Journal of Supramolecular Structure 8:455–463 (1978)
Normal and Abnormal Red Cell Membranes 177–185

Spectrin Binding and the Control of Membrane Protein Mobility

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Transmembrane proteins of the human erythrocyte show restricted in-plane mobility. Many of the restrictions on mobility are attributable to the molecules of spectrin which are located on the protoplasmic surface of the erythrocyte membrane. These molecules are elongate, form end-to-end heterodimer associations, and bind selectively to protein (or proteins) accessible on inside-out, but not right-side out, membrane vesicles.

Key words: protein mobility, spectrin shape, spectrin binding

Our knowledge of the interactions that restrict the mobility of membrane macromolecules is limited for the most part to conclusions drawn from indirect experiments in complex systems. For example, interactions between transmembrane elements and extrinsic fibrous or fiber-forming elements are commonly evoked [1–4] to explain the response of cell surface elements to cytochalasin, colchicine, or polyvalent ligands, but simpler experimental systems are required if underlying biochemical and molecular organization is to be understood. The human erythrocyte membrane, which can be readily purified with a defined orientation, provides an ideal starting point for such studies.

Two kinds of evidence indicate that the transmembrane integral proteins of the human erythrocyte are mobile but that this mobility is restricted. The first kind of evidence comes from experiments [5] in which fluorescently labeled and unlabeled erythrocytes are fused and the movement of fluorescent surface elements is observed by light microscopy. The results (Table I) show that the intrinsic proteins of the red cell membrane exhibit a lateral mobility which is comparable to, but somewhat slower than, that observed for proteins of other plasma membranes measured by similar fusion techniques. The second kind of evidence comes from experiments in which intramembrane particles due to intrinsic proteins are observed by electron microscopy using freeze-fracturing methods [6, 7]. The results of these experiments suggest that the extrinsic protein spectrin, perhaps together with erythrocyte actin, forms a fibrous meshwork that restricts the lateral mobility of the erythrocyte intrinsic membrane proteins.

Received for publication May 5, 1978; accepted May 31, 1978.

0091-7419/78/0804-0455$02.00

TABLE I. Minimum Diffusion Coefficients for Fluorescently Labeled Integral Membrane Proteins

Temperature (°C)	Fused erythrocytes ($cm^2 \cdot s^{-1}$)	Mouse-human heterokaryons ($cm^2 \cdot s^{-1}$)
37	4×10^{-11}	9×10^{-11}
30	3×10^{-11}	–
23	6×10^{-12}	–
0	No movement of label observed	

Diffusion coefficients were calculated from the time required for at least 90% of the initially fused cells to show total intermixing (uniform fluorescence to the human eye) of the labeled membrane proteins. Results for fused erythrocytes are from Fowler and Branton [5]; results for mouse-human heterokaryons are estimated from the data of Edidin and Wei [25].

The notion that spectrin can form a meshwork that restricts or regulates the motion of other membrane macromolecules assumes that spectrin is an elongate molecule that binds to sites at the protoplasmic surface of the erythrocyte membrane [8]. These assumptions can now be confirmed.

SPECTRIN SHAPE

It has been known for some time that spectrin, especially when in the presence of other membrane proteins such as erythrocyte actin, can self-associate to form fibrous aggregates [9]. However, the shape of individual spectrin molecules has been difficult to study. Negative staining for electron microscopy usually aggregates spectrin molecules, and indirect physical methods using analytical ultracentrifugation have produced conflicting results [10–16]. Nevertheless, a consistent picture of the molecule is beginning to emerge from electron microscopy of shadowed molecules [17], light scattering studies [18], and from electrooptical analysis [19]. When the purified, approximately 9S, spectrin heterodimer (one molecule of band 1 and one molecule of band 2; total molecular weight ~460,000) is visualized by low-angle shadowing (Fig 1), it appears as a very flexible molecule approximately 1,000 Å in length. Its constituent monomer polypeptides are often individually visible, lying partially separated from one another or twisting around each other. The purified 12S tetramer is formed by the end-to-end association of two heterodimers, with minimal or no overlap. These electron microscopic results are consistent with the light scattering and birefringence relaxation measurements which show that spectrin heterodimers in solution also have contour lengths in excess of 1,000 Å [18, 19].

