectrin-based erythrocyte membrane skeleton is believed to play an essential role in maintaining the shape, integrity and elasticity of erythrocytes (for review see e.g.: [1]). The membrane skeletal meshwork is formed by long (maximal length approx. 200 nm) spectrin tetramers connected at their ends by junctional complexes consisting of a single actin filament (37 nm, 13 actin subunits) [2] and other proteins: protein 4.1 and adducin as well as dematin (protein 4.9) [3]. In the observed images of the spread cytoskeletons, spectrin tetramers are either bare or associated with one, or a pair, of distinct globules 9–12 nm in diameter that were suggested to be ankyrin or ankyrin/anion transporter protein complexes [2, 4]. Ankyrin was identified as a high affinity spectrin-binding protein in the erythrocyte membrane [5–7]. Isolated ankyrin binds spectrin and the cytoplasmic domain of anion transporter [8]. Since spectrin is known to interact directly with the lipid domain either in natural membranes [9, 10] or in model systems [11–13] it is suggested that, apart from the high affinity binding sites, there are lower affinity receptors for spectrin that appear to be aminophospholipids. The question which we address in this communication concerns the interrelationship between these two types of receptors for spectrin (i.e. ankyrin and aminophospholipids) on the cytoplasmic surface of erythrocyte membrane. Our data indicate that phospholipids (particularly phosphatidylethanolamine) may, at least in part, occupy the same binding site as ankyrin.

As it is shown in Fig. 1A, isolated ankyrin is able to inhibit binding of purified spectrin to PE/PC[1]-containing liposomes. However, about 40% of binding was insensitive to inhibition by ankyrin. It should be noted that the half maximal effect was observed at ankyrin concentration of about 100 nM, in agreement with a dissociation constant for the spectrin–ankyrin complex [6]. Ankyrin bands were not present in the Coomassie stained gels obtained after electrophoresis of the liposome pellets, indicating absence of the ternary complex phospholipid–spectrin–ankyrin as well as absence of direct binding of ankyrin to liposomes. The results of the experiments presented in Fig. 1B indicate that, when ankyrin was added after preincubation of spectrin with phosphatidylethanolamine/phosphatidylcholine-containing liposomes, the final effect was smaller pointing to the existence of several binding sites. The inhibitory effect of ankyrin on the phosphatidylserine/phosphatidylcholine-containing vesicle binding by spectrin (Fig. 1C) was much smaller.

This effect seems to be in good agreement with our data on the effect of PE-containing vesicles on intrinsic fluorescence of spectrin [13]. To our knowledge this is the first demonstration that the binding sites for ankyrin and phospholipids (mainly phosphatidylethano-

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[1]Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecylsulfate.

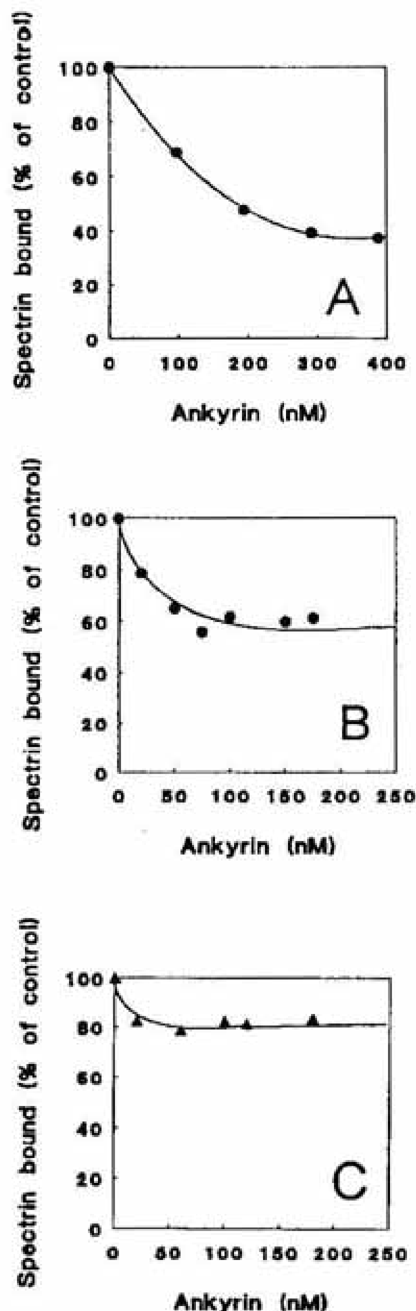

Fig. 1. Ankyrin inhibits binding of spectrin to liposomes.