SPECTRIN BINDING

The association of spectrin with the red cell membrane has been studied with a binding assay [20] using radiolabeled, purified spectrin and spectrin-depleted vesicles derived from erythrocyte ghosts. Spectrin binds selectively to inside-out but not right-side-out vesicles. The association between spectrin and the vesicles is slow, pH- and salt-

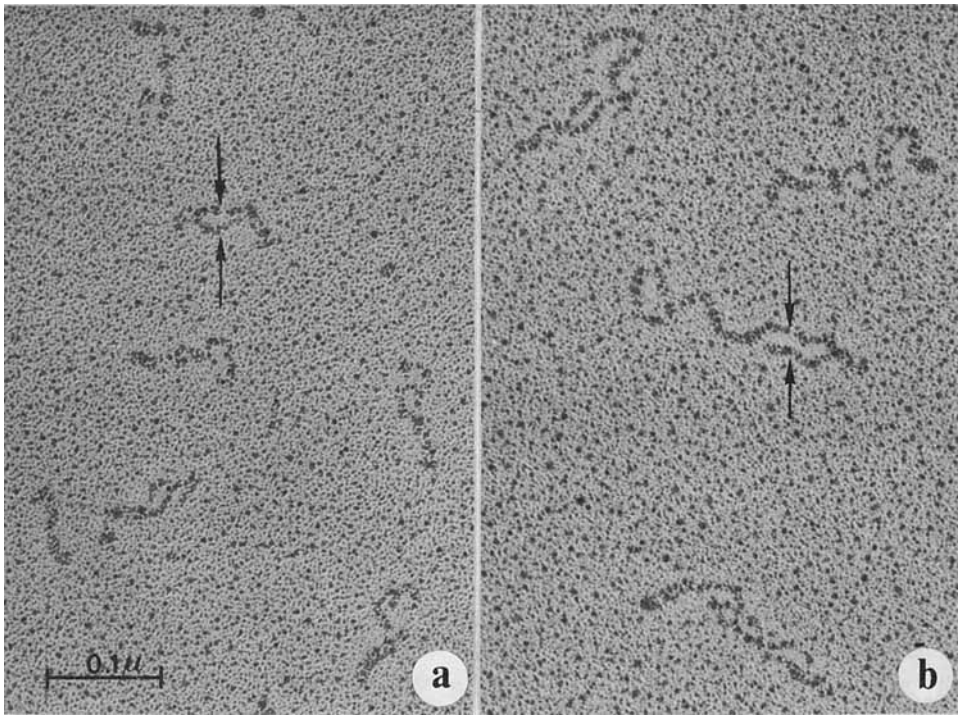

Fig 1. Spectrin heterodimers (a) and tetramers (b). The heterodimers and tetramers were purified on a Sepharose 4-B column according to Ralston, Dunbar, and White [15] and Pt-C replicas of the dried molecules were prepared by low-angle, rotary shadowing. The arrows point to a region of a heterodimer (a) or tetramer (b) where the constituent monomer polypeptides are individually visible (From the work of Shotton, Burke, and Branton [17], with permission. (Magnification: × 170,000, a and b.)

dependent, and saturable. Partial proteolysis of the vesicles indicates that binding depends upon a protein (or proteins) that is attached to the membrane.

These experiments further suggest that under controlled proteolysis, the binding protein can be cleaved so as to release the fragment with which spectrin associates. Indeed, during partial proteolysis with α-chymotrypsin, water-soluble polypeptides are released from the membrane which inhibit binding of spectrin to spectrin-depleted vesicles [20]. Diethylaminoethanol (DEAE) cellulose chromatography of these soluble polypeptides resolves a 72,000-dalton polypeptide which competitively inhibits binding of spectrin to spectrin-depleted vesicles [21]. This 72,000-dalton polypeptide also forms a complex with spectrin in solution and thus appears to be the membrane attachment site for spectrin [21].

This solubilized 72,000-dalton polypeptide has been used to evaluate the role spectrin plays in restricting the lateral mobility of intrinsic membrane proteins [22]. The 72,000-dalton polypeptide causes a rapid and complete dissociation of ^{32}P-spectrin from inside-out vesicles, and when included in the cytoplasmic space of fused erythrocytes, it increases the mobility of their integral membrane proteins [22]. These results confirm that the association of spectrin with its anchorage site plays a role in limiting the lateral mobility of integral membrane proteins. However, when the 72,000-dalton polypeptide was included in the cytoplasmic space of the fused cells, the integral membrane protein

mobility was still considerably slower than expected from unrestricted diffusion. Thus, interactions other than those between spectrin and its membrane attachment site may also regulate the mobility of the major integral proteins.

The membrane protein which is the source of the 72,000-dalton attachment site is not known. One way the parent protein is being identified is the preparation of antisera against the solubilized fragment (Bennett V, personal communication). Such antisera should react with the parent protein. Another approach to the identification of the spectrin-binding protein(s) is suggested by the observation [23, 24] that when erythrocytes or erythrocyte ghosts are extracted with nonionic detergents, an insoluble proteolipid "shell" is formed which contains – in addition to lipid, spectrin, and actin – a few other major polypeptides visible on SDS polyacrylamide electrophoresis.

When erythrocytes are extracted with 0.5% Triton X-114 in 10 mM 4-(2-hydroxyethyl)-1-pereazineethanesulfonic acid (Hepes), 2.5 mM reduced glutathione, 0.5 mM ethylene glycol bis(β-aminoethyl ether) N,N′-tetracetic acid (EGTA), and 70 mM KCl, several classes of proteolipid shells are formed which differ in the relative proportions of the major polypeptide species (Goodman, unpublished data). These classes can be separated by sedimentation through a 10–55% sucrose gradient at 120,000g for 15 h. The major class of proteolipid shells sediment to a density of approximately 1.190 g/ml, while most of the free Triton X-114 and unbound protein remain on top of the gradient. These gradient-purified shells have a diameter of 6–8 μ and appear to be composed of small lipid vesicles (1,000–3,000 Å in diameter) clumped within a dense reticulum of filamentous material (Fig 2a). Freeze-fracture of these shells shows the clustered lipid vesicles, some of which contain aggregated intramembrane particles (Fig 2b).

The proteolipid shells purified on the sucrose gradient have a protein composition consisting primarily of spectrin, erythrocyte actin, and polypeptides migrating in the band 3 region and the band 4 region* (Fig 3). The same major bands are found after electrophoresis in sodium dodecyl sulfate (SDS) independently of whether the shells are derived from untreated or chymotrypsin-treated erythrocytes (in which the bulk of band 3 is eliminated). This indicates that the primary component in the band 3 region of the shells is distinct from the bulk of band 3. It has been referred to as band 3′ [24], although its origin and relation to bands existing in the native membrane are unclear. A polypeptide migrating at the same molecular weight as band 3′ is sometimes seen in ghosts derived from chymotrypsin-treated cells, but not if the ghosts are solubilized in SDS as soon as they are produced (as in Fig 3, gel E). Thus, 3′ may itself be derived from some other polypeptide. The fact that the band 3 region of the proteolipid shells contains slightly more protein when derived from untreated rather than chymotrypsin-treated erythrocytes may indicate that a small amount of residual band 3 is also present in shells derived from untreated erythrocytes.

Dialysis of the proteolipid shells against low-ionic-strength buffer at pH 8.2 releases the spectrin and actin, and the vesicles unclump. Electron micrographs of this material

*We do not distinguish bands 4.1 and 4.2 in the proteolipid shells because in some preparations both 4.1 and 4.2 are visualized, while other preparations contain only 4.2. We currently believe that during the triton extraction, band 4.1 is cleaved by an endogenous protease.