Bovine erythrocyte spectrin was purified as was described previously [14]. Erythrocyte membrane ankyrin was purified essentially according to Hall & Bennett [15], except that chromatography was carried out on a 40 ml DEAE-Sephacel (Sigma) column using linear gradient (0.2–0.5 M) of NaCl. To avoid the presence of traces of spectrin, ankyrin was purified by gel filtration on Sephacryl S-200 column (1 × 35 cm) equilibrated with 0.6 M KI in 10 mM Na_2HPO_4, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4, Frozen and thawed liposomes were prepared according to Hope *et al.* [16] using the buffer: 5 mM Tris, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol, 1 mM NaN_3, pH 7.6 (test buffer) containing 20% Dextran T-40. The incubation mixture (400 ml) contained: liposomes (1 mg phospholipid), 150 nM purified spectrin dimer and indicated ankyrin concentrations. After 60 min incubation at room temperature (20°C) the sample was overlayered on a 600 µl cushion of 0.5% Dextran in the test buffer and centrifuged at 30000 × *g* for 6 min. The liposome pellets were analyzed electrophoretically in SDS-polyacrylamide gel [17]. Spectrin bands from Coomassie blue stained gels were cut out and extracted with dimethylsulfoxide and the absorbance at 595 nm was measured *versus* the "background" gel slice. A standard curve using pure spectrin was prepared. A, Inhibition of spectrin binding to the vesicles prepared from PE/PC (60:40) by ankyrin; B, ankyrin partly dissociates spectrin-PE/PC liposome complex. Experiments were performed as described above (A) except that spectrin was pre-incubated for 30 min with liposomes before ankyrin addition. C, The effect of ankyrin on complex formation between spectrin and PS/PC (60:40) liposomes. Experimental details as in A except that PS/PC-containing liposomes were used.

lamine), are at least in part, overlapping. Previously found [18] the proteolytic fragment of spectrin which showed strong affinity to hydrophobic agaroses was suggested to be a fragment of β subunit containing ankyrin binding site. Kennedy *et al.* [19] showed that the ankyrin-binding domain was formed by the entire 15 repeat segment of β subunit. This site contains also a highly conserved domain encompassing first 33 residues of 15th repeat unit and extending over 3 residues of the 14th segment that is highly conserved (about 90% identity) between red blood cell spectrin and nonerythrocyte spectrin (Ma, Y., Zimmer, W.E. & Goodman, S.R., personal communication). Physical properties of this fragment seem to be in good agreement with the phospholipid (phosphatidylethanolamine) binding ability. Of this sequence 20 residues are hydrophobic according to the consensus hydrophobicity scale [20] ($\Delta\mu^o > 0$). There are at least several situations in red blood cell when ankyrin is absent, e.g. some of spectrin tetramers are devoid of the 9–12 nm globular particles [2, 5]. Ankyrin is among the last components to continue to be synthesized and assembled to the membrane skeleton (for review see [21]). Due to genetic deficiency erythrocytes of mutant mice, whose erythroblasts fail to synthesize ankyrin but still accumulate approx. 50% of spec-

trin [22]. Affinity of ankyrin for spectrin is reduced upon phosphorylation of ankyrin [23]. The physiological meaning of the described events would be that, in certain situations, phosphatidylethanolamine-rich membrane domains would serve as "anchors" substituting for ankyrin to ensure the preservation of mechanical properties of spectrin tetramer in the cytoskeletal meshwork.

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