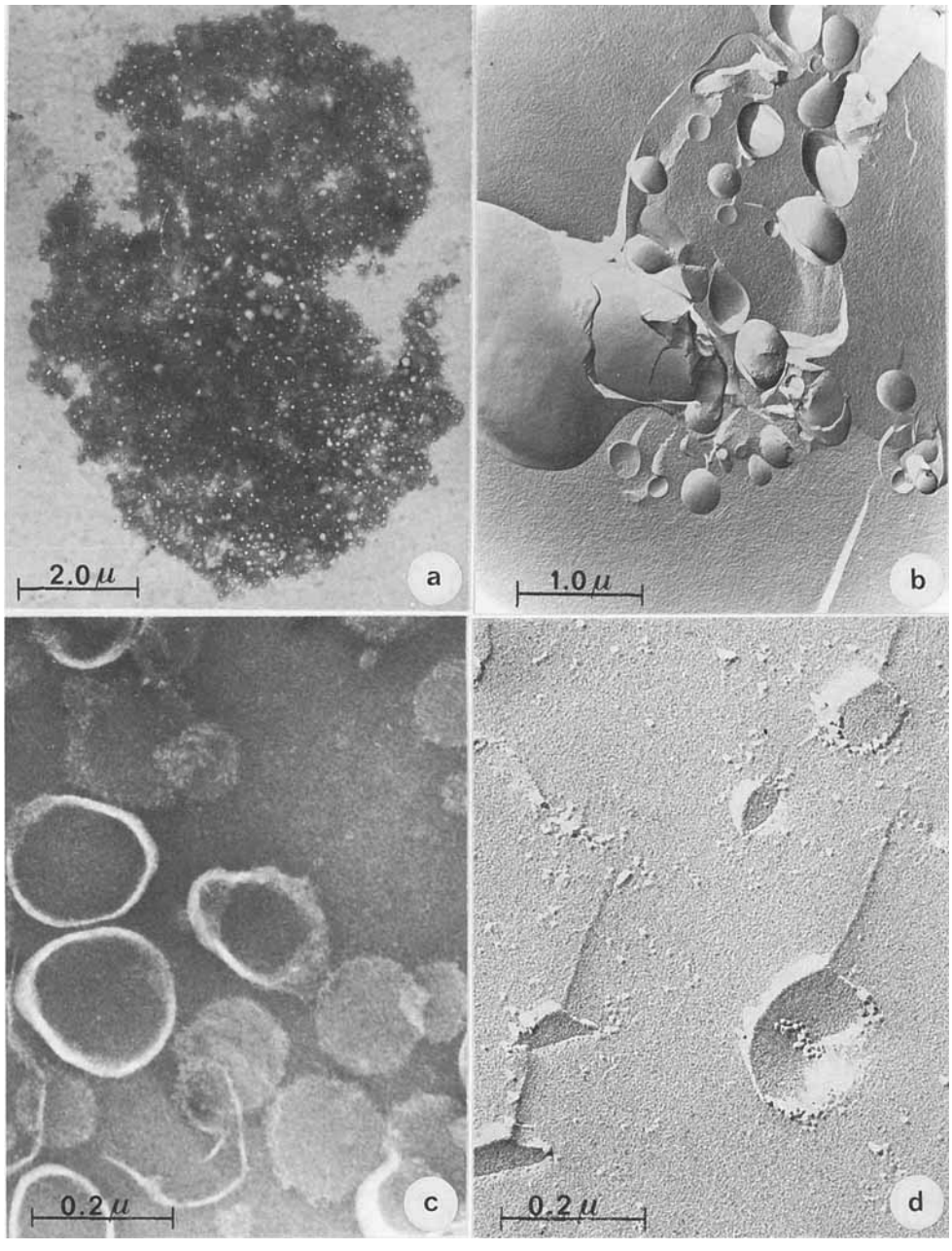

Fig 2. Electron micrographs of the proteolipid shells negatively stained (a and c) or freeze-fractured (b and d). a,b) Proteolipid shells from untreated erythrocytes; c,d) spectrin-depleted proteolipid vesicles obtained by dializing proteolipid shells against 0.3 mM Hepes, 0.2 mM EGTA, 0.2 mM adenosine triphosphate, 0.2 mM dithiothreitol (DTT), pH 8.2, over night at 4°C and sedimented at 120,000g for 60 min. From work in preparation (Goodman et al, 1978). Photographs by D. Anderson. (Magnification: a, × 9,100; b, × 18,200; c,d, × 100,000.)

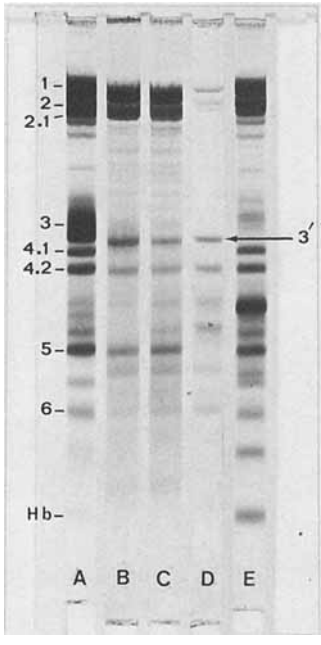

Fig 3. Polyacrylamide gel electrophoresis of ghosts and proteolipid shells. Gel A) ghost protein from untreated erythrocytes; B) proteolipid shells derived from untreated erythrocytes; C) proteolipid shells derived from chymotrypsin-treated erythrocytes (200 μg/ml α-chymotrypsin, 60 min at 37°C); D) spectrin- and actin-depleted proteolipid vesicles derived from chymotrypsin-treated erythrocytes; E) ghost protein from chymotrypsin-treated erythrocytes. Electrophoresis was performed in sodium dodecyl sulphate as in Yu, Fischman, and Steck [23], and the major bands were labeled as in that reference.

reveal a mixture of freely suspended vesicles and amorphous fibrillar material. The large (6–8 μ) structures of clustered vesicles are no longer found. The vesicles can be separated from the spectrin and actin by differential centrifugation at 120,000g for 60 min. The pellet of this centrifugation consists of freely suspended 1,000- to 3,000-Å proteolipid vesicles (Fig 2c). Freeze-fracture of this preparation demonstrates freely suspended vesicles, many of which contain highly clustered intramembrane particles (as in Fig 2d). Electrophoresis of these vesicles after low ionic strength dialysis indicates that at least 95% of the spectrin and all visible actin have been removed. Band 3′, band 4, and traces of residual spectrin are the major components found on SDS acrylamide gels (Fig 3, gel D). The biochemical and morpholigic experiments taken together strongly suggest that the 1,000- to 3,000-Å vesicles contain bands 3′ and 4, while the amorphous filamentous material represents a spectrin-actin complex.

The fact that spectrin, band 3′, band 4, and actin are found complexed in the shells has interesting implications but does not represent proof of their association in vivo. To ask whether bands 3′ and 4 might represent the spectrin attachment site, we isolated ^{32}P-spectrin by the technique of Bennett and Branton [20] and attempted to rebind ^{32}P-spectrin to spectrin-depleted proteolipid vesicles and intact proteolipid shells containing their full complement of spectrin. Binding studies were conducted at 0°C for 90 min under the ionic and pH conditions found to be optimal for spectrin binding to spectrin-depleted inverted vesicles [20]. After the incubation, the components were separated by velocity sedimentation through a 10–50% sucrose gradient at 70,000g for 45 min. Under

these conditions, free ^{32}P-spectrin remains at the top, while ^{32}P-spectrin which is bound to vesicles migrates into the gradient. When ^{32}P-spectrin was incubated with the spectrin-depleted proteolipid vesicles, a large peak of ^{32}P-spectrin comigrated on the gradient with the vesicles (Fig 4a), just as when ^{32}P-spectrin was bound to spectrin-depleted inverted vesicles (Fig 4b). If proteolipid shells with their full complement of spectrin are incubated (at a protein concentration which makes the shell spectrin concentration equal to the

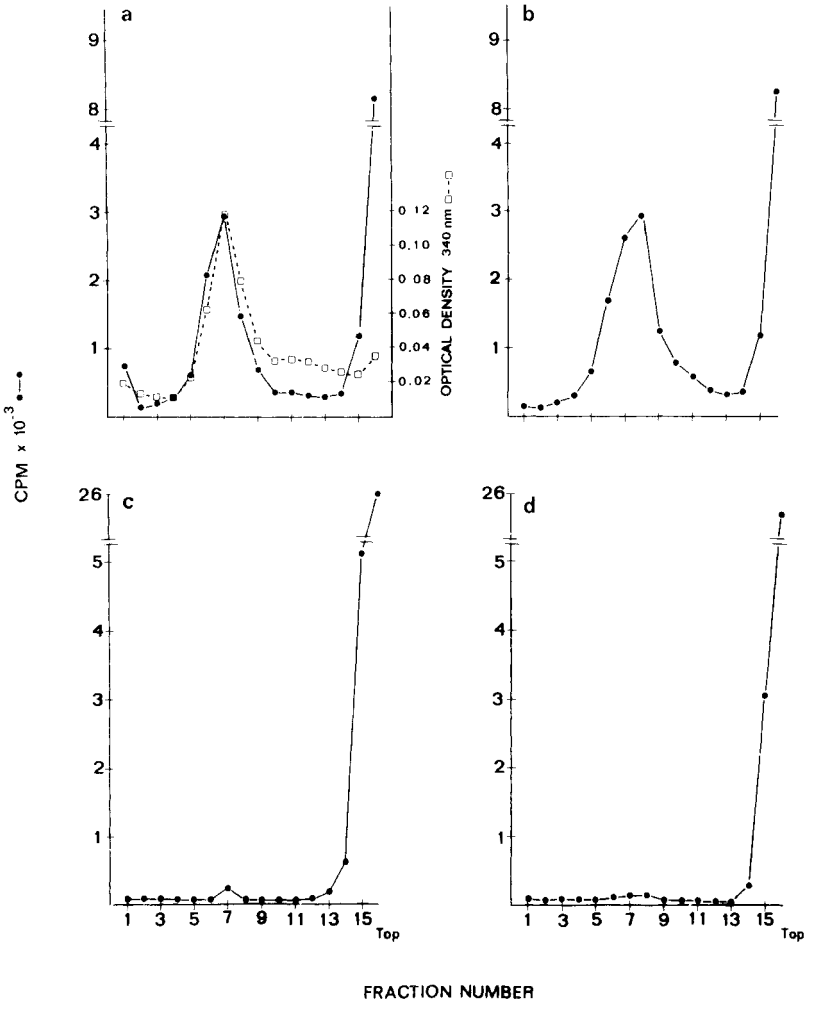

Fig 4. Spectrin binding studies. ^{32}P-spectrin (34 μg; 1,322 cpm/μg) was incubated for 90 min at 4°C in a final volume of 1 ml of buffer containing 0.7 mM $NaPO_4$, 20 mM KCl, 0.6 mM DTT, 0.3 mM $MgCl_2$, 2% sucrose, pH 7.6, with a) spectrin-depleted proteolipid vesicles (1 mg membrane protein), b) spectrin-depleted inverted vesicles (1 mg protein), c) proteolipid shells (100 μg protein), and d) spectrin depleted proteolipid vesicles (1 mg protein), but with heat-denatured (5 min at 55°C) ^{32}P-spectrin. The incubation mixture was layered onto a 13-ml 10–50% sucrose gradient and bound spectrin was separated from free spectrin by centrifuging at 70,000g for 45 min (SW40 rotor, 4°C). Sixteen fractions were dripped directly into counting vials and 10 ml of a counting cocktail of Aquasol containing 80 ml/liter water and 50 ml/liter glacial acetic acid was added. Radioactivity was determined with a Beckman scintillation counter.

residual spectrin concentration in the spectrin depleted triton vesicles) with ^{32}P-spectrin, they bind less than 10% of the ^{32}P-spectrin that binds to spectrin-depleted vesicles (Fig 4c). Therefore, the primary binding of spectrin to the spectrin-depleted proteolipid vesicles is not to the small residue of remaining spectrin in these vesicles. Heat-denatured ^{32}P-spectrin does not bind to the spectrin-depleted vesicles (Fig 4d). Therefore, the binding depends on spectrin's normal quaternary structure and does not seem to be nonspecific. Furthermore, ^{32}P-spectrin will not bind to vesicles which have been pretreated with 1 μg/ml chymotrypsin (30 min, 0°C; data not shown). Therefore, the binding of spectrin appears to be to protein in the vesicles.

These results suggest that in the proteolipid shells the 89,000-dalton polypeptide we call band 3′, perhaps complexed to band 4, is the spectrin-binding site. Although the possibility that a minor protein species present in the shells may actually be involved in the binding of spectrin cannot be ruled out by these experiments, it appears unlikely because only spectrin, bands 2.1 and 3′, and band 4 in the spectrin-depleted vesicles are large enough proteins to give rise to the 72,000-dalton fragment reported to be a water-soluble portion of the binding protein [21]. The provenance of band 3′ remains to be explored. It may itself be a cleavage product of a larger polypeptide. A 72,000-dalton chymotryptic fragment can be generated from spectrin-depleted proteolipid vesicles obtained from untreated erythrocytes or erythrocytes pretreated with 200 μg/ml α-chymotrypsin at 37°C for 2 h (Goodman et al, manuscript in preparation). In addition, the binding of ^{32}P-spectrin to spectrin-depleted vesicles is the same regardless of whether the vesicles are obtained from chymotrypsin-treated or untreated erythrocytes. Therefore, the small residue of band 3 remaining in shells derived from untreated erythrocytes cannot be the binding site (Goodman, unpublished observation).

Although further work is clearly required to complete our understanding of the interactions that control erythrocyte membrane protein mobility, it is evident that this experimental system is one in which a detailed molecular approach is feasible. Solubilization of the 72,000-dalton attachment site for spectrin has led to the first reported isolation of a membrane attachment site for a major cytoskeletal protein [21], but the same approach could be, and no doubt will be, applied to other proteins in other cells.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health, the National Science Foundation, and the Department of Energy. S.R. Goodman is supported by a postdoctoral stipend from the National Institutes of Health.

